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3D-microanatomy of the mesopsammic Pseudovermis salamandrops Marcus, 1953 from Brazil (Nudibranchia, Gastropoda)

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3D-microanatomy of the mesopsammic *Pseudovermis salamandrops* Marcus, 1953 from Brazil (Nudibranchia, Gastropoda)

Katharina M. Jörger · Tina Stoschek · Alvaro E. Migotto · Gerhard Haszprunar · Timea P. Neusser

Abstract Species of the nudibranch Pseudovermidae Thiele, 1931 are rare but conspicuous inhabitants of the marine mesopsammon. Their characteristic vermiform body with reduced cerata and acorn-shaped head lacking appendages is well adapted to life in the interstices of sand grains. Traditionally, species descriptions are based mainly on external morphology and radula characteristics; knowledge on their anatomy is scarce. Here we provide the first microanatomical redescription of a member of Pseudovermidae based on 3D-reconstruction from histological semi-thin section series. The present study on *Pseudovermis salamandrops* Marcus, 1953 reveals several discrepancies to the original description especially within the complex triaulic genital system (i.e., absence of a connection between vas deferens and kidney, presence of a receptaculum seminis and a large muscular penial sheath gland). We also add microanatomical details such as the presence of gastroesophageal ganglia in the central nervous system, described for the first time in Pseudovermidae. Concluding from the nematocysts found in the cnidosacs of *P. salamandrops*, this species is a cnidarivore which likely preys on various meiofaunal cnidarians. We show that microanatomical redescriptions of poorly known Pseudovermidae are needed to gather comparative data as a backbone to place these neglected meiofaunal slugs in a phylogeny and trace their evolutionary pathway into the mesopsammon. Traditional characters used for species delineation are insufficient to diagnose *Pseudovermis* and an integrative approach is needed to reliably address pseudovermid diversity in the future.

Keywords Meiofauna · Cnidosacs · Cnidarivore · Aeolidoidea · Sea slug

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Introduction

Sediment covered ocean floors form one of the largest habitats on Earth and potentially presented stable environmental conditions to its inhabitants over long geologic timescales (Rundell and Leander 2010). One of the major restrictions of the mesopsammic habitat is the limited space available, which is reflected in conspicuous similarities in body plans among interstitial molluscs and many other metazoa (e.g., vermiform body shape and frequent lack of appendages). Recent molecular phylogenetic analyses reveal part of the similarities in morphology and anatomy as convergent adaptations to the mesopsammon–termed among slugs as the 'meiofaunal syndrome' (Brenzinger et al. 2013a)–and support the hypothesis of several independent evolutionary pathways of heterobranch slugs into the interstitial (Jörger et al. 2010; Schrödl et al. 2011). Pseudovermidae Thiele, 1931 (Nudibranchia, Gastropoda) are one of the best adapted of these meiofaunal lineages in terms of body shape (Swedmark 1964, 1968; Brenzinger et al. 2013a). They have an elongated vermiform body with few short and slender or knob-like reduced cerata and an acorn-shaped head, which lacks oral tentacles and rhinophores. With their worm-like appearance, they were at first assigned to turbellarian flatworms in the end of the 19th century, resembling instead one of the first discovered

members of the molluscan meiofauna (see Kowalevsky 1901). Traditionally, Pseudovermidae are placed among cladobranch Aeolidoidea (e.g., Kowalevsky 1901; Thiele 1931; Bouchet and Rocroi 2005). In the first cladistic analyses on nudibranch relationships, the monophyly of Aeolidoidea was not well supported (Wägele and Willan 2000) and reanalyses of the dataset including additional taxa like *Hancockia* Gosse, 1877 further questioned aeolidoid monophyly (Martin et al. 2009). Molecular phylogenetics have contributed to an ongoing rearrangement in nudibranch systematics (e.g., Pola and Gosliner 2010; Wägele et al. 2014) and in the relationships of several aeolidoidean subclades (see e.g., Carmona et al. 2013). First preliminary data, which included Pseudovermidae into a phylogenetic analysis of aeolidoids based on two mitochondrial markers, recovered them basal to other aeolidoids and dentronotaceans (Alejandrino 2007), but reanalyses with larger taxon sampling on an extended set of molecular markers are overdue to evaluate the systematic relationships of Pseudovermidae.

Besides the likely epibenthic and not strictly interstitial *Embletonia pulchra* (Alder & Hancock, 1844) (Embletoniidae), *Pseudovermis* Pereyaslavtzeva, 1891–the monotypic genus of the family–is exclusively mesopsammic and presents the only known lineage of nudibranch gastropods restricted to the interstitial habitat (Swedmark 1964; Arnaud et al. 1986). These minute 'pseudo-worms' are easy to recognize in the field due to their characteristic body shape, but detailed accounts on their anatomy are nevertheless scarce. Most of the 17 described species are diagnosed only via external morphology and by details of radula and mandibles (see e.g., Fize 1961; Salvini-Plawen and Rao 1973; Hughes 1991); their anatomical diversity is largely unknown. This partially relates to former taxonomic tradition and partially to the rarity of these slugs, which are often only found as singletons and seldomly occur in high densities (see e.g., the ecological account of Poizat (1983)). The lack of comparative microanatomical data currently impedes the placement of Pseudovermidae into a phylogeny of Nudibranchia via cladistic analyses and the lacking knowledge on sister-group relationships evidently complicates interpretations on the evolution of these unique mesopsammic nudibranchs.

Due to the minute body size anatomical investigation via direct dissection is problematic if not impossible and data derived from older e.g., paraffin-based histology do not provide the accuracy needed to resolve all organ systems in full detail (Neusser et al. 2006). In the past years, several studies have demonstrated the power of advanced 3D-reconstructions based on serial semi-thin sections to present the microanatomy of different groups of sea slugs detailed and traceable for future research (see e.g., DaCosta et al. 2007; Martynov and Schrödl 2011). Microanatomical revisions combined with molecular data have raised doubts on the reliability of external morphology as mere character sets for species delineation

especially in mesopsammic taxa, in which the habitat constrains external morphology (Eder et al. 2011; Neusser et al. 2011). Representatives from most meiofaunal slug lineages have been redescribed successfully via 3D-microanatomy (such as Acochlidia (Jörger et al. 2008; Neusser et al. 2009a; Neusser et al. 2009b), Sacoglossa (Rückert et al. 2008), Cephalaspidea (Brenzinger et al. 2013b) or Rhodopemorpha (Brenzinger et al. 2011; Brenzinger et al. 2013a)). These data allow for interpretations on the processes leading to present day morphologies and provide comparative data to discuss the role of certain organ systems for the habitat shifts of sea slugs into the mesopsammon. Progenesis (i.e., accelerated sexual development resulting in the sustaining of morphological features of larvae or juveniles) has been discussed as a principle in the evolution of meiofaunal taxa (Westheide 1987). For example, the aberrant morphology of Acochlidia has been interpreted as paedomorphic (Jörger et al. 2010), such as several anatomical features (e.g., pentaganglionate nervous system) in *Helminthope* (Rhodopemorpha) (Brenzinger et al. 2013a). The role of progenesis versus the development of novel morphological inventions in Pseudovermidae still remains to be investigated.

Direct observations on their biology and ecological interactions within the meiofaunal communities in the marine mesopsammon are difficult due to minute body sizes and the common negative phototaxis of mesopsammic organisms. The scarce knowledge is usually based on incidental observations under laboratory conditions or indirectly inferred from morphological analyses. *Pseudovermis* retained–albeit partially weakly developed–cerata from its suspected aeolidoidean ancestry (Salvini-Plawen and Sterrer 1968). Pseudovermid cerata are reported to contain cnidosacs with cleptocnides (i.e., nematocysts taken up from their prey) (Kowalevsky 1901; Marcus 1953; Challis 1969). The presence of cnidosacs was discussed as a potential apomorphy for Aeolidoidea (Wägele and Willan 2000), but comparative studies showed that they are actually quite heterogenous regarding structure, function, and, potentially, evolution (Martin et al. 2009; Martin et al. 2010). They probably serve as a storage device for nematocysts taken up with the food and may be actively reused for defense (Wägele and Klussmann-Kolb 2005). This unique feature offers indirect information on the biology and on food sources of Pseudovermidae, which can contribute to the understanding of the mesopsammic food web and predator–prey interactions in this ecologically little explored habitat.

