Cuticular structures in the copulatory apparatus of *Planorbis planorbis* (Linnaeus, 1758) (Gastropoda: Pulmonata: Planorbidae)

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The morphology of the copulatory apparatus and associated cuticular structures in *Planorbis planorbis* was studied by light microscopy, SEM, TEM and confocal laser scanning microscopy. The significance of these cuticular structures for the taxonomic status of the species and for the systematics of the family Planorbidae in general is discussed.

Key words: molluscs, morphology, stylet, taxonomy, *Planorbis*

INTRODUCTION

Planorbis planorbis (Linnaeus, 1758) is one of the best known and most widely distributed species living in freshwater habitats in Europe. For most of the scientists of the Old World this species has become a model for studying morphology, ecology, karyology, and physiology of the family Planorbidae Rafinesque, 1815 (Matzke, 1959; Alekseev & Antipin, 1976; Stadnichenko, 1990; Costil & Daguzan, 1995). There is a large number of papers discussing the anatomy of *P. planorbis* (Baker, 1945; Hubendick, 1955; Starobogatov, 1958a, 1958b, 1967; Likharev & Starobogatov, 1967; Meier-Brook, 1976; Soldatenko & Starobogatov, 2000) that dedicate special attention to the description of the reproductive system and, specifically, to the anatomy of the copulatory apparatus (the characters of the copulatory apparatus are widely used in planorbid systematics). All authors agree that the penis lacks stylet and the opening of vas deferens (spermatic duct) is terminal.

Planorbis seems to be the only genus within the tribe Planorbini Rafinesque, 1815 (subfamily Planorbinae Rafinesque, 1815) that lacks penial stylet; all other members of this group of closely related genera (*Anisus* Studer, 1820; *Armiger* Hartmann, 1840; *Choanomphalus* Gerstfeldt 1852; *Gyraulus* Agassiz in Charpentier, 1837), which have been described in details, have well-developed cuticularized stylet-like structures at the tip of the penis (Baker, 1945; Hubendick, 1955, 1957; Odhner, 1956; Hudec, 1967; Meier-Brook, 1983; Prosorova & Starobogatov, 1997; Soldatenko & Sitnikova, 2009). This seemingly exceptional status of *P. planorbis* suggests the possibility that the stylet was overlooked in this species due to its extremely small size.

The purpose of this paper is to determine whether or not *P. planorbis* has cuticularized structures in the copulatory apparatus and, thus, to further our understanding of the phylogenetic relationships within the Planorbidae.

MATERIAL AND METHODS

Material consisted of the samples of *P. planorbis* in the collections of the Zoological Institute of Russian Academy of Sceinces, St. Petersburg (ZISP) and of the specimens kept in the private collection of the first co-author (EVS). The material came from the following localities:

ZISP 204; Leningrad Prov., Ivan-Gorod, a pool 1.5 km from the town near the household plots, 15 June 1978; coll. Ya.I. Starobogatov; ZISP 205; Ryazan Prov., 5 km from Kleniki, Pra R., May 1979; coll. M.N. Zatravkin;

