Anatomy and Ultrastructure of the Female Reproductive System of *Pleioplana atomata* (Platyhelminthes: Polycladida)

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ABSTRACT The ultrastructure of the female reproductive system of the polyclad flatworm *Pleioplana atomata* is described. Numerous ovaries are scattered throughout the entire body but are mainly concentrated on the dorsal side. Within an ovary, a germinative zone with oogonia and prefolicular cells is located in the dorsal part of the ovary. The remaining part of the gonad is filled with previtellogenic and early vitellogenic oocytes enwrapped by follicular cells. During previtellogenesis, oocytes produce numerous eggshell globules, which are distributed into the cortical area of the cell in later stages. Eventually, these globules release their contents into the space between the eggshell cover and oolemma. Similar types of globules are also found in others flatworms, and may represent useful phylogenetic characters. Entolecital, vitellogenic oocytes pass to paired uteri, where vitellogenesis is completed. The remainder of the female reproductive system consists of paired thin uterine ducts that join a vagina. The distal part of the long, curved vagina forms a large Lang's vesicle, while the proximal part is connected to a female atrium leading to a female gonopore. We hypothesize that Lang's vesicle functions in the digestion of excess sperm received. Two kinds of different shell (cement) glands that release their secretion into the vagina are identified. Both are unicellular glands and each gland cell connects to the lumen of the vagina via an individual canal. Similar glands in other acotylean polyclads have been implicated in the formation of eggshell covers. J. Morphol. 270:337–343, 2009.

KEY WORDS: Platyhelminthes; Polycladida; oogenesis; reproductive system; *Pleioplana atomata*

Polyclad flatworms are considered to be at the archoophoran level of gonad and egg organization, producing entolecithal eggs, having no vitellaria and having a primitive developmental pattern (Hyman, 1951; Karling, 1967; Gremigni, 1983). *Pleioplana atomata*, free-living, simultaneous hermaphroditic flatworm, possesses fully functional male and female reproductive systems. Data concerning the anatomy and ultrastructure of the polyclad female reproductive system are limited to only a few species (Boyer, 1972; Domenici et al., 1975; Ishida et al., 1981; Ishida and Teshirogi, 1986) despite the fact that this structure has been considered as one of the most variable and complex among turbellarians (Gremigni, 1983).

Recent systematics of Polycladida is based on reproductive structure morphology at the light microscopy level (for review see: Faubel, 1983, 1984; Prudhoe, 1985; Rieger et al., 1991). In the few species investigated to date, female gonads are homocellular, where each ovarian follicle develops from a single oogonial cell and is surrounded by somatic follicle cells (Rieger et al., 1991). Using light and transmission electron microscopy, we investigated the entire female reproductive system of *Pleioplana atomata*, a locally abundant intertidal polyclad in the Northeast United States. Additionally, we were interested in the phylogenetic utility of morphological data of the female reproductive system.

MATERIALS AND METHODS

Adult specimens of *Pleioplana atomata* (Müller, 1776) Faubel 1983 were collected at Odiorne Point State Park Beach in New Hampshire, USA (N 43° 02.395′; W 70° 42.899′) from the intertidal zone during early spring 2006. Animals were fixed in 2.5% glutaraldehyde in PBS for 24 h at room temperature and postfixed for 12 h in 1% OsO_4. After dehydration in a graded ethanol series followed by a final dehydration step in propylene oxide, the specimens were embedded in EMbed 812. Semi-thin sections were stained with epoxy tissue stain and observed using light microscopy. Thin sections were contrasted with uranyl acetate and lead citrate and examined with a LEO 822 omega transmission electron microscope.

RESULTS

The female reproductive system of *Pleioplana atomata* consists of numerous ovaries distributed throughout the entire body, but concentrated along the dorsal side.

