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Really a "secondary gill under the skin"? Unveiling "dorsal vessels" in freshwater slugs (Mollusca, Panpulmonata, Acochlidimorpha)

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Abstract

The freshwater slugs of the genus *Acochlidium* (Heterobranchia, Gastropoda, and Acochlidimorpha) are peculiar, one to two centimeter sized animals found only in small coastal rivers and streams of Southeast Asian and Western Pacific islands. When first described by Bücking, the author observed a branching "net of dendritic vessels connected to the heart," which he assumed to have replaced the original gastropod gill. In the present study, we compare the renopericardial systems of four *Acochlidium* species in microanatomical, histological and ultrastructural detail and identify where exactly the enigmatic, subepidermal "dorsal vessels" connect to the renopericardial system to examine if they can really function as a gill. *Acochlidium* have elaborate renopericardial systems compared to their ancestrally marine and also freshwater relatives. The primary site of ultrafiltration is the epicardium of the atrium with podocytes as usual for gastropods. The "dorsal vessels" in *Acochlidium* are extensions of the outer epithelium of the pericardial cavity and represent true vessels, that is, coelomatic channels, having an endothelium with podocytes. Hence, they considerably enlarge the site of ultrafiltration increasing the pericardial surface. "Dorsal vessels" in *Acochlidium* are therefore not homologous to externally similar morphological structures in Sacoglossa (marine panpulmonate slugs and snails). The multiplication of renopericardioducts in *Acochlidium* is a unique feature within Mollusca that enhances the negative pressure necessary for ultrafiltration in the thin, tube-like dorsal vessels and as a consequence the transport of primary urine from the pericardium to the kidney. The circulatory and excretory systems in *Acochlidium* are adaptations to a lifestyle in their freshwater environment in which snail bodies are hyposmotic and accrue considerable influx of surplus water into the body, which needs to be expelled.

KEYWORDS
Acochlidium, excretion, habitat shift, invertebrates, metanephridia, ultrastructure

1 | INTRODUCTION

Habitat shifts from sea to freshwater or land and vice versa were rare events in animal evolution. Beyond competition with well-adapted co-existing resident species, land invasions require adaptive evolution to life in physically different conditions (Vermeij & Dudley, 2000). Molluscs are among the most successful animal groups and among them, Gastropoda (snails and slugs) inhabit (almost) all habitats on Earth (e.g., Ponder et al., 2019). The Panpulmonata is the most species-rich and anatomically extremely diverse gastropod clade with several independent invasions of freshwater and terrestrial habitats in its evolutionary history (Jörger et al., 2014). Therefore, Panpulmonata are especially suited as model group to explore morphological adaptations, which come along with habitat shifts from sea to land or to freshwater (e.g., Krug et al., 2022).

Pulmonate heterobranch gastropods are known for having branching vessels associated with the renopericardial system that are altogether situated in the mantle roof (Brace, 1977; Haszprunar, 1985) and were often regarded to aid in oxygen uptake (Barker, 2001). In the largest panpulmonate subclade Stylommatophora the mantle cavity has even become modified into a functional "lung" with vessels closely associated with the kidney (e.g., Barker, 2001). Molecular phylogenetics completely changed the understanding of "pulmonate" relationships within the heterobranch system (e.g., Jörger et al., 2010) and among others show two groups of seaslugs to be more closely related to stylommatophoran landsnails than to other seaslugs (e.g., nudibranchs or seahares). Interestingly, both of these panpulmonate taxa, the Sacoglossa and the Acochlidimorpha, were described to have some kind of 'dorsal vessels' visible through the skin of their posterior body. Similar vessels are otherwise unusual for the several thousand species of "seaslugs," the only exception is the cladobranch nudibranch *Trivettea papalotla* (Bertsch, Á. Valdés, Gosliner, 2009) (see Bertsch et al., 2009; Hulett et al., 2015).

Function and homology of the subepidermal organ among Sacoglossa, Acochlidimorpha and other panpulmonates remained speculative due to the lack of reliable primary data and inconsistent naming among lineages (Neusser et al. [2019] for review of terminology). Recently, Neusser et al. (2019) showed that "dorsal vessels" in the sacoglossan *Elysia viridis* (Montagu, 1804) are in fact channeled dorsal hemolymph sinuses without endothelium and thus, the term "vessel" is misleading in *E. viridis*. The study underlined the need for comparative microanatomical and ultrastructural investigation of "dorsal vessel systems" in other panpulmonate lineages.

Within Acochlidimorpha, "dorsal vessels" were recently reported for the deep-sea species *Bathyhedyle boucheti* Neusser, Jörger, Lodde-Bensch, Strong & Schrödl, 2016 (Neusser et al., 2016), semiterrestrial Aitengidae (Neusser et al., 2011) and, in traditional literature, for the freshwater slug *Acochlidium amboinense* Strubell, 1892 (Bücking, 1933). In his gross-anatomical study of *A. amboinense*, Bücking (1933) described a "net of dendritic vessels connected to the heart," which he assumed to have replaced the gill in these otherwise gill-less animals. Acochlidimorpha comprise the only known freshwater slugs with the possibly extinct small, interstitial *Tantulum* from the Caribbean (Neusser & Schrödl, 2007) and the large-sized, benthic Acochlidiidae (*Strubellia*, *Wallacellia*, *Palliohedyle* and *Acochlidium*) from the tropical Indo-Pacific (e.g., Schrödl & Neusser, 2010). While *Strubellia* and *Wallacellia* are already known in 3D-microanatomical detail and lack dorsal vessels (Brenzinger, Neusser, Glaubrecht, et al., 2011; Brenzinger, Neusser, Jörger, et al., 2011; Brenzinger et al., 2021), detailed morphological or ultrastructural data on rare *Palliohedyle* and relatively widespread *Acochlidium* are still lacking.

In this study, we comparatively examine the circulatory and excretory systems of four species (one undescribed) of the genus *Acochlidium*. We discuss the peculiarities in the circulatory and excretory systems of *Acochlidium* in light of the adaptation to a limnic lifestyle, the function of "dorsal vessels" in acochlidimorph freshwater slugs and its homology to other vessel-like structures traditionally described.