The present study provides a first detailed 3D-microanatomical redescription of a member of Pseudovermidae as a first step towards an in-depth revision of this neglected mesopsammic lineage. *Pseudovermis salamandrops* Marcus, 1953 was recollected at its type locality at Ilhabela, São Paulo, Brazil (see Marcus 1953). Currently, it represents the only known *Pseudovermis* species

in the Western Atlantic, avoiding taxonomic problems resulting from conflicting literature data and, therefore, allowing the critical evaluation of the taxonomic characters used for species delineation in Pseudovermidae. Implications of the microanatomy for the biology and evolution of these mesopsammic nudibranchs are discussed.

Material and methods

Sampling and fixation

Four specimens of *Pseudovermis salamandrops* Marcus, 1953 were collected at and near the type locality (Marcus 1953) during the 'Taxonomy and diversity of marine Meiofauna' workshop at the Centro de Biologia Marinha of the University of Sao Paulo, Brazil in October 2012. They were found at two subtidal collecting sites at Itaçucê, São Sebastião (23°49′55.70″S; 045°26′35.72″W; workshop Station 17, at 7 m depth, shell gravel collected by scuba diving by Maikon Di Domenico, Katrine Worsaae and Marco Curini-Galletti) and Parcel do Julião, Ilhabela (23°51′13.26″S; 045°25′2.64″W; Station 22, at 4–5 m depth, shell gravel collected by scuba diving by Gustavo Fonseca and Marco Curini-Galletti). The specimens were extracted from sand samples applying a careful decantation technique in $MgCl_2$-seawater solution as described by Schrödl (2006). Collected specimens were documented alive via light-microscopy (bright-field and differential interference contrast) using a Zeiss Axiophot 2 with mounted Sony HDR-XR250 and Zeiss Discovery V20 with mounted Nikon D5100. Prior to fixation, specimens were relaxed using 7 % $MgCl_2$ solution and subsequently transferred to 96 % ethanol for molecular analyses and trialdehyde for histology.

Microanatomy

One mature specimen fixed for histology was post-fixed in 1 % OsO_4 and embedded in Epon epoxy resin. Heidemarie Gensler (LMU) serially sectioned the specimen at 1.5 µm with a MT7000 ultramicrotome (RMC) using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland). The ribboned sections were stained with methylene blue-azure II according to Richardson et al. (1960). The object slides were scanned with an Olympus® dotSlide microscope (.vsi format) and, subsequently, digital images of each section were recorded with OlyVIA® 2.4 software (Olympus Soft Imaging Solutions GmbH). All photographs (.tif format) were edited (i.e., contrast enhanced, converted to grey scale and reduced in resolution) in Adobe Photoshop®. The 3D-reconstruction of the microanatomy of *P. salamandrops* was performed in AMIRA® 3D-rendering software 5.2. (VSG, Visualization

Sciences Group SAS, United States), largely following the outline described by Ruthensteiner (2008).

Results

External morphology

Body size of extended specimens of *Pseudovermis salamandrops* varied between 2 and 5 mm (Fig. 1a). The adult specimen described herein measured up to 3.5 mm when crawling and 1.8 mm in the contracted and fixed state. The worm-like body is highly flexible and contractible (Fig. 1b). In living specimens the body is translucent whitish, with the light yellowish colored and black spotted digestive gland shining through the epidermis (Fig. 1a). A movie of a living *P. salamandrops* is provided in Additional material 1. The head lacks appendages (i.e., labial tentacles or rhinophores) and is anteriorly broadened and rounded (Fig. 1a, b). Eyes are lacking. The epidermis of the head is densely ciliated. The foot is not clearly separated from the rest of the body but detectable as heavily ciliated gliding sole which extends from the mouth opening nearly all the way to the posterior end of the animal. On histological sections, the gliding sole is bordered by paired rows of single celled glands, which accumulate near the mouth opening (Fig. 2c) and towards the posterior end. Typically, in the first pair of cerata, which is located directly behind the head, both cerata are positioned oppositely, the following cerata are arranged alternately (Fig. 1a, b). In the largest collected specimen (see Fig. 1a, b; Additional material 1), there are eight cerata on the left side of the animal and six on the right side. The sectioned smaller, but also mature specimen, bears four cerata on each side. The cerata are finger-shaped and decrease in size towards the posterior end of the animal (Fig. 1a).

Microanatomy

All major organ systems of *P. salamandrops* were reconstructed based on the histological section series. An overview on the microanatomy and the relative position of the organ systems is given in Fig. 1c.

Nervous system The central nervous system (CNS) is highly concentrated and lies post-pharyngeally (see Fig. 1c). It comprises the paired cerebro-pleuro-visceral ganglia, pedal ganglia and buccal ganglia, the latter having a tiny gastroesophageal ganglion attached dorsally (Fig. 3a, b; for identification of the ganglia, see Discussion). The cerebro-pleuro-visceral ganglia are the largest ganglia (approx. 90 µm) of the CNS and are connected via a thick commissure (Fig. 3a, b), which passes the esophagus dorsally. There is a double connective between the cerebro-pleuro-visceral
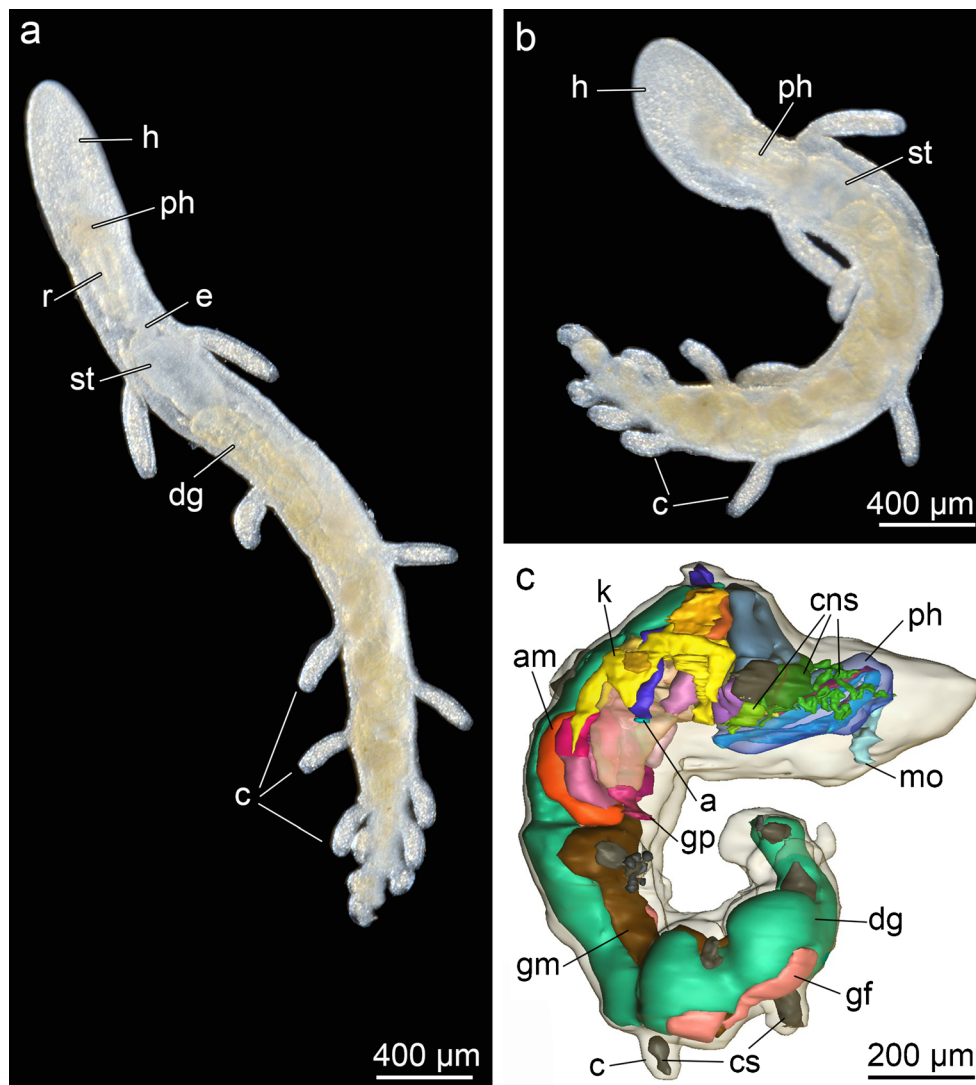
Fig. 1 External morphology and microanatomy. **a**, **b** Light-microscopic image of living specimen. **a** extended while gliding. **b** slightly contracted state. **c** 3D-reconstruction (right view) showing all organ systems. *a* anus; *am* ampulla; *c* ceras; *cns* central nervous system; *cs* cnidosac; *dg* digestive gland; *e* esophagus; *gf* female gonad; *gm* male gonad; *gp* gonopore; *h* head; *k* kidney; *mo* mouth opening; *ph* pharynx; *r* radula; *st* stomach

ganglion and the pedal ganglion, i.e,. the cerebro-pedal and pleuro-pedal connectives (see Fig. 3c). The pedal ganglion is located ventrally and slightly posteriorly of the cerebro-pleuro-visceral ganglion. No visceral nerve cord and no eyes were detected. Each of the paired statocysts contains one statolith and is positioned dorsolaterally of the pedal ganglion and innervated via the thin static nerve by the cerebral ganglion. Two major cerebral nerves arise from each cerebral ganglion (one dorsally and one more lateroventrally) and pass anteriorly (Fig. 3a). Each nerve bifurcates directly at its root with the cerebro-pleuro-visceral ganglion (see Fig. 3a, b). These cerebral nerves are partially swollen in their anterior course. The swellings are identified herein as 'accessory ganglia' (Fig. 3a, d). The pedal ganglia (approx. 80 μm) are connected via a strong pedal commissure and a second thin commissure, herein identified as parapedal commissure