Each ovary possesses a small, delicate oviduct. Oviducts merge and connect to one of paired, wid-

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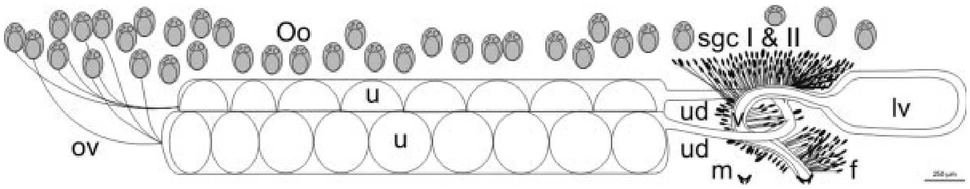


Fig. 1. Schematic reconstruction of the female reproductive system of *Pleioplana atomata*. Reconstruction from 1 μm thick serial sagittal sections. f, female atrium; lv, Lang's vesicle; m, male atrium; Oo, ovaries; ov, oviduct; sgc I & II, shell gland cell types I & II; u, uterus; ud, uterine ducts; v, vagina.

ened segments called the uteri. The uteri begin near the anterior part of the pharynx and extend on either side of the pharynx toward the posterior end of the body. At the level where the pharynx ends, the uteri form thin uterine ducts, which eventually join each other just below the upper part of vagina and thus, forming a very short unpaired segment, which connects to the ventral side of the upper vagina. The long (~1 mm) and folded vagina connects the female atrium with a long (~1 mm), well-developed Lang's vesicle. Numerous shell glands (cement glands) of two different types empty their secretions into the vagina (see Fig. 1).

The ovaries consist of developing, previtellogenic oocytes surrounded by somatic, follicular cell(s). There is a single germinative zone mainly in the dorsal part of each ovary (Fig. 2A). The entire gonad is separated from the parenchyma by a basal lamina that is also separated from the germ cells by a thin layer formed by the follicular cells (Fig. 2B). Oogonia or very young, previtellogenic oocytes, as well as a few somatic (prefollicular) cells, are present in the germinative zone of an ovary. Young germ cells have a large, round nucleus (12 μm in diameter) surrounded by a relatively thin rim of electron-lucent cytoplasm. Small mitochondria (0.3 μm) are located in proximity of the nuclear envelope. These cells are enwrapped by follicular cells that contain more electron-opaque cytoplasm (Fig. 2C). The central and ventral parts of the ovary contain the growing previtellogenic oocytes. Their diameter is about 200 μm, and the nuclei (50 μm in diameter) contain a large (10 μm in diameter) nucleoli (Fig. 2A). The electron-lucent nucleoplasm surrounds the large, grainy electron-dense nucleolus (Fig. 2D). The cytoplasm of these previtellogenic oocytes is more electron opaque, with numerous mitochondria, free ribosome, and a well-developed ER system forming large, round structures (Fig. 2D, E). Large globules of different electron opacity appear in the cytoplasm (Fig. 2A, E–G). The growing oocytes are surrounded by somatic cells with a flattened nucleus and electron-dense cytoplasm. The nuclei of these cells are located between the oocytes and a basal lamina, and they possess numerous condensed chromatin. The cytoplasm of these cells is more electron-dense than the cytoplasm of the oocytes and is filled with free ribosomes, rER, mitochondria, and small vesicles (0.5 μm in diameter) containing electron-dense material (Fig. 2G, H).

The growing vitellogenic oocytes pass through the oviducts to the paired uteri, where they are stored and where vitellogenesis is completed. During this process, the oocytes are growing, and they are densely packed into the uteri. Their nuclei with large nucleoli are centrally located in the maturing cell. Electron-opaque globules are located in the cortical area of the cells, whereas densely packed yolk granules (Fig. 3A) fill most of the central part of the cell. The cytoplasm also contains rER, in addition to numerous free ribosomes. The walls of the uteri are very thin (0.2 μm) and are composed of highly flattened cells. The nuclei (7 μm in length) of these cells are flattened as well and are filled with large amounts of heterochromatin (Fig. 3B).

The long, paired uterine ducts begin on the posterio-dorsal part of the uteri and extend toward the posterior part of the body. They join and form a very short unpaired segment that enters the ventral side of the vagina close to Lang's vesicle. The ducts are about 50 μm in diameter and their walls consist of two parts. The outer part is a multilayered (about 5 layers) muscular cover. The alternating layers of muscle fibers run longitudinally or circularly around the duct. The inner part is separated from the muscular layer by a basal lamina and consists of highly polarized cells. The basal part of these cells is greatly folded and contains ribosome-rich cytoplasm and numerous mitochondria. The nuclei occupy the central parts of the cells. They are large and rounded with large amounts of heterochromatin. The central and apical parts of these cells contain electron-dense cytoplasm and numerous vesicles with electron-opaque material. The sizes of the vesicles vary (0.3–1 μm) and their content is probably released into the duct lumen (Fig. 3C). The apical plasmalemma of these cells forms numerous microvilli directed into the lumen of the duct. Connections between cells in their apical parts are fortified by well-developed desmosomes. The lumen of the resting duct is

