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A caenogastropod in 3D: microanatomy of the Munich endemic springsnail *Sadleriana bavarica* Boeters, 1989

(Caenogastropoda, Hydrobiidae)

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Comparative 3D-microanatomy reconstructed from histological sections has become a powerful method for investigating anatomical details of small animals, and has been particularly applied to heterobranch gastropods. Here we present first comprehensive 3D data on a member of the Caenogastropoda, the putative sister clade of Heterobranchia. The limnic hydrobiid snail *Sadleriana bavarica* was collected from its type locality, the Brunnbach, a creek within the city limits of Munich, Germany. External features are described and compared with the holotype; *S. bavarica* is supported as a valid species based on morphological evidence, but further morphoanatomical examination of allosperm receptacles and molecular analyses are required. Five specimens were embedded in epoxy resin, sectioned serially, and described histologically. With the software Amira 3D models of all major organ systems were generated. Anatomy is compared to other caenogastropods and heterobranchs; special reference is given to the central nervous system. The arrangement and homology of cerebral nerves are discussed, contrasting earlier opinions on the origin and evolution of tentacular nerves.

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Introduction

The vast majority of gastropods is grouped into the sister taxa Caenogastropoda and Heterobranchia, together forming the taxon Apogastropoda (Salvini-Plawen & Haszprunar 1987); these relationships were supported by multi-locus sequence analyses (Stöger et al. 2013). Caenogastropods include over 120 families, and about 60 % of all living gastropod species (Ponder et al. 2007, Strong 2003). Although mainly marine, caenogastropods have undergone several successful radiations into freshwater, including the species-rich family Hydrobiidae Troschel, 1857 (Strong 2003, Ponder et al. 2007, Strong et al. 2008). Hydrobiidae is considered to be one of the largest gastropod families with more than 400 genera and over 1250 species assigned (Strong et al. 2008, Wilke et al. 2013). Because most hydrobiids are small animals, living in small and isolated freshwater bodies such as artesian springs, caves, or crevices in groundwater, their collection – especially of live specimens – is often difficult (Glöer 2002, Ponder & Clark 1990). Most hydrobiids show a dextrally coiled, smooth, valvati- to slightly turriform shell and a head with a distinct snout and a pair of long and thin tentacles (Glöer 2002), as do other closely

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related taxa. Currently, only few anatomical autapomorphies are described to distinguish Hydrobiidae, e. g. the presence of a closed ventral wall of the female capsule gland (Wilke et al. 2013). Gross-anatomical information is available from dissections of several hydrobiids (e. g. Hershler & Davis 1980, Hershler & Ponder 1998), but detailed histological or microanatomical information on tiny organs and structures is largely lacking.

Shell features have been used as taxonomic characters for central European snails for a long time. However, taken alone they can be misleading and are often not reliable to distinguish on a species level (Haszprunar & Koller 2011). Individuals of a single species, for example, may develop different shells, size and shapes in dependency of their habitats (ecomorphs) (Glöer 2002). Particularly in hydrobiids, the taxonomy is primarily shell-based, and recent molecular studies suggest that their traditional taxonomy is widely deficient (Criscione & Ponder 2013, Wilke et al. 2013).

Hydrobiids of the genus *Sadleriana* Clessin, 1890 are small freshwater snails characterized by a round shell, open umbilicus and reddish-brown operculum (Clessin 1890, Glöer 2002). There are only 8 species assigned to *Sadleriana* currently (Szarowska & Falniowski 2013) and their taxonomy is still unclear. The genus itself was originally described as a subgenus of *Lithoglyphus* C. Pfeiffer, 1828 by Clessin (1890; see Szarowska & Wilke 2004) and considered as a distinct genus by Giusti & Pezzoli (1980). One species, *Sadleriana pannonica* (Frauenfeld, 1865) from the eastern parts of Slovakia and Hungary, recently was transferred to the genus *Bythinella* Moquin-Tandon, 1856 (Szarowska & Wilke 2004, Wilke et al. 2013). The distribution of the genus *Sadleriana* is largely restricted to limestone areas of southern Europe, with species in e. g. Italy, Slovenia and Croatia (Szarowska & Wilke 2004, Szarowska & Falniowski 2013). That is why the affiliation of *S. bavarica* Boeters, 1989, a single species occurring north of the Alps, to this group was in doubt.

Sadleriana bavarica is a highly endemic species that occurs only within the city limits of Munich, Bavaria, where it is found only in a short (approx. 3 kilometer long) cool stream leading from a small groundwater spring into the Isar river (Seidl & Colling 1986, Boeters 1989, Glöer 2002, Szarowska & Wilke 2004). This habitat (the "Brunnbach") is regarded to be an isolated remnant of glacial deposits stemming from the Riß period, and is thus older than comparable habitats in the southern proximity, which were remodelled by moraines during the subsequent Würm glaciation (Sedlmeier & Schwab 2006, LBV 2007). Seidl and Colling (1986) originally determined their specimens to belong to an isolated population of *Sadleriana fluminensis* (Küster, 1853), a species otherwise found south of the Alps in Slovenia. Further analyses by Boeters (1989) revealed morphological differences between *S. bavarica* and *S. fluminensis*, namely conchological aspects and characters of the male and female genital systems.

3D-microanatomy reconstructed from serial histological sections, e. g. using the software Amira, has shown its power for the detailed investigation and visualization of smaller gastropods (Neusser et al. 2006, DaCosta et al. 2007). Generating anatomical 3D models of minute structures from labelled semi-thin histological slices this method shows higher resolution, accuracy and reproducibility compared to the traditional examination via dissection, which is not always efficient and usually destructive to small specimens. To date, 3D-microanatomical studies of apogastropods are mainly restricted to Heterobranchia (e. g. Neusser et al. 2009, Haszprunar et al. 2011, Martynov et al. 2011, Brenzinger et al. 2013a,b, Hawe et al. 2013, Kohnert et al. 2013). Altnöder et al. (2007) examined a juvenile eulimid parasite using Amira software, but, to our knowledge, adult representatives of typical snail-like caenogastropods are still unexplored.

In this paper, we undertook a histological and 3D-microanatomical study of the hydrobiid *S. bavarica* for two reasons: 1) to evaluate and supplement the original description, especially soft-body characteristics, and 2) to investigate the anatomy of a typical caenogastropod as basis for comparison to microanatomically better-known heterobranch taxa. Of special interest is obtaining reliable information on the central nervous system and on cerebral nerves, in order to evaluate contradictory hypotheses by Huber (1993) and Staubach (2008) on the homology and evolution of apogastropod nervous systems.

Materials and methods

Specimens of *Sadleriana bavarica* were collected by hand from stones and submerged driftwood from the Brunnbach, its type locality and single known habitat. Specimens were taken close to the Brunnbach spring (Herzogpark area, 48°9'21"N, 11°36'45"E) and towards St. Emmeram (16.08.2013, 01.03.2014; end of stream: 48°10'45"N, 11°37'34"E) near the river Isar in Munich (31.05.2012, 16.08.2013 and 01.03.2014). Collection permits were obtained as part of the project Barcoding Fauna Bavarica (BFB).

Live specimens and the habitat were documented with digital cameras. For histology, specimens were relaxed with menthol and preserved in formalin (2.5 %). For 3D reconstruction five specimens were embedded in epoxy-blocks (Epon, block-numbers: 8W8, 8W9, 9W0, 9W1, 9W2; collection numbers ZSM Mol 20131107–

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20131111). All but the first block were trimmed and sectioned serially with a microtome using a HistoJumbo diamond knife (Diatome, Biel, Switzerland), following the method described by Neusser et al. (2006) and Ruthensteiner (2008) (8W9, 9W0, 9W1: section thickness 1.5 µm, 9W2: 2.0 µm). The ribbons were collected on microscope slides, stretched by heat, stained with aqueous methylene blue/azure-II (after Richardson et al. 1960), and sealed with araldite resin. The slides of a male specimen (9W2, cross section) and a female specimen (9W0, longitudinal sections) were chosen for 3D-reconstruction. For photography a ProgResC3 ccd camera (Jenoptik, Jena, Germany), mounted on a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany), was used. In case of the male specimen (9W2) the complete animal (5×) and, its anterior body containing the nervous system and buccal organs (20×) were photographed. The female specimen (9W0) was photographed under a 20× lens. Using Adobe Photoshop (Adobe Systems, Mountain View, CA), the photographs were stack processed (resized, changed to greyscale, unsharp-masked) and imported into Amira 5.2 software (Visage Imaging, Berlin, Germany). For the male (9W2) a resolution of 1200×890 pixels (complete animal), and 2080×1542 pixels (nervous system) were used. The female slides (9W0) were imported with a resolution of 2080×1542 pixels. Photographs were aligned and for each image-stack, the organs were labelled manually onto the sections, using different colours. Rendered 3D models of the organ systems were created for both specimens. In specimen 9W2 the complete 3D model is based on 507 photographs, with every second section being used. Details of the nervous system were analysed in a separate aligned stack (307 photos, every section used). The model of 9W0 based on longitudinal sections consists of 373 slides, with every second section used. For labelling, specimens were chosen according to their state of fixation and clarity of organ structures: habitus, digestive system and mantle cavity are reconstructed from the male specimen (9W2). Pericardial complex and central nervous system were reconstructed based on the female specimen (9W0). The reproductive system was reconstructed from a male and an immature female specimen, for that reason, structures like the bursa copulatrix and the receptacula could not be investigated.

