



3D RECONSTRUCTION OF THE ANATOMY OF THE  
OVOVIVIPAROUS (?) FRESHWATER GASTROPOD *BORYSTHENIA*  
*NATICINA* (MENKE, 1845) (ECTOBRANCHIA: VALVATIDAE)

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ABSTRACT

Because of its apertural shape and an unusual reproductive mode, *Borysthenia naticina* (Menke, 1845) has always held a special position within the Valvatidae. The species is known to be viviparous, whereas all other valvatids produce spawn. Living animals from the Bavarian Danube river provided the opportunity to investigate the structure of the genital system in relation to this special mode of reproduction. We applied 3D surface- and volume-rendering techniques using AMIRA<sup>®</sup>, based on histological semithin slices. We also provide SEM photos of the taenioglossate radula of this species. Contrary to our expectations, there were no special structures (e.g. brood pouch) in the genital system that could be connected with such an accelerated early ontogeny. In general, only minor anatomical differences were found between *Borysthenia naticina* and other valvatid species. The previous reports of ovoviviparity need at least to be modified, because true spawn was observed, although the embryos were close to hatching. The generally accepted taxonomic status of *Borysthenia* as a genus distinct from *Valvata* is doubtful, and the family Valvatidae is in need of a thorough systematic revision.

INTRODUCTION

The concept of a monophyletic group Heterobranchia (consisting mainly of all opisthobranch and pulmonate groups) was first presented by Gray (1840). It was later revived by Haszprunar (1985a), who added several, formerly ‘mesogastropod’ groups like the Valvatoidea (=Ectobranchia), Rissoellidae and Architectonicoidea. The clade Heterobranchia has been confirmed by all subsequent phylogenetic analyses, based on both morphological and molecular data (Salvini-Plawén & Haszprunar, 1987; Haszprunar, 1988b; Bieler, 1992; Ponder & Lindberg, 1997; Colgan *et al.*, 2003; Dayrat & Tillier, 2003; McArthur & Harasewych, 2003; Grande *et al.*, 2004; Aktipis *et al.*, 2008; Wägele *et al.*, 2008; Dinapoli & Klussmann-Kolb, 2010; Jörger *et al.*, 2010).

In contrast, the phylogenetic position and content of the most basal heterobranch clades (often summarized as paraphyletic ‘Allogastropoda’ or ‘Heterostropha’) are still under discussion. Based on molecular analyses some clades (e.g. Pyramidelloidea) have been transferred to the Euthyneura, while others (e.g. Rhodopidae and Acteonoidea) have been added (Göbbeler & Klussmann-Kolb, 2010; Jörger *et al.*, 2010; Wilson, Jörger, & Schrödl, 2010; Dinapoli, Zinssmeister, & Klussmann-kolb, 2011; Schrödl *et al.*, 2011).

Based on detailed anatomical and ontogenetic studies, Rath (1986), Salvini-Plawén & Haszprunar (1987) and Haszprunar (1988b) were the first to consider the freshwater family Valvatidae, formerly classified as ‘primitive mesogastropods’, to be the most basal clade of the Heterobranchia. This view has also been supported by conchological, morphological and spermatological data on marine relatives of this family, namely Cornirostridae, Orbitestellidae, Xylodisculidae and Hyalogyrinidae (e.g. Healy, 1990, 1993; Ponder, 1990a, b; Warén, 1992; Haszprunar *et al.*, 2011) and by molecular data. Indeed, there is considerable agreement (although consensus is not complete, see Dinapoli & Klussmann-Kolb, 2010) that Ectobranchia (which we prefer as a nonranked taxon over Valvatoidea, because of Echinodermata: Asteroidea: Valvatida) represent the first extant offshoot of the heterobranch Gastropoda.

Among the Ectobranchia the Valvatidae Gray (1840) are somewhat set apart by their exclusively freshwater or brackish habitat, in which they have persisted at least since the Jurassic (Bandel, 1991). There are about 60 valid species, currently classified in about 16 supraspecific taxa of uncertain validity, which are spread over the northern hemisphere.

Most available data on the anatomy of the family are restricted to the relatively large (5 mm) and widespread species *Valvata piscinalis* O.F. Müller, 1774 (e.g. Bernard, 1890;

Garnault, 1890; Starmühlner, 1952; Cleland, 1954; Rath, 1986; Falniowski, 1989a, b, 1990). Furrow (1935) provided data on the genital anatomy and gametogenesis of *V. tricarinata* Say, 1817. In her largely unpublished PhD thesis, Rath (1986) investigated the pallial and genital anatomy and histology of several valvatiid species, based on serial sections, and provided detailed data on the ontogeny of *V. piscinalis*. Tair-Abbaci & Garric (2012) provided histological data on the gonad development of *V. piscinalis*. Healy (1990, 1993) studied the sperm ultrastructure of *V. cristata* O.F. Müller, 1774. Falniowski (1989a, b, 1990a) provided data on shell structure, radula, and genital anatomy on several valvatiid species.

The shell of valvatiids is known to be variable (e.g. Steussloff, 1922; Haas, 1938; Hanson, Euliss, & Mushet, 2002; Scholz & Glaubrecht, 2010), but the protoconch and radula are quite uniform throughout the family. Aside from *V. piscinalis*, the anatomy of most valvatiid species has remained remarkably unstudied, except for the recent works by Boeters & Falkner (1998) and Glöer & Pešić (2008, 2012), in which soft-body characters were used for species delimitation.

Recent molecular studies have shown that shell morphology is often entirely inconsistent with molecular data in delimitation of species or (sub)genera (Hauswald, Albrecht, & Wilke, 2008). Thus it is likely that molecular data will significantly change the taxonomy of Valvatiidae.

*Borysthenia naticina* (Menke, 1845) was originally described as *Valvata naticina* from the Danube river near Budapest (Hungary). However, Menke (1845: 129) noted that this species had a different aperture from typical *Valvata*. Crosse (1869: 382–383, pl. 13: Fig. 5) described *V. jelskii* from the Dniestre river near Kiev (Ukraine) and this taxon was made the type species of a new genus, *Jelskia*, by Bourguignat (1877: 92). Because *Jelskia* Bourguignat (1877) is preoccupied by *Jelskia* Taczanowski, 1871 (Chelicerata: Araneae), the generic name was replaced with *Borysthenia* by Lindholm (1913: 167).

Lindholm (1927) reviewed *V. naticina* and its relatives and proposed synonymy between *V. naticina* and *V. jelskii*. Because of differences in shell thickness, aperture morphology, operculum structure and an assumed ovoviviparous mode of reproduction (based on a personal communication from his friend Velitchkowsky, who claimed the presence of well-developed embryos in the genital system), he placed *V. naticina* within *Valvata* in the subgenus *Borysthenia*. Currently (Sitnikova, Starobogatov, & Chernogorenko, 1986; Kantor *et al.*, 2011), four nominal extant species plus two extinct taxa are recognized in the genus *Borysthenia*: *B. naticina* (Menke, 1845), *B. menkeana* (Jelski, 1863), *B. jelskii* (Crosse, 1863), *B. alligans* (Lindholm, 1927) and the fossils *B. goldfussiana* (Wüst, 1901) and *B. intermedia* Kondrashov, 2007. However, only *B. naticina* is unquestionably valid and the whole discussion on species delimitation over decades has been based mainly on the shell, operculum and radula. Recently, Niero & Bodon (2011) presented the first anatomical data on *B. naticina*, but until now a comprehensive investigation of the anatomy and histology of this species has been lacking.

The main goal of this study is to investigate the anatomy of *B. naticina* by three-dimensional reconstruction and histological techniques. The functional and systematic significance of these data are outlined. This study adds to the recent one on the rhipidoglossate Hyalogyrinidae (Haszprunar *et al.*, 2011) and will be continued by a similar study on Xylodisculidae to provide data for a thorough comparative morphological study of all ectobranch gastropods.

## MATERIAL AND METHODS

### *Specimens, fixation and histology*

Three specimens (two adults, one juvenile) of *Borysthenia naticina* were investigated in detail. Details on sampling location,

collector, date of collecting and treatment are summarized in Table 1. The collected specimens were photographed alive and kept alive for some days in native water.