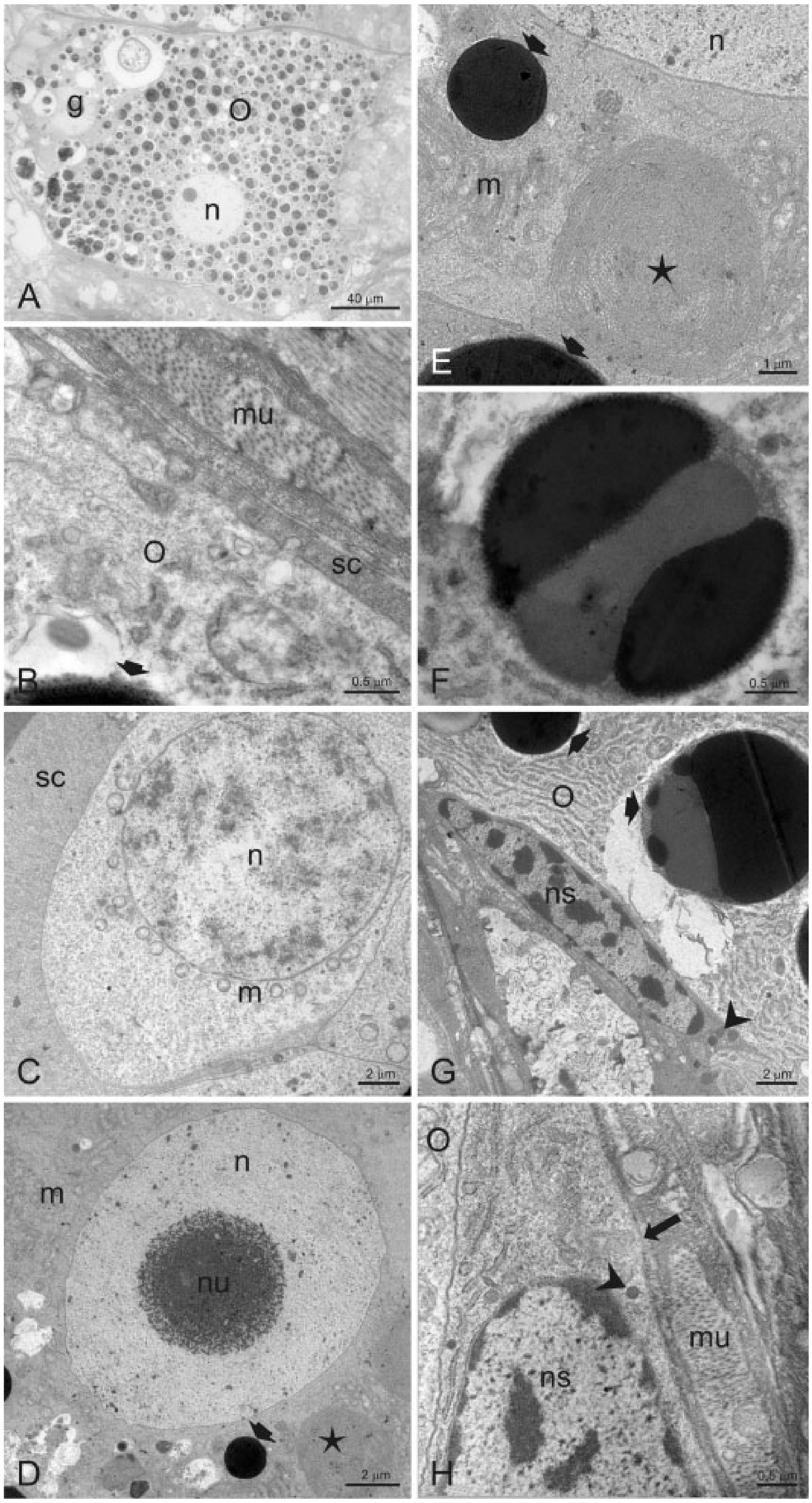
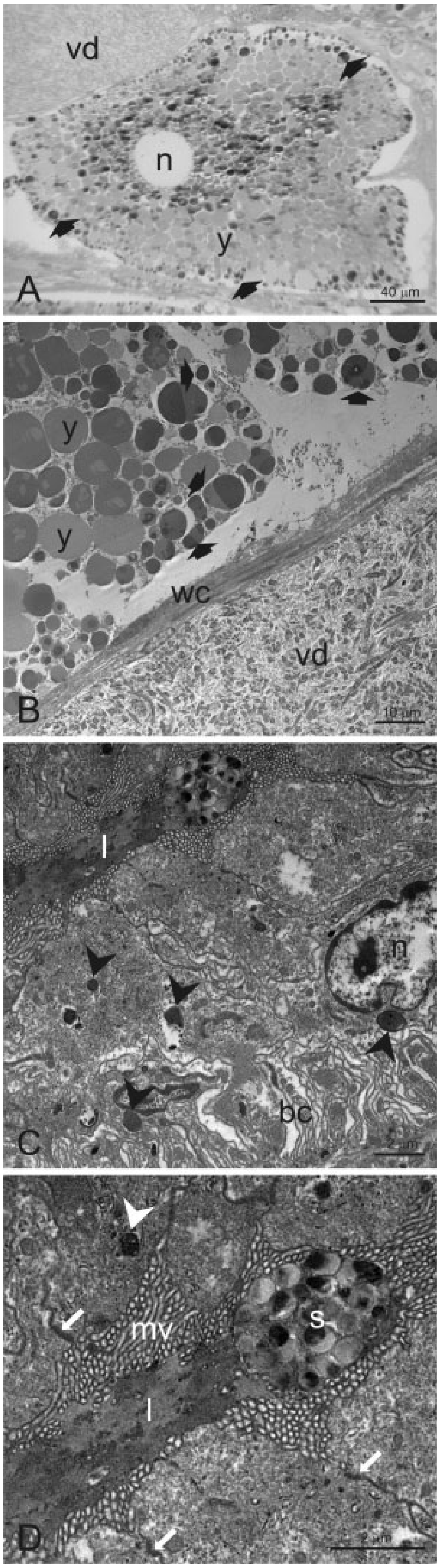