2 | MATERIAL AND METHODS

2.1 | Sampling of specimens

Living specimens of *Acochlidium* ssp. were collected on several occasions on different Indo-Pacific islands (see Table 1), in small streams and rivers with slight current between the river mouth to over 1 km upstream. They were picked by hand from the underside of small rocks in 0–1 m depth. Specimens were fixed for anatomical and ultrastructural studies. Additionally, one alcohol-preserved paratype specimen of *Acochlidium bayerfehlmanni* from Palau, originally collected by Frederick M. Bayer and H. Adair Fehlmann in 1957, was obtained from the National Museum of National History, Washington DC, USA for semithin sectioning. An overview of the material used in this study is given in (Table 1).

2.2 | Fixation and embedding

Before fixation, specimens were anaesthetized using nicotine or menthol crystals. The fixation for anatomical or ultrastructural studies was in Bouin's fluid and ethanol (75% or 80%) or in glutardialdehyde (2.5% and 4%) buffered with 0.2 mol L^{-1} sodium cacodylate (pH 7.2).

Later, the specimen of *A. bayerfehlmanni* fixed in Bouin's solution was washed with 80% ethanol overnight. The specimens fixed in glutardialdehyde were post-fixed in the laboratory in buffered 1% OsO_4 for 1 h in the dark. All specimens were decalcified in 1% ascorbic acid overnight and dehydrated in a graded series of acetone solution (30, 50, 70, 90, and 100%). Specimens were finally embedded in Spurr's low-viscosity resin (Spurr, 1969) and left to polymerize at 60°C for 24 h.

TABLE 1 Overview of the material used in present study.

Species	Museum number	Sampling locality	Sampling date/ collector	GPS coordinates	Fixation	Material
Acochlidium amboinense Strubell, 1892	ZMB Moll. 193942	Eastern part of Leihitu, at Watatiri, road Passo-Natsepa, Maluku Utara, Ambon, Indonesia	10.2008/MG	−3.617533 128.271033	75% ethanol	ss 2 µm
Acochlidium bayerfehlmanni Wawra, 1980	USNM 575737	Arakitaoch Stream, North Fork, Airal Munic, Babelthaup Island, Palau	09.1957/B&F		75% ethanol	ss 2 µm, 3D
Acochlidium bayerfehlmanni Wawra, 1980	ZSM Mol-20071898	Ngatpang waterfall, Tabecheding River, State of Ngatpang Babelthuap Island, Palau	11.1997/YK	7.452778 134.528889	Bouin's fluid	ss 2 µm
Acochlidium fijiense Haynes and Kenchington, 1991	ZSM Mol-20071097	Lami River, Lami District, Viti Levu, Fiji	08.2006/MS	−18.1045 178.401333	2.5% glu	ss 1.5 µm, us
Acochlidium sp.	ZSM Mol-20071919	Mamara River, near Mamara, 10 m from river mouth, Guadalcanal, Solomon Islands	10.2007/KJ, YK	−9.4025166667, 159.8899666667	4% glu	embedded, vr, µCT

Abbreviations: B&F, Frederick Bayer & Adair Fehlmann; glu, glutardialdehyde; KJ, Katharina Jörger; MG, Matthias Glaubrecht; MS, Michael Schrödl; ss, semithin sections; us, ultrathin sections; USNM, National Museum of National History, Washington DC, USA; vr, volume rendering; YK, Yasunori Kano; ZMB, Moll Museum für Naturkunde Berlin, Germany; ZSM, Bavarian State Collection of Zoology Munich, Germany; 3D, 3D-reconstruction.

2.3 | Semi-thin sectioning and ultrathin sectioning

We examined histological series of four specimens belonging to three *Acochlidium* species. Semi-thin sections of 1.5 or 2 µm were prepared using a RMC MT-7000 ultramicrotome with a diamond knife (Histo Jumbo, Diatome). Contact cement was applied on the lower cutting edge according to the method described by Ruthensteiner (2008) to form ribbons. Sections were stained with methylene blue-azure II (Richardson et al., 1960). The section series of the voucher of *A. bayerfehlmanni* is deposited at the NMNH. The section series of *A. amboinense* is deposited at the Museum für Naturkunde. The other section series are deposited at the Mollusca Section of SNSB-ZSM (the Bavarian State Collection of Zoology; see Table 1 for voucher numbers).

For the examination of the ultrastructure of the circulatory and excretory systems of *Acochlidium fijiense*, ultrathin sections were prepared from a single semithin section of series ZSM Mol-20071097. The method largely followed Handschuh et al. (2013). The region of interest was located on the semithin section. To detach the selected section from the section series, the glue to the neighboring sections was cut with a razorblade and the section was covered with a drop of water. Edges of the section, which were not released by the water only were carefully detached with an eyelash. An empty resin bloc with a smooth surface (cut with a diamond knife) was prepared. The detached section was transferred with an eyelash into a drop of water onto the resin bloc. The bloc with the section was dried at 60°C for 24 h. Then the bloc was trimmed and ultrathin sections of approx. 80 nm (pale gold reflection) were prepared using a RMC MT-7000 ultramicrotome with a diamond knife (Ultra 35°, Diatome, Biel, Switzerland). Ultrathin sections were collected on copper slot grids covered with a thin layer of formvar and subsequently stained with uranyl acetate and lead citrate after Reynolds (1963) to enhance the contrast. The sections were analyzed using a FEI Morgagni transmission electron microscope.

2.4 | Digital imaging and 3D-reconstruction with Amira®

Glass slides with sections were scanned with the 10x or 20x objective of an Olympus® dotSlide microscope (*.vsi format). Scanned images of slides were loaded into the imaging software OlyVia® (Olympus Soft Imaging Solutions GmbH) and individual digital images were taken (*.tif format). These images were converted to grayscale format (8 bit), contrast enhanced and unsharp masked using a sharpening filter in Adobe Photoshop® (Adobe Systems Software Ireland Limited).

