Journal of Microscopy and Ultrastructure 4 (2016) 184–194

Contents lists available at ScienceDirect

Journal of Microscopy and Ultrastructure

journal homepage: www.elsevier.com/locate/jmau

Original Article

Ultrastructure of ovotestis of young and adult pulmonate mollusk, *Macrochlamys indica* Benson, 1832

Soumen Roy[a], Korak Kanti Chaki[a], Tapas Chandra Nag[b], Kamales Kumar Misra[c,*]

[a] *Department of Zoology, City College, Kolkata, India*
[b] *Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India*
[c] *Department of Zoology, RBC College, Naihati, West Bengal, India*

ARTICLE INFO

Article history:
Received 3 January 2015
Received in revised form 27 February 2016
Accepted 5 March 2016
Available online 15 March 2016

Keywords:
acinar boundary
interstitial cell
periacinar cell
protandrous
Sertoli cell

ABSTRACT

Macrochlamys indica is a hermaphrodite terrestrial pulmonate mollusk. Transmission electron microscope studies were done on the ovotestis of young and adult (older) *M. indica* which are elaborated in this paper.

The ovotestis contains numerous lobes each of which contains many ovoid shaped acini which are occupied by stages of spermatogenesis and a single oocyte. In younger snails, the acini contain stages of developing spermatogenesis, whereas each acinus of older snails is composed of single large oocyte and few stags of spermatogenesis. The number of Sertoli cells is high in the acini of younger snails than in older snails. Details of the cellular organization of the Sertoli cell are described. Some long thin threads extend from the acinar boundary to acinar lumen. The anterior end of these threads is either free or directly connected to the developing cells of spermatogenesis. There are two types of cells in the interacinar space of the ovotestis in both younger and older snails. One cell is small oval interstitial cell and other is thin elongated periacinar cell. The acinar boundary contains secretory cells with deeply stained nucleus. In the acinus of older snails, the Sertoli cells do not form any barrier between oocyte and spermatogenic cells.

Functions of the periacinar cell and interacinar cell are discussed. It is found that the spermatogenesis is highly active in younger snails with single dormant oocyte while process of oogenesis predominates in the older snails. The reproductive strategy of this pulmonate species thus depends on the individual's body size and their maturity.

1. Introduction

Pulmonates are hermaphrodites and the reproductive organ, the ovotestis is embedded within the digestive gland. The ovotestis consists of lobes; each of which contains numerous ovoid shaped acini. Interacinar spaces are filled with cellular components of loose connective tissues, and blood vessels. The hermaphroditic duct is formed by the fusion of several acinar ducts into which acini open [1].

The ovotestis of hermaphroditic mollusks is the source of both oocytes and sperms [2–5]. The sperm cells differentiate and mature through sequential stages of spermatogenesis. In each acinus, mainly four types of cells are found: male and female germ cells, Sertoli cells, and follicle cells [1,5]. Among several other factors, body size has an important influence on the type and numbers of gametes produced in several hermaphroditic snail species [6–8]. The small-body snails act as males and larger ones as females, and even in copulation, body size determines the

* Corresponding author at: 156/84 Banerjee Para Road, Behala, Kolkata 700060, India.
E-mail address: misrakk@vsnl.com (K.K. Misra).

http://dx.doi.org/10.1016/j.jmau.2016.03.001
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sex identity of the partners [6]. It was observed that there are differences in body sizes between potential mates and male/female differentiation [9–11]. It was established that in the early part of life, hermaphroditic snails act as male and simultaneous female for later periods of life [12–18]. It was reported that the albumen gland is a diagnostic marker of male/female distinction in hermaphroditic snails [19]. The male snails have small albumen glands whereas female snails have large albumen glands [20] because the albumen gland provides nutrition to the developing oocytes.