Fig. 2. *Pleioplana atomata*, **A:** Photomicrograph of a cross section through the ovary. **B:** Periphery of the ovary. **C:** Previtellogenic oocyte. **D:** Nucleus of previtellogenic oocyte. **E:** Details of previtellogenic oocyte. **F:** Cortical granule of differing electron opacity. **G, H:** Details of ovarian wall. g, germinative zone of ovary; m, mitochondria; mu, muscle; n, nucleus; ns, nucleus of somatic cell; nu, nucleolus; o, previtellogenic oocyte filled by cortical granules; sc, somatic cell; arrowhead, vesicles with electron-dense material in somatic cell, long arrow, basal lamina; short arrow, cortical granule; star, round structures of ER.



shrunken and filled by electron-dense material of different opacity, as well as vesicle-like structures filled with smaller, electron-dense vesicles. The latter structures look similar to secretions of one of the shell glands (type I; Fig. 3D).

The vagina is a long s-shaped duct that connects Lang's vesicle to the female genital pore. Lang's vesicle is a large structure located posterior to the female genital pore. Its wall is thick (50 µm) and consists of numerous, elongated cylinder-shaped gland cells. The basal part of these cells rests on a basal lamina (Fig. 4A). Their cytoplasm is electron-opaque and filled with free ribosomes, mitochondria, rER, and vesicles filled with electron-dense material (0.5 to 5 µm in diameter). Nuclei (10 µm in diameter) are oval and usually contain large nucleoli (Fig. 4B). The apical parts of these cells possess numerous microvilli that are directed into the lumen of the organ. The apical connections between these cells are fortified by well-developed desmosomes (Fig. 4C).