The circulatory and excretory systems of the paratype of *A. bayerfehlmanni* were 3D-reconstructed using the software Amira® 5.2 (now Thermo Fisher Scientific). The imported images were automatically aligned and the alignment corrected manually where necessary; structures were mapped by hand and interpolated where possible. Segmentation and reconstruction of the organs largely followed Ruthensteiner (2008).

2.5 | Microcomputed tomography and volume rendering

One specimen of *Acochlidium* sp. from Solomon Islands (ZSM Mol-20071919), post-fixed with osmium tetroxide and embedded in Spurr's epoxy resin, was scanned using a Nanotom m (phoenix/X-ray) micro CT after manually removing areas of empty epoxy resin surrounding the specimen with a razorblade. The raw data were loaded in the 3D-reconstruction software Amira® and volume rendering performed to show organ systems in situ, with some transparency (default settings and color map except α scale 0.083333).

3 | RESULTS

3.1 | Microanatomy, histology, and ultrastructure of the circulatory and excretory systems

All comparatively investigated species of *Acochlidium* have circulatory and excretory systems that are visible in large parts from the exterior and situated at the right side of the visceral sac; the dorsal vessels also expand across much of the dorsal side (Figure 1a,b). The thin-walled, spacious pericardium surrounds a well-developed heart (Figures 1c–e, 2, and 3a–e). For describing the renopericardial complex we used terminology following Bartolomaeus (1996) and Schmidt-Rhaesa (2007): the outer epithelium of the pericardial cavity is the border towards the primary body cavity. The inner epithelium of the pericardial cavity covers the heart and is called "epicardium."

The heart is composed of a thin-walled atrium and a thick-walled ventricle (Figure 3a,c) in *A. amboinense* and *A. fijiense*. However, the separation in two chambers was not distinctly visible in *A. bayerfehlmanni* (Figure 3b,d). The highly folded epicardium of the atrium is composed of podocytes with pedicels (Figure 4c,d). The thin diaphragms that span the slits between the pedicels could not be detected. The epicardium of the ventricle in *A. fijiense* is highly muscular (Figure 4e) and consists of epithelio-muscle cells that are connected to each other by belt desmosomes and that are tied to the basal lamina by hemidesmosomes (Figure 4f). The myocardium of the ventricle is made up of mesenchymal muscle cells embedded in collagen fibers in the primary body cavity (Figure 4e). The aorta (Figures 1d and 2) emerges from the ventricle anteriorly and extends to the head.

Branches of dorsal vessels (Figures 1b,c and 2) are extensions of the posterior part of the outer epithelium of the pericardial cavity. These dorsal vessels are lined with a thin endothelium (Figure 3f and 4b). The latter contains podocytes (Figure 4 g,h) and resembles the epicardium of the atrium.

The pericardium is connected to the kidney by a multitude of well-developed, strongly ciliated renopericardioducts (rpd; Figures 1e, 2, and 3a,c,d). These are funnel-shaped epithelial ducts with compound cilia directed from the pericardium into the excretory system, towards the proximal, narrow part of the kidney (Figures 1e, 2, and 3d). We found a minimum of 14 such rpd in a juvenile specimen of *A. fijiense* and 42 rpd in a mature adult of *A. bayerfehlmanni* (see Table 2 for variability).

The complex kidney is long and undulated and followed by a long, looped nephroduct. The kidney is internally divided into a proximal, narrow lumen and a distal, wide and highly vacuolated lumen (Figures 2 and 3). One leads into the other only in the posterior part of the kidney, so that excretory fluids run in a hairpin-shape first posteriorly through the proximal part and then anteriorly through the distal part. The transition from the kidney to the nephroduct lies in the anterior end of the kidney. The ciliated nephroduct (Figures 1b-d, 2, and 3b-d) is approximately as wide as the proximal part of the kidney; it lies to the right of the kidney and also has an undulated, hairpin-like shape. The distal part of the nephroduct is then dilated and forms an upward loop (Figures 1c,d, 2, and 3a) that touches the outer epithelium of the pericardial cavity. The nephropore finally opens ventrolaterally to the exterior (Figure 3e), slightly posterior and lateral to the anus. An overview of the ontogenetical stages and number of renopericardioducts is compiled in Table 2.

4 | DISCUSSION

4.1 | Peculiarities of the renopericardial system in *Acochlidium*

The renopericardial system in *Acochlidium* shows common features of molluscan circulatory and excretory systems, that is, a metanephridial system (in the sense of Bartolomaeus and Ax [1992]) with podocytes and the heartbeat creating overpressure necessary for ultrafiltration (e.g., Schmidt-Rhaesa, 2007). Our data confirm that, in *Acochlidium*, the epicardium of the atrium can be considered as the primary site of ultrafiltration. The direction of ultrafiltration here is from the hemolymph-filled lumen of the atrium (primary body cavity) through the basal lamina and ultrafiltration slits into the voluminous lumen of the pericardium (secondary body cavity). This site of ultrafiltration is in concordance with other gastropods investigated in ultrastructural detail (e.g., Brenzinger et al., 2013; Fahrner & Haszprunar, 2001, 2002a; 2002b; Luchtel et al., 1997; Neusser et al., 2019) and Mollusca in general (e.g., Andrews, 1988; Luchtel et al., 1997; Ponder et al., 2019). An alternative ultrafiltration site was discussed to be between the conspicuous epicardial cells located instead on the ventricle in the closely related freshwater genera *Strubellia* and *Wallacellia* and the brackish-water *Pseudunela espiritusanta* (Brenzinger, Neusser, Glaubrecht, et al., 2011; Brenzinger, Neusser, Jörger, et al., 2011; Neusser & Schrödl, 2009; Brenzinger et al., 2021). However, ultrastructural analyses to confirm or reject this latter hypothesis are still lacking. In *Acochlidium*, the structure of the epicardium of the ventricle composed of epithelio-muscle cells resembles that of the marine cephalaspidean seaslug *Philinoglossa helgolandica* (see Bartolomaeus, 1996).