Systematics

Caenogastropoda Cox, 1960
Sorbeoconcha Ponder & Lindberg, 1997
Truncatelloidea Gray, 1840
Family Hydrobiidae Stimpson, 1865
Subfamily Belgrandiinae de Stefani, 1877

Genus *Sadleriana* Clessin, 1890

Type species: *Sadleriana fluminensis* (Küster, 1853) (as *Paludina*), by original designation.

Sadleriana bavarica Boeters, 1989

Holotype: HYD1008, stored at the Senckenberg Museum of Natural History, Frankfurt (Figs 1A–C). – Material examined: 5 specimens (ZSM Mol 20131107–2013111), collected at the type locality (Brunnbach, Munich). Embedded in epoxy resin, 4 of them sectioned. Several further specimens (two lots: ZSM Mol 20131112, 20131113).

Natural habitat

In its natural habitat (Fig. 1D), *S. bavarica* is found abundantly in shallow water (10–40 cm), dwelling actively at day on rocks and driftwood that are covered by a biofilm of green algae and diatoms (Fig. 1E). Male and female specimens of *Sadleriana bavarica* occur mixed with individuals of another hydrobiid species (here tentatively identified as *Bythiospeum* sp.). Specimens of *S. bavarica* are most abundant close to the Brunnbach spring.

External morphology

Sadleriana bavarica has a thick and brown shell between 3 to 4 mm in height and 3 to 3.5 mm in width, with 3.5 or 4 rapidly increasing whorls. The umbilicus is open and slotted. In most specimens the shell is covered in green algae (Fig. 1F–I). A corneous, reddish-brown operculum is attached to the foot-end's upper side measuring 1.1 to 1.7 mm (Figs 3B,G; op). The soft body is black, except for the foot sole and the tentacle bases. The body is subdivided into a dextrally coiled visceral sac, which lies inside the shell curling up to the apex, and a broad muscular head-foot. The foot is broadened at the front and contains a well-developed foot gland (propodial gland), which is 0.6 mm width and 0.5 mm height (Figs 2F, 3A,C,G; fg, fgo). The head is clearly differentiated from the foot and possesses a pair of thin tentacles (0.6 mm long), which cannot be retracted. Eyes are located at the outer side of each tentacle base (Figs 2G, 3A,C,D; ey, tn). Here there is also a cushion-like batch of vacuolated cells. The snout is long, flexible, and protrusible (Figs 3C,D; sn).

Mantle cavity and pallial organs

The mantle cavity and pallial organs were reconstructed from the male specimen (9W2). The mantle cavity fills nearly half of the first whorl and then narrows to its rear parts increasingly (Figs 2B, 3D; mc). On its left side, the ctenidium is formed by 8 leaflets each of about 0.2 mm length (Figs 2A,B, 3A–C; ct, ctf). Behind the ctenidium, there is a voluminous semicircular mantle gland (length: 0.75 mm, height: 0.2 mm) (Figs 2B,D, 3A,C,D; mg). Histologically, the gland consists of a strip of particularly tall epidermal

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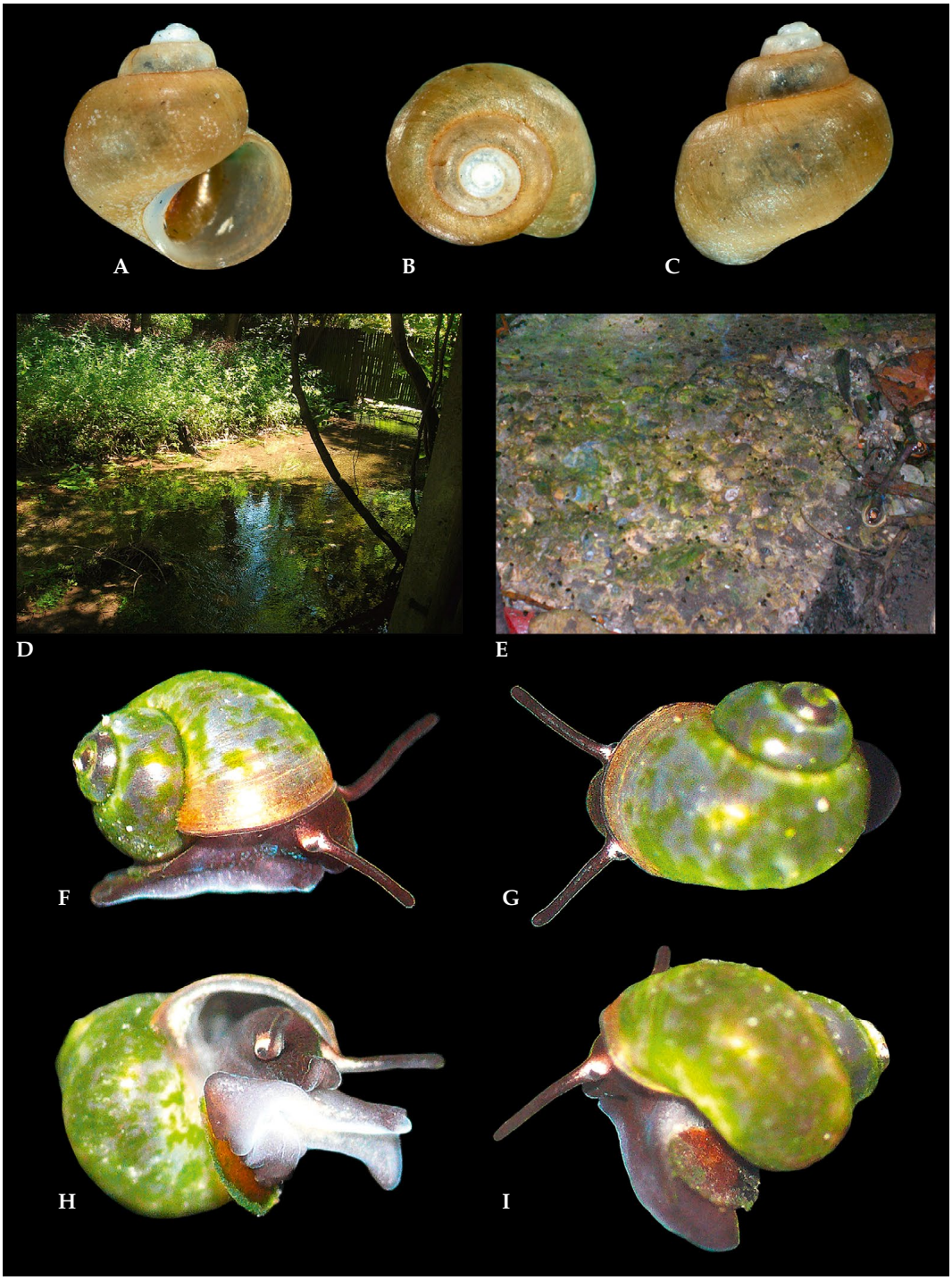

Fig. 1. *Sadleriana bavarica*. External morphology and habitat. **A–C.** Holotype of *Sadleriana bavarica*, Senckenberg Museum Frankfurt (HYD1008). Photos by Sigrid Hof, section malacology, SMF. **D–E.** Natural habitat of *Sadleriana bavarica*, Brunnbach, Munich. **F–I.** Living specimens. Shell diameter approximately 2 mm.