The vagina is a long, curved duct with a thick muscular cover. This cover (~2 µm thick) consists of a few muscle layers that surround the duct longitudinally and circularly. The remainder of the wall is built of a single cell layer, separated from the muscular layer by a basal lamina (Fig. 4D). The cytoplasm of these cells is electron dense, filled with free ribosomes and rER, as well as numerous mitochondria. The cells also contain numerous vesicles of electron-opaque material. The centrally located nuclei are round (~5 µm in diameter) containing large amounts of heterochromatin (Fig. 4D, E). The apical plasmalemma forms numerous microvilli directed into the vaginal lumen. The latter is filled by electron-opaque material in which vesicles (Fig. 4F) and striated oval structures (Fig. 4F inset) are submerged. Numerous shell gland cells (cement glands) release their secretions into the lumen of vagina (Fig. 4G). We observed two different types of shell gland cells, which are scattered throughout the parenchyma surrounding the area of the vagina and Lang's vesicle. The shell gland cells of type I possess rounded nuclei (7 µm in diameter) with distinct nucleoli, cytoplasm filled by free ribosomes and a large number of vesicles (0.5–2 µm in diameter) filled by material of differing electron density. The shell gland cells of type II have an electron-lucent cyto-

Fig. 3. *Pleioplana atomata*, **A:** Photomicrograph of a cross section though a vitellogenic oocyte in the uterus. **B:** Details of the periphery of a vitellogenic oocyte and the uterine wall. **C, D:** Details of uterine duct. bc, basal, folded part of cells; l, lumen of the duct; mu, muscle; mv, microvilli; n, nucleus; s, secretion of shell glands; vd, vas deferens with sperm; wc, uterine wall cells; y, yolk granules; arrowheads, vesicles with electron-dense material in uterine duct cells; long arrow, desmosome; short arrow, cortical granule.