Specimen 1 was relaxed by adding menthol crystals to the water and then preserved in 80% alcohol. Later it was post-fixed in Bouin's fluid [concentrated formalin, saturated picric acid and concentrated acetic acid in the proportions 15:5:1 (Romeis, 1989)], which also dissolved the shell. After dehydration in a graded acetone series the specimen was embedded in paraffin, then serially sectioned at 5- $\mu$ m thickness (MT-7000 ultramicrotome) and stained with Azan reagents (Heidenhain, 1915; Romeis, 1989). Because paraffin sections are compressed or deformed in various ways, 3D reconstruction and volumetric measurements are practically impossible (Ruthensteiner & Heß, 2008).

Specimens 2 and 3 were treated as follows. After initial preservation in 100% alcohol (originally for DNA preservation) they were transferred to 1% ascorbic acid (1–3 d according to shell thickness and size) for decalcification. After dehydration in a graded acetone series, they were transferred to Epon A [Glycider 100 and DDSA (dodecenylsuccinic anhydride) in the proportions 31:50]. Semithin sectioning (1  $\mu$ m) was performed after Ruthensteiner & Heß (2008) with a MT-7000 ultramicrotome. The ribbons of sections were mounted on glass slides and stained with Richardson's reagent (Richardson, Jarrett, & Finke, 1960).

The semithin section series were all photographed with bright-field illumination under a standard light microscope (Olympus CX41, PlanN 4x/0.10 objective, Olympus DP 25 camera) and saved as TIFF files. These digital images were pre-processed in Adobe Photoshop CS4 with automatic white balance; when present, dust around specimens was erased; images were converted from RGB to greyscale (8 bit) and inverted. Using Amira<sup>®</sup> software computer-aided 3D reconstructions (surface models) were generated by individually marking and labelling the different organs on each section, as described by Heß *et al.* (2008). After smoothing, a realistic reconstruction of the specimen could be achieved. In addition, we created interactive 3D models for the pdf publication as described by Ruthensteiner & Heß (2008) and Ruthensteiner, Bauemler, & Barnes (2010).

For histological details, additional photos were taken by bright-field and phase-contrast illumination. Due to greyscale inversion, volume-rendering techniques (cf. Handschuh, Schwaha, & Metscher, 2010; Ruthensteiner *et al.*, 2010) could be applied in order to generate more illuminating pictures.

Specimens 4 and 5 were prepared for SEM investigation following Geiger *et al.* (2007) to obtain SEM photos of the hard structures, i.e. radula, shell and operculum. After critical-point drying the specimens were mounted on aluminium stubs with adhesive carbon tabs, sputtered with gold using a Polaron SEM coating system, and investigated with a LEO 1430 VP SEM at 15 kV.

All prepared specimens have been deposited in the collections of the Zoologische Staatssammlung München (ZSM; Table 1).

## RESULTS

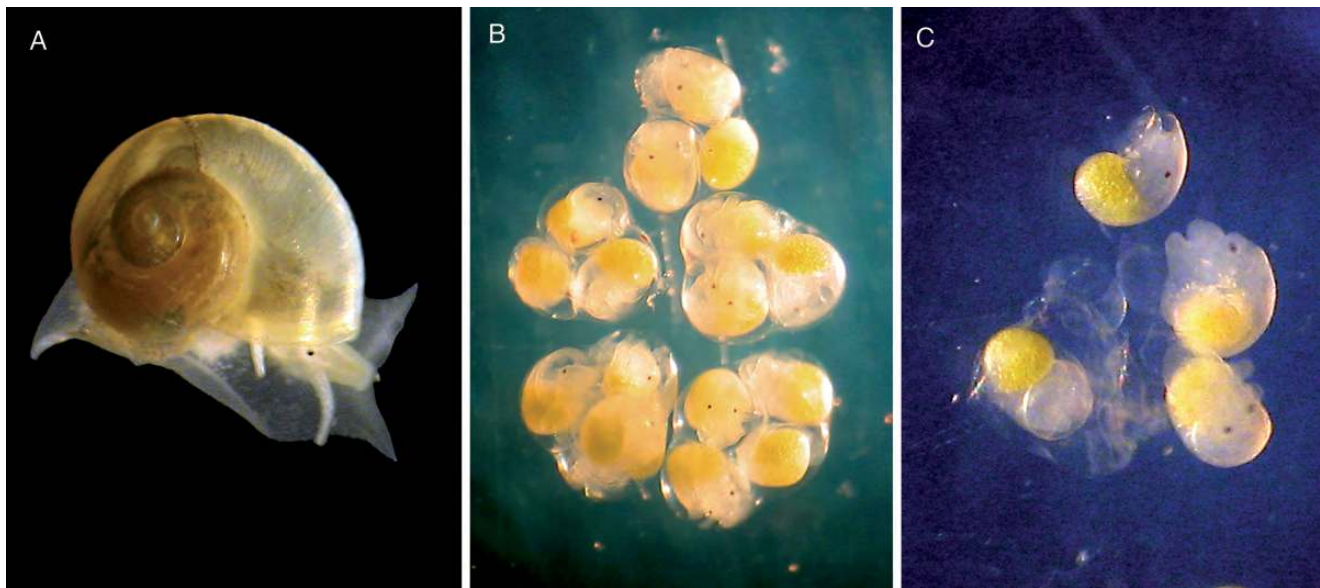
### *Observations of living animals*

The whitish soft body of the living animal (Fig. 1A) has a broad foot with a laterally bifid propodium, a pronounced tapered snout, paired cephalic tentacles with pigmented eye at their inner bases and a densely ciliated pallial tentacle at the right mantle edge. The penis is usually folded back into the mantle cavity.

The specimens (kept alive by M. Colling, see Acknowledgements) deposited several egg capsules, each containing 4–6 well-developed

**Table 1.** Details of all investigated specimens of *Borysthenia naticina*

Specimen number	Sampling date	Location	Collector	Number of sections or SEM image	Slice thickness	Developmental stage	Preservation	Staining	ZSM Mol inventory number
1	04.11.2003	sampling site 31.45/1 Danube	G. Falkner	570	6 $\mu\text{m}$	adult	80% ethanol	Azan	20120138
2	28.10.07/ 08.06.07	lateral basin of Danube, 2 km NNE Aicha, Germany	M. Colling	1225	1.5 $\mu\text{m}$	adult	100% ethanol	Richardson	20120139
3	23.05.2004	lateral basin of Danube, 2 km NNE Aicha, Germany	M. Colling	304	$\sim 1 \mu\text{m}$	juvenile	100% ethanol	Richardson	20120140
4	28.10.07/ 08.06.07	lateral basin of Danube, 2 km NNE Aicha, Germany	M. Colling	radula		adult	100% ethanol		20120139
5	28.10.07/ 08.06.07	lateral basin of Danube, 2 km NNE Aicha, Germany	M. Colling	shell, operculum		adult	100% ethanol		20120139

**Figure 1.** Images of *Borysthenia naticina* in different stages. **A.** Living adult size about 4 mm). **B.** Clutches of egg capsules (each about 1.5 mm) with three embryos each. **C.** Juveniles shortly after hatching (each about 0.5 mm). Photographs by M. Colling (see Acknowledgements).

embryos (diameter about 1.5 mm) (Fig. 1B). The embryos lacked any trace of a velum, but had well-pigmented eyes and a yellow-brown, yolky midgut gland visible through the transparent shell. The embryos hatched three days after spawning (Fig. 1C).