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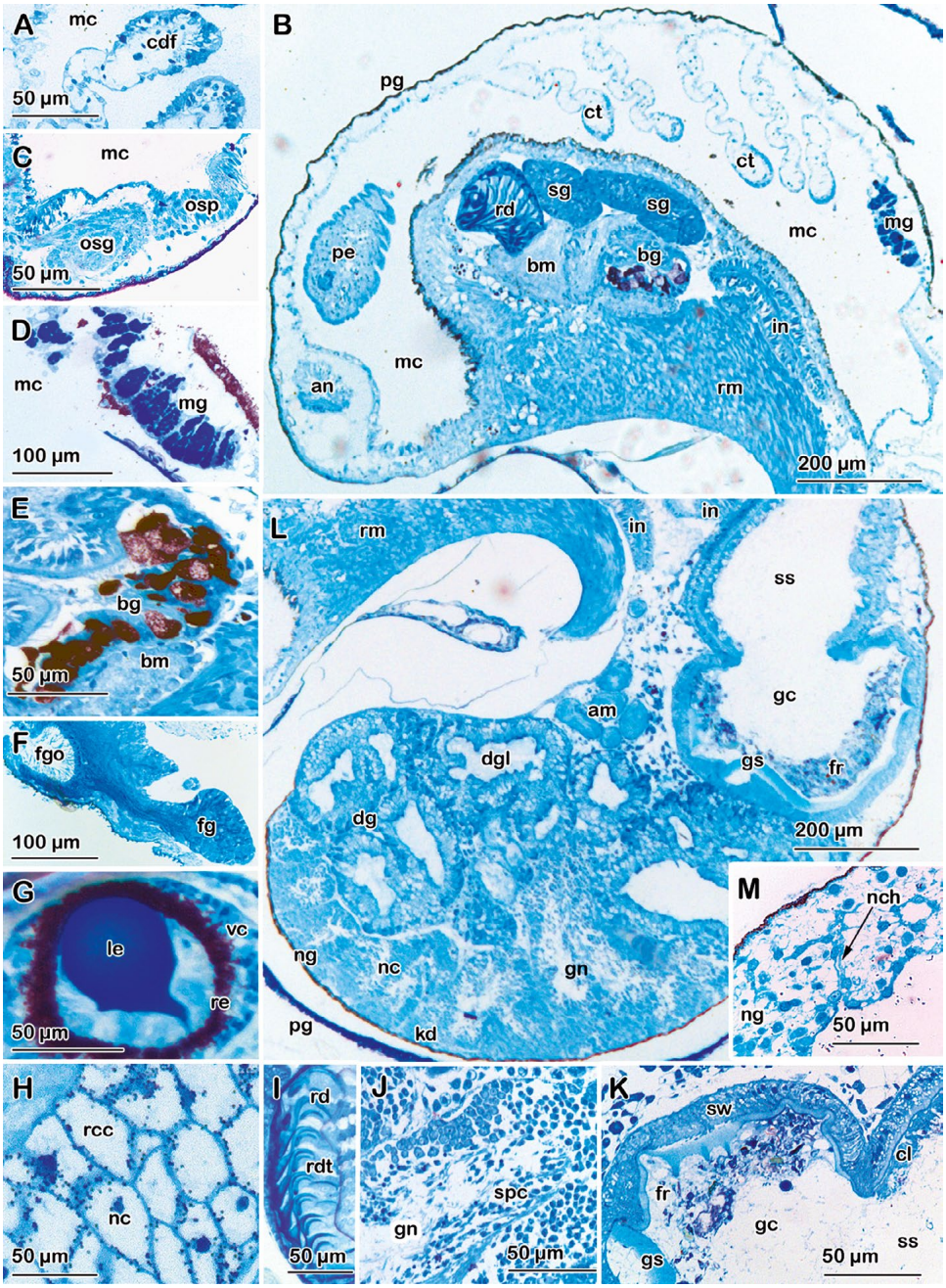

Fig. 2. *Sadleriana bavarica*. Histology, cross sections; overview and details. **A.** Ctenidium. **B.** Anterior part, overview. **C.** Osphradium and connected ganglion. **D.** Mantle gland. **E.** Buccal gland. **F.** Foot gland. **G.** Eye. **H.** Radula cartilage. **I.** Radula. **J.** Gonad. **K.** Stomach. **L.** Posterior overview. **M.** Nephridial gland. Abbreviations: **am**, ampulla; **an**, anus; **bg**, buccal gland; **bm**, buccal mass; **cdf**, ctenidium filament; **cl**, ciliata; **ct**, ctenidium; **dg**, digestive gland; **dgl**, digestive gland lumen; **fg**, foot gland; **fgo**, foot gland opening; **fr**, food remains; **gc**, gastric chamber; **gn**, gonad; **gs**, gastric shield; **in**, intestine; **kd**, kidney; **le**, lens; **mc**, mantle cavity; **mg**, mantle gland; **nc**, nucleus; **nch**, nephridial channel; **ng**, nephridial gland; **osp**, osphradium; **osg**, osphradial ganglion; **pe**, penis; **pg**, pigments; **rcc**, radula cartilage cell; **rdt**, radula tooth; **sg**, salivary gland; **ss**, style sac; **vc**, visual cells.

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cells that are stained in a very dark blue (Fig. 2B,D). Two distinct body openings lead into the mantle cavity's right part: the anus opens into the right corner of the mantle cavity (Figs 2B, 3A; an); in males, the genital opening is located on the right side of the head-foot. The osphradium is a crescent-shaped, ciliated groove with tall yet narrow epithelial cells situated on the anterior left side of the mantle cavity roof, anterior to the gill; there is an oval ganglion just below the epithelium (Fig. 2C; osp and osg). The pericardial complex is located posteriorly, dorsally and to the left.

Circulatory and excretory systems

The pericardial complex comprises the main organs of the circulatory and excretory systems and is located at the posterior left of the mantle cavity, near the mantle gland (Fig. 3E,F). The kidney measures 1.8 mm in length and 0.43 mm in width. It is characterized by a vacuolated and unstained epithelium (Fig. 2L). Superior to the kidney there is a nephridial 'gland', a mass that contains loosely organized, irregular and unstained cells with darker blue nuclei (Fig. 2M); the part of the kidney in contact with this structure is thin and not vacuolated, forming short, apparently blind-ending ducts that project into the nephridial gland (Fig. 2M; nch and ng). On its posterior side, the kidney is attached to a thin-walled pericardium, which surrounds a two-chambered heart: a thicker-walled ventricle and an auricle (Fig. 3E,F).

Digestive system

The digestive system consists of a short pharynx, followed by the short esophagus leading into the stomach, which is connected to a voluminous digestive gland. The intestine runs from the stomach to the mantle cavity's right side.

The mouth opening lies medially on the tip of the snout and leads into a wide pharynx. The pharynx contains a pair of small chitinous jaws, which are fused dorsally and located just behind the mouth opening, and two pads of epithelial single-celled glands in the posterior lower part. The radula (Figs 2I, 4B) is quite long (about 1 mm) and shaped like a question mark. It extends through much of the snail's headfoot and is equipped with roughly 60 rows of teeth which are stained in a dark blue. The radula is bedded on two lower cartilaginous pillows and is topped by a smaller upper cartilage, all characterized by voluminous unstained cells with big and well-apparent nuclei and minute darker granules (Figs 2H, 4B). Two long (ca. 0.8 mm), tube-like salivary glands lie on the pharynx and open nearby the mouth opening. Histologically, they are glandular with numerous small vesicles and stained blue. Centrally, the salivary glands have a narrow lumen (Fig. 4A,C,E). The pharynx narrows to a ciliated oesophagus leading into the stomach. The stomach wall is muscular and thick (66 µm). The stomach is separated into a smaller, ciliated, upper part (style sac) and a bigger lower bag (gastric chamber) (Fig. 4A,C–E). The latter is equipped with a light blue-stained, angular and cuticular shield that carries a strongly elevated ridge (Figs 2L, 4D; gs). The stomach's interior is voluminous and shows some amorphous remains of food, including abundant shells of diatoms (Fig. 2K,L). Attached to the stomach, the big digestive gland extends as a spiral to the apex. The digestive gland cells are stained bright with large vacuoles; the gland itself has thick walls and a big lumen which distinguish it from the finer structured gonad, with which it is interlaced (Figs 2J, 5A–C). The ciliated intestine leaves the stomach centrally and features a single loop that is about 2.5 mm long and quite thick (0.2 mm). The loop is thick-walled and muscular, and at some parts bulging, with irregular surface, due to food pellets inside. Narrowing slightly, it opens into the right hand side of the mantle cavity (anus) (Figs 3A,B, 5A–C).

Reproductive system

The male reproductive system comprises a gonad, a prostate and a penis (Fig. 5A–C). The gonad is slightly coiled, spacious, overlaying the digestive gland. It is histologically characterized by a finely dotted appearance, and moderately stained (Fig. 2J). Emerging from the gonad, a thin-walled proximal male gonoduct first forms an undulated ampulla (84 µm wide) and then a rather straight vas deferens portion, which leads into the posterior end of prostate. The prostate is kidney-shaped, measuring 0.49 mm in length and 0.23 mm in width (Fig. 5A–C). Its lumen is slightly stained, surrounded by a thick wall of blue stained cells. The distal vas deferens is a long, thin, and darkly staining tube (45 µm thick) opening at the tip of the penis (Fig. 5B). The penis is about 1 mm long, flat and tapering towards its tip. The outer surface is rough and covered by concentric and regular folds (Fig. 5A–C); the distalmost vas deferens is not demarcated externally (see Discussion). The female reproductive system is not described here, as examined individuals were immature, with indistinctly developed reproductive organs.

Central nervous system

The central nervous system consists of paired pedal and cerebropleural ganglia, and a smaller pair of buccal ganglia. The nerve-ring is circumoesopha-

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geal and epiathroid. The visceral loop is short, with three ganglia that are close together (Figs 6A, 7): on the left and right, the respective sub- and supraesophageal ganglia are closely annexed anteriorly to the cerebropleural ganglia. The middle, visceral ganglion is situated slightly to the right. From the supraesophageal ganglion emerges a long connective that runs to the left, where it carries the osphradial ganglion (Figs 6A, 7; but see Discussion). The osphradial ganglion carries two nerves, one of which runs to the osphradium, where it carries another, distal ganglion just below the osphradial epithelium (Fig. 2C). Histologically, the ganglia are characterized by distinct neurons in the periphery and lighter-stained nerve fibers in the centre.