There are some unique features in the circulatory and excretory systems of *Acochlidium*. One peculiarity in *Acochlidium* (and

presumably also the poorly-known closely related genus *Palliohedyle*) is the presence of dorsal vessels connected to the outer epithelium of the pericardial cavity forming branching extensions. We show here that these structures are lined by a thin endothelium with basal lamina towards the outside and are therefore true vessels. Therein, they differ from the recently investigated so-called 'dorsal vessels' in marine *Elysia* slugs (Sacoglossa), which are not lined by an endothelium with basal lamina and are thus only channeled

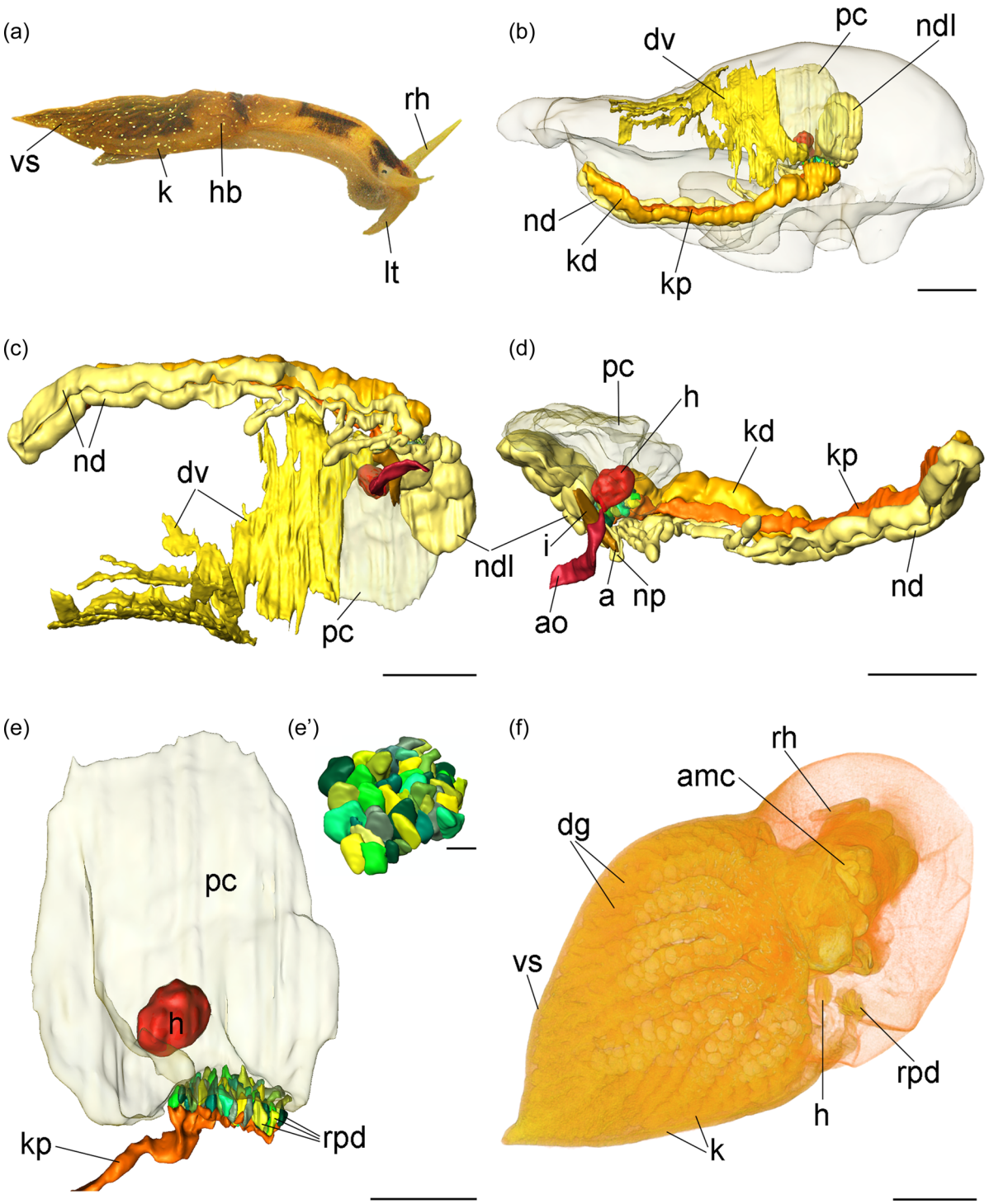

FIGURE 1 (a) Living specimen of *Acochlidium* sp. from Papua New Guinea (approx. 1 cm, right view). (b–e') 3D-reconstructions of *A. bayerfehlmanni* from Palau Island: the circulatory and excretory systems (b, right ventral view; c, ventral view; d, left view), the pericardium with heart (e, right view) and the multiplication of renopericardioducts (e'). (f) Volume rendering of *Acochlidium* sp. from Solomon Islands (dorsal view). Scale bars: 1 mm (b–d, (f), 500 µm (e), 100 µm (e'). a, anus; amc, anterior male copulatory organs; ao, aorta; dg, digestive gland; dv, dorsal vessel; h, heart; hb, heart bulb; i, intestine; k, kidney; kd, distal part of kidney; kp, posterior part of kidney; lt, labial tentacle; nd, nephroduct; ndl, distal loop of nephroduct; np, nephropore; pc, pericardium; rh, rhinophore; rpd, renopericardioduct; vs, visceral sac.