There is controversy as to whether each acinus contains only one oocyte or more than one. Previous studies on *Lymnea stagnalis* reported the occurrence of more than one oocyte in an acinus [21–23], whereas only one oocyte was observed in each acinus of the ovotestis of *Achatina fulica* [5]. Thus, the aims and objectives of the present study on *Macrochlamys indica*, a terrestrial pulmonate mollusc, were: (1) to describe the microanatomical structure of acini between younger and older snails to clarify the male/female distinction; (2) to find the location and arrangement of male and female germ cells in an acinus of the ovotestis; (3) to confirm the number of oocytes per acinus; (4) to characterize the different developmental stages of spermatogenic cells and Sertoli cells; (5) to describe the nature of compartmentation between male and female gametes; and (6) to establish the presence of interstitial and periacinar cells in the interacinar space. The hypothesis of the present work is that younger snails perform only male gametogenesis, while oogenesis predominates in older snails, as was physiologically advocated earlier [19].

2. Materials and methods

2.1. Sampling and rearing of experimental animals

Healthy active *M. indica* were collected from fields during the rainy season (Figure S1; https://en.wikipedia.org).

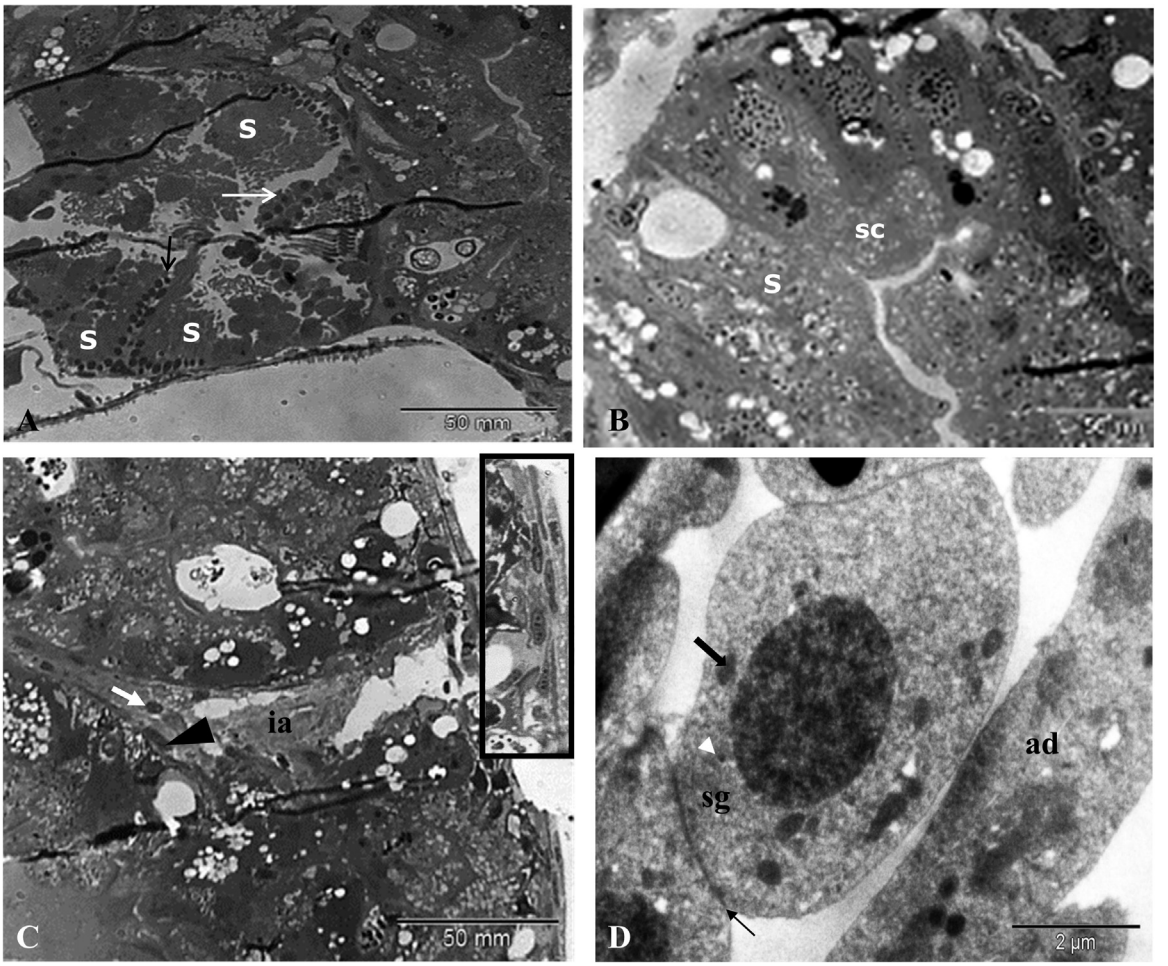

Fig. 1. Semithin section of ovotestis of younger *Macrochlamys indica*. (A) An acinar portion showing various developing stages (white arrow) of only spermatogenesis. The Sertoli cell pockets separate different forms of spermatogenic development (black arrow). (B) Elongated columnar Sertoli cells are lined on the acinar inner wall and spermatocyte is located between the Sertoli cells. (C) Oval interstitial cells (white arrow) at interacinar zone and thin periacinar cells (arrow head). Enlarged view of an acinar portion showing outer boundary wall and many periacinar cells are in box. (D) Transmission electron micrograph of ovotestis of younger snails, showing typical spermatogonial cells with numerous mitochondria (bold arrow), membrane-bound vesicle (arrow head) and junctions of adjacent cells (thin arrow)