The pedal ganglia are biggest (length: 0.44 mm, width: 0.23 mm) and interconnected by a single, short and thick (80 µm) commissure (Figs 6A–C, 7). Each pedal ganglion bears three nerves, the two thick, anterior ones carry a small ganglion each (ca. 60 µm in diameter; Fig. 7). Attached to the upper, posterior surface of the pedal ganglia are the two statocysts (0.13 mm diameter; Fig. 6B), with a single spherical statolith. The static nerve was not detected. The paired cerebropleural ganglia (length: 0.43 mm, width: 0.16 mm) are connected to each other via a long commissure (connective width: 60 µm), and to the pedal ganglia by two connectives per side, the cerebropedal and pleuropedal connectives (Figs 6, 7). Three nerves emerge from each cerebral ganglion (Figs 6A,C, 7): nerve 1 (N1, 30–35 µm thick) emerges anteroventrally and innervates the sides of the snout, nerve 2 (65 µm thick; a fused N2+N3, see Discussion) innervates the tentacle and the eye after splitting into three branches. The third nerve (N4, 30 µm thick) emerges at the base of N2 and also innervates the snout. Paired buccal ganglia (0.12 mm) are located in front of the cerebral ganglia and are connected to each cerebral ganglion by a single, short connective (Figs 6A,C, 7); each buccal ganglion carries a nerve that runs to the sides of the pharynx (Figs 6A, 7).

Discussion

Remarks on taxonomy

Our specimens are identified as *Sadleriana bavarica* Boeters, 1989, for they were found at the type locality (Brunnbach) in the same habitat, and they agree in external characters. According to the original description, the shell of *Sadleriana bavarica* is pressed conical with 3.5 to 4 whorls and a size from 3.5 to 4 mm in width, with an umbilicus that opens into a crescent-bordered channel, while the operculum is reddish brown. This description agrees with the studied holotype (HYD1008; shell height = 3.4 mm, shell width = 2.8 mm). All those features coincide with the specimen studied herein, which also fit the shell-based diagnosis of the genus *Sadleriana* by Clessin (1890). Boeters' description of *Sadleriana bavarica* also includes some reproductive characters. The smooth, flat and distally rounded appearance of the penis described from dissected specimens by Boeters (see Fig. 5E) differs from our 3D-reconstruction, which shows a tapering penis covered by regular folds (Fig. 5A). These folds could be explained as a contraction artefact during fixation of not optimally relaxed specimens, or may alternatively be a permanent feature. Having regular folds, the reconstructed penis of *Sadleriana bavarica* thus comes closer to the drawing of a *S. fluminensis* penis by Küster (1862) (see Fig. 5D), but still is distinct.

In the case of female specimens, Boeters (1989) differentiated between *Sadleriana bavarica* and its congeners using the shape and length of the bursa copulatrix and the receptaculi. Boeters emphasized the unequal length of the two receptaculi of *S. bavarica*, in contrast to other species where the length of the receptaculi is approximately similar. Receptaculi could not be found in the single immature female specimen that was investigated histologically, so we cannot evaluate the presence and potential variation of such characters in *Sadleriana bavarica*.

Both the somewhat variable penis shape and the difficulties investigating females, however, point to the general taxonomic problem that reproductive characters depend on ontogenetic stages, reproductive condition and preparation or relaxation of the specimens available for study. In order to study the range of morphological variation of putative hydrobiid species, careful micro-dissectings such as performed by Boeters (1989) need be efficiently applied to an adequate number of specimens and stages. Additional histological and 3D microanatomical examination of at least a few representatives is demanding but useful even within a merely taxonomic framework, providing accurate and permanent structural information, and supplementing often ephemeral gross-morphological observations. Once comparative molecular data is available on relevant species, barcoding approaches may be more efficient to identify ontogenetic stages of hydrobiids. For purposes of species discovery, i. e. species delimitation, we think integrative approaches will become more useful (e. g. Jörger et al. 2012), and recently, methods have been developed to use sequences as characters in descriptions of morphologically more or less cryptic species (Jörger & Schrödl 2013) that should be applied to elusive hydrobiids also.

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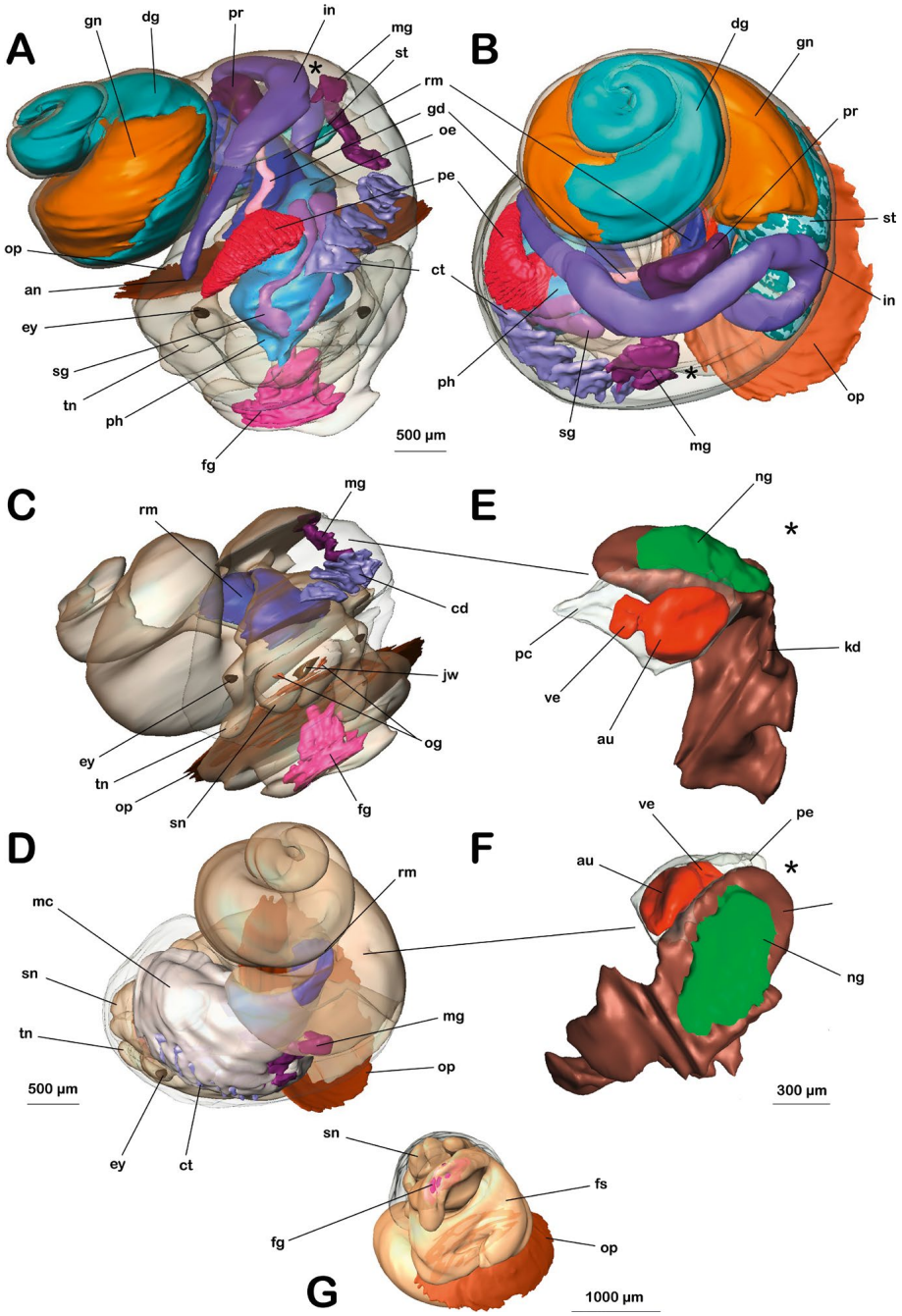

Fig. 3. *Sadleriana bavarica*. Three-dimensional reconstruction of general anatomy and pericardial complex. **A.** Frontal overview. **B.** Dorsal overview. **C.** Mantle cavity; frontal view. **D.** Mantle cavity; dorsal view. **E.** Pericardial complex; lateral view. **F.** Pericardial complex; dorsal view. **G.** Habitus; ventral view. Abbreviations: **an**, anus; **au**, auricle; **ct**, ctenidium; **dg**, digestive gland; **ey**, eye; **fg**, foot gland; **gd**, gonoduct; **gn**, gonad; **in**, intestine; **jw**, jaw; **kd**, kidney; **mg**, mantle gland; **ng**, nephridial gland; **oe**, oesophagus; **og**, oral gland; **op**, operculum; **pc**, pericardium; **pe**, penis; **ph**, pharynx; **pr**, prostate; **rm**, retractor muscle; **sg**, salivary gland; **sn**, snout; **st**, stomach; **tn**, tentacle; **ve**, ventricle.