(Fig. 3a, e). Three pedal nerves were detected emerging from each pedal ganglion, which all lead ventrally (Fig. 3c). The small buccal ganglia (approx. 25 μm) (Fig. 3e, f) are located postero-ventrally to the cerebro-pleuro-visceral ganglia and antero-dorsally to the pedal ganglia (Fig. 3a, b). The cerebro-buccal connective (Fig. 3c, e) is comparably thin and slender. A thin buccal nerve innervates the pharynx. A tiny gastroesophageal ganglion (approx. 15 μm) (Fig. 3a, f) is attached dorsally to each buccal ganglion.

Digestive system (including cnidosacs) The mouth opening is located ventrally in the mid-head region (see Figs. 1c and 2a). The digestive tract comprises the short oral tube, the muscular pharynx, which contains the mandibles and the radula, the short esophagus, the spacious stomach, the well-developed digestive gland and intestine (see Fig. 2a). The transition of the oral tube
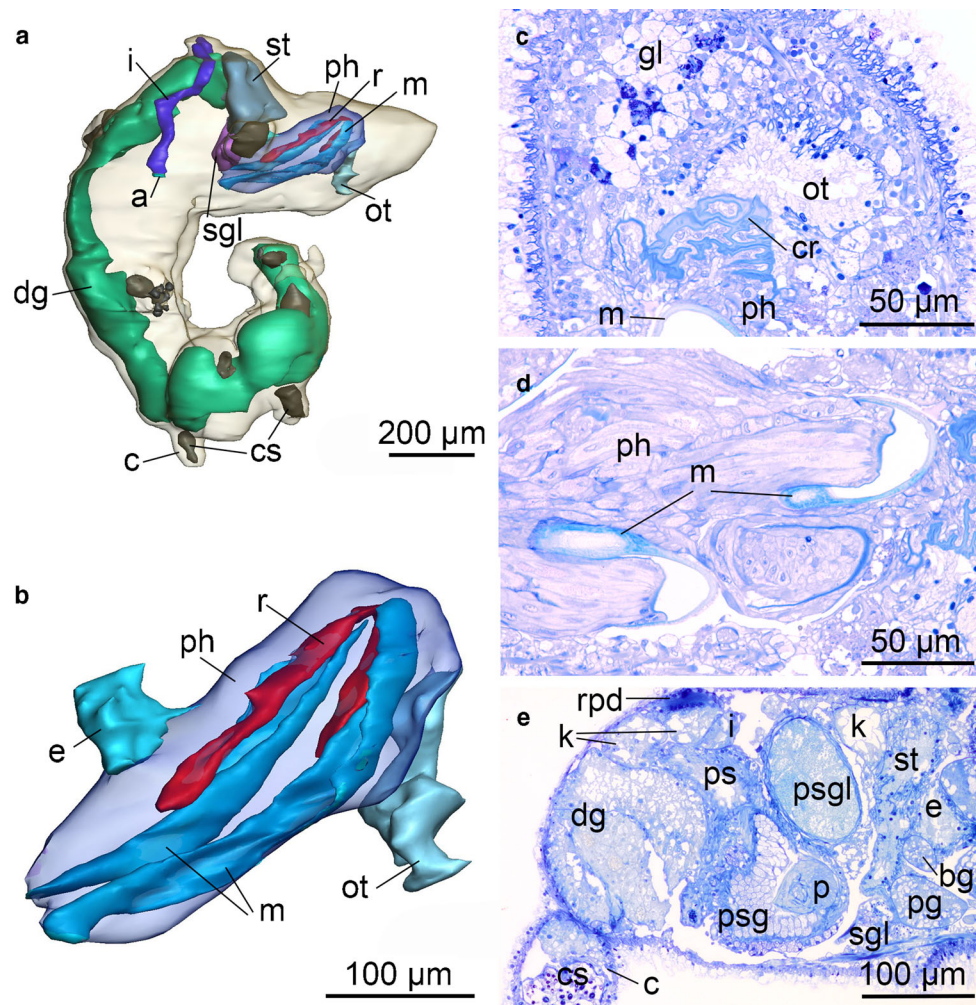
Fig. 2 Digestive system. **a**, **b** 3D-reconstructions (right view), **c–e** Histological longitudinal sections. **a** Position of the digestive system within the body. **b** Pharynx with mandibles and radula. **c** Cuticular ring anterior to the pharynx. **d** Mandibles within the pharynx. **e** Stomach and digestive gland. *a*, anus; *bg* buccal ganglion; *c*, ceras; *cr* cuticular ring; *cs*, cnidosac; *dg*, digestive gland; *e* esophagus; *gl* gland cells in the anterior foot region; *i*, intestine; *k* kidney; *m* mandible; *ot*, oral tube; *p* penis; *pg* pedal ganglion; *psgl* penial sheath gland; *ph*, pharynx; *ps* penial sheath; *psg* glandular part of penial sheath; *r*, radula; *rpd* renopericardioduct; *sgl*, salivary gland; *st*, stomach

to the pharynx is marked by a folded cuticular ring (Fig. 2c). The bulbous pharynx contains the two chitinous mandibles (= jaws) and the hook-shaped radula (Fig. 2a, b), with the formula 1.1.1. Light-microscopy of the radula shows a rhachidian tooth with a large and pointed central cusp and 4–5 lateral denticles, which increase in size towards the outer most; the lateral tooth is narrow, slightly curved and pointed (see Additional material 1). The inner borders of the mandibles are thickened (Fig. 2d). The short esophagus with thin epithelium emerges postero-dorsally from the pharynx (Fig. 2b) and opens into the spacious stomach (Fig. 2a), which has a large lumen and the inner side of the epithelium is covered with mucus (Figs. 3c and 4c). A pair of roundish salivary glands lies posterior to the esophagus ventrally to the stomach (Fig. 2a). No salivary ducts were detected. The cells of the salivary glands are vacuolous and contain some dark blue stained droplets (Fig. 2e). On the left lateral dorsal side of the stomach emerges

the intestine (Fig. 2a). Ventrally the stomach widens into the large sac-like, unbranched digestive gland. The digestive gland occupies large parts of the posterior body cavity (Fig. 2a), and it slightly protrudes into six out of eight cerata in the sectioned specimen (see an example in Fig. 6c). The epithelium of the digestive gland is glandular (Figs. 2e and 4f) and bears some vacuoles containing small yellowish light-refracting roundish structures (see Fig. 6d) and especially in the vicinity of the cerata dark blue staining nematocysts (Fig. 6c, d). The intestine emerges from the stomach on the dorsal left side, passes to the right of the animal and then runs ventrally (Fig. 2a). It is a thin tube and its epithelium is heavily ciliated (Figs. 4c and 5b). The anus (Figs. 1c and 2a) opens on the right mid side of the animal to the exterior opposite of the second left ceras.