ad = acinar boundary; ia = interacinar zone; S = Sertoli cell; sc = spermatocyte; sg = spermatogonia.

The pulmonates aestivate for 8–9 months and become active at the onset of the rainy season, so these snails are available only in the rainy season. A group of 20 younger (1.1–1.2 cm in shell diameter with small albumen gland) and 20 older (2.1–2.2 cm in diameter of shell with large albumen gland) specimens were selected for the study. They were acclimatized in separate cages. The specimens were provided with leafy vegetables and water was sprayed regularly to maintain humid ambience.

2.2. Histological analysis

Small samples of ovotestis were fixed in aqueous Bouin's solution (12 hours), dehydrated, embedded in paraffin, and 5-μm-thick sections were cut. These sections were stained with hematoxylin and eosin. Sections were observed under a light microscope for examining cellular parameters. The stage micrometer (0.01 mm; Erma, Tokyo, Japan) and ocular micrometer (1 ocular division = 4.35 μm in 40× magnification) were used to measure cellular parameters.

2.3. Semithin sections and TEM preparation

For ultrastructural studies, ovotestes of active *M. indica* were dissected out and fixed in a mixture of 3% glutaraldehyde and 2% paraformaldehyde for 4 hours at 4 °C in 0.1 M phosphate buffer (pH 7.2). After dehydration in acetone, the tissues were embedded in Araldite CY 212. Semithin sections (1 μm) were stained in 0.25% toluidene blue [24]. Thin sections (60–70 nm) were stained with uranyl acetate (0.5%) and lead citrate (0.5%) and examined in a Morgagni 268D electron microscope operated at 80 kV (Fei, Eindhoven, The Netherlands) and Technai G^2 electron microscopes operated at 120 kV.

3. Results

3.1. Structures of ovotestis

The ovotestis of *M. indica* is composed of many lobes; each of which contains numerous acini. The acini are ovoid to semicircular in shape (Figure 1A). The acini have a homogeneous matrix and a lumen at their center. The acinus of