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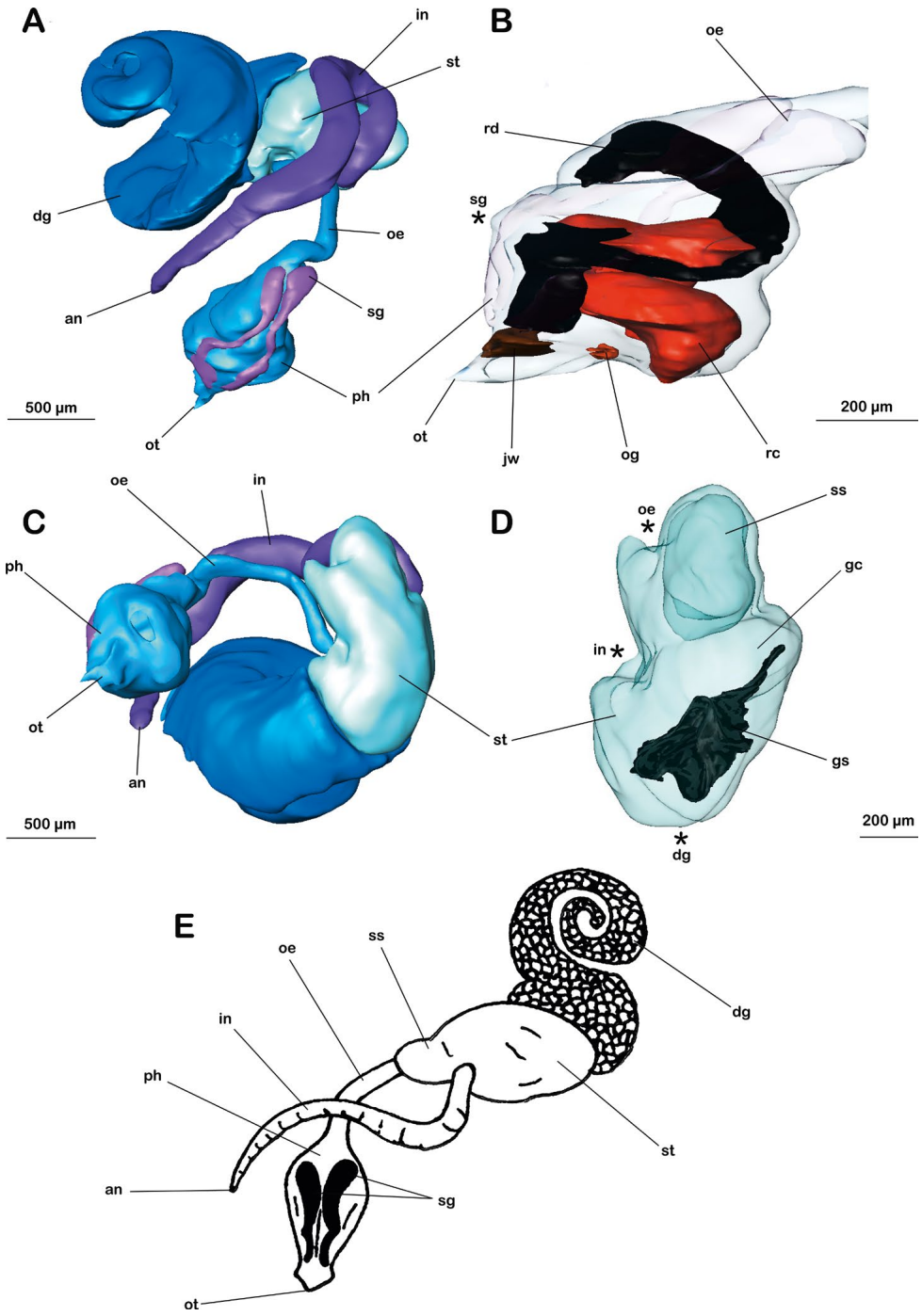

Fig. 4. Three-dimensional reconstruction and schematic overview of *Sadleriana bavarica*. digestive system. **A.** Dorsal overview. **B.** Lateral view of buccal organs in the pharynx. **C.** Ventral overview. **D.** Stomach. **E.** Digestive system schematic view. Abbreviations: **an**, anus; **dg**, digestive gland; **gc**, gastric chamber; **gs**, gastric shield; **in**, intestine; **jw**, jaw; **oe**, oesophagus; **og**, oral gland; **ot**, oral tube; **ph**, pharynx; **rc**, radula cartilage; **rd**, radula; **sg**, salivary gland; **ss**, style sac; **st**, stomach.

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Comparative morphology

This is the first detailed 3D-microanatomical approach on a typical, i.e. snail-like caenogastropod. As shown for Heterobranchia, this approach based on serial semithin sections is powerful for revealing full-scale histological and anatomical data on tissues and organs even from small specimens, and is also suitable for minute caenogastropods such as hydrobiids. However, also macroscopic caenogastropods or other animals, or complex parts thereof, e.g. central nervous systems, can be promising targets of comparative morphological studies using that technique. In the following we use the detailed histological and microanatomical data from *Sadleriana bavarica* as a representative of Caenogastropoda for comparisons with their heterobranch sister group, which has already been much better explored from a microanatomical point of view.

External morphology

The head of *Sadleriana bavarica* bears long and narrow cephalic tentacles, with eyes on swellings at their outer bases, as usual for caenogastropods (Ponder et al. 2007), but similarly present in lower heterobranchs and certain "archaeopulmonate" panpulmonates ('Basommatophora', see e.g. Nordsieck 1993). The wedge-like broadened propodium includes a well-developed propodial gland (Ponder & Lindberg 1997). It was suggested that the propodium, with a distinct anterior pedal gland opening beneath it into a groove, is a synapomorphy of all gastropods other than adult patellogastropods (Ponder & Lindberg 1997). This character has become lost or modified in some 'higher' heterobranchs (Ponder & Lindberg 1997).

Mantle cavity and pallial organs

The mantle cavity of *Sadleriana bavarica* contains a single monopectinate ctenidium on its left side, as usual in caenogastropods (Ponder & Lindberg 1997, Ponder et al. 2007). A central rachis is not present, and the gill leaflets are attached directly to the roof of the mantle cavity. The ctenidia of the herein investigated specimens have 8 lamellas with apical ciliary bands. This contradicts Boeter's (1989) description of 15 lamellas in specimens with the same size, which might be a matter of intraspecific variability.

Behind the ctenidium of *Sadleriana bavarica*, towards the right side, a well-developed gland is located. This 'mantle gland' or 'hypobranchial gland' is usually assumed to be a homologous structure throughout the gastropods (Ponder & Lindberg 1997). Because of the reduction of the mantle cavity organs, in caenogastropods, there is only a single hypobranchial gland left (Ponder 2007). In Heterobranchia, there is higher complexity with several described types of 'mantle cavity glands' that are histologically different (Wägele et al. 2006, Brenzinger et al. 2014). While the largest structure is commonly called hypobranchial gland in the literature, it is not clear whether smaller, additional glandular structures and cell types found in some taxa are derived from the same stock of 'hypobranchial' gland cells.

The anus of *Sadleriana bavarica* opens into the right part of the mantle cavity, as it does in many shelled heterobranchs. According to Ponder and Lindberg (1997), at least in caenogastropods this results from the extension of the rectum along the right side of the mantle cavity, correlated with the loss of the right ctenidium.

The osphradium of *Sadleriana* is relatively large (compared to similarly-sized heterobranchs) but otherwise morphologically similar to that of other caenogastropods (e.g. *Hydrobia ulvae* as examined by Haszprunar 1985a).

Pericardial complex

On the left side of the mantle cavity, *Sadleriana bavarica* possesses a voluminous 'kidney' (Ponder & Lindberg 1997, Ponder 2007). The organ partly extends into the left side of the mantle cavity roof, and is histologically characterized by its typical uniform and vacuolated appearance of the excretory tissue as known for other caenogastropods (Strong 2003).

The kidney is topped by a gland-like structure, with unclear homology and function. Fretter & Graham (1962) described a nephridial 'gland' of caenogastropods as a mass of connective tissue and muscle fibers between kidney and pericardium, lined with ciliated cells and penetrated by haemocoelic spaces. According to Strong (2003), the histology of the nephridial gland is remarkably uniform across the caenogastropods. Ponder & Lindberg (1997) suggest that the caenogastropod 'nephridial' or 'renal' gland is probably not homologous to the nephridial glands of other groups, such as vetigastropod Trochoidea, for there are different regions in which these supposed nephridial glands are located. Similar organs have been reported for some 'lower' heterobranch clades (e.g. Haszprunar 1985c, Hawe et al. 2014). Furthermore, organs of similar histology, comparable position (associated with the circulatory or excretory systems) and thus perhaps comparable function are found in some more derived heterobranchs, e.g. as the so called 'blood gland' of nudibranchs (Schrödl & Wägele 2001, Wägele 2004). The latter was shown to be a loose aggregation of rhogocytes and connective tissue by Fahrner & Haszprunar (2002). Interestingly,

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none of these so-called 'glands' seems to be a real gland composed of a secretory, glandular cell type. In caenogastropods, tissue on top of the kidney forms a distinct, superficially gland-like structure, which sometimes consists of a simple mass or a single series of lamellae (Ponder & Lindberg 1997). This surface enlargement may be the same as seen in *Sadleriana bavarica*, where the kidney epithelium forms crypts extending into the nephridial gland. Whether these organs have homologues throughout Gastropoda remains to be investigated.