#### Shell and operculum

The whole shell is whitish-yellow; the investigated specimen measured 3.85 mm in diameter. The orthostrophic protoconch measured 460  $\mu\text{m}$  and consists of about 4 whorls with slowly increasing diameter (Fig. 2C, D). The apex is slightly raised and stands about 80  $\mu\text{m}$  above the teloconch. There is a clear borderline between protoconch and teloconch based on different

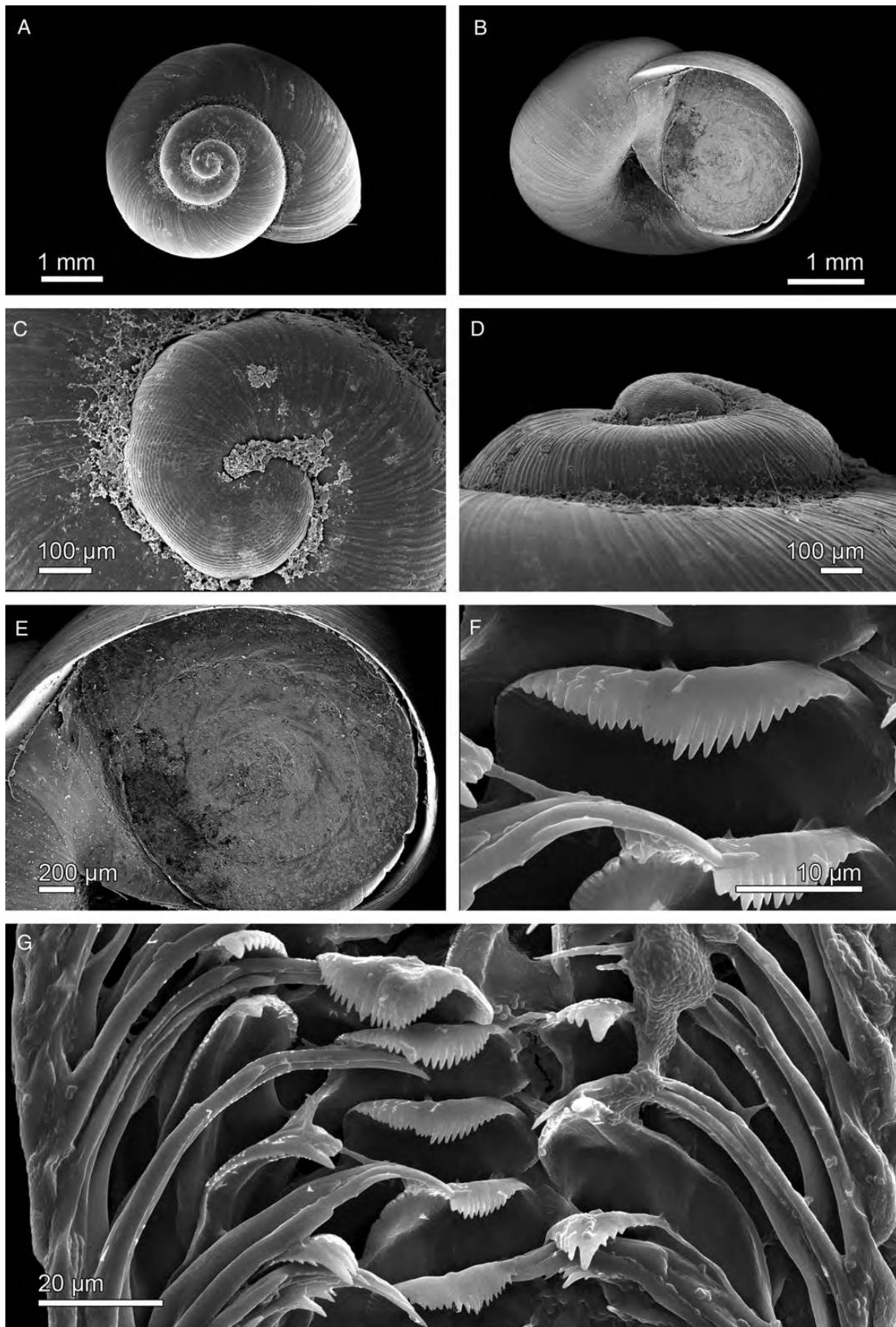
surface patterns (Fig. 2C). In addition to the initial radial growth lines (which continue on the first whorl of the teloconch) the protoconch shows longitudinal lines too, producing a reticulate surface. The orthostrophic teloconch consists of about 3.5 whorls and has a deep umbilicus. The aperture is approximately circular and slightly pointed at the apical side (Fig. 2A, B).

The whitish, slightly oval and spiral operculum is 2.11 mm in diameter and has 5 whorls (Fig. 2B, E).

#### Body morphology and histology

The body is divided in a cephalopodium and a visceropallium. The snout is slightly bifid. Two cephalic tentacles (contracted





**Figure 2.** SEM images of *Borysthenia naticina*. **A.** Complete shell viewed from right side. **B.** Complete shell showing aperture. **C.** Protoconch viewed from above. **D.** Side view of protoconch. **E.** Operculum with five whorls. **F.** Rachidian tooth. **G.** Portion of radula across complete width.

due to fixation) emerge from the head in a dorsomedian position and are sparsely ciliated. A single, densely-ciliated pallial tentacle arises at the junction of the right mantle edge and cephalopodium (Fig. 3G).

The penis is long, situated at the posterior base of the right cephalic tentacle and is reflected backwards into the right side of the mantle cavity (Fig. 3G). All appendages are filled with large blood sinuses and have a thin outer muscular layer.

The propodium is significantly bifid, the foot sole is strongly ciliated and epipodial tentacles are lacking. In the anterior part of the cephalopodium, a single but prominent pedal gland can be found, starting in front of the pedal ganglia and occupying the whole anterior foot in the propodial lobes.

#### Mantle cavity

The structures within the mantle cavity are described in the same order as the circulation of water through the cavity. The mantle cavity reaches deep into the first whorl of the body. The whole epithelium of the mantle roof is underlain by blood sinuses.

At the anterior left side, the inconspicuous osphradium is located in the mantle cavity roof and is supplied by the underlying osphradial ganglion. Next, the gill is situated medio-dorsally in the mantle cavity. It contains two large blood sinuses (the right afferent sinus and the left efferent sinus) and has a variable number of alternating, densely-ciliated lamellae with prominent blood sinuses (Fig. 4A). Bursicles or skeletal rods are absent. The opening of the ureter is situated behind the gill on the left side and of the rectum on the anterior right side. The posterior end of the mantle cavity is occupied by the huge left kidney. A so-called pigmented mantle organ was not found.

#### Heart, circulatory and excretory systems

The monotocardian heart and adjacent kidney are both located in the left mantle roof behind the gill (Fig. 3A). Due to fixation artefacts, a detailed description of auricle and ventricle cannot be provided. From the central part of the pericardium a relatively large (about 230  $\mu\text{m}$  in length), ciliated pericardial duct leads to the single left kidney. The kidney is huge and fills nearly half of the posterior end of the first whorl, being situated between the efferent gill and kidney sinuses, the rectum and the pericardium (Fig. 3A–C). From the pericardial duct it leads first backwards, then makes a narrow loop and leads forwards again. The volume between the two turns is filled with blood in the visceral sinus. The kidney has a uniform, nonciliated epithelium. The most distal part of the kidney functions as a ureter, runs forwards and has a medio-dorsally situated nephropore.

#### Genital tract

The hermaphroditic gland fills the outer parts of the last whorl (Fig. 5A). It is composed of an outer ovarian and an inner testicular portion (Fig. 6A). Within the hermaphroditic gland, eggs of various sizes were present, but eggs were not found in the rest of the female genital system. The eggs have a maximum diameter of about 240  $\mu\text{m}$  and are very yolky (Fig. 6A, C). Only euspermatozoa were found, and were present in both the hermaphroditic gland and in the vesicula seminalis (Fig. 6A, B).

The hermaphroditic duct acts as a vesicula seminalis and leads forward to the left side, then splits into two different ducts: the vas deferens and the oviduct (Fig. 5B, C). The oviduct, a slightly elongate structure rich with glandular cells, leads forwards into the bursa copulatrix, which also receives the contents of the albumen gland and the bifid capsule gland (Figs 5B, 6D, F). The bursa copulatrix opens into the short vaginal duct and from there into the posterior right side of the mantle cavity.

The vas deferens continues forwards in the centre of the first whorl up to the base of the penis, and from there to its tip

(Fig. 5C). At the beginning of the male system the prostate duct, with a prominent prostate gland at its distal end, opens into the vas deferens. In the head region the vas deferens is surrounded by thick circular muscles (Fig. 6E). In the contracted condition the penis is bent backwards within the mantle cavity, extending almost to its posterior end.