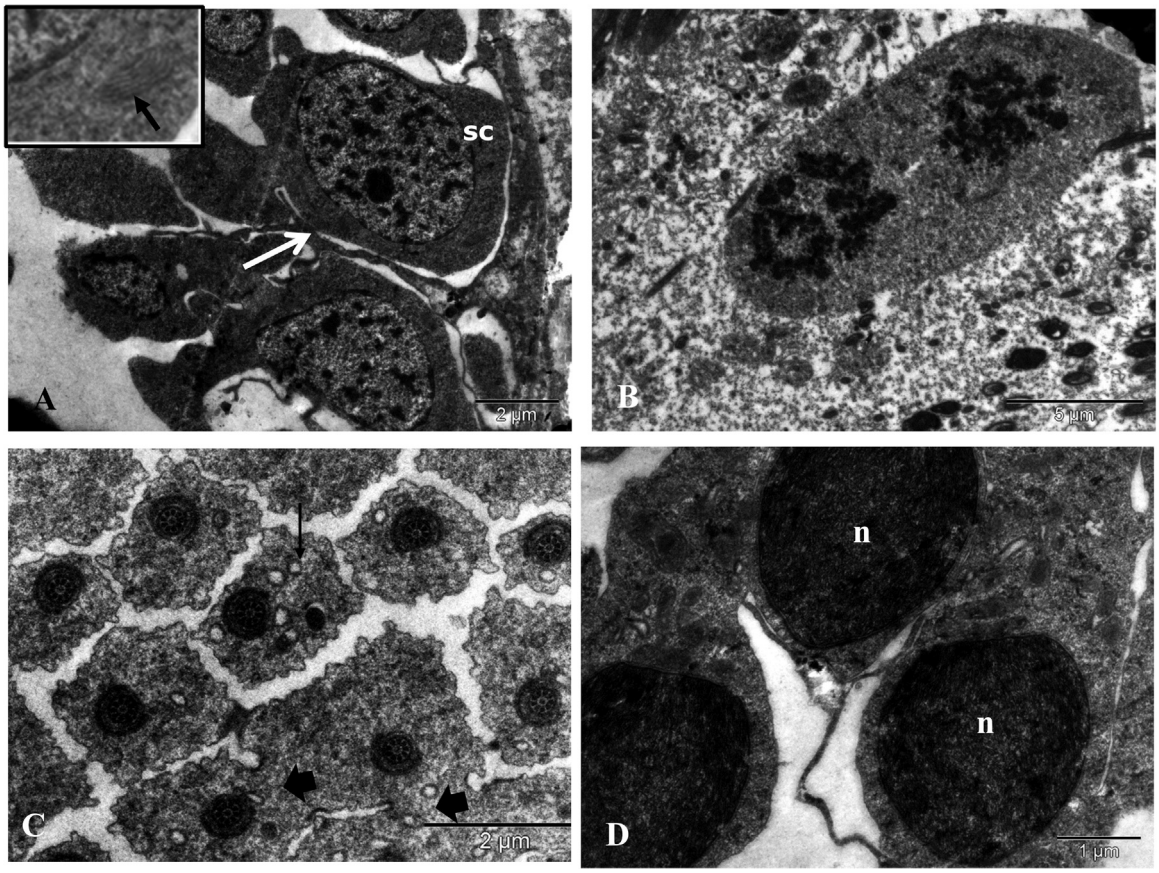

Fig. 2. Transmission electron microscopy of ovotestis of younger snails. (A) Developing spermatocytes possess cytoplasmic connection between adjacent cells (white arrow) with numerous mitochondria and stack of Golgi bodies. A magnified view of the Golgi stack (arrow) in spermatocyte is shown in box. (B) Spermatocyte showing nuclear division. (C) Developing spermatid showing finger like projections around the cell membrane with some syncytial cytoplasmic bridge (bold arrow). The cytoplasm consists of Golgi vesicles (thin black arrow). (D) Magnified view of some developing spermatids head. n = nucleus; sc = spermatocyte.