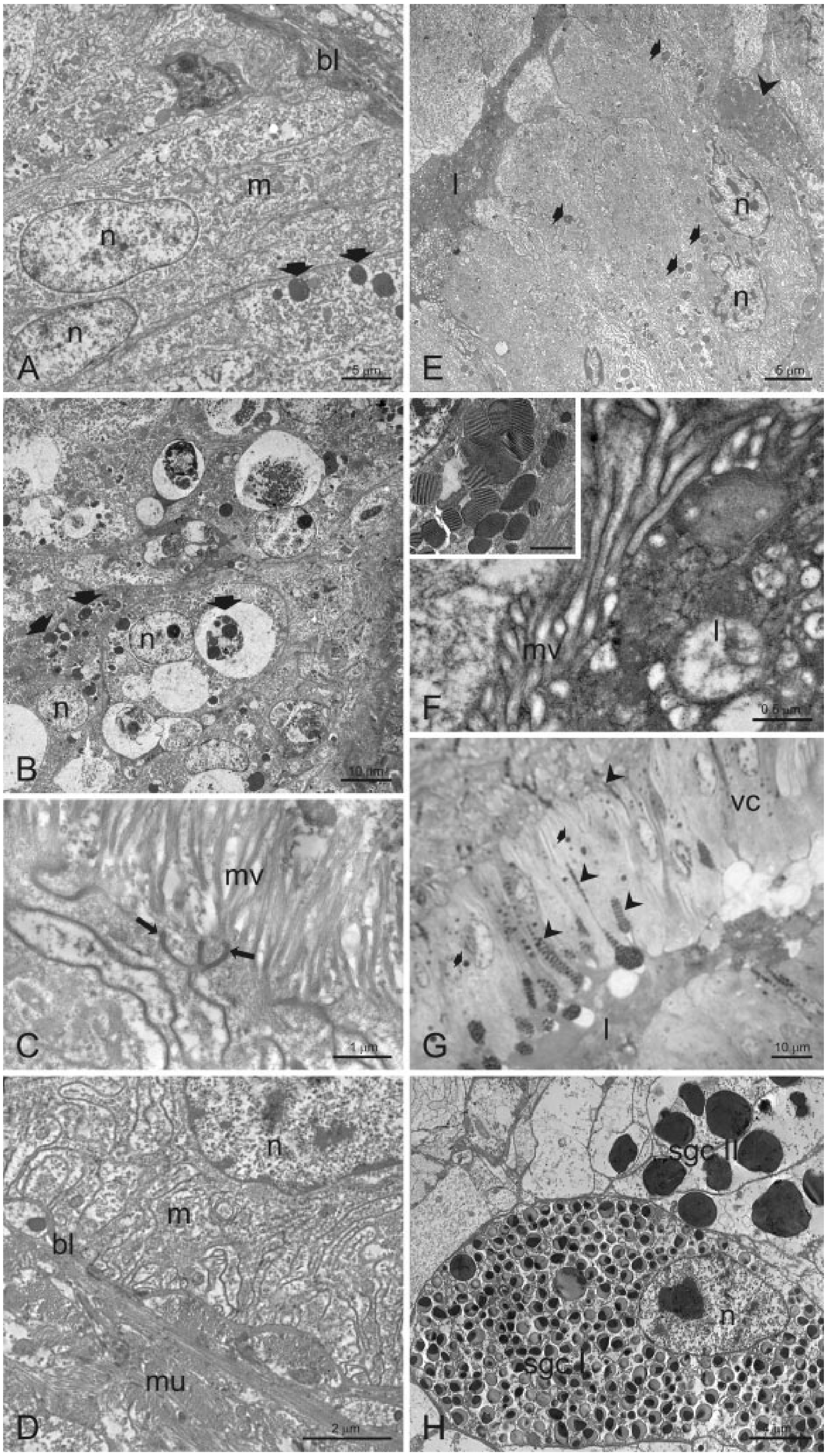


Fig. 4. *Pleioplana atomata*, **A:** Cross section through basal part of Lang's vesicle wall cell. **B:** Transverse section through the central part of a Lang's vesicle cell. **C:** Cross section through apical part of a Lang's vesicle cell. **D:** Basal part of a vaginal cell and surrounding muscles. **E:** Cross section through vagina. **F:** Details of the apical part of a vaginal wall cell and vaginal lumen. Inset: striated oval structures in vagina lumen (scale bar: 2 μm). **G:** Photomicrograph of a cross section trough vagina. **H:** Cross section through shell gland cells with two different types of secretions. bl, basal lamina; l, vagina lumen; m, mitochondria; mu, muscles; mv, microvilli; n, nucleus; sgc I, shell gland cell type I; sgc II, shell gland cell type II; vc, vagina cells; arrowhead, shell gland canal; long arrow, desmosomes; short arrow, vesicles with electron-dense material.

plasm filled by large (3–5 μm in diameter) vesicles with electron-dense homogenous material (Fig. 4H). In the gland canals and the vaginal lumen, the material inside the vesicles of shell gland type II becomes striated. Each gland cell has a separated canal that pierces though the vaginal wall and releases its secretions directly into the vaginal lumen (Fig. 4G). The vagina ends in the female atrium, which leads to the female gonopore.

DISCUSSION

The female reproductive system of *Pleioplana atomata* consists of three main parts: gonads, canal system, and accessory glands. The numerous, widespread female gonads are of the saccular and follicular type (Rieger et al., 1991). Two levels of female gonad organization are recognized in Platyhelminthes, namely Archoophora and Neoophora (Hyman, 1951; Gremigni, 1983, 1988). The archoophoran type is considered the plesiomorphic and is also common in many other primitive groups outside the Platyhelminthes (Karling, 1967; Gremigni, 1988). All members of this level of organization have simple female gonads, which are composed only of ovaries containing entolecithal eggs and lacking vitellaria. In contrast, the Neoophora is considered a monophyletic taxon with the heterocellular condition of the female gonad a derived, autoapomorphic character (Ehlers, 1986; Gremigni, 1988). All studied members of Neoophora have complex female gonads composed either of germovitellaria or separate germaria with oocytes and vitellaria with vitelline or yolk cells (Gremigni, 1988).

Polycladida as representatives of Archoophora, possess simple ovaries producing entolecithal eggs. Oogonia or very young oocytes usually are located in the dorsal part of the gonad, and it appears that each ovarian follicle develops from a single oogonial cell enwrapped by a follicular cell (or cells) at the beginning of oogenesis. During the early stages of oogenesis (previtellogenesis), an oocyte produces numerous electron-dense granules that are widespread throughout the entire ooplasm. During vitellogenesis, these granules move and become more regularly distributed (usually in a single row) in the cortical ooplasm. Granules-like these have been described under different names in several polyclads species: *Prostheceraeus floridanus* (cortical granules; Boyer, 1972), *Notoplana alcinoi* (eggshell globules, Domenici et al., 1975; Gremigni, 1988), and *Pseudostylochus* sp. and *Planocera multitentaculata* (shell-forming granules; Ishida et al., 1981; Ishida and Teshirogi, 1986; Ishida, 1989). Cytochemical tests revealed that at least in one species, these inclusions are composed mainly of proteins and polyphenols (Domenici et al., 1975). These compounds are the main component of most platyhelminth eggshells. Moreover, Ishida (1989) showed that in *Pseudocstylochus* sp. the contents of the shell-forming granules is discharged and will form the eggshell shortly after egg lying. Similar electron-dense globules are also present in *P. atomata*, but their ultrastructure is slightly different. Mostly they consist of an electron-opaque core surrounded by a thin rim of granular material. However, we often found globules consisting of three different types of material of different electron opacity. Because the origin and distribution of these globules are similar to that described for other species, we hypothesize that they play the same role in eggshell formation.