#### Alimentary tract

Compared with vetigastropods, neritimorphs and caenogastropods, the structure and shape of the whole gut are quite simple and the pharynx is short (330  $\mu\text{m}$ ). The simple jaw consists of two rows of cuticular teeth. The taenioglossate radula reaches deep into the ventrally located end of the pharynx (Figs 3E, 4E). The radula emerges from a short, ventrally situated radula sheath. A dorsal food groove is present. True radular cartilages are lacking and are replaced by a compact mass of cross-striated musculature.

The simple, nonglandular, ciliated oesophagus leads backwards and opens into the distinct stomach (Fig. 4D). Parallel to the oesophagus are the tubes of the paired salivary glands, consisting of polyploid cells (Fig. 4F), which open into the pharynx. The inner cell layer of the posterior part of the oesophagus is star-shaped in cross section and lacks glands.

The stomach fills nearly half of the first whorl (Fig. 3E, F). There are no specialized glandular cells or a stomach shield, although a style sac and stylus are present (Figs 3E, 4G, K). The whole epithelium of the stomach is densely ciliated. Two prominent openings lead from the stomach into the two (anterior and posterior) voluminous digestive glands, which (together with the hermaphroditic gland) fill the upper whorls. The digestive glands and the stomach are embedded in parenchyma. The thick intestine emerges on the left anterior side of the stomach and is relatively short and continued by the rectum. By the middle of the intestine a prominent typhlosole is formed, which runs along to the middle of the rectum. Intestine and rectum are S-shaped in dorsal view, with an anterior anal tube (Fig. 3E, F). This tube is highly ciliated and opens into the anterior right part of the mantle cavity.

The radula (Fig. 2F, G) is taenioglossate with a radula formula of 2-1-1-1-2. The trapezoidal central tooth has a pronounced median cusp, which is significantly longer and broader than the up to 12 smaller lateral cusps. Each lateral tooth also possesses a protruding cusp on the outer side. The laterals narrow towards the outer sides; the inner sides are rounded with smaller cusps. The marginal teeth are similar, shaped like bent bristles and lacking visible cusps.

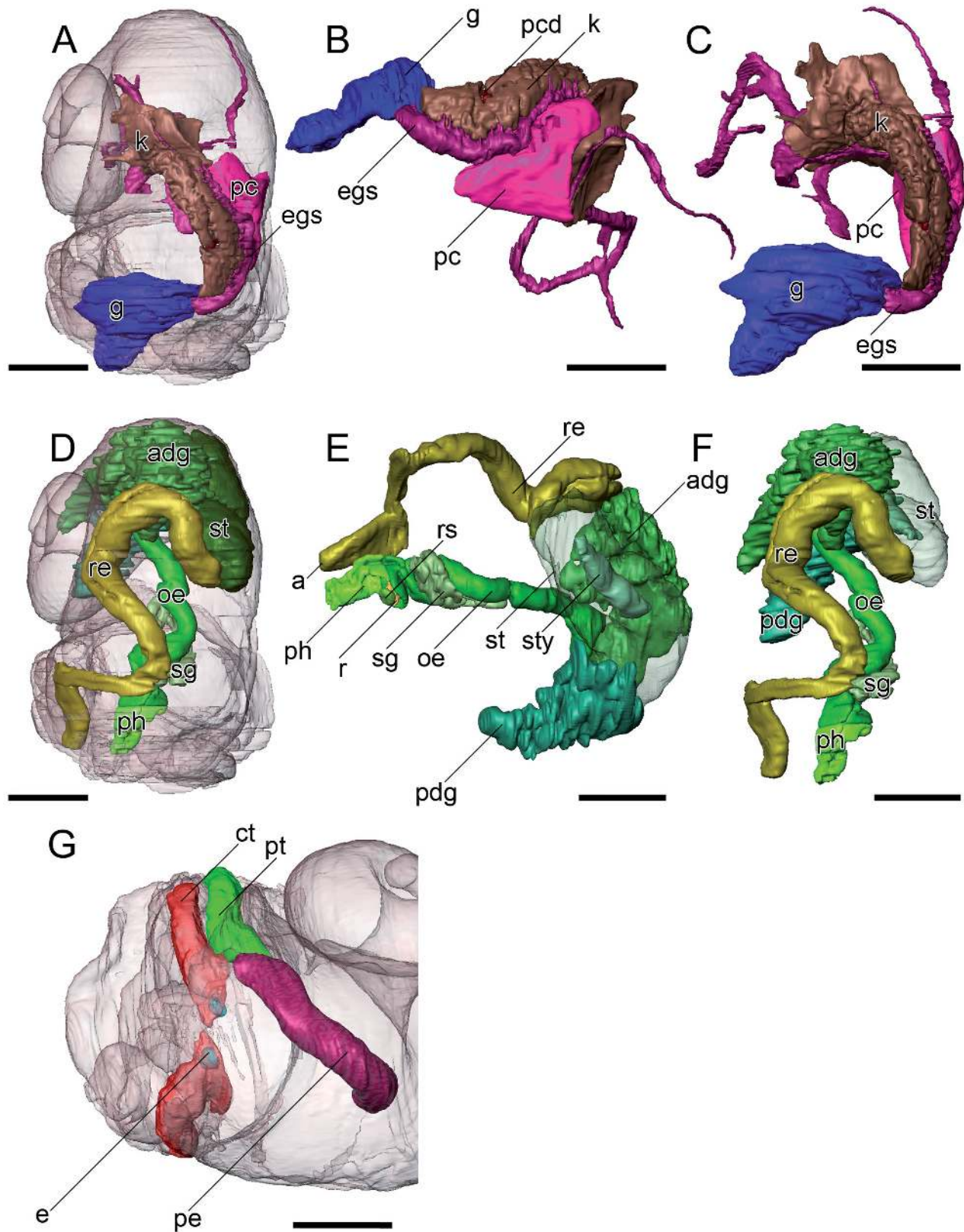
#### Nervous system

*Borysthenia naticina* has an epiathroid and streptoneurous nervous system (Fig. 7). The cerebral and pleural ganglia are fused; the resulting cerebropleural ganglia are located behind the buccal complex on the dorsal side of the oesophagus and are interconnected by a short commissure. The eyes are directly supplied by a single nerve each from the cerebropleural ganglia, which also supplies the bifurcate cephalic tentacle nerves and the statocysts, which are situated on the inner side of the pedal ganglia.

Two single nerves emerge from the cerebropleural ganglia and run within the buccal protractor muscles to reach the buccal ganglia. The buccal ganglia are situated at the transition between the oesophagus and pharynx and are interconnected by a short commissure. Paired nerves lead from the buccal ganglia backwards alongside the oesophagus to the stomach.