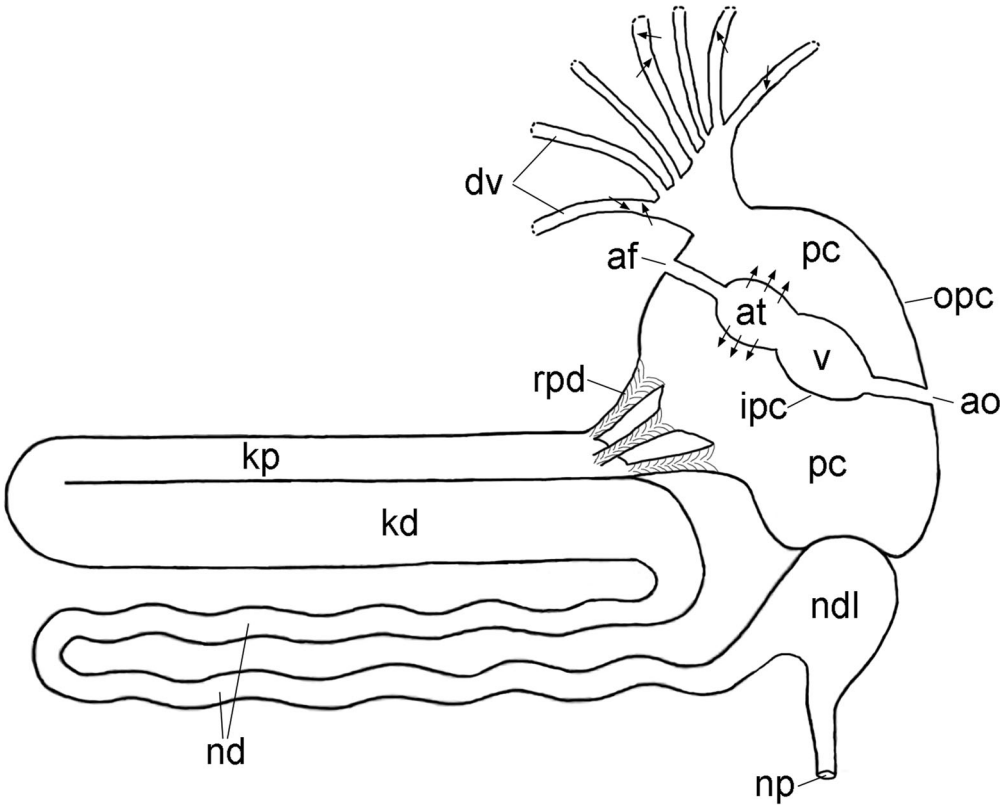

FIGURE 2 Schematic overview of the circulatory and excretory systems. The arrows indicate the direction of ultrafiltration. Not to scale. af, afferent hemolymph flow; at, atrium; ao, aorta; dv, dorsal vessel; ipc, inner epithelium of pericardial cavity; kd, distal part of kidney; kp, proximal part of kidney; nd, nephroduct; ndl, distal loop of nephroduct; np, nephropore; opc, outer epithelium of pericardial cavity; pc, lumen of pericardium; rpd, renopericardioduct; v, ventricle.

hemolymph spaces connected to the atrium and need to be correctly termed "dorsal hemolymph sinuses" (Neusser et al., 2019). The presence of podocytes in the endothelium of the dorsal vessels implies that the site of ultrafiltration in *Acochlidium* is considerably enlarged compared to closely related taxa without these vessels. The direction of ultrafiltration in the dorsal vessels is from the hemolymph-filled primary body cavity through the basal lamina and ultrafiltration slits into the lumen of the dorsal vessels being part of the pericardium and hence, secondary body cavity. Species with podocytes present in the outer epithelium of the pericardial cavity are scarce within Mollusca. Such an ultrastructure was only reported in the nudibranch *Hypselodoris tricolor* (see Fahrner & Haszprunar, 2002b), in Cephalopoda as appendages of the wall of the branchial "hearts" (e.g., Schipp & Hevert, 1981) and in some freshwater Bivalvia as "pericardial glands" (e.g., Andrews & Jennings, 1993; Meyhöfer et al., 1985). The increase in the surface area of the ultrafiltration sites may be due to higher rates of ultrafiltration and metabolism in predatory Cephalopoda and sponge-feeding *Hypselodoris*. In contrast, the well-developed pericardial glands in freshwater bivalves are discussed as adaptations to a freshwater environment (Andrews & Jennings, 1993; Meyhöfer et al., 1985). "Pericardial glands" were also reported in other dorid nudibranchs (e.g., Schrödl & Wägele, 2001; Wägele et al., 1999), however, their ultrastructure and function is unclear. Here, we consider the dorsal vessels in *Acochlidium* as adaptations to the limnic lifestyle, in which high ultrafiltration rates are necessary to overcome the osmotic stress caused by constant influx of freshwater into the slug's body.

Most marine acochlidimorphs have simple renopericardioducts (Schrödl & Neusser, 2010). In contrast, the well-developed, funnel-shaped renopericardioducts in *Acochlidium* resemble the so-called syrinx in nudibranchs (e.g., Wägele & Willan, 2000), which was discussed as apomorphy for nudibranchs by Dayrat and Tillier (2002). Another eye-catching feature in the microanatomy of *Acochlidium* species is the multiplication of renopericardioducts. A clear pattern explaining the number of renopericardioducts in our examined specimens was not yet evident to us; the number might increase during ontogeny as seen in *A. bayerfehlmanni*, in which the examined juvenile had only 26 renopericardioducts, but an examined adult had a "field" of 42 renopericardioducts. However, the juvenile of *A. bayerfehlmanni* had more renopericardioducts as did mature adults of *A. amboinense* and *Acochlidium* sp. from the Solomon Islands examined by us, possibly indicating the number of renopericardioducts is (also) a species-specific feature. More *Acochlidium* specimens of different species and diverse ontogenetical stages need to be examined for clarification. We hypothesize that the well-developed,