The first ceras to the right is situated nearby the stomach. The posteriormost ceras on the right is separated from the digestive system by the male part of the gonad. In the remaining cerata the
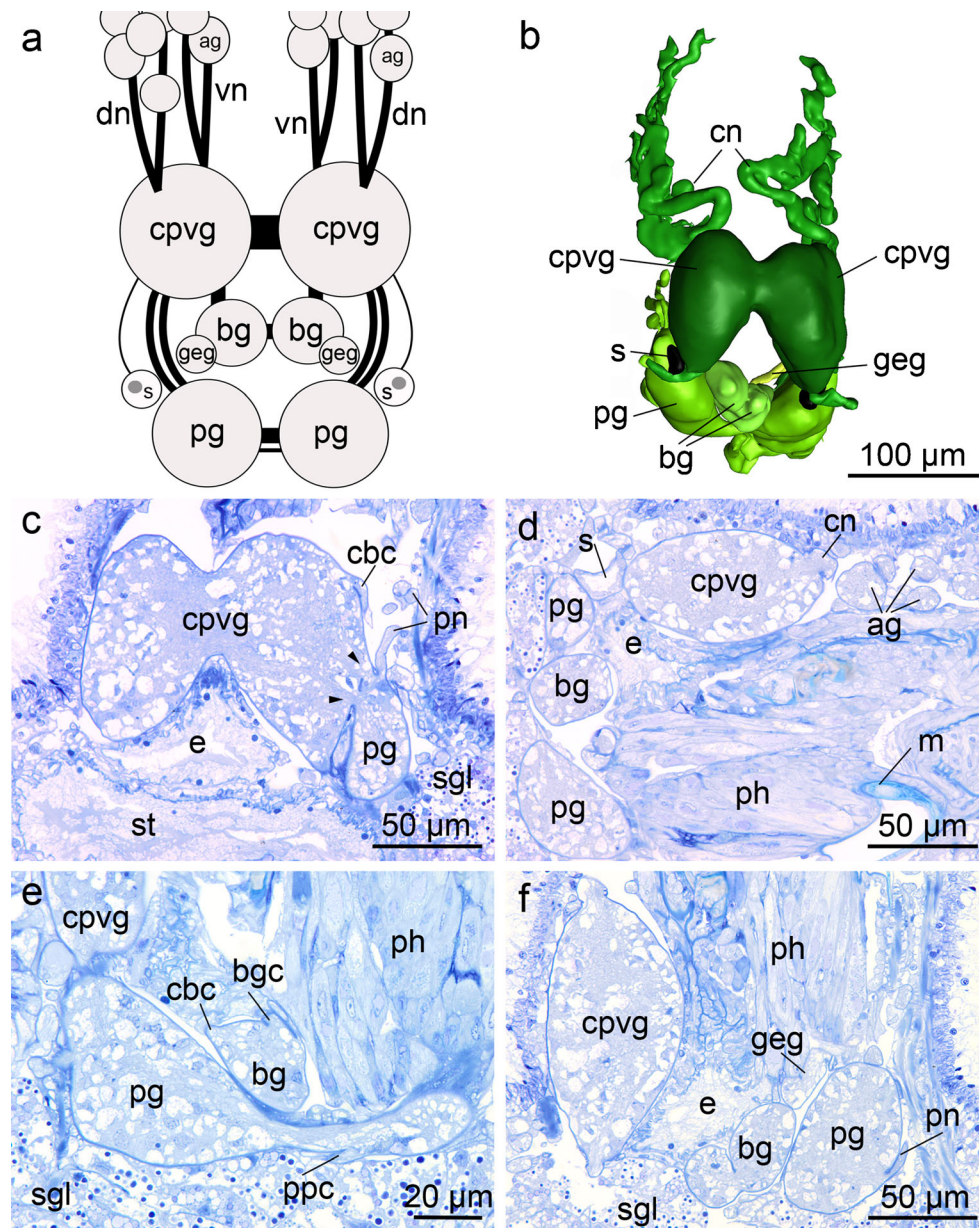
Fig. 3 Central nervous system (CNS). **a** Schematic overview, nerves omitted for clarity except for cerebral nerves (dorsal view, not to scale). **b** 3D-reconstruction (dorsal view, accessory ganglia omitted). **c–f** Histological longitudinal sections (**c**, **e** anterior side of the animal facing upwards. **d**, **f** anterior side facing to the right). **c** Cerebro-pleuro-visceral ganglia with double connective to the pedal ganglion (see *arrow heads*). **d** CNS with accessory ganglia and statocyst. **e** Pedal ganglia with parapedal commissure. **f** Buccal and gastroesophageal ganglia. *ag* accessory ganglion; *bg*, buccal ganglion; *bgc* buccal-gastroesophageal connective; *cbc* cerebro-buccal connective; *cn*, cerebral nerve; *cpvg*, cerebro-pleuro-visceral ganglion; *dn*, dorsal cerebral nerve; *e*, esophagus; *geg*, gastroesophageal ganglion; *m* mandible; *pg*, pedal ganglion; *ph*, pharynx; *pn* pedal nerve; *ppc*, parapedal commissure; *s*, statocyst; *sgl*, salivary gland; *st*, stomach; *vn*, ventral cerebral nerve

digestive gland approaches or slightly protrudes into the lumen of the cerata (Fig. 6c). Each ceras contains a cnidosac (Fig. 6a, c), which is surrounded by a thin epithelium and filled with numerous large cnidophages (Fig. 6c), which each contain several (unfired; i.e., histologically intact) nematocysts (Fig. 6b). No connecting duct between digestive tract and cnidosacs could be detected, nor a pore at the tip of the ceras opening to the exterior. However, some cerata are ruptured at the tip, releasing the cnidophages (Fig. 6d). Using light-microscopy, we observed

at least four distinct undischarged capsule types densely packed within cnidosacs. The largest (11.0–12.5×9.7–11.2 μm) type of nematocyst was almost roundish with a tubule evenly coiled perpendicular to the main axis of the capsule, identified as holotrichous isorhiza (haplonemes). The most common nematocyst (7.1–10.8×4.8–8.4 μm) was oval with a protruding anterior end and a shaft in the center of the capsule (possibly a stenotele). Among these two types, there were a few unidentified bean-shaped capsules (7.5×3.3 μm) and several smaller
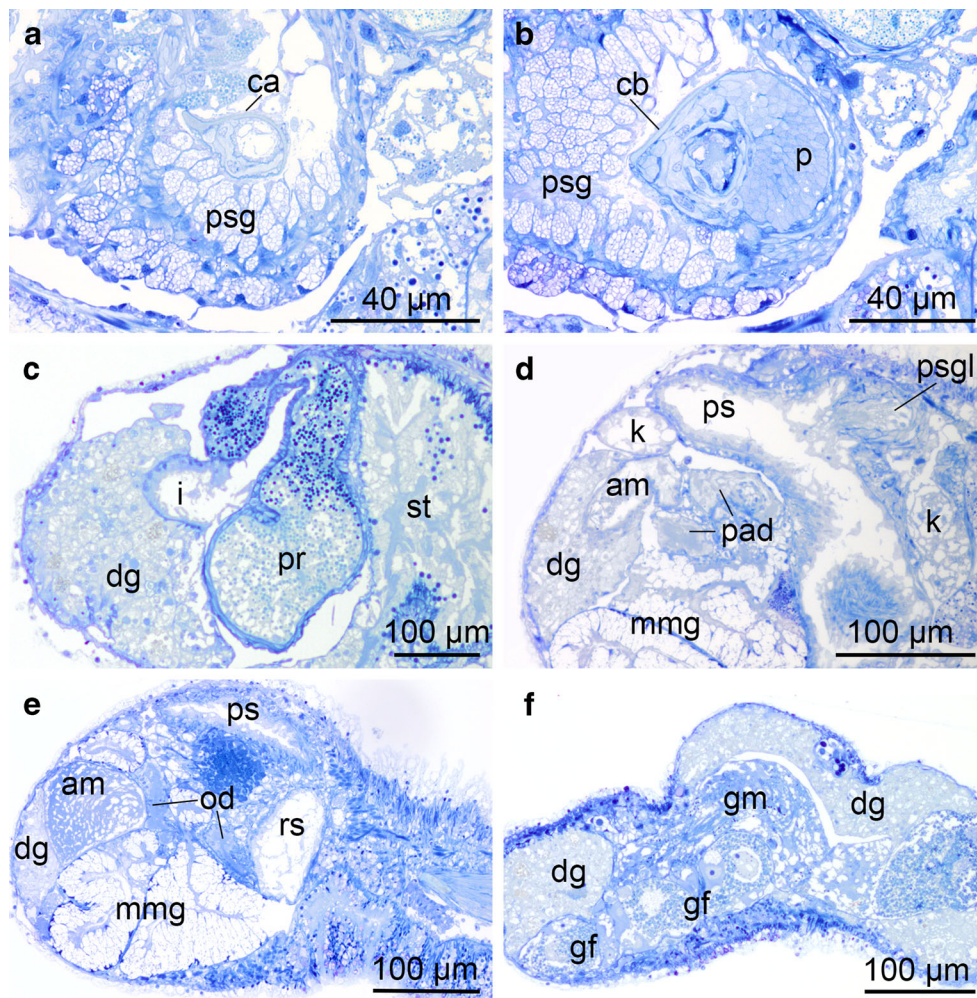
Fig. 4 Histological sections of the genital system. **a** Tip of the penis with cuticular lining. **b** Base of the penis cuticular lining. **c** Prostate. **d** Penial sheath, penial sheath gland and ampulla. **e** Receptaculum seminis and membrane-mucus gland. **f** Gonad with male and female follicle. *am* ampulla; *ca* cuticular lining at the apical tip of the penis; *cb* cuticular lining at the base of the penis; *dg* digestive gland; *gf* female gonad; *gm* male gonad; *i* intestine; *k* kidney; *mmg* mucus-membrane gland; *od* oviduct; *p* penis; *pad* post-ampullary duct; *pr* prostate; *ps* penial sheath; *psg* glandular part of penial sheath; *psgl* penial sheath gland; *rs* receptaculum seminis; *st* stomach