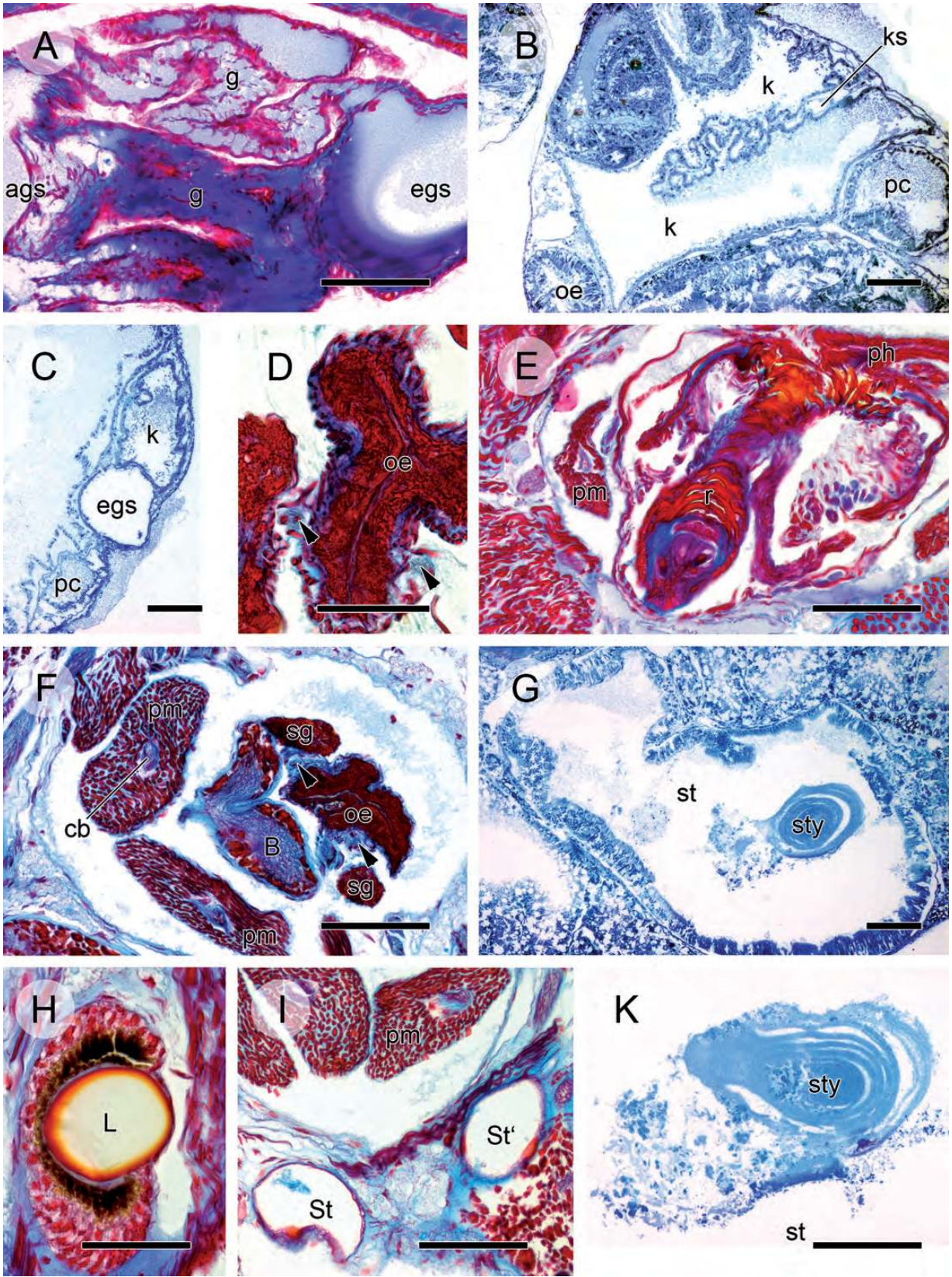
Two connectives on each side (one for the cerebral, one for the pleural ganglion) lead from each cerebropleural ganglion to the ventrally positioned pedal ganglion. There is a single pedal





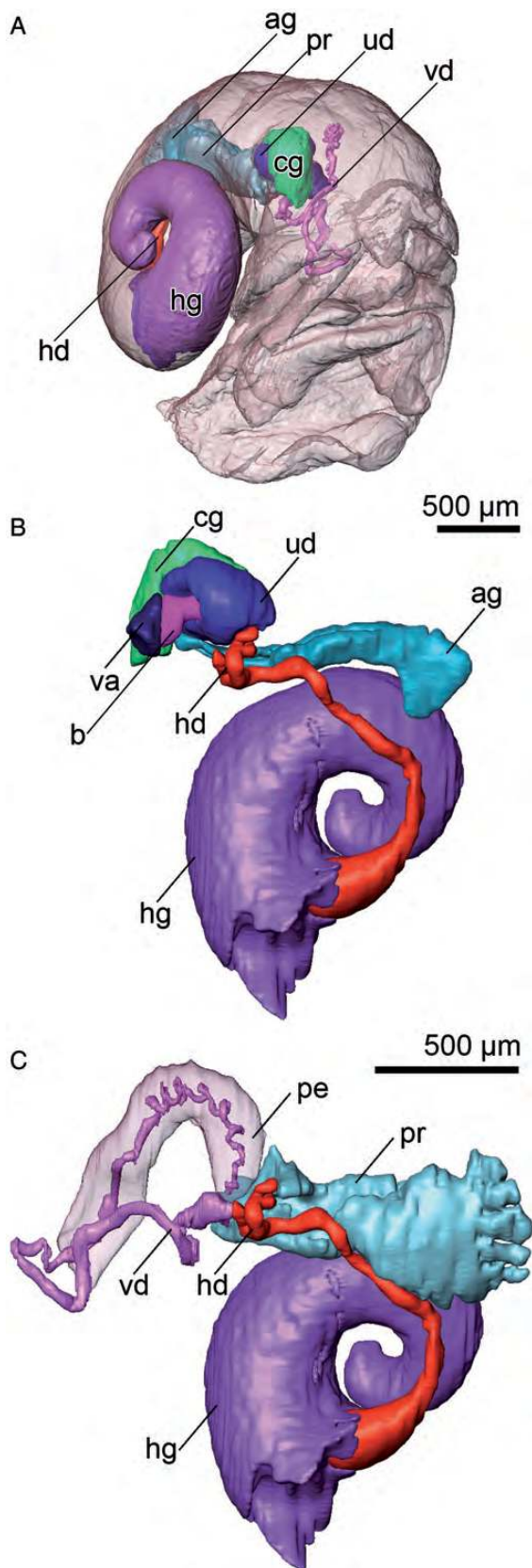
**Figure 3.** 3D reconstructions of the body, including the alimentary tract and excretory system of *Borysthenia naticina*. **A–C.** Excretory system. **D–F.** Alimentary tract. **G.** Position of tentacles. Abbreviations: a, anus; adg, anterior digestive gland; ct, cephalic tentacle; e, eye; egs, efferent gill sinus; g, gill; k, kidney; oe, oesophagus; pc, pericardium; pcd, pericardial duct; pdg, posterior digestive gland; pe, penis; ph, pharynx; pt, pallial tentacle; r, radula; re, rectum; rs, radula sheath; sg, salivary glands; st, stomach; sty, style. Scale bars: 500  $\mu\text{m}$ . The interactive 3D model of *B. naticina* can be accessed by clicking on the figure (Adobe Reader v. 7 or higher required). To rotate model drag with left mouse button pressed; to shift model use same action plus ctrl; to zoom use mouse wheel (or change default action for left mouse button). It is also possible to select or change transparency of components in the model, to switch between prefab views and to change surface visualization (e.g. lighting, render mode, crop etc.).





**Figure 4.** Histological semithin sections of the body of *Borysthenia naticina*. **A.** Gill. **B.** Kidney and surrounding structures. **C.** Excretory system. **D.** Oesophagus. For comparison with Architectonicidae see Haszprunar (1985c: fig. 25). **E.** Radula within pharynx. **F.** Buccal apparatus. **G.** Stomach. **H.** Eye showing lens surrounded by photoreceptor and pigment cells. **I.** Statocysts. **K.** Stylus within stomach. Abbreviations: ags, afferent gill sinus; B, buccal ganglia; cb, cerebropleural-buccal connective; egs, efferent gill sinus; g, gill; k, kidney; L, lens; oe, oesophagus; pc, pericardium; ph, pharynx; pm, buccal protractor muscle; r, radula; sg, salivary glands; St/St', right/left statocyst; st, stomach; sty, stylus; arrowheads, oesophageal nerves. Scale bars: **A–C, E–G, I, K** = 100  $\mu\text{m}$ ; **H, D** = 50  $\mu\text{m}$





**Figure 5.** 3D reconstruction of the genital system of *Borysthenia naticina*. **A.** Overview of the genital system. **B.** Female part. **C.** Male part. Abbreviations: ag, albumen gland; b, bursa; cg, capsule gland; hd, hermaphroditic duct; hg, hermaphroditic gland; pe, penis; pr, prostate gland; u, upper bursa duct; va, vagina; vd, vas deferens.

commissure. From each pedal ganglion two pedal nerves arise, which supply the whole muscular foot.

Adjacent to the right cerebropleural ganglion, the supraoesophageal ganglion is located and extends posterior to the left. From here two neural connections split off. While the first one leads to the small osphradial ganglion in the mantle roof (situated on the left), the second one continues backwards through the visceropallium to the visceral ganglion.

From the left cerebropleural ganglion, a single neural connective runs ventrally and then below the oesophagus and then turns backwards on the right to the suboesophageal ganglion. From there the remaining part of the visceral loop is represented by a thin connective leading to the visceral ganglion.