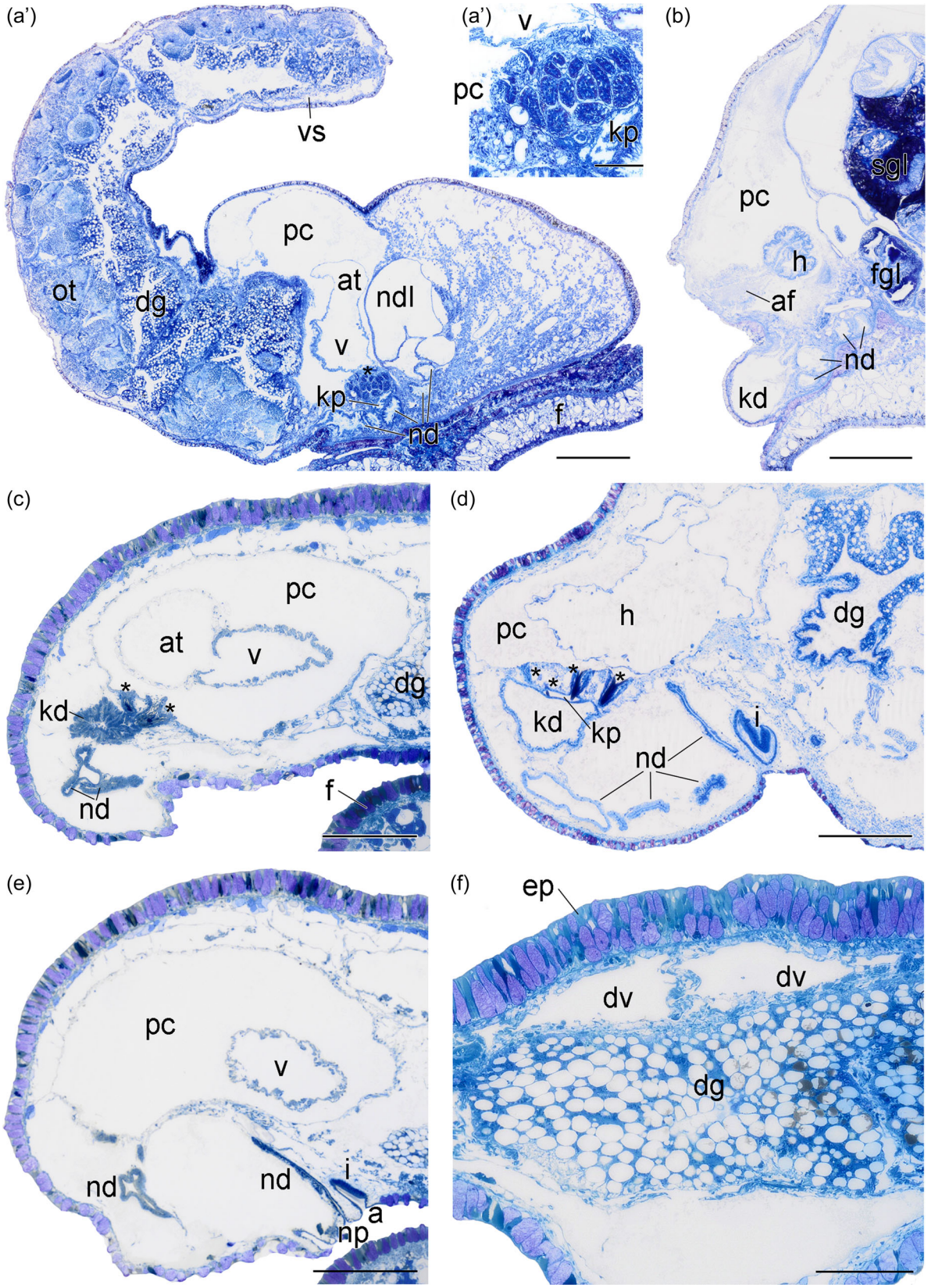

FIGURE 3 Histological longitudinal (a) and cross-sections (b–f) of *Acochlidium*. (a, a') Multiplication of renopericardioducts (*A. amboinense*). (b) Afferent hemolymph flow to the heart (*A. bayerfehlmanni*). (c) Two-chambered heart (*A. fijiense*). (d) Kidney and nephroduct (*A. bayerfehlmanni*). (e) Anus and nephropore (*A. fijiense*). (f) Subepidermal dorsal vessels (*A. fijiense*). Scale bars: 500 μm (a,b), 200 μm (c-e), 100 μm (a',f). a, anus; af, afferent hemolymph flow; at, atrium; dg, digestive gland; dv, dorsal vessel; ep, epidermis; f, foot; fgl, female gland; h, heart; i, intestine; kd, distal part of kidney; kp, proximal part of kidney; nd, nephroduct; ndl, distal loop of nephroduct; np, nephropore; ot, ovotestis; pc, pericardium; sgl, salivary gland; v, ventricle; vs, visceral sac; *, renopericardioduct.