The number of auricles varies within the molluscs from one to four and is usually correlated with the number of gills (Ponder & Lindberg 1997). In caenogastropods, there is thus a single auricle combined with a single left ctenidium (Ponder 2007) as observed in *Sadleriana bavarica*. This condition can be regarded as a shared character of Apogastropoda. Variability exists in the orientation of the heart, especially in partially detorted heterobanchs. Blood vessels such as an aorta could not be reconstructed from the present material.

Digestive system

In most gastropods (see Warén & Bouchet 1990), there is a single pair of fused, dorso-lateral jaws, in the buccal cavity. Histologically, minute rod-like structures can be detected at the jaws of *Sadleriana bavarica*. This agrees with the hypothesis of Ponder & Lindberg (1997) based on Starmühlner (1969), that the jaws of caenogastropods in general and in particular those of the sorbeoconchans (Strong 2003) are at least partially composed of rods, which is also the case in opisthobranchs and lower heterobranchs (Mikkelsen 1996, Wägele & Willan 2000).

The radula of *Sadleriana bavarica* shows a plesiomorphically taenioglossate condition; it bends longitudinally and enables the marginal teeth to sweep inwards. This 'flexoglossate' radula was regarded as a synapomorphy of gastropods (Golikov & Starobogatov 1975, Ponder & Lindberg 1997), with patellogastropods establishing a stereoglossate condition independently (see also Stöger et al. 2013).

In the case of *Sadleriana bavarica*, the radula lies on top of two cartilaginous cushions, and is topped by a smaller third cartilage. All cartilages of *Sadleriana* show the typical large cells embedded in extracellular matrix, forming homogeneous units (see Hall 2005, Schmidt-Rhaesa 2007). The unpaired dorsal cartilage is unusual, as most caenogastropods possess only a single pair of buccal cartilages (Strong 2003, Ponder 2007). Structural diversity of cartilages was found to be not directly related to feeding ecology, and therefore regarded as a potentially useful character for phylogenetic study (Golding et al. 2009).

Usually, the salivary glands of gastropods open dorsally into the buccal cavity near the connection to the oesophagus (Ponder & Lindberg 1997). In *Sadleriana bavarica*, they open into the pharynx much more anteriorly, nearby the mouth opening, instead.

The stomach of *S. bavarica* is separated into a smaller (= style sac) and a bigger part (= gastric chamber) that has an angular gastric shield. According to Strong (2003) and Salvini-Plawen (1988), a gastric shield might be the antagonist of a protostyle within the style sac. In *S. bavarica*, there was no protostyle found, which could be a matter of fixation. The stomach of heterobranchs is much simpler (gastric shields and a style sac have been lost except for in the lower heterobranch Valvatoidea; e. g. Hawe et al. 2014), but larger forms have sometimes re-evolved a more complex stomach with at least epithelial folds (e. g. the sea hare *Aplysia*). As usual in gastropods, oesophagus and digestive gland ducts open into the gastric chamber and the intestine leaves the stomach anteriorly (Ponder & Lindberg 1997, Strong 2003), a situation which could be observed in *Sadleriana bavarica* as well.

Reproductive system

As other caenogastropods, *Sadleriana bavarica* is a dioecious species. The condition found in the investigated specimens generally agrees with the usual situation in hydrobiids (Radoman 1983). Both sexes possess a voluminous gonad, which is located at the upper whorls of the shell, and a gonoduct leading to the genital opening at the right hand side of the mantle cavity. Male snails have a rather simple prostate and penis, as described from histological reconstruction herein, while females, according to Boeters (1989), develop a bursa copulatrix and two receptaculi. Histological and functional details of the female reproductive system could not be investigated yet. The number, structure and distribution of allosperm receiving organs within heterobranchs is highly heterogeneous, and their homology is controversely discussed (e. g. Wägele & Willan 2000 versus Valdés et al. 2010). The homology of allosperm receptacles across major apogastropod groups remains to be evaluated based on microanatomical detail.

Central nervous system

The central nervous system, e. g. the arrangement of the circum-oesophageal ganglia, has always been considered important for gastropod classification (e. g. Ponder & Lindberg 1997). The nervous system of *Sadleriana bavarica* is concentrated, with well-defined pairs of cerebropleural and pedal ganglia, as typical in caenogastropods (Ponder et al. 2007).

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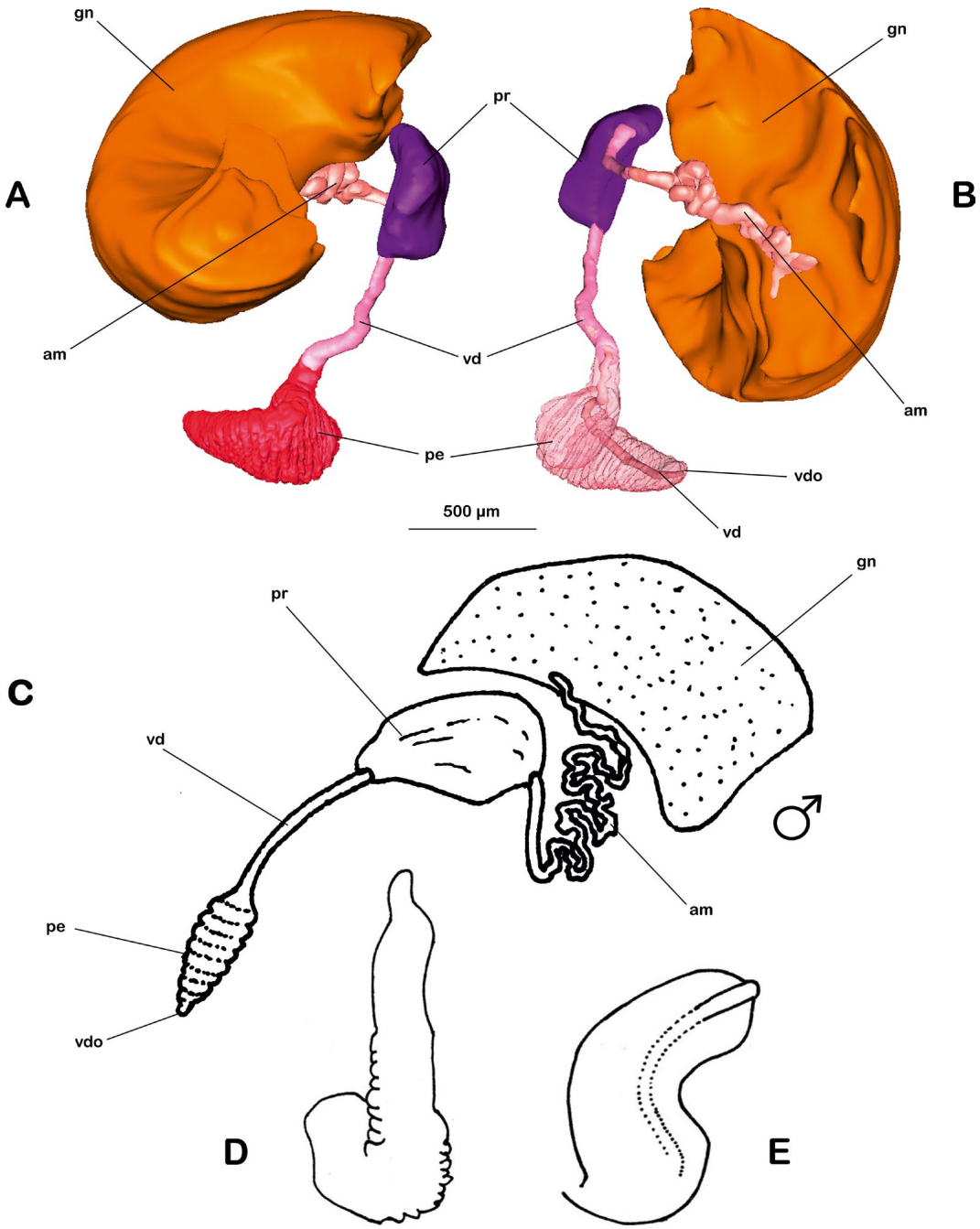

Fig. 5. Three-dimensional reconstruction and schematic overview of the male reproductive system of *Sadleriana bavarica*. **A.** Dorsal view. **B.** Ventral view. **C.** Schematic overview. **D.** Penis of *Sadleriana fluminensis* modified after Radoman (1983). **E.** Penis of *Sadleriana bavarica* modified after Boeters (1989). Abbreviations: **am**, ampulla; **gd**, gonoduct; **gn**, gonad; **pe**, penis; **pr**, prostate; **vd**, vas deferens; **vdo**, vas deferens opening.