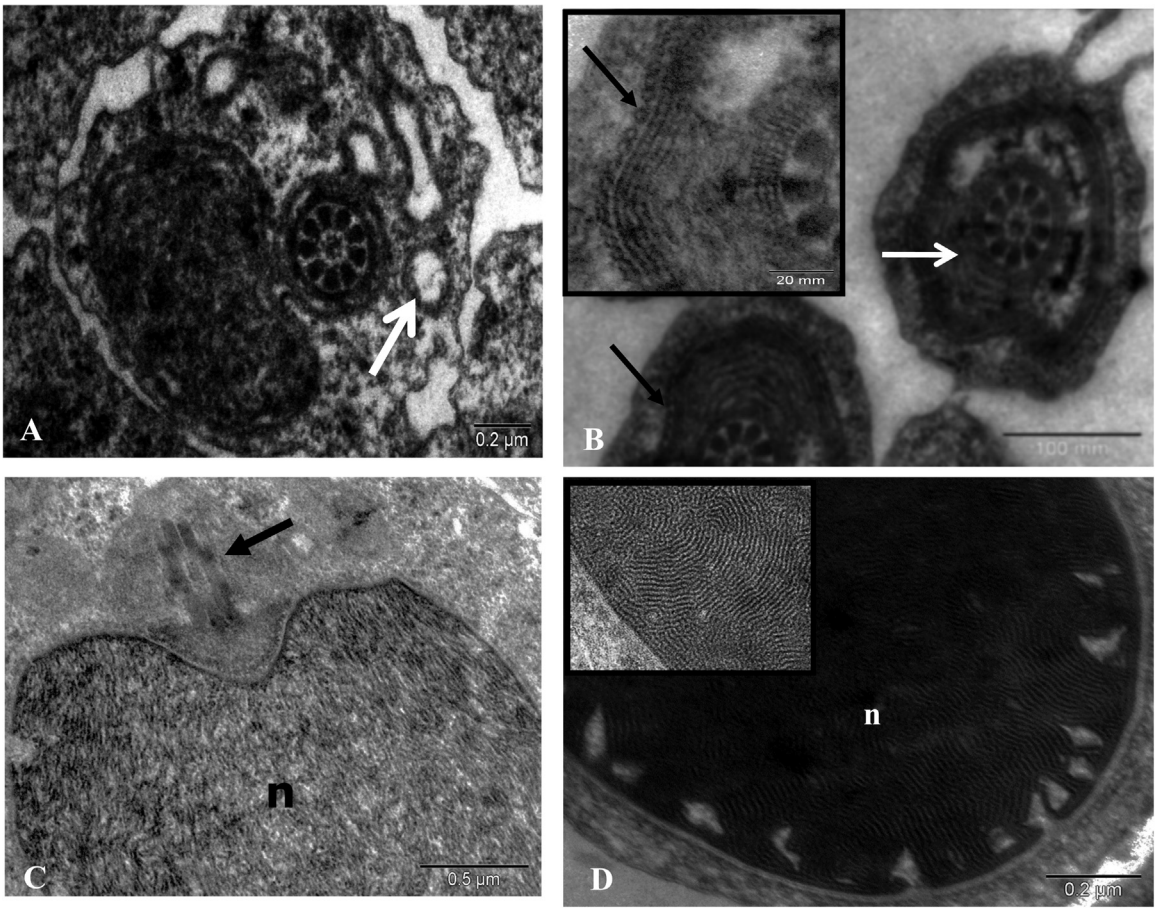

Fig. 3. Ovotestis of younger snails. (A) A more developed form of spermatid showing 9+2 microtubular arrangement in tail with modified nucleus and Golgi vesicles (white arrow). (B) The central canal of spermatid tail is surrounded by two sets of circular layers and is separated by little cytoplasm. The outer one is covered by an electron-lucent beaded layer (black arrow). A portion between two sets of layers is filled by fibrous structure (white arrow). (C) Tail bud (arrow) is formed from the posterior end of the nucleus. (D) Nuclear condensation of spermatid showing the specific patterns of its arrangement. Magnified view of a portion of spermatid nucleus showing the pattern of chromatin material in box.
n = nucleus.

the younger snails contains only distinct different stages of spermatogenesis (Figure 1A). In older snails, each acinus contains one large oocyte and a few cells of spermatogenesis.

3.2. Ovotestis of younger snails

3.2.1. Spermatogenesis

The acini with an average size of 298.71 μm × 240.69 μm are densely packed with different differentiating stages of spermatogenesis (Figure 1A–C).