#### Sense organs

The cephalic tentacles and the osphradium have been described above.

Two pigmented and well-visible eyes lie at the inner bases of the cephalic tentacles (Figs 3G, 4H) and are embedded in the body surface. Each eye is provided with a cornea and a prominent lens. The paired statocysts (diameter 100 µm; Fig. 4I) are situated in between and slightly dorsal to the pedal ganglia and each contains a single statolith.

## DISCUSSION

#### General remarks

For direct anatomical comparison within the Valvatidae, we use the main references (genera/species in alphabetical order):

*Borysthenia naticina*: Niero & Bodon (2011);  
*Valvata piscinalis*: Bernard (1890); Cleland (1954); Garnault (1890); Rath (1986);  
*V. tricarinata*: Furrow (1935);  
*V. cristata*, *V. pulchella* Studer, 1820 (= *V. studeri* Boeters & Falkner, 1998), *V. relicta*, *V. hirsutecostata*, *V. stenotrema*, *V. rhabdota*: Rath, 1986.

For direct anatomical comparison within the Ectobranchia, we use the main references (families in alphabetic order):

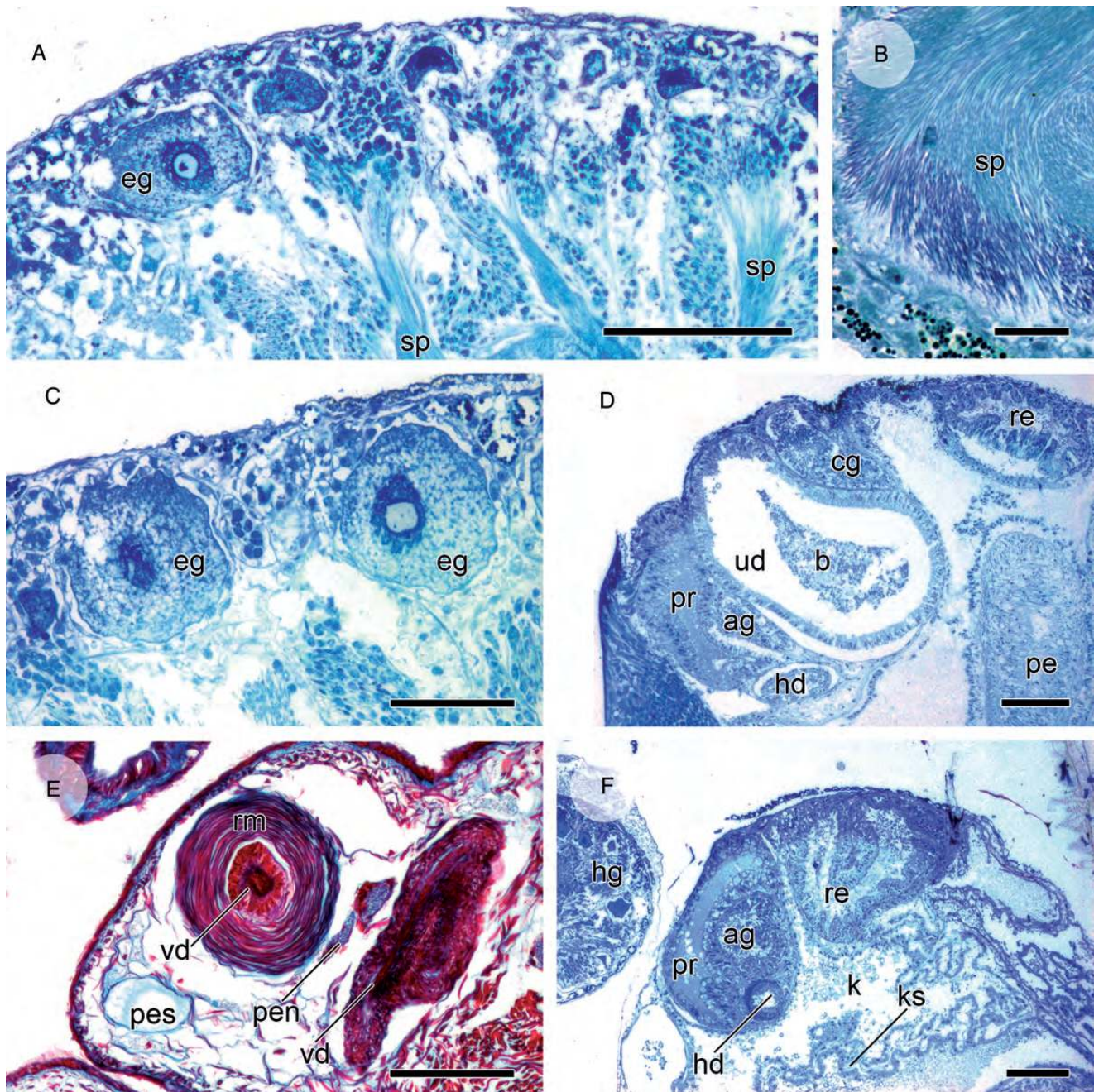
Cornirostridae Ponder, 1990: Ponder, 1990a, 1991; Bieler, Ball, & Mikkelsen, 1998;  
 Hyalogyrinidae Warén & Bouchet, 1993; Warén, Gofas, & Schander, 1993; Warén & Bouchet, 1993; Haszprunar *et al.*, 2011;  
 Orbitestellidae Iredale, 1917 (synonym Microdisculidae Iredale & McMichael, 1964): Ponder, 1990b, 1991; Warén & Bouchet, 2001;  
 Valvatidae O.F. Müller, 1840: Bernard, 1890; Furrow, 1935; Yonge, 1947; Starmühlner, 1952; Cleland, 1954; Johansson, 1956; Sitnikova, 1984; Rath, 1986, 1988; Falniowski, 1989a, b, 1990a;  
 Xylodisculidae Warén, 1992; Marshall, 1988; Warén, 1992; Høisaeter & Johannessen, 2002.

This list also shows the significant progress in our knowledge of ectobranch anatomy during the last 25 years, in particular concerning the marine relatives of the freshwater Valvatidae.

#### Shell and operculum

The difference in the aperture mentioned by Lindholm (1927) between *Valvata* (circular) and *B. naticina* (nearly circular, but pointed at apical side), as well as a slightly more depressed apex in *B. naticina*, has been confirmed by Niero & Bodon (2011) and our SEM photos (Fig. 2B). However, shells of at least some valvatid species are highly variable (e.g. Steusloff, 1922; Haas, 1938;





**Figure 6.** Histological sections of the genital system of *Borysthenia naticina*. **A.** Overview of hermaphroditic gland. **B.** Spermatozoa in vas deferens. **C.** Detailed view of eggs within hermaphroditic gland. **D** and **F.** Sections of the proximal genital tract. **E.** Vas deferens within penial base, surrounded by muscular layer. Abbreviations: ag, albumen gland; b, bursa; cg, capsule gland; eg, egg; hd, hermaphroditic duct; hg, hermaphroditic gland; k, kidney; ks, kidney sinus; pe, penis; pen, penis nerve; pes, penis sinus; re, rectum; pr, prostate gland; rm, ring muscle of vas deferens; sp, sperm; ud, upper bursa duct; vd, vas deferens. Scale bars: **A, D–F** = 100  $\mu\text{m}$ ; **C** = 50  $\mu\text{m}$ ; **B** = 20  $\mu\text{m}$ .