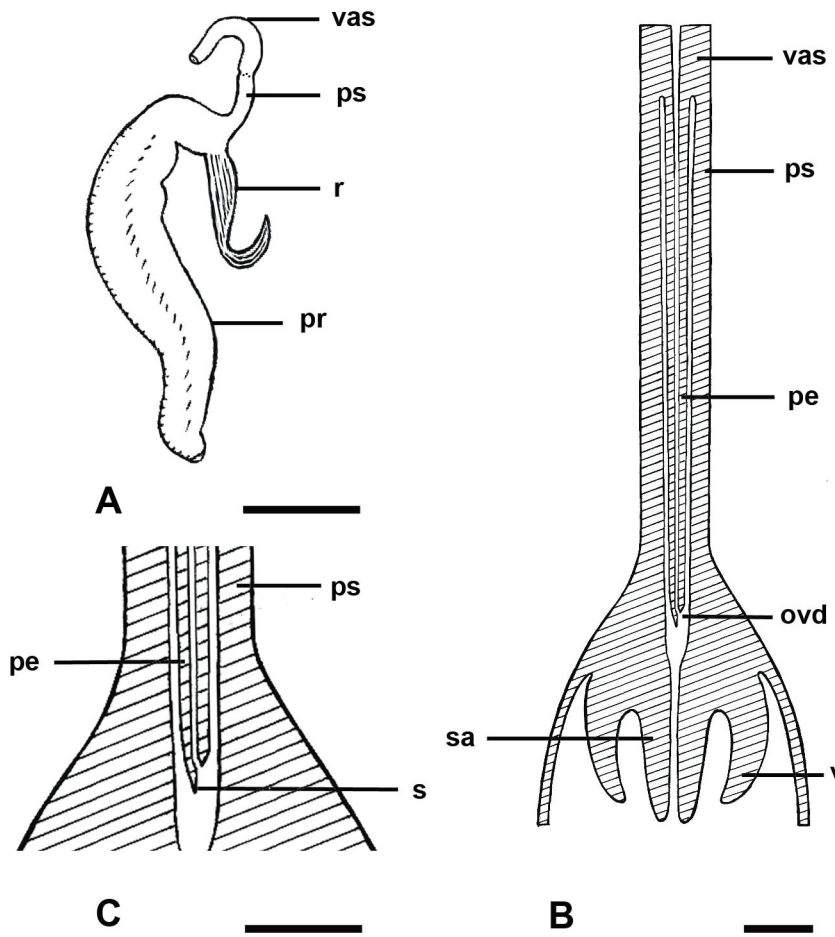

Fig. 1. Copulatory apparatus of *Planorbis planorbis*: **a** – general view; **b** – diagrammatic representation of longitudinal section through penial sac and upper part of prepucium; **c** – diagrammatic representation of penial sac (enlarged view of b). ovd – opening of vas deferens; pe – penis; pr – prepucium; ps – penial sac; r – retractor; s – stylet; sa – sarcobellum; v – velum; vas – vas deferens. Scale bars: a – 1 mm; b, c – 100 μm.

EVS; Smolensk Prov., Demidovskiy Distr., at Przhevalskoye, a branch of Mutnoye-Rytoye L., 24 June 1999; coll. E.V. Soldatenko;

EVS; Smolensk Prov., Demidovskiy Distr., at Przhevalskoye, Sapsho L., 4 July 1994; coll. E.V. Soldatenko;

EVS; Leningrad Prov., Pavlovsk, Pavlovsky Park, Slavyanka R., 29 July 2008; coll. E.V. Soldatenko.

Twenty five adult specimens were dissected, their internal organization was studied and then their copulatory apparates were removed for further examination (Fig. 1a-c). The extracted copulatory apparates and their fragments were examined using different methods.

Sixteen specimens were prepared as unstained whole mounts. These whole-mount preparations were examined and photographed on a Leica DMLS-2 microscope equipped with a CCD camera; the line drawings were copied from photographs according to their original proportions.

Five specimens were prepared for scanning electron microscopy. Each extracted penis was dehydrated in ethanol, air dried for 20 min in hexamethyldisilazane (Bock, 1987), and then coated with platinum in a HITACHI IB-5 ion sputter. The distal ends of the penises were viewed on a HITACHI S-570 scanning electron microscope.

For confocal microscopy, two specimens were fixed in 4% paraformaldehyde in 0.1M PBS for 1 h at room temperature, rinsed in 0.1M PBS (3 times for 15 min), permeabilized for 1 h in PBS containing 0.2% Triton X-100, rinsed again shortly in the same buffer and then transferred to phalloidin-TRITC (Sigma) for 1 h. After that, the specimens were washed again 3 times for 15 min in the same buffer, mounted in 80% glycerol on glass slides, and viewed on a Leica TSC SP5 microscope.

For anatomical observations, serial semi-thin sections of extracted copulatory apparates were stained with toluidine blue and then viewed and photographed on an Amplival and Leica DM LS-2 microscopes.

For TEM study, a standard double fixation in glutaraldehyde and osmium tetroxide was used. Samples were initially fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) for 2-4 h or were kept in fixative for several months. The samples were then washed in several changes of 0.2 M phosphate buffer, postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 12 h to overnight, dehydrated in graded ethanol or acetone series, and finally embedded in an araldite mixture. Serial ultrathin sections were obtained on a Leica UC-6 ultramicrotome, stained with uranyl acetate and lead citrate, and examined on a LEO-900 transmission electron microscope at 50-80 kV.