Spermatogonial cells are round with a prominent centrally placed circular nucleus (Figure 1D). A prominent cell junction is seen between adjacent cells. The nucleus contains homogeneous nuclear materials. The cytoplasm of these cells consists of mitochondria and membrane-bound vesicles (Figure 1D). The developing spermatocytes are large, 9.8 ± 1.1 μm in diameter and irregular in shape, and contain large nuclei (Figure 2A). The chromatin materials are dispersed throughout the nucleoplasm. Karyokinesis is observed in primary spermatocytes (Figure 2B). Spermatids are usually developed near the acinar lumen. These cells having numerous finger-like projections around the cell membrane (Figure 2C). The nuclei of spermatids are more condensed than spermatocytes and arranged in an anteroposterior axis (Figures 2D and 3A–D). An acrosomal cap was found at the anterior end of the spermatid nucleus (Figure 4A). The initiation of tail formation was found to form the posterior end of the nucleus (Figure 3C). The microtubules in the tail were surrounded with two sets of fibrous canals (Figure 3B). First, the inner canal was composed of three to four circular layers. These layers were connected to each other by several vertical short arms. Second, the outer canal was also composed of three to four circular layers and was surrounded by a prominent peripheral beaded layer, just beneath the cell membrane. The cytoplasm of the spermatids contained Golgi vesicles, few lysosomes and rough endoplasmic reticulum (RER; Figure 2C). A prominent cytoplasmic bridge, syncytial in nature, was found between adjacent developing spermatids (Figure 2C). The matured sperm measured 105.12 ± 10 μm in length and were inserted into Sertoli cells by their head (8.7 μm × 2.2 μm in size). We also observed that a batch of developing cells at different stages of