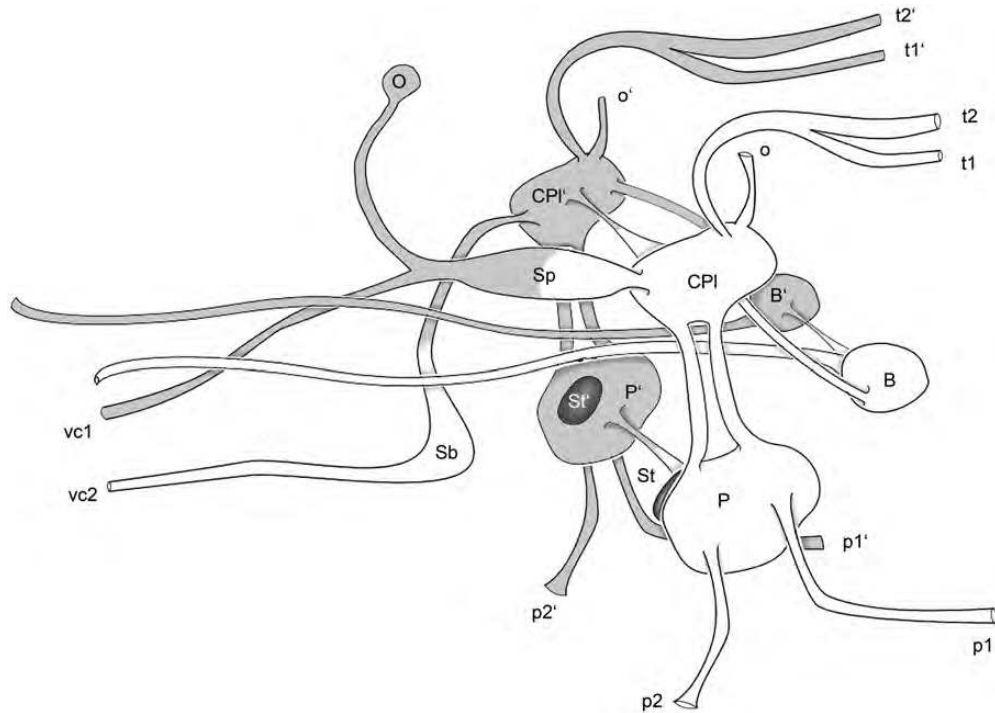
Hanson *et al.*, 2002; Scholz & Glaubrecht, 2010). Moreover, the molecular (COI gene) study by Hauswald *et al.* (2008) revealed different gene sequences in cases of similar shells and identical sequences in cases of clearly different shells. Thus, it is more than doubtful that shell morphology alone is truly significant for species delimitation and higher systematics within the Valvatidae.

Protoconch sculpture is quite uniform throughout the family and is clearly different from those of hydrobiids, which otherwise may have very similar shells (Hershler & Ponder, 1988; Falniowski, 1989a, b, 1990b, 1992a, b). Orthostrophic protoconchs are typical for all valvatids and are generally found in

freshwater or terrestrial Heterobranchia (i.e. pulmonate taxa). A hyperstrophic protoconch (i.e. heterostrophic relative to teleoconch shell) is typical of most marine Heterobranchia, but there are some orthostrophic exceptions such as Rissoellidae and Umbraculomorpha (Haszprunar, 1985a,b,c, 1988a,b; Robertson, 1993; Haszprunar *et al.*, 2011).

According to Falniowski (1990a: 83), who studied *Borysthenia naticina* (figs 264–272), *V. piscinalis* (figs 231–238) and *V. pulchella* (figs. 247–256) by SEM, “the teleoconch inner structure [of *B. naticina*] differs markedly from the one described for the other valvatid species under consideration”. If these differences





**Figure 7.** Schematic illustration of the nervous system of *Borysthenia naticina*, viewed from the right side. Abbreviations: B/B', right/left buccal ganglion; CPI/CPI', right/left cerebropneural ganglion; O, osphradial ganglion; o/o', right/left optical nerve; P/P', right/left pedal ganglion; p1/p1', right/left anterior pedal nerves; p2/p2', posterior pedal nerves; Sb, suboesophageal ganglion; Sp, supraoesophageal ganglion; St/St', right/left statocyst; t1/t1' and t2/t2', right/left pair of tentacle nerves; vc1 and vc2, nerves of the visceral loop leading towards visceral ganglion.

could be shown to be consistent within the Valvatidae, shell structure might become diagnostic for *Borysthenia* and perhaps for other groups of valvatids.

Differences in the number of opercular whorls (max. 3.75 for Russian/Ukraine *Borysthenia*, 5 in *Valvata* species) was one of the main characters used to separate the two (sub)genera by Lindholm (1927). However, Niero & Bodon (2011) described 4.5 opercular whorls in specimens from Italy and our specimens from Bavaria show 5 whorls. This might be a matter of growth or might reflect different populations, but in any case this character clearly cannot be used for generic separation. There is no consistent pattern of opercular whorls among outgroups or among other basal Heterobranchia.

#### *Body morphology, histology and pedal characters*

In all valvatids, a ciliated pallial tentacle is present, as seen in *B. naticina* (e.g. Haszprunar, 1988b; Ponder, 1991). Yonge (1947) and Starmühlner (1952) considered that the main function of this pallial tentacle is to provide a water current through the mantle cavity, being an analogue of the ciliary ridges that can be found on each side of the mantle cavity in other basal heterobranchs (Haszprunar, 1985b,c).

Among the Ectobranchia only Valvatidae, Cornirostridae and Orbitestellidae show a true, prominent penis, whereas the other clades (Hyalogyrinidae and Xylodisculidae) lack a copulatory organ. The position of the penis, arising behind the right cephalic tentacle, is exactly the same in all valvatids studied (Furrow, 1935; Rath, 1986; Falniowski, 1989b, 1990a).

Both foot and snout are more or less bilobed, as in most basal Heterobranchia. As usual for Valvatidae and most other allogastropods there is only one, prominent pedal gland, whereas Architectonicidae and Omalogyridae have an additional posterior pedal gland.

#### *Mantle cavity*

The specific shape and structure of the gill of *B. naticina* resembles those of the undetermined *Valvata* specimen from Cairo described by Rath (1986). Significant differences from typical ctenidia and its mode of development (Rath 1986) suggest a secondary nature of the valvatid gill (Haszprunar, 1988b; Rath, 1988; Ponder & Lindberg, 1997). The structure of the valvatid gill is highly variable and depends on size (for example the number and shape of lamellae, cf. Falniowski, 1990a). Accordingly, details of the gill cannot be used as significant characters for defining genera.

On the other hand, the presence of a pallial tentacle and a gill, which can be extended out of the mantle cavity, both provide significant synapomorphies shared among all ectobranch clades, namely Hyalogyrinidae (Haszprunar *et al.*, 2011), Cornirostridae (Ponder, 1990a), Xylodisculidae (Warén, 1992; personal observation), and Valvatidae. The Orbitestellidae, which lack a gill (Ponder, 1990b; personal observation), should be placed outside the Ectobranchia (e.g. Dinapoli & Klussmann-Kolb, 2010; Jörger *et al.*, 2010; Dinapoli *et al.*, 2011; Göbbeler & Klussmann-Kolb, 2011; Schrödl *et al.*, 2011).

#### *Alimentary tract*

Hensche (1866: 102) first described and depicted the radula of *B. naticina*. However, his species determination appears doubtful, since he mentioned up to 60 eggs in his specimens from Skirwith (today Borovoe on the Rusné river, on the border between Russia and Lithuania). The radula of our Bavarian specimens shows a small central cusp on the rachidian tooth, whereas those of Hensche (1866) and of Niero & Bodon (2011) have a much larger median cusp as otherwise usual in *Borysthenia* (e.g. Sitnikova *et al.*, 1986) and several *Valvata* species. The SEM images of Niero & Bodon (2011) also show that the marginals



may bear small denticles, as in *Valvata* species. However, in all cases the outer marginals of *Borysthenia* are slender and lack inner denticles, whereas in *Valvata* species they are much broader and are denticulate also on the inner side (SEM studies by Falniowski, Economou-Amilli, & Anagnostidis, 1988; Falniowski, 1989a, b, 1990a; Anistratenko *et al.*, 2010).

A taenioglossate-like radula is typical for all Valvatidae and several other basal heterobranch groups (e.g. Haszprunar, 1985b, c, 1988b; Falniowski, 1990a, b; Ponder, 1991; Haszprunar *et al.*, 2011). Haszprunar *et al.* (2011) assumed that the rhipidoglossate radula of Hyalogyrinidae was plesiomorphic, because of the striking similarities to some ‘archaeogastropod’ taxa; if so, the evolution of a taenioglossate-like type (i.e. the loss of most marginal teeth) must have evolved independently in Valvatidae, Cornirostridae (Ponder, 1990a), Rissoellidae (Ponder & Yoo, 1977), Mathildidae and Architectonicidae (Haszprunar, 1985b, c; Bieler, 1988) and Caenogastropoda.