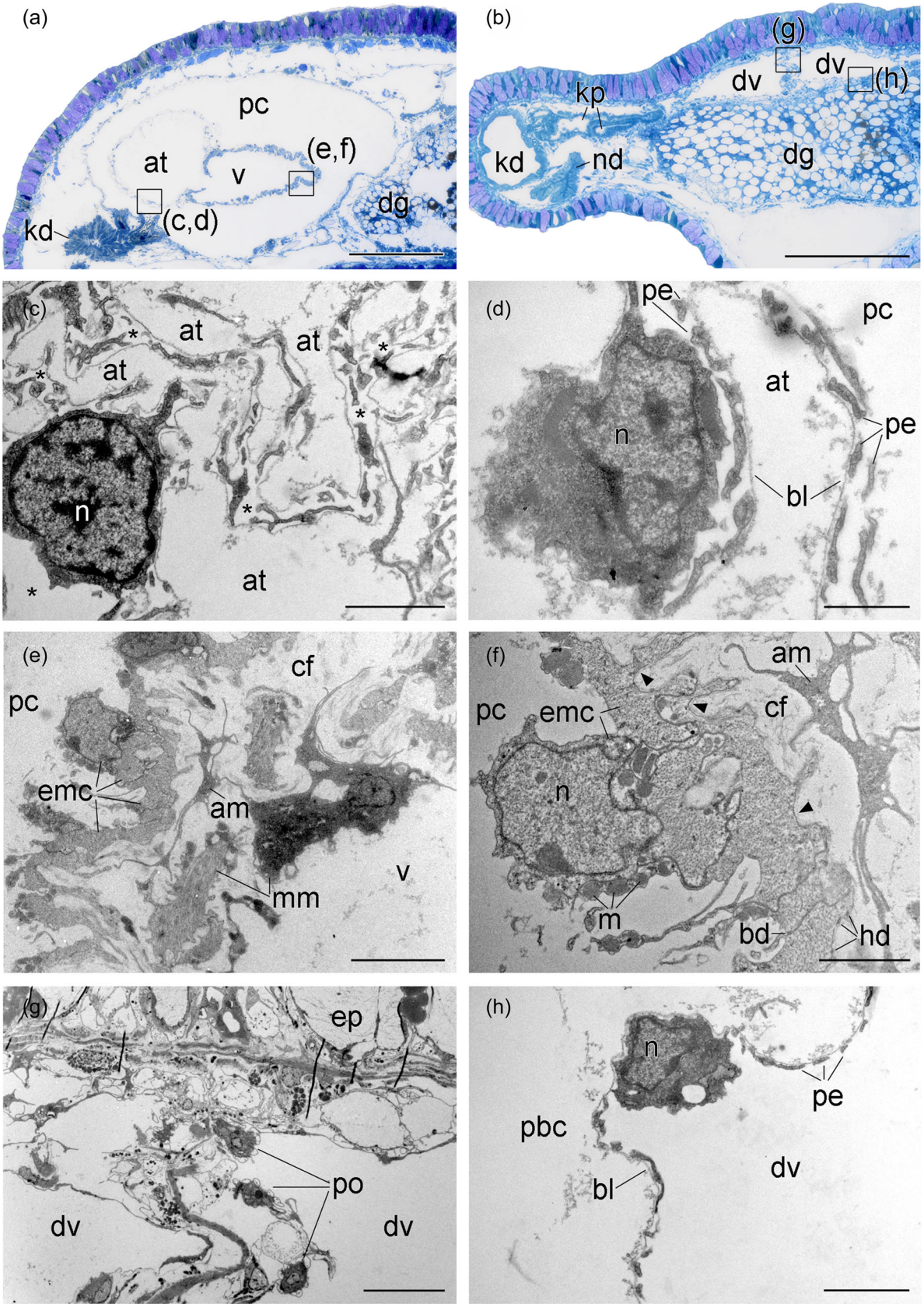

FIGURE 4 (See caption on next page).

TABLE 2 Ontogenetical stages and number of renopericardioducts in the examined *Acochlidium* species.

Species	Museum number	Ontogenetical stage	N° of rpd
Acochlidium amboinense Strube, 1892	ZMB Moll. 193942	Adult, mature	Approx. 20
Acochlidium bayerfehlmanni Wawra, 1980	USNM 575737	Adult, mature	42
Acochlidium bayerfehlmanni Wawra, 1980	ZSM Mol-20071898	Juvenile	26
Acochlidium fijiense Haynes and Kenchington, 1991	ZSM Mol-20071097	Juvenile	14
Acochlidium sp. Solomon Is.	ZSM Mol-20071919	Adult, mature	Approx. 16

Note: Rpd renopericardioduct. USNM National Museum of National History, Washington D.C.; ZMB Moll Museum für Naturkunde Berlin, Germany; ZSM Bavarian State Collection of Zoology Munich.

ciliated renopericardioducts produce a negative pressure in the pericardial system and thereby enable ultrafiltration into the narrow, tube-shaped dorsal vessels. Furthermore we assume that the multiplication of renopericardioducts in *Acochlidium* speeds up the transport of primary urine from the pericardium into the kidney. This is required because limnic molluscs are exposed to permanent inflow of water by osmosis (Ponder et al., 2019) and therefore, the production rate of hyposmotic primary urine must be larger than in marine slugs (e.g., Strong et al., 2011 on shell-bearing cerithioid gastropods); in consequence a more powerful transportation system is required.

There are usually one or two renopericardioducts present in Mollusca (review in Ponder et al., 2019); the multiplication of renopericardioducts beyond that number in *Acochlidium* species is to our knowledge a unique feature within Mollusca and was not reported before for any molluscan taxa.

The presence of dorsal vessels and the multiplication of well-developed renopericardioducts in *Acochlidium* are most likely morphological adaptations to life in a freshwater environment, yet, the congeners *Strubellia* and *Wallacellia* (no data on *Palliohedyle* available) lack both anatomical features and apparently manage to cope with the osmotic stress in a different way (even though heart and kidney are similarly developed).

Therefore, the question remains, why *Acochlidium* developed additional morphological structures as adaptations to life in freshwater while other genera did not and whether these adaptations might be related to environmental factors. One explanation may be the shape of the visceral sac. While the latter is roundish and elongated in *Strubellia* and *Wallacellia* (Brenzinger, Neusser, Glaubrecht, et al., 2011; Brenzinger, Neusser, Jörger, et al., 2011; Brenzinger et al., 2021), the visceral sac of *Acochlidium* is flat and leaf-like and thus has a larger surface-to-volume ratio. Depending on the body size of the slug, the surface of the visceral sac may be considerably larger when leaf-like and hence, also the amount of freshwater inflow by osmosis. In this scenario, the dorsal vessels and the multiple renopericardioducts would be advantages in a freshwater environment. Another explanation may be related to the distribution. Among the entire range of Indo-Pacific Islands where acochlidimorphs are known, different genera of these freshwater slugs rarely co-occur in the same river or stream, examples are known from Sulawesi with co-occurring *Acochlidium* and *Palliohedyle* (see Jörger et al., 2014) and from Ambon Island with *A. amboinense*, *Strubellia paradoxa* and *Wallacellia siputbiru* (Brenzinger et al., 2021; Strubell, 1892). On the Solomon Islands, *Acochlidium* and *Strubellia* are recorded, but do not appear to reliably co-occur in the same rivers. On other Indo-Pacific islands records are restricted to *Acochlidium* (Indonesia, Papua New Guinea, Fiji and Palau; e.g., Bayer & Fehlmann, 1960; Benthem Jutting, 1955; Haynes & Kenchington, 1991; Wawra, 1979,1980; own unpubl. data) or to *Strubellia* on Vanuatu (see Brenzinger, Neusser, Jörger, et al., 2011; Haynes, 2000). Although our own sampling of acochlidimorph freshwater slugs always followed the same method, it cannot be ruled out that our sampling efforts are incomplete and biased, for example, due to different diurnal rhythms of the slugs and/or different habitat choices in different river sections. An alternative explanation is that the different genera tolerate different degrees of water hardness, that is, the concentration of soluble minerals in the water. While salinity was regularly measured during sampling, water hardness was not monitored, thus, this hypothesis must be confirmed during future sampling events. Yet, for example, existing data report that *Strubellia* was often found in rivers whose beds were dominated by limestone (Brenzinger, Neusser, Jörger, et al., 2011; Haynes, 2000) and therefore in water rich in minerals where the osmotic gradient is lower. In contrast, in water low in minerals—as flowing through areas