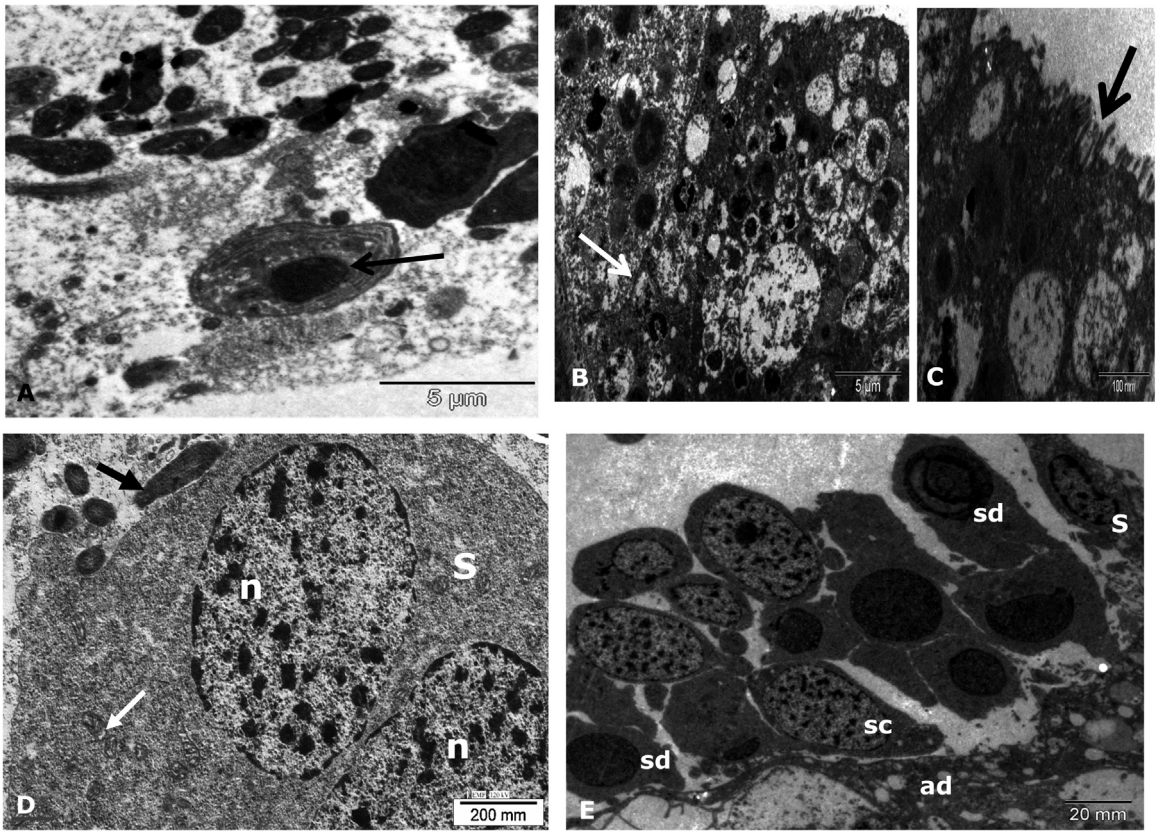

Fig. 4. Ovotestis of younger snails. (A) Acrosomal cap at the anterior end (arrow) of the nucleus in a developing spermatid. (B) Columnar Sertoli cells showing their cellular junction (white arrow). (C) The numerous microvilli at the anterior tip of Sertoli cells. (D) Sertoli cells showing their dividing stage with karyokinesis. The spermatid (black arrow) is inserted into the membrane of the Sertoli cell. White arrow indicates mitochondria. (E) Acinar portion showing acinar boundary, spermatocytes, spermatid and Sertoli cell.
ad = acinar boundary; n = nucleus S = Sertoli cell; sc = spermatocyte; sd = spermatid.

spermatogenesis was separated from each other by Sertoli cell pockets (Figure 1A).

3.2.2. Sertoli cells

The acinar space of the ovotestis was occupied by elongated, columnar Sertoli cells (46.8 µm ×25.56 µm) with nuclei (diameter: 14.49 µm; Figure 1B). All acini had numerous Sertoli cells that spanned the basement membrane to the acinar center and contained microvilli at their anterior end (Figures 4B and 4C). The intimate associations between Sertoli cells divided the acinar space into some pockets or incomplete compartments. The different stages of developing and dividing male germ cells were contained in Sertoli cell pockets, whose openings were directed towards the acinar center (Figure 1A). In the developed acini, Sertoli cells were of varying sizes, and most of them measured 40.59 µm × 30.45 µm but had no definite shape (Figure 1C). The cytoplasm of the Sertoli cells was packed with both electron-dense and -lucent granules, mitochondria and membrane-bound vacuoles of different sizes (Figures 4B and 4C). In some acini, Sertoli cells showed nuclear division (Figure 4D). Tight junctions between Sertoli cells and Sertoli and germ cells were prominent (Figures 2A, 4B and 4D).

3.2.3. Oogenesis

No female gametes were found in an acinus (Figures 1A and 1C).

3.2.4. Acinar boundary

In the ovotestis, each acinus was surrounded by a membrane-bound acinar boundary (Figures 2A, 4E , and 5A). The boundary of the acinus was composed of nutritive cells with elongated, deeply stained nuclei (Figures 5B–D). The nutritive cells were secretory in nature and packed with abundant mitochondria and RER (Figure 5D). Their secretions were released into the acinar lumens as small, drop-like, membrane-bound secretory vesicles (Figure 5C). Several membrane-bound thin threads were elevated from the acinar boundary to the lumen. These threads were either free or usually connected to the cell membrane of developing spermatogenic cells (Figure 5A). Numerous mitochondria were located at that secretory area of the cell.

3.2.5. Interacinar components

The interacinar space was filled with connective tissues, loose cells, and blood vessels. The space contained two types of cells. One type was small and oval (10.87 µm × 7.97 µm), and interstitial cells were dispersed throughout the interacinar spaces (Figure 5B). The cytoplasm of the