nematocysts (6.2–6.7×4.1–4.7 μm) that looked like desmonemes. We noted a variation in type and size of the encountered nematocysts in a second specimen. The nematocysts observed here were fewer in number and smaller in size (desmonemes: 4.2–4.8×3.0–3.1 μm; stenoteles: 7.7–8.8×4.7–6.9 μm; bean-shaped: 6.6–6.7×3.0–3.2 μm); the large
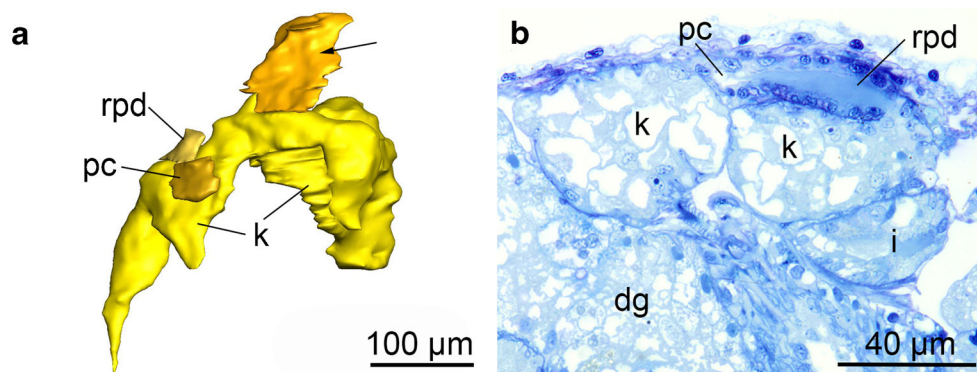

Fig. 5 Excretory and circulatory systems. **a** 3D-reconstruction (dorsolateral right view, *arrow*, unidentified part of the kidney), **b** Histological section. *dg*, digestive gland; *i*, intestine; *k*, kidney; *pc*, pericardium; *rpd*, renopericardioduct
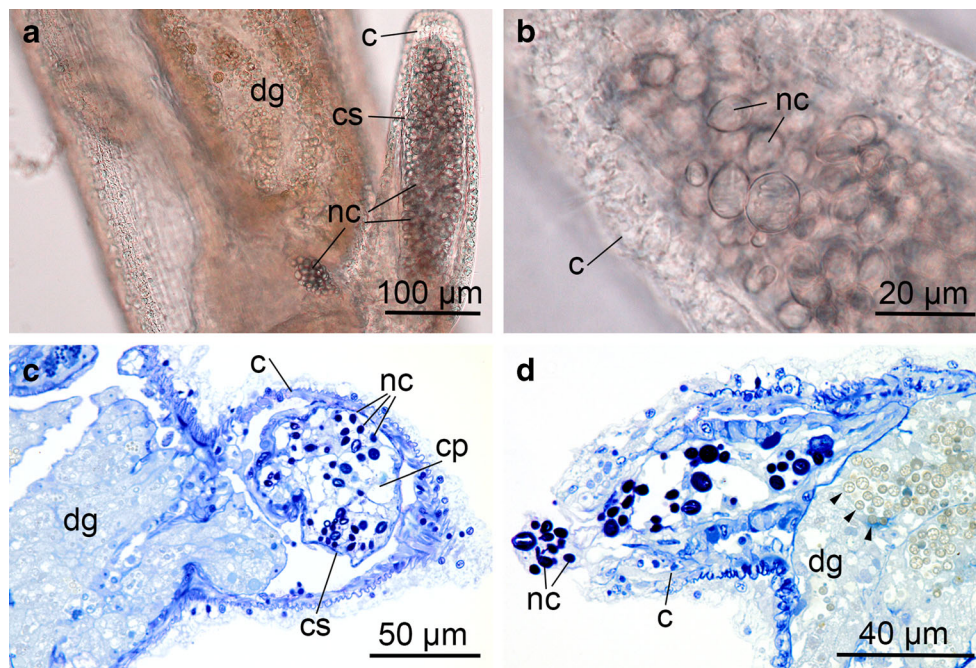
Fig. 6 Cerata with cnidosacs. **a, b** Light-microscopical images. **c, d** Histological sections. **a** Ceras with cnidosac, showing nematocysts also in the stomach. **b** Cnidosac filled with nematocysts. **c** Digestive gland extending into the ceras. **d** Ruptured ceras and cnidosacs (*arrow heads* unidentified yellowish structures in digestive gland cells). *c* ceras; *cp* cnidophage; *cs* cnidosac; *dg* digestive gland; *nc* nematocyst

putative holotrichous isorhiza present in the other specimen was not observed.

Excretory and circulatory systems The excretory and circulatory systems are located at the right side of the middle of the body (Fig. 1c). The kidney is an elongated bent sac (Fig. 5a) with glandular epithelium (Fig. 5b). The small and inconspicuous pericardium is situated dorsally to the kidney and connected to the latter by the short and narrow renopericardioduct (with ciliated epithelium) (Fig. 5a, b). Further comparative and ultrastructural investigation is needed to clarify whether this ciliated renopericardioduct represents a syrinx. The renopericardioduct is located between the kidney and the body wall and bears a small lumen (Fig. 5a, b). No heart could be detected by light-microscopy of living specimens (see Additional material 1) or on the histological sections. No nephroduct could be detected histologically. A thin-walled, yet unidentified structure of unclear origin is situated dorsally to the kidney (see arrow in Fig. 5a).

Reproductive system The reproductive system of *Pseudovermis salamandrops* is hermaphroditic and has a triaulic condition (Fig. 7a). The male part of the genital system comprises the male follicle of the gonad, the vas deferens, the prostate and the penis surrounded by a penial sheath and an attached penial sheath gland. The female part of the genital system comprises the female follicles of the gonad, the oviduct, the receptaculum seminis and two histologically distinct nidamental glands. The male and female parts of the genital system connect close to the hermaphroditic genital opening (see Fig. 7a), which is located on the right side of the body posteriorly to the anus (Fig. 1c).