RESULTS

SEM images of the distal end of the penis in *P. planorbis* show that the tip of the penis bears a trough-shaped lamella. The lamella is slightly curved and conically tapering toward the apex. The opening of vas deferens is subterminal and lies approximately 10-15 μm from the tip of the penis. The distal end of vas deferens opens into a furrow on the concave surface of the lamella; the furrow runs along the middle of the lamella, following its curve, and ends near the tip of the lamella on the inner side of the curve (Fig. 2a). The shape of the lamella is consistent with that of a plate-like cuticular stylet.

Light microscopy of unstained whole mounts reveals a cuticular layer, more evident at the tip of the penis and extending into more proximal portions of the penis. The furrow is clearly visible (Fig. 2b). Light microscopy could not confirm or disprove the presence of a cuticular stylet; however, the pointed shape of the penis tip indicates that it might be rigid in structure.

The presence of the stylet in *P. planorbis* was confirmed by fluorescence microscopy using phalloidin-TRITC staining. Phalloidin specifically binds filamentous actin and reveals cell outlines (actin cytoskeleton), leaving cuticular structures unstained. Fig. 2c shows the bright-field image (left) and corresponding phalloidin fluorescence image (right) of one optical section through the penis. The tip of the penis is clearly seen on the bright-field image; cell outlines on the fluorescence image are visible along the entire length of the penis, but the penis tip remains unstained. This might be an indication that the tip of the penis bears a structure (the stylet) composed primarily of extracellular matrix material. The exact size of the stylet is difficult to determine, because the stylet may be partly surrounded with soft tissues.

The ultra-thin sections through the penis tip studied with TEM confirm that the stylet, despite its small size, has a complex shape and structure. The stylet itself is an internal cuticular apodeme, ending somewhat below the penis tip, and, just as the rest of the penis, covered on the outside with a thin, somewhat corrugated, cuticle, a derivative of the epithelial cells of the penis (Fig. 2d, 2e). The length of the stylet is 7-10 μm.