Comparable globules are present in the Neoophora, where they are produced by oocytes and vitelline cells and are thought to play a similar role as described earlier. According to Gremigni (1988), the ultrastructure of these globules may be used as a valuable character in phylogenetic analyses, but special precautions need to be taken. The globules must be at the same stage of ooctye development as well as the technique and staining used must be the same. Because of the fact that these globules are probably homlogous in different species and because there are distinct differences in their ultrastructure, we agree with this assessment.

Vitellogenesis in *Pleioplana atomata* begins in the ovary and continues as the oocytes move through the canal system and into the uteri. As has been described for other archoophorans, the entolecithal oocytes are producing yolk globules and lipid droplets. The archoophoran type of female gonads is considered a symplesiomorphy (Karling, 1974), and is not limited to flatworms. It is of no systematic value in defining platyhelminth relationships and today, is used in a purely descriptive fashion (Karling, 1967, 1974; Gremigni, 1988).

The ultrastructure of the thin, smooth walls of the uteri indicates that these organs function in the storage of maturing oocytes. However, the ultrastructure of the uterine ducts differs significantly. During the resting stage, the wall cells are producing electron-dense material that is probably discharged into the lumen of the ducts. Moreover, in the lumen of the distal part of these ducts, secretions of type I shell glands have been found. We did not find any gland cell canals that release their contents into this part of the female tract. Therefore, the only way that such secretions could enter the uterine ducts is via the vagina. These findings suggest that eggshells in *Pleioplana atomata* start to form early, probably in the last section of the paired uterine ducts. Ishida (1989) described eggshell formation in two polyclad species *Pseudostylochus* sp. and *Planocera multitentaculata*. In both species, the eggshell granules produced by shell gland cells were released into the vagina, where the shell gland substance covered the egg. After oviposition, the shell-forming granules release their content into the space between the oolemma and the newly formed shell gland cover. In *P. atomata*, each single egg is surrounded by the newly formed envelope (Rawlinson et al., 2008), which is similar to the condition found in *Pseudostylochus* sp. but not in *P. multitentaculata*. In this latter species, a common envelope surrounds several eggs. In *P. atomata* the process of eggshell formation appears to begin earlier than those in *Pseudostylochus* sp. and *P. multitentacuata*. According to Ishida (1989), eggshell formation is completed after oviposition when release of the shell-forming granules and tanning of the eggs occur. We suspect that a similar process takes place in *P. atomata*, although this hypothesis requires further study. *P. atomata* lays numerous batches of eggs. The number in the batch ranges from several to a few hundreds eggs, and all of them are covered by an amorphous material forming a plate-like structure. We hypothesize that it is the vaginal wall cells that produce this covering material, although this supposition needs further investigation.

The distal part of the vagina forms a special structure called Lang's vesicle. Lang's vesicle is characteristic for many acotylean polyclads, although its function is still unknown. Sperm received during copulation are often found in Lang's vesicle, but its role as a seminal receptacle has not been proven (Kato, 1940; Galleni and Gremigni, 1989). Another possible role for Lang's vesicle is its involvement in the digestion of excess sperm and prostatic secretion (Bock, 1913). This function is supported by the fact that in some representative polyclads (*Taenioplana teredini, Enterogonia pigrans, Mucroplana caelata, Asolenia deilogyna* [Faubel, 1983; Prudhoe, 1985]) the female reproductive tract is connected to the intestine. This connection may indicate that Lang's vesicle is indeed involved in the digestion of sperm and/or seminal fluid. The wall cells of Lang's vesicle in *Pleioplana atomata* clearly have a secretory function; thus, we favor the digestive role of this organ.

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