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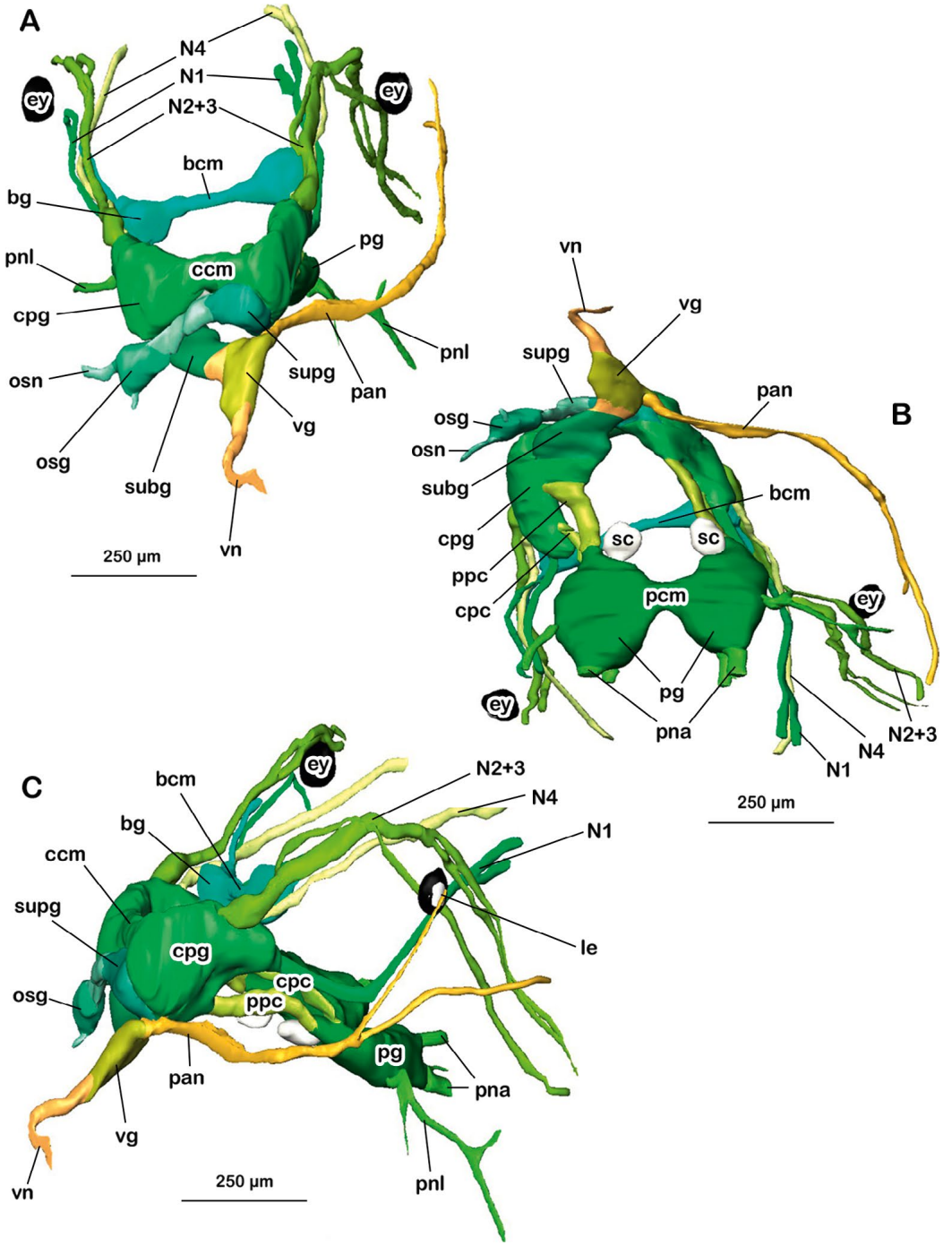

Fig. 6. Three-dimensional reconstruction of *Sadleriana bavarica* central nervous system. **A.** Frontal view. **B.** Posterior view. **C.** Lateral view. Abbreviations: **bcm**, buccal commissure; **bg**, buccal ganglion; **ccm**, cerebral commissure; **cpc**, cerebro-pedal connective; **cpg**, cerebro-pedal ganglion; **le**, lens; **osg**, osphradial ganglion; **osn**, osphradial nerve; **pan**, pallial nerve; **pg**, pedal ganglion; **pna**, anterior pedal nerve; **pnl**, lateral pedal nerve; **subg**, suboesophageal ganglion; **supg**, supraoesophageal ganglion; **sc**, statocyst; **vg**, visceral ganglion; **vn**, visceral nerve.

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We identified the presence of cerebropleural ganglia and the location of the pleural portion of the latter according to the presence of two close-by connectives to the pedal ganglia, a condition also found in numerous Heterobranchia (e. g. Brenzinger et al. 2013a,b). Regarding the pleural ganglia as not externally demarcated, the three ganglia on the visceral nerve loop are the following (from left to right): a suboesophageal ganglion (lying rather ventral), a visceral ganglion (with thick nerve running posteriorly), and a supraesoophageal ganglion (lying more dorsally). The first and last are anteriorly more or less completely fused with the pleural ganglia, and the supraesophageal ganglion additionally carries an osphradial ganglion, which is shifted to the left and is connected to a further, peripheral ganglion. Therefore, two ganglia are associated with the osphradium: one just below the sensory epithelium (osg1 herein), and the aforementioned one connected to the supraesophageal ganglion (osg2).

Save the additional ganglion found underneath the osphradial epithelium, this condition is morphologically essentially identical to what was reported for hydrobiids (e. g. Davis 1967: pl. 20, Hershler & Davis 1980). The ganglion connected to the visceral loop but shifted to the side (left side in caenogastropods, right side in many heterobranchs) is generally referred to as the osphradial ganglion in the literature (e. g. Haszprunar 1985a). Our nomenclature of the visceral loop ganglia is in conflict with descriptions in the literature: Davis (1967) called the single left ganglion (osg2 herein) the supraesophageal ganglion, and the one on the visceral loop 'right pleural ganglion' (supraesophageal ganglion herein). The same nomenclature could be used for *Sadleriana*, where the 'sole' osphradial ganglion would then be the subepithelial one (osg1), but this would be inconsistent with our detection of the right pleural ganglion as part of a fused cerebropleural ganglion. Further research is necessary to homologize heterobranch and caenogastropod ganglia and to correlate terminology.

Interpretational difficulties also pertain to the nerves found herein. From the right side, a single long nerve arises between the supraesophageal and visceral ganglion, which leads into the mantle cavity. The visceral ganglion bears a single nerve that runs into the visceral sac, which is therefore the visceral nerve as in other gastropods; no nerves in the subesophageal ganglion were detected. Anterior accessory ganglia are reported for other hydrobiids ("propodial" ganglia by Hershler & Davis 1980). Accessory ganglia are present on the cerebral nerves of many heterobranchs, but pedal accessory ganglia are rare and were reported only for some lower heterobranch Rhodopemorpha (see Brenzinger et al. 2013b).

Cerebral nerves

In *S. bavarica*, three pairs of cerebral nerves were found. Staubach (2008) and Klussmann-Kolb et al. (2013) comparatively investigated the cerebral nerves of several heterobranchs and the caenogastropod *Littorina* using stain backfilling techniques. We follow the authors' nomenclature of cerebral nerves here, as the nerves in *Sadleriana* fit in size and position with those described for *Littorina littorea* (Linnaeus 1758) and other Apogastropoda: an 'oral' one (N1), which innervates the lip and anterior head region, a very thick tentacle nerve (a fused N2+N3 according to Staubach 2008) with basal thickening (not a ganglion), and the N4 (as 'Nclc') innervating the anterior head region (snout in *Sadleriana*). The thick N2+N3 of *S. bavarica* innervates the head tentacle, i. e. is the "tentacular" or "tentacle" nerve, soon branching and innervating the eye as well and dividing in three further branches. In a phylogenetic context, a bifurcate tentacular nerve as found in *S. bavarica* has been thought to be typical for Apogastropoda (Haszprunar 1985b, 1988a,b, Salvini-Plawen & Haszprunar 1987, Salvini-Plawen 1988, Ponder & Lindberg 1997). However, an analysis by Strong (2003) revealed that there is an unappreciated diversity of branching patterns in Caenogastropoda, including trifid, quadrifid and multifid tentacle nerves in addition to the commonly recognized single and bifid patterns. In a previous study, Huber (1993) examined the nervous systems of many Heterobranchia and some caenogastropods, including the littorinoidean *Skeneopsis planorbis* (Fabricius, O., 1780), and found a somewhat different situation. Huber (1993) described a thick tentacle nerve ('te' therein), but additionally an optic nerve (op) that arises directly beneath from the cerebral ganglion, instead of branching from the tentacle nerve as in *Sadleriana bavarica* and *Littorina littorea*. Furthermore in *Skeneopsis planorbis*, there are four 'labial' nerves (li, ls, le, lm), instead of only two (i. e. N1, N4). The homology of these nerves cannot be clarified definitely here, but there might be some evidence due to the position of origin at the cerebral ganglion described by Huber: Referring to its anterior origin, N1 might refer to Huber's nerve 'le', while N4 might be the homologue of 'ls'. The differences in the setting of cerebral nerves might have observational or systematic reasons. In traditional systematics all three mentioned species belong to the superfamily Littorinoidea, molecular phylogenetic studies are necessary to clarify their actual relationships within Caenogastropoda.