FIGURE 4 Ultrastructure of the circulatory and excretory systems of *Acochlidium fijiense*. (a, b) Overviews of the region of interest (histological cross-sections at the right side of the visceral sac). (c) Podocyte of the highly folded epicardium of the atrium. (d) Pedicels and basal lamina of podocyte of atrium. (e) Epicardium and myocardium of ventricle. (f) Epithelio-muscle cell of epicardium of ventricle. (g) Endothelium of dorsal vessel. (h) Close-up of a podocyte of dorsal vessel. Arrow heads indicate the basal lamina. Scale bars: 200 µm (a, b), 10 µm (g), 5 µm (e), 2 µm (c, f, h), 1 µm (d). am, amoebocyte; at, atrium; bd, belt desmosome; bl, basal lamina; cf, collagen fiber; dg, digestive gland; dv, dorsal vessel; emc, epithelio-muscle cell; ep, epidermis; hd, hemidesmosome; kd, distal part of kidney; kp, proximal part of kidney; m, mitochondrium; mm, mesenchymal muscle cell; n, nucleus; nd, nephroduct; pbc, primary body cavity; pc, pericardium; pe, pedicel; po, podocyte; v, ventricle; *, pericardium.

with granitic or volcanic rock base—the osmotic gradient may be expected to be higher. Consequently, there would be larger water inflow into the slug and higher rates of ultrafiltration would be necessary, explaining why *Acochlidium* species may be more common in areas with soft water as *Strubellia*. However, microhabitat choice and ecological needs or dissimilarities between different freshwater slugs are not investigated enough. In general, the surface-volume-ratio of freshwater slugs is inconvenient in smaller specimens and especially juveniles resulting in a higher water inflow in relation to larger or adult specimens (Haszprunar, 2017; Howard et al., 2020). Dorsal vessels therefore are obviously not mandatory for expelling surplus water, but provide an additional, efficient tool for a slug's life in freshwater.

4.2 | Homology of "dorsal vessel systems"

"Dorsal vessel systems" have been traditionally described in panpulmonate Sacoglossa and Acochlidimorpha, as well as one dendronotoidean nudibranch. The function and homologies of the "dorsal vessels" in aforementioned nudibranchs are entirely unclear as the histology and ultrastructure are unknown. Recently, Neusser et al. (2019) showed that the subepidermal structures in sacoglossan *Elysia* are channeled hemolymph spaces without endothelium and hence incapable of ultrafiltration; thus not homologous with the real 'dorsal vessels' in freshwater *Acochlidium* that are coelomatic channels. Within Acochlidimorpha, there now seem to be at least two types of morphologically different "dorsal vessel systems" with presumably different functions. In contrast to freshwater *Acochlidium* where the vessels emanate from the pericardium, superficially similarly branching "dorsal vessels" in (semi)terrestrial Aitengidae and deep-sea Bathyhedylidae are instead connected to the kidney (Neusser et al., 2011, 2016). We expect that "dorsal vessels" in Aitengidae and Bathyhedylidae show differing ultrastructure and thus may be convergently shaped structures with different functions. To date, none of these structures appear to be homologous to gills and related in oxygen uptake as was the original idea of Bücking (1933), but dedicated studies highlight the novelty that evolved multiple time during the numerous habitat switches that occurred during the evolution of Panpulmonata.

5 | CONCLUSIONS

We show that the circulatory and excretory systems of *Acochlidium* have morphological structures traditionally regarded as adaptations to a life in freshwater, that is, a complex, internally divided kidney and a long, looped nephroduct. Additionally, they have morphological features unique to this genus to cope with the osmotic stress: (i) the presence of dorsal vessels that are extensions of the outer epithelium of the pericardial cavity with podocytes and therefore the enlarged site of ultrafiltration and (ii) the multiplication of renopericardioducts that supposedly produce a negative pressure enabling the ultrafiltration and allow for a fast transportation of the primary urine from the pericardium to the kidney.

The dorsal vessels in the freshwater slug *Acochlidium* are coelomatic channels and, hence, true vessels rejecting earlier homology assumptions between sacoglossan and acochlidimorph 'dorsal vessels'. This study underlines the need for comparative ultrastructural analyses of poorly-known morphological structures that highlight ecological, evolutionary and biological diversity of these otherwise rarely studied animals.

AUTHOR CONTRIBUTIONS

Katharina M. Jörger and Michael Schrödl organized sampling trips and collected specimens. Timea P. Neusser designed the study, prepared semithin section series, supervised the 3D reconstruction, and analyzed histological data. Katharina M. Jörger and Timea P. Neusser analyzed the ultrastructural data. Bastian Brenzinger helped in embedding specimens, analyzed µCT data and prepared volume rendering images. Timea P. Neusser wrote the original draft, Bastian Brenzinger/Katharina M. Jörger/Michael Schrödl improved and edited early manuscript versions. All authors approved the last manuscript version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The histological section series are deposited at the Mollusca Section of the Bavarian State Collection of Zoology, Munich, the Museum für Naturkunde, Berlin and the National Museum of Natural History, Washington D. C., USA. The Three-dimensional reconstruction data are deposited at the repository of Systematic Zoology at the Biocenter of the Ludwig-Maximilians-University Munich.

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