All heterobranch gastropods, except some pulmonate species, share the presence of a muscular cushion that replaces true, primary, odontophoral cartilages, which can be found in most ‘archaeogastropods’ and caenogastropods (e.g. Haszprunar, 1985a, c, 1988b; Salvini-Plawén & Haszprunar, 1987; Ponder, 1991).

Most other features of the anterior alimentary system (e.g. polyploid salivary glands, buccal connective within radular protractor muscle, buccal nerve along oesophagus) are constant within basal Heterobranchia (Haszprunar, 1988b; Ponder, 1990b, 1991; Haszprunar *et al.*, 2011) and were among other characters first used to support the classification of Valvatidae within the basal Heterobranchia (Salvini-Plawén & Haszprunar, 1987; Haszprunar, 1988b). A gastric shield with a tooth is shared with Cornirostridae, Orbitestellidae and Hyalogyrinidae (no data so far available on Xylodisculidae). This feature is common among the ‘archaeogastropod’ and many caenogastropod taxa and is thus a plesiomorphic character among the Ectobranchia, having been lost in most other Heterobranchia. The presence of an intestinal typhlosole, another plesiomorphic feature, has also been recorded in *V. piscinalis* (Cleland, 1954).

#### *Nervous system and sense organs*

There is no significant difference in the gross nervous system between *Borysthenia* and other valvatids.

The epiathroid and streptoneurous nervous system is typical for all allogastropod Heterobranchia and sorbeoconch Caenogastropoda. Furthermore, as in certain *Hyalogyrina* species (Haszprunar *et al.*, 2011), single nerves are present in the buccal protractors of *B. naticina*. In this case, they connect the cerebropleural and the buccal ganglia. Haszprunar *et al.* (2011: 231) argued this to be a “synapomorphy of Heterobranchia and, thus, a plesiomorphy for Ectobranchia”, because it is also found in Architectonicidae, Mathildidae and Pyramidellidae (Haszprunar, 1985b, c, 1988b; Haszprunar *et al.*, 2011). The quite unusual paired nerve, which leads alongside the oesophagus, is also present in Architectonicidae (Haszprunar, 1985c).

#### *Genital tract*

The complex hermaphroditic genital system of *Borysthenia* is nearly identical with those of *Valvata* species (Bernard, 1890; Garnault, 1890; Furrow, 1935; Cleland, 1954; Rath, 1986; Sitnikova *et al.*, 1986; Niero & Bodon, 2011). This pertains to both the overall course of the various ducts and also its histological structure. In contrast to the findings of Niero & Bodon (2011), the existence of a Garnault’s duct (an additional connection between the hermaphroditic duct and albumen gland) is not traceable in our material of *Borysthenia*, or in all other

examined valvatids (e.g. Cleland, 1954; Rath, 1986, 1988). Only euspermatozoa were found, as typical for all Valvatidae (Kohnert & Storch, 1984; Healy, 1990; Tair-Abbaci & Garric, 2012).

Based on the presence of nearly mature embryos in the genital tracts, Velitchkowsky (in Lindholm, 1927) and, most recently, Niero & Bodon (2011) assumed that *B. naticina* is an ovoviviparous species. Until now this assumption has not been tested. However, the data presented here cast doubt on the interpretation of ovoviviparity, because observations of specimens in captivity showed normal development of embryos within clutches in most cases. Egg capsules laid by the Bavarian *B. naticina* contained a few (4–6), already well developed embryos, which hatched three days after spawning (Fig. 1B, C).

The number of eggs per capsule and timing of development vary significantly among valvatid species. Whereas the numerous (up to 40) eggs of *V. piscinalis* need 15–30 days until hatching, the North American *V. sincera* has only 2–4 eggs/embryos (no clear data) per capsule, which hatch in 5–8 days (Heard, 1963). The latter case is already close to the condition found in *B. naticina*, although we cannot entirely exclude that other populations might show true ovoviviparity. Interestingly, Hensche (1866) noted up to 60 eggs per capsule in his *B. ‘naticina’* specimens from the Memel region, a number that appears to exclude ovoviviparity. Accordingly, ovoviviparity cannot be used to define the genus *Borysthenia*.

Falniowski (1990a) has shown that the external structure of the penis is in most cases of minor interest, due to its large variability with e.g. age, season and physiological condition. Furthermore, proportions and shape can change with fixation. The position of the penis, reaching the posterior end of the mantle cavity, was described by Hensche (1866) for *B. naticina* and also by Rath (1986) and Cleland (1954) for *V. piscinalis*, suggesting that this is the resting state of the penis. Internal characters can be more useful for species taxonomy. A ring of circular musculature around the middle part of the vas deferens was also found in various species studied by Rath (1986), namely *V. piscinalis*, *V. cristata*, *V. pulchella*, *V. stenotrema* and *V. hirsutecostata*. Indeed, this extended coating of muscle at the base of the penis may serve as a synapomorphy for Valvatidae. Ponder (1990b) mentioned in *Cornirostra pellucida* only that “the vas deferens becomes muscular”. Since series of histological sections are not available, it is not possible to say whether this is the same structure seen in the Valvatidae. If so, this shared character would agree with the molecular analysis of Dinapoli & Klusmann-Kolb (2010), supporting a closer relationship between Valvatidae and Cornirostridae compared with the other families within the Ectobranchia (Orbitestellidae and Hyalogyrinidae), a finding also supported by Haszprunar *et al.* (2011).

#### *Heart, circulatory and excretory system (renopericardial system)*

The position of the single (left) kidney behind the gill in the pallial roof, as well as the monotocardian heart, are typical for all basal heterobranchs (Haszprunar, 1985b, c, 1988b; Ponder & Lindberg, 1997; Haszprunar *et al.*, 2011). The large size and bent shape of the kidney’s lumen have also been described in *V. piscinalis* (Bernard, 1890; Cleland, 1954; Rath, 1986) and reflect the freshwater habitat with increased osmoregulatory requirements. Within the Ectobranchia the Valvatidae are the only freshwater group; therefore, the presence of a large kidney represents another synapomorphy of the family.

#### *Systematic considerations*

The main focus of this work was to investigate the anatomical differences between *B. naticina* and other valvatid species, in

order to validate generic or even subfamilial separation (Sitnikova *et al.*, 1986).

Summing up, there is an overall striking similarity in anatomy between various *Valvata* species and *B. naticina*, raising doubts concerning their generic (or higher) separation. The morphology of the shell aperture (adapically pointed vs. round), marginal radular teeth (slender vs. broad and denticulate) and (possibly) internal shell structure still define the genus as a probably monophyletic assemblage, whereas the character of ovoviviparity is now very doubtful. The molecular phylogenetic analysis by Hauswald *et al.* (2008) focused on the 'endemic' species of Lake Ohrid and Lake Prespa and showed substantial disagreements between shell morphologies, gill anatomies (Rath, 1988) and the molecular data. Accordingly, it is doubtful whether Valvatinae (contrasting with Borystheniinae) or any of the proposed (sub)genera are monophyletic taxa. There is little doubt that a comprehensive, integrative analysis of valvate systematics, based on both phenotypic and genotypic characters, is badly needed.

Aside from molecular data, the close relationship of the Valvatidae with the remaining Ectobranchia is based mainly on the distinctive, shared gill-type (extendable, bipectinate, ciliated, lacking bursicles and skeletal rods, showing shape variation; cf. Haszprunar *et al.*, 2011; the only exceptions are the gill-less Orbitestellidae, cf. Ponder, 1990a, b) and the right pallial tentacle(s). Robust inference of relationships between the ectobranch families requires anatomical investigations of more taxa (in particular of Xylodisculidae, which are already underway) and molecular data.

As recently outlined by Haszprunar *et al.* (2011) the position of the Ectobranchia as the earliest offshoot of the Heterobranchia is well founded on both phenotypic and genotypic data. In contrast, the relationships of the remaining basal heterobranch groups are still far from being resolved and will be a challenge for future research.

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