Homology and evolution of the tentacle nerve

There is an important question behind evaluating the homology and distribution of cerebral nerves

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in apogastropods. Huber (1993) considered that a nervus tentacularis, i.e. a tentacle nerve as in *Sadleriana*, is present in Caenogastropoda, lower heterobranchs, Rissoellidae, Pyramidellidae, Pulmonata and Thecosomata, and that this nerve is homologous to the Nclc (N4 herein) of some headshield-bearing opisthobranchs. In contrast, Staubach (2008) proved unambiguously by tracing nervous features by immunocytochemistry, that the caenogastropod tentacle nerve contains all neurons and axons of the separate nerves N2 (= labiotentacular nerve) and N3 (= rhinophoral nerve) of euthyneurans and acteonoids (Staubach 2008: figs 33 and 45), but no parts of the N4 (Nclc). This clearly contradicts Huber's assumption of direct homology of the tentacle nerve with the Nclc, and there is no indication for even just partial homology yet. Since caenogastropods and lower heterobranchs possess a thick tentacle nerve (N2/N3), its presence is likely symplesiomorphic for heterobranchs and caenogastropods. In stark contrast to paradigms conveyed by Huber (1993), this means that ancestral apogastropods already had a rhinophoral nerve, which was basally combined with the labial tentacle nerve. We suggest that head tentacles innervated by both the N2 and N3, such as in *S. bavarica*, serve the special sensory functions of both of these nerves, i.e. short and long distance chemosensory perception.

In the light of modern hypotheses on euthyneuran relationships (e. g. Jörger et al. 2010, Schrödl et al. 2011a,b, Brenzinger et al. 2013b, Wägele et al. 2014), we hypothesize that the ancestrally combined apogastropod N2/N3 became fully separated in an ancestral euthyneuran, or in the common ancestor of euthyneurans and acteonoideans. In groups with 2 pairs of head tentacles (or separated anterior and posterior sensory organs sensu Klussmann-Kolb et al. 2013), e. g. most nudibranchs, the N2 innervates the anterior labial tentacles, while the N3 innervates proper rhinophores, organs specialized to short and long distance olfactory functions, respectively. Several instances of secondary fusion of nerves and/or tentacles may have occurred, e. g. thecosomatous pteropods show a potentially paedomorphic tentacle nerve (Kubilius et al. in press). Within panpulmonates, in most acochlidians the separate N2 and N3 innervate different tentacles, while in several sacoglossans they jointly innervate a single pair of head tentacles (Huber 1993, Jensen et al. in press) or a flattened head without tentacles in *Gascoignella* (Kohnert et al. 2013). Ancestrally separate nerves apparently have fused to form a bifurcating, pseudoancestral tentacular nerve (N2/N3) again in (non-monophyletic) "archaeopulmonate" groups.

Conclusion and outlook

This study presents a full anatomical 3D model of *Sadleriana bavarica*, thus providing an (almost) complete anatomical description of a member of Hydrobiidae, which are so far mainly described on the basis of shells, and the first comprehensive histological and microanatomical 3D data of a typical snail-like caenogastropod.

In order to further elucidate the species status and specific characters of *Sadleriana bavarica*, in particular mature female specimens remain to be analysed anatomically, showing the arrangement, shape and variation of allosperm receptacles. In addition to macropreparations, histological 3D models need to be reconstructed, to evaluate functions and homologies. SEM studies exploring the details and variability of hard structures, such as shell and radula, are warranted as well. In addition, molecular investigations have to be considered. Currently, *Sadleriana bavarica* is regarded as an endemic taxon with divergent, extremely restricted relictual habitat. COI barcodes from *S. bavarica* and Slovenian and Croatian *S. fluminensis* show minimum uncorrected pairwise distances of 2 percent (source: BOLD Barcode of Life Data Systems, and GenBank; Swarowska & Wilke 2004, Swarowska & Falniowski 2013). This currently neither supports nor rejects the morphology-based idea that *Sadleriana bavarica* is specifically distinct rather than an isolated population of *Sadleriana fluminensis*.

Furthermore, the detailed 3D-microanatomical data on *S. bavarica* is a first set of data for minute Caenogastropoda which can be used for comparison to other taxa including their histologically more comprehensively explored sister, the Heterobranchia. The potential of detailed comparative work is exemplified here briefly discussing the homology of nephridial glands or visceral loop ganglia. It is important to note that gross-anatomical dissectings or paraffin-based histology, which were standard methods for examining macroscopic caenogastropods, may not be sufficient to reveal all delicate features. Also, as shown from comparative studies of pulmonates and opisthobranchs (e. g. Jörger et al. 2010, Schrödl et al. 2011a) to discover overlooked aspects and to understand functions, homology and evolution of structures, it is crucial to compare them across conventional taxon borders.

Considering modern tree hypotheses and comparative research on cerebral nerves (Staubach 2008) clearly challenges Huber's (1993) paradigms on the evolution of apogastropod cerebral nerves and tentacles. Of particular interest to us is the homology of caenogastropod and heterobranch tentacular nerves with the labiotententacular plus rhinophoral nerves

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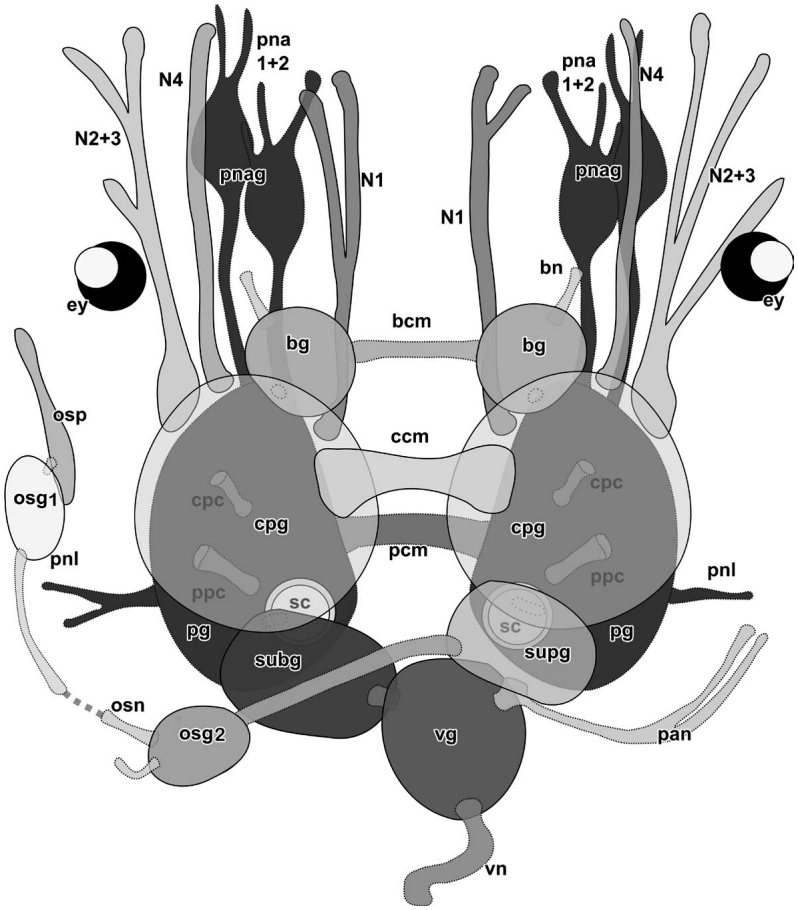

Fig. 7. *Sadleriana bavarica*. Schematic overview of central nervous system. Dorsal view, anterior at top. Lower-lying structures with darker shading. Abbreviations: **bcm**, buccal commissure; **bg**, buccal ganglion; **ccm**, cerebral commissure; **cpc**, cerebro-pedal connective; **cpg**, cerebro-pedal ganglion; **le**, lense; **osg1**, distal osphradial ganglion; **osg2**, proximal osphradial ganglion; **osn**, osphradial nerve; **pan**, pallial nerve; **pg**, pedal ganglion; **pna**, anterior pedal nerve; **pnl**, lateral pedal nerve; **subg**, suboesophageal ganglion; **supg**, supraoesophageal ganglion; **sc**, statocyst; **vg**, visceral ganglion; **vn**, visceral nerve.

of acteonoideans and many euthyneurans (Staubach 2008), implying separation(s) and multiple secondary fusions during heterobranch evolution. Because of their similar posterior position on the head, innervation of the tentacles by the N3 (plus N2), similar association with the eye (nerve), and continuous distribution across the apogastropod tree, we suggest that caenogastropod and lower heterobranch head tentacles are at least partly homologous to opisthobranch rhinophores, to pyramidellid posterior tentacles, to basommatophoran-style pulmonate tentacles, and also to systellommatophoran and stylommatophoran "eye-stalks". The evolution of caenogastropod and heterobranch cerebral nerves and head tentacles appears to be excitingly different from conventional views, and remains to be studied in more anatomical and comparative detail.

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