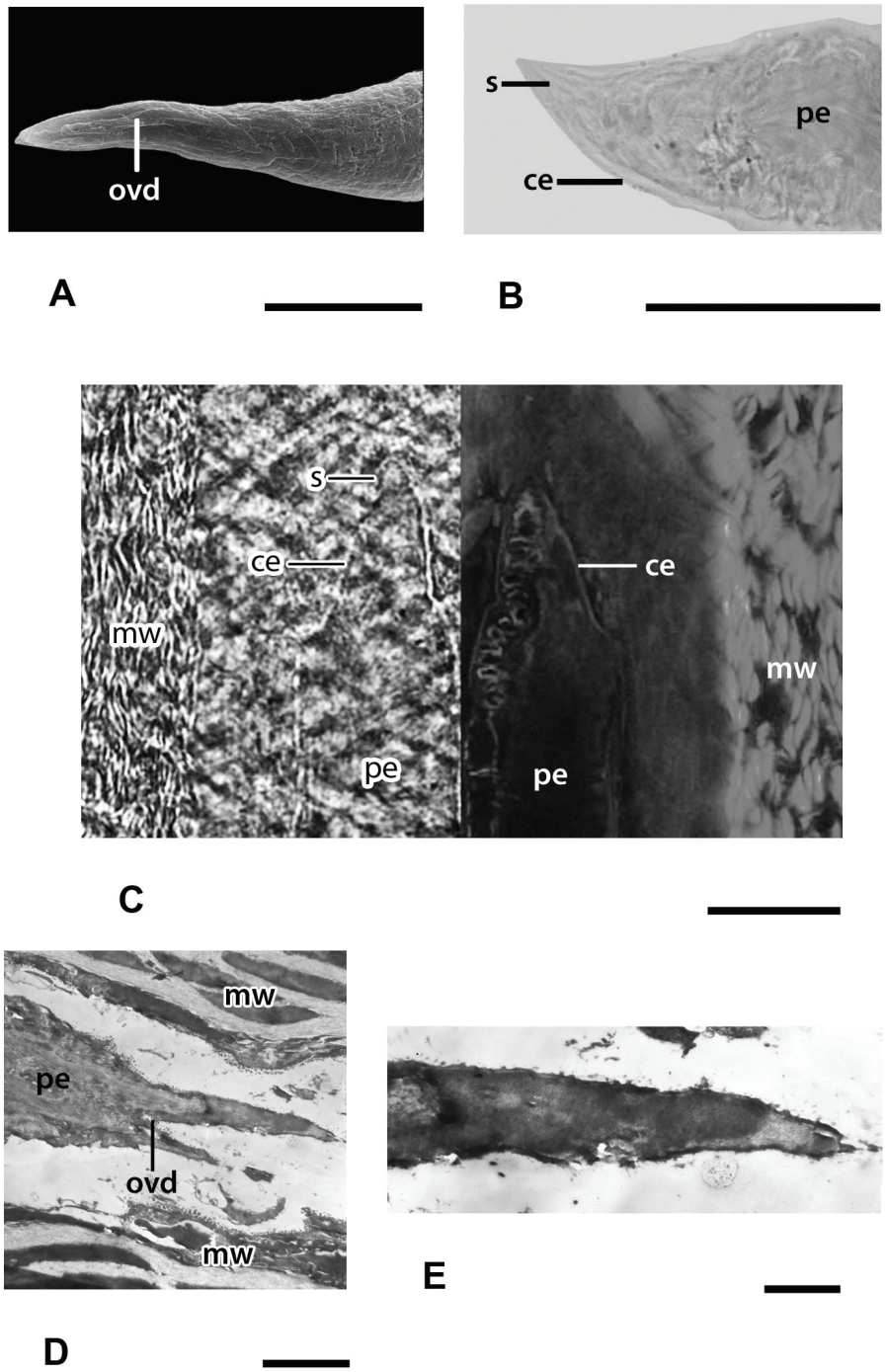

Fig. 2. Penis of *Planorbis planorbis*: **a** – SEM; **b** – light micrograph of whole mount; **c** – confocal micrographs: bright-field (left), phalloidin-stained (right). Images are mirror-reversed for easier comparison; **d, e** – TEM. ce – cuticular epithelium; mw – muscular wall of penial sac; ovd – opening of vas deferens; pe – penis; s – stylet. Scale bars: a – 30 μm, b – 50 μm, c – 25 μm, d – 5 μm, e – 1 μm.

DISCUSSION

Previous morphological descriptions of *P. planorbis* (Baker, 1945; Hubendick, 1955; Starobogatov, 1958a, 1958b, 1967; Likharev & Starobogatov, 1967; Meier-Brook, 1976; Soldatenko & Starobogatov, 2000) relied largely on light microscopy techniques. Unfortunately, the commonly used histological methods are not sensitive enough to detect a small cuticular structure in the copulatory apparatus of this species. The light micrographs made from the whole mount preparations or from histological sections, could give a wrong impression that the opening of vas deferens is terminal, especially since the distal portion of the penis is often bent backwards, or squeezed.

The novel research techniques have substantially expanded our ability to reveal and describe even the most obscure or incipient structures. A combination of modern research techniques used in this study (SEM, TEM and fluorescent histochemistry) shows that the distal end of the penis in *P. planorbis* bears a small cuticular structure in the form of a plate-like (i.e., not rolled into a tube) stylet, somewhat curved inward on the sides, and tapering to a pointed tip. Our results explain the close affinity of the genus *Planorbis* to other stylet-bearing forms, which is supported both by morphology-based phylogenetic reconstructions (Hubendick, 1955; Starobogatov, 1958a, 1967; Meier-Brook, 1976) and by recent molecular phylogenetic data (Albrecht et al., 2007). We, however, cannot agree with the conclusion made by Albrecht et al., 2007, that *Planorbis* is a more derived phylogenetic lineage than *Choanomphalus* (which has a hollow conical stylet), and that the penial cuticular structure was secondarily reduced in *Planorbis*. The study of stylets in other genera of the Planorbidae – *Anisus, Armiger*, *Choanomphalus, Gyraulus*, *Kolhymorbis* Starobigatov et Streletzkaja, 1967 (Soldatenko, unpublished data) – shows that plate-like and conical stylets are likely to be two different lineages of stylet evolution.

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