The hermaphroditic gonad is large and occupies most of the posterior part of the body next to the digestive gland. It is well-separated into the female portion (divided into several female follicles separating oocytes in various stages of maturation (approx. 25 μm)) and the male one on the right side of the animal (one elongated male follicle containing the autosperm) (Figs. 4f and 7a, b). The gonad passes into the large, tubular ampulla, which is packed with randomly orientated sperm (Figs. 4d, e and 7a). The post-ampullary gonoduct splits into the vas deferens and the oviduct (Figs. 4d and 7a, b). The short and narrow vas deferens leads into the tubular prostate (Figs. 4c and 7a, b) from which the small distal vas deferens connects to the muscular penis. The latter is lined on one side with a thin cuticle which slightly extends over the tip of the penis (Figs. 4a, b and 7d). The penis is surrounded by a loose thin-walled penial sheath (Fig. 7d), which is glandular at the base (Fig. 4a, b). A sac-like penial sheath gland (Fig. 7a–c) discharges its content into the distal part of the penial sheath. This conspicuous muscle-lined gland is filled with very fine dark blue droplets (Fig. 4d). The penial sheath joins the hermaphroditic duct just at the common hermaphroditic genital opening (Figs. 1c and 7a). The proximal oviduct is comparably long. The small, sac-like and ciliated receptaculum seminis (Fig. 4e) branches off from the oviduct (Fig. 7c). No allosperm was encountered in the receptaculum seminis. The distal oviduct portion bearing two discernable female
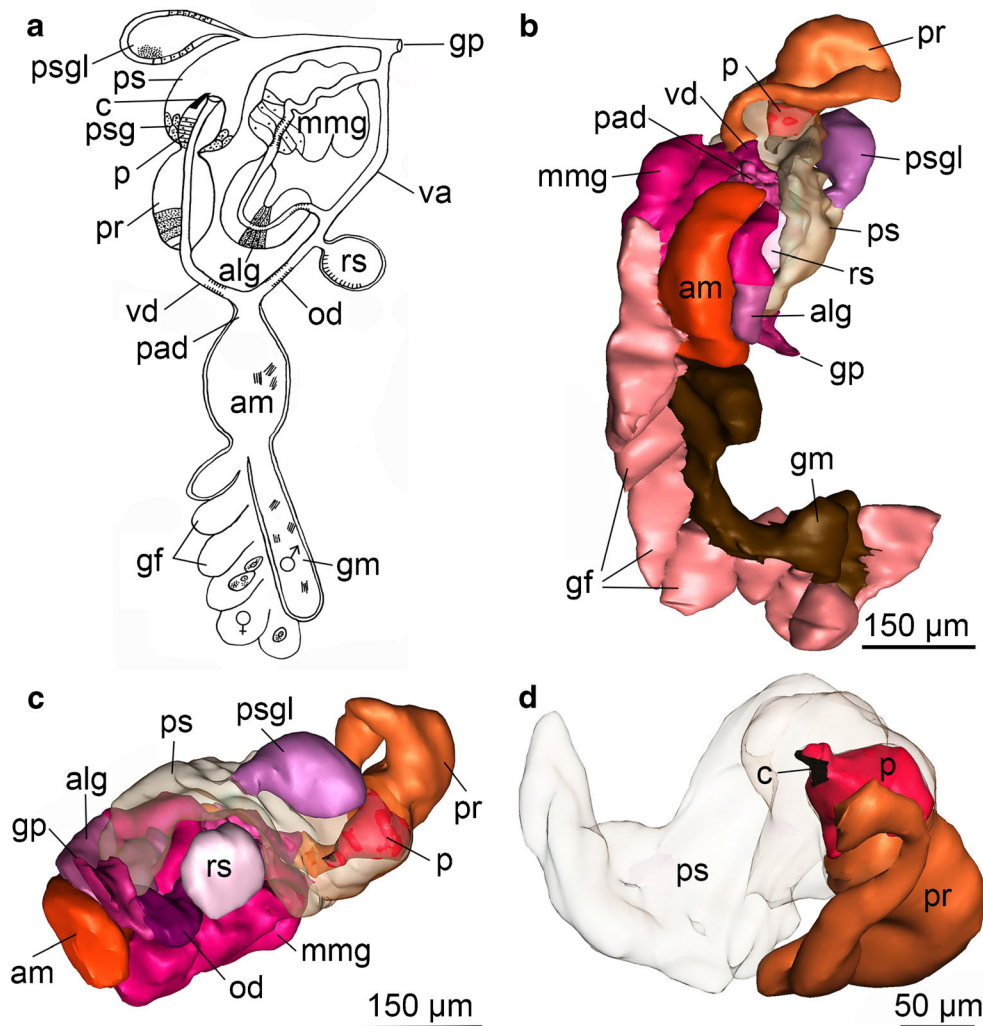
Fig. 7 Genital system. **a** Schematic overview (not to scale), **b–d** 3D-reconstructions. **b** Complete genital system (dorsolateral right view). **c** genital system without gonads (ventral view). **d** Copulatory organ (right view). *alg* albumen gland; *am* ampulla; *c* cuticular lining of penis; *gf* female gonad; *gm* male gonad; *gp* gonopore; *mmg* membrane-mucus gland; *od* oviduct; *p* penis; *pad* post-ampullary duct; *pr* prostate; *ps* penial sheath; *psg* glandular part of penial sheath; *psgl* penial sheath gland; *rs* receptaculum seminis; *va* vagina; *vd* vas deferens

glands, is separated from the vagina, and leads to a partially triaulic condition. The proximal female gland is smaller and tube-like with elongated wedge-shaped cells filled with dark blue staining granules, identified as albumen gland. The distal female gland is larger with large vacuolous cells and identified as membrane-mucus gland (Figs. 4d, e and 7a–c). Oviduct and vagina unite leading to the hermaphroditic gonopore.

Discussion

Species delineation in Pseudovermidae

Traditionally, species delineation in Pseudovermidae relies on characters from the external morphology (e.g., number, size, and relative position of cerata were regarded of considerable importance), as well as radula and mandibles (see Salvini-Plawen and Sterrer 1968; Urgorri et al. 1991). As reported previously and shown in Fig. 1a, b for *Pseudovermis salamandrops*, the body of *Pseudovermis* is contractile and body size can vary in extended vs. contracted states of an individual. The number of cerata varies in the ontogeny of *Pseudovermis* (Fize, 1961; Challis, 1969; Salvini-Plawen, 1991), but additionally also intraspecifically in adult specimens of different sizes (present study and Marcus (1953) on *P. salamandrops*). Prominent, slender and finger-like cerata can be clearly distinguished from non-prominent inconspicuous ones and appropriately serve as major distinguishing feature in keys to Pseudovermidae (see Salvini-Plawen and Sterrer 1968; Urgorri et al. 1991). Slight modifications in their appearance, however, are problematic for taxonomy due to their contractibility and potential artifacts during fixation. This should be taken into account especially when comparing data from living animals to

descriptions based entirely on fixed material. External differences among Pseudovermidae, if consistent and real, can be useful in some instances, but are insufficient for reliable species discrimination in others.

The radula formula of *Pseudovermis* is 1.1.1; the rhachidian tooth is characterized by one large central cusp bordered by 3–6 lateral denticles, which often increase in size towards the outermost (see Additional material 1); only *P. hancocki* Challis, 1969 lacks the prominent central cusp (Challis 1969). The lateral teeth are sharply pointed and can additionally bear 1–2 lateral and pointed denticles (Salvini-Plawen and Sterrer 1968; Challis 1969; Salvini-Plawen 1991; Urgorri et al. 1991). Based on the existing records, interspecific variation is limited to number and relative size of denticles on rhachidian and lateral teeth (Urgorri et al. 1991; table 1 for summary). Several studies report, however, an intraspecific or even intraindividual variation in these characters, i.e., in the number of denticles on the rhachidian tooth (Marcus and Marcus 1955; Salvini-Plawen and Sterrer 1968; Challis 1969; Salvini-Plawen and Rao 1973; Salvini-Plawen 1991) and on the lateral teeth (Hamatani and Nunomura 1973; Urgorri et al. 1991). The variability of mandible morphology (the denticulation of the anterior edge and its enforcement and elongation) is not sufficiently explored as yet to judge the taxonomic value. Challis (1969) criticized rightly the use of divergences in the shape of sectioned mandibles as taxonomic character due to its dependence on the cutting plane (see also Fig. 2d herein). To further evaluate the value of the characters from radulae and mandibles for species delineation in Pseudovermidae 1) reinvestigation via SEM is needed especially for species with light-microscopy based descriptions only and 2) it is inevitable to critically evaluate in a comparative approach whether the intraindividual/intraspecific variation of these characters exceeds the interspecific ones.

Because of the potential limitations of traditional taxonomic characters, described *Pseudovermis* species need to be reexamined. Especially the taxonomic validity of clusters of potentially co-occurring European species only diagnosed by minor differences of the characters listed above need to be critically reinvestigated using additional characters. This concerns mainly the cluster of European species bearing eyes but lacking prominent cerata, i.e., *P. paradoxus* Pereyaslavtzeva, 1891, *P. schulzi* Marcus & Marcus, 1955, *P. kowalevskyi* Salvini-Plawen & Sterrer, 1968, *P. boadeni* Salvini-Plawen & Sterrer, 1968, *P. setensis* Fize, 1961 and *P. thompsoni* Salvini-Plawen, 1991. Microanatomical features could provide additional characters for species delineation. Based on available comparative data, details of the genital system (e.g., presence of penis, cuticular lining of the penis) seem promising but need to be carefully evaluated due to the probably high ontogenetic variability. Putative high intraspecific variation is further complicated by morphological similarity among taxa. Cryptic species were revealed in meiofaunal Acochlidia and

assumed to present the rule rather than the exception across meiofaunal taxa with low dispersal abilities (Neusser et al. 2011; Jörger et al. 2012; Jörger and Schrödl 2013). Accounting for the likeliness for cryptic species also in *Pseudovermis*, an integrative approach (i.e., combining information from all available sources e.g., morphology, microanatomy, molecular data or biogeography) will be needed to reliably address the worldwide diversity of *Pseudovermis* in future research.

Microanatomy of *Pseudovermis salamandrops*

Our redescription of *P. salamandrops* corrects the original description in several aspects, mainly of the reproductive system, and adds some new data especially to the nervous system.

The nervous system of *P. salamandrops* reconstructed herein (see Fig. 3a) confirms with the outline given in the original description (Marcus 1953) and the general bauplan of the central nervous system of other *Pseudovermis* (Marcus and Marcus, 1955; Challis, 1969; Salvini-Plawen, 1991; Huber, 1993). The cerebral ganglion fuses early in development with the pleural ganglion, based on ontogenetic data from aeolidoidean nudibranchs (Tardy 1970, 1974; Carroll and Kempf 1994). The fusion of the two ganglia is still indicated in adult specimens of *P. salamandrops* by the presence of two connectives to the pedal ganglion (i.e., cerebro-pedal and pleuro-pedal, see Fig. 3c). Later in ontogenetic development of aeolidoids these two connectives frequently fuse to one (Tardy 1970), a condition also reported from the nervous system of *P. mortoni* (Challis, 1969). In *P. thompsoni* the two connectives are partially fused (Salvini-Plawen 1991). Later in aeolidoidean ontogeny the ganglia of the visceral nerve cord also fuse with the cerebro-pleural ganglia (Tardy 1970; Kristof and Klussmann-Kolb 2010). Based on the pentaganglionata hypothesis (Haszprunar 1985) and ontogenetic studies on aeolidoidean Nudibranchia, the ganglia of *Pseudovermis* which were traditionally and in related taxa identified as cerebro-pleural ganglia, were termed as cerebro-visceral ganglia by Huber (1993). Offering a term which unifies terminology, we refer to the cerebro-pleural/cerebro-visceral ganglia of *P. salamandrops* as cerebro-pleuro-visceral ganglia. Unfortunately, we were unable to detect the remainder of the visceral cord, probably due to its reduced diameter and lack of ganglia (e.g., Schmekel 1985). Gastroesophageal ganglia are reported herein for the first time in Pseudovermidae, but reinvestigation of the other species is needed because the ganglia might have been overlooked due to their small size. We detected two major bifurcating nerves emerging anteriorly from the cerebral ganglion, one dorsally and one in a more lateroventral position (see Fig. 3a). According to previous studies on the nervous system of *Pseudovermis* (Salvini-Plawen, 1991; Huber, 1993), the dorsal nerve herein likely corresponds to

the rhinophoral nerve (*nervus rhinophoralis*) and the ventral one to the labiotentacular nerve (*nervus labiotentacularis*). This terminology by Huber (1993) was synonymised as N3 and N2 respectively in an attempt to homologize cerebral nerves across 'Opisthobranchia' via axonal projection patterns (Klussmann-Kolb et al. 2013). But homologization especially in aberrant taxa with reduced head appendages still remains problematic. In contrast to the original description, the cerebral nerves of *P. salamandrops* bear accessory ganglia, as described for some other *Pseudovermis* (e.g., *P. paradoxus* as 'foliaceous groups' (Kowalevsky 1901) or *P. thompsoni* (Salvini-Plawen, 1991)) but absent from others (e.g., *P. artabrensis* (Urgorri et al., 1991)). Accessory ganglia (defined as accumulations of nervous tissue surrounded by a thin connective tissue, but lacking the characteristic division between cortex and medulla) are commonly found in mesopsammic interstitial slugs (see e.g., Neusser et al. 2006; Jörger et al. 2008; Brenzinger et al. 2013a) and were argued to present an adaptation to the mesopsammic environment by enhancing the abilities to cope with and process the stimuli of this three-dimensional habitat (Brenzinger et al. 2013a).

Excretory and circulatory systems of *P. salamandrops* are reduced and simplified. The only trace of a circulatory system is a small and inconspicuous pericardium, which is characteristic for *Pseudovermis* (Salvini-Plawen, 1991). We were unable to detect a 'pericardial gland', a very unusual feature described in the original description as a mug-shaped cuticularized part around the distal part of the pericardium (40–50 μm in diameter). An unidentified structure potentially attached to the kidney found in the examined specimen herein requires reexamination when additional material is available to clarify its potential relation to the excretory system. But due to its position and uncuticularized appearance, it certainly does not relate to the 'pericardial gland' described by Marcus (1953). Due to the minute size of the pericardium, electron microscopy is needed to supplement the microanatomy provided and to clarify the site of ultrafiltration respectively to the presence of podocytes in these animals.

The redescription of the digestive system of *P. salamandrops* is in concordance with the original description. From a systematic point of view, the holohepatic, unbranched digestive gland is of special interest as it is highly unusual among Aeolidoidea, which usually bear a cladohepatic digestive gland (Wägele and Willan 2000). The putative plesiomorphic state of the pseudovermid digestive gland might indicate a basal position of Pseudovermidae to remaining Aeolidoidea. The simple arrangement of the digestive gland, however, might also result from progenetic effects or could present a regressive feature affected by miniaturization. The branching of the digestive gland was discussed as an adaptation to increase the surface area for digestion (Wägele and Willan 2000), which might be dispensable in minute sea slugs. We did not observe a connecting duct between the digestive gland and

the cnidosacs, nor a terminal pore for expulsion of the nematocysts (see Fig. 6c, d). Both features are usually present in aeolidoidean cnidosacs (see e.g., Martin et al. 2010) and the pseudovermid state observed herein rather represent the cnidosac-structure of dendronotacean *Hancockia* sp., raising doubts on the systematic placement of Pseudovermidae within Aeolidoidea.

The presented redescription of the genital system of *P. salamandrops* shows several discrepancies to the original description. The latter (see Marcus 1953) lacked a receptaculum seminis and a penial sheath gland, and Marcus described an unusual connection between vas deferens and excretory system as well as an additional connection between the albumen and the membrane-mucus gland. None of the latter two features was detectable in our specimen. A connection between the male gonoduct and the kidney is indeed extraordinary and its functional significance questionable, thus, it probably rather presented an artifact than an ontogenetic stage or paedomorphic feature showing the two systems connected. Challis (1969) regarded the sac-like appendage of the oviduct proximal of the nidamental glands as fertilization chamber. Despite of the absence of allosperm inside, it is identified herein as receptaculum seminis, because of its ciliary epithelium and relative (proximal) position in the system. Moreover, Marcus (1953) described separate, though directly neighboring, male and female genital openings, while in our specimen both ducts clearly unite right before leading into the common hermaphroditic genital opening (see Fig. 7a). Either the close vicinity led to a misinterpretation or the genital openings are variable in different stages of sexual maturity. Other triaulic genital system with separated or common genital opening are described for *P. axi* Marcus & Marcus, 1955, *P. schulzi* and *P. mortoni* (Marcus and Marcus, 1955; Challis, 1969). The partial triauly reported for *Pseudovermis* is not fully consistent, however, with the definition of triauly by Ghiselin (1965), requiring fully separated gonoducts. Triauly is considered the apomorphic stage in Nudibranchia and most cladobranch sea slugs show a diaulic condition (Wägele and Willan 2000). Among Pseudovermidae such a potentially plesiomorphic diaulic genital system is only known for *P. thompsoni* (Salvini-Plawen, 1991). But with the few comparative data available at present, an evolutionary interpretation on the differences in the genital systems of *Pseudovermis* is difficult and more data covering the difference in sexual development are needed. Reinvestigation of the 'cuticular penis' (Marcus 1953) of *P. salamandrops* showed a cuticular lining of one side of the penis, which slightly projects beyond the tip of the penis (see Fig. 7a). This cuticular lining does not resemble a hollow stylet; it is solid and the sperm is discharged via an opening at the tip of the muscular penis. The cuticular lining and spine might serve for anchoring and better fixation of the penis during copulation or alternatively may serve as stabilization of the penis during hypodermic injection. In aphallic species of *Pseudovermis* 2–3 tubular blind

sacs filled with secretory products emerge from the distal part of the male gonoduct (Marcus and Marcus 1955; Salvini-Plawen 1991), these are absent in species with penis which probably transfer sperm via copulation (Challis 1969; present study). These blind sacs might serve as accessory storage containers for spermatophores. Despite the still unclear phylogenetic affinities of *Pseudovermis*, (reciprocal) copulation probably presents the plesiomorphic state in *Pseudovermis* as it is the most common mode of sperm transfer in Nudibranchia (Rivest 1984) and, therefore, likely the outgroup state. Sperm transfer via spermatophores or hypodermic injection in *Pseudovermis* might present an adaptation to the interstitial environment, which is hypothesized to favor fast and imprecise modes of sperm transfer (Jörger et al. 2009).

Adaptations to the mesopsammon

Next to *Helminthope psammobionta* Salvini-Plawen, 1991, members of the *Pseudovermidae* are considered to have the best adapted body plan for life between sand grains among meiofaunal slugs (Swedmark 1968; Brenzinger et al. 2013a). This pseudovermid body plan can be interpreted as a paedomorphic feature based on histological studies by Tardy (1970), who described the ontogeny of the nudibranch *Aeolidiella alderi* (Cocks, 1852) in remarkable detail. An early juvenile stage after settlement and metamorphosis of the veliger larvae closely resembles the external morphology of *Pseudovermis* with an elongated worm-shaped body, few cerata and a simple roundish head prior to the formation of oral tentacles and rhinophores (Tardy 1970). Several nudibranchs are temporarily mesopsammic, i.e., early juvenile stages inhabit the interstitial before changing to a benthic lifestyle (pers.obs.). *Pseudovermis* might have evolved from an dexiarchian ancestor via a progenetic step, as suspected for several other meiofaunal slugs (Jörger et al. 2010; Brenzinger et al. 2013a) and discussed as general driving force of meiofaunal evolution (Westheide 1987). Progenesis might have also affected the excretory and circulatory systems potentially explaining the reduction of the pericardial/nephridial system. The nervous system of *Pseudovermis* is further developed than the early juvenile stage of *Aeolidiella alderi*, which was described by Tardy (1970) to resemble the pseudovermid body plan. In contrast to other meiofaunal slugs (e.g., Rhodopemorpha (Brenzinger et al. 2013a) or Acochlidia (Neusser and Schrödl 2007)) the ganglia of the visceral nerve cord are probably fused with the cerebro-pleural ganglia in *Pseudovermis*, which presents a derived stage based on the pentaganglionate hypothesis (Haszprunar 1985). But Tardy (1970) states that this developmental step in the central nervous system of aeolidoidean nudibranchs proceeds quickly and varies in the ontogenetic development between the different individuals.

Some additional adaptations to life in the interstitial spaces of sand grains are visible in *Pseudovermis*. Only *P. mortoni* has intraepidermal calcareous spicules developed (Challis 1969), which is characteristic for many other meiofaunal slug lineages (Brenzinger et al. 2013a). Based on current knowledge, these spicules present an apomorphy of *P. mortoni* only and do not characterize the genus *Pseudovermis*. The ability to adhere to sand grains is, however, present in most pseudovermids (see e.g., Hamatani and Nunomura 1973; Salvini-Plawen 1991), likely the posterior accumulation of pedal glands are transformed into a posterior adhesive gland.

Biology of *Pseudovermis*

Most aeolidoidean Nudibranchia feed on Cnidaria and are not only able to prevent nematocysts from exploding while feeding but also to store the eaten nematocysts and retain them as functional (see e.g., Grosvenor 1903; Schmekel and Portmann 1982). In general, part of the uptaken nematocysts are digested but a part is transported to the digestive gland, phagocytosed by digestive gland cells (cnidophages) and stored in cnidosacs. The latter are defined as muscular-lined storage organs at the tip of body appendages (cerata) (Martin 2003; Martin et al. 2009). The identification of the stored nematocysts to a cnidarian taxon allows linking the nudibranch to its prey (Grosvenor 1903; Kälker and Schmekel 1976). The food sources of *Pseudovermis* have long been a matter of speculation, especially with regards to rarity of mesopsammic cnidarians for a putative cnidarivore (Challis 1969). Based on the co-occurrence and similarities of the nematocysts Challis (1969) indirectly concluded that *Pseudovermis mortoni* probably feeds on the polyp *Euphysa* sp. (Corymorphidae, Hydrozoa; as *Heterostephanus* sp.) and Boaden (1961) suggested the interstitial medusa *Halammohydra vermiformis* Swedmark & Teissier, 1957 (Halammohydridae, Hydrozoa) as potential food source of *P. boadeni*. The only direct feeding records in Pseudovermidae exist of *P. thompsoni*, which was caught in the act feeding on *Halammohydra schulzei* Remane, 1927 and *H. octopodides* Remane, 1927 (Salvini-Plawen, 1991). Marcus (1953) suggested that the two types of nematocysts she identified in *P. salamandrops* might belong to *Psammohydra nanna* Schulz, 1950 (Boreohydridae, Hydrozoa), but without having found both species to co-occur. In the present study, we also found no directly co-occuring cnidarians, but at two neighboring stations an unidentified species of *Halammohydra* sp. (station 24) and *Pinushydra chiquitita* Bouillon & Grohmann, 1990 (Corymorphidae, Hydrozoa) (station 30) were encountered (AEM, unpublished data). The interstitial hydroid *Nannocoryne mammylia* Bouillon & Grohmann, 1994 (Corynidae, Hydrozoa) was described for the neighboring

state of Rio de Janeiro (Bouillon and Grohmann 1994) and hydroids of small size living attached to sand grains were also found in our sampling region (AEM, own observations). Unfortunately, nematocysts could not be identified with certainty to species level in light-microscopic observations of living *P. salamandrops*. The cnidomes of the specimens of both *Halammohydra* sp. and *Pinushydra chiquitita* found in the region comprise stenoteles and desmonemes as identified in *P. salamandrops*. *Pinushydra chiquitita* has also a bean-shaped microbasic eurytele that might correspond to the bean-shaped capsules found in one of the specimens of *P. salamandrops*. The sizes of these three types of nematocysts (desmonemes, stenoteles, microbasic euryteles) match approximately with the sizes of the ones in the cerata of *P. salamandrops*, but small differences prevent an assignment with certainty. Stenoteles are also present in the Corynidae *Nannocoryne mammylia* (see Bouillon and Grohmann 1994), as well as in other families of the suborders Aplanulata and Capitata, including those that have interstitial representatives or are small and live buried in the sediment, as Acaulidae (*Acaulis*, *Acauloides*), Boreohydridae (*Boreohydra*, *Psammohydra*), Corymorphidae (*Euphysa*, *Pinushydra*), Protohydridae (*Protohydra*), and Tricyclusidae (*Tricyclusa*). Interstitial cnidarians are still poorly known. They can pass undetected due to their minute size, fragility, and difficulty to extract from sediment. There could be, thus, a yet unknown fauna of mesopsammic cnidarians in the studied region that can be used as food source by *P. salamandrops*, which potentially preys on various interstitial cnidarians. The yellowish, light-refractive granules found in some digestive gland cells of *P. salamandrops* and previously reported for *P. mortoni* (Challis, 1969) might relate to zoochlorellae or zooxanthellae ingested together with the cnidarians food and not relate to excretory cells as previously interpreted (Graham 1938; Challis 1969).

The (exclusive) cnidariovory of *Pseudovermis* is contradicted by records on *P. japonicus* Hamatani & Nunomura, 1973 which lacked cnidosacs or traces of nematocysts in its digestive tract (Hamatani and Nunomura 1973). This phenomenon was additionally reported in some individuals of *P. thompsoni* (Salvini-Plawen, 1991) and juveniles of *P. setensis* (Fize, 1961) and is known for other aeolidoidean nudibranchs, which prey on other animals but not cnidarians (Grosvenor 1903). This goes along with various direct observations on different *Pseudovermis* species attacking and feeding on other meiofaunal slugs that are microhedylid Acochlidia (Kowalevsky 1901; Fize 1961; Challis 1969; own observations). Thus, *Pseudovermis* seems to be a facultative cnidarivore (Fize 1961) and, based on present knowledge might not be specialized on one mesopsammic cnidarian, but rather omnivorously takes advantage of different food sources according to availability.

Conclusions

The microanatomy of *Pseudovermis salamandrops* presents a combination of potential paedomorphic features (general body plan) with simplified organ systems on the one hand (circulatory and excretory systems) and organ systems, which show a high anatomical complexity on the other hand (i.e., the triaulic reproductive system). More comparative data on Pseudovermidae and their potential sister groups are needed to evaluate the plesiomorphic versus apomorphic state of the observed characters and to place them in an evolutionary context. Several anatomical details do not support the traditional placement of Pseudovermidae within cladobranch Aeolidoidea (e.g., arrangement of cerata, unbranched, holohepatic digestive gland, cnidosacs morphology). At least some of these characters might, however, be affected by progenesis and/or miniaturization, potentially misleading systematic conclusions. This underscores once more the need for an integrative approach in future research to address the evolution of Pseudovermidae.

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