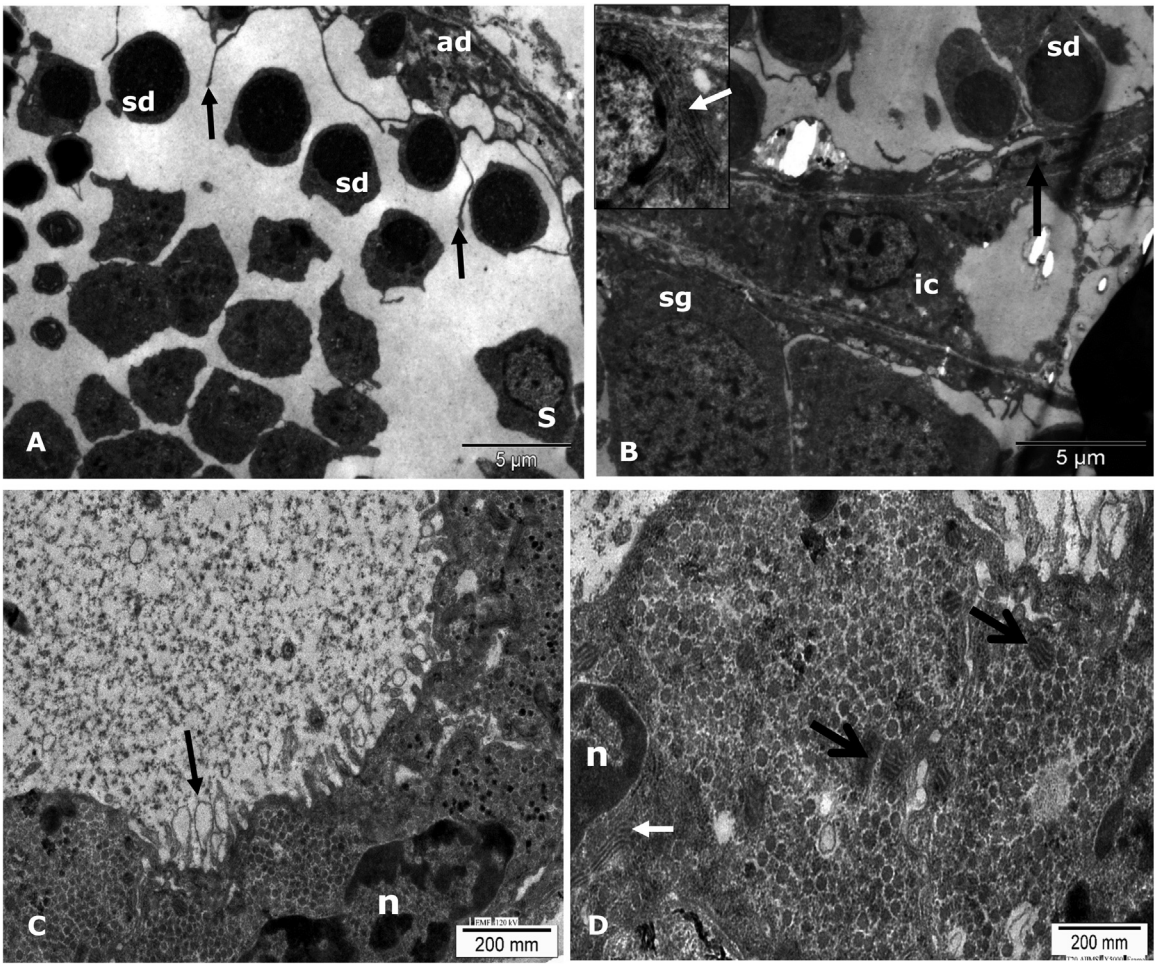

Fig. 5. Ovotestis of younger snails. (A) Portion of acinar space showing spermatids connected to thin threads (arrow) of acinar boundary. (B) Interacinar space between two acinar boundaries showing interstitial cell. Magnified view of the cytoplasmic part of an interstitial cell showing the RER (white arrow) is shown in box. A prominent elongated nutritive cell is shown in the acinar boundary (black arrow). (C) Acinar boundary with nutritive cell. Numerous membrane-bound secretory vesicles (arrow) are secreted into the acinar lumen. (D) Higher magnification of a portion of C, showing the secretory region packed with numerous mitochondria (black arrow) and RER (white arrow).
ad = acinar boundary; ic = interstitial cell; n = nucleus; RER = rough endoplasmic reticulum; S = Sertoli cell; sd = spermatid; sg = spermatogonia.

interstitial cells was composed of prominent RER as well as electron-dense secretory granules (Figure 5B). The second type was thin, comparatively elongated (11.57 μm × 5.07 μm), periacinar cells. The periacinar cells were closely attached to the acinar outer boundary wall. The junction between the periacinar cells and acinar outer walls was fibrous in nature (Figures 6A–E).

3.3. Ovotestis of older snails

3.3.1. Spermatogenesis

The acini of older snails were composed of few spermatogenic cells (Figures 7A–B). Like younger snails, the structure of spermatogenic cells was almost the same in the acini of older snails. The distribution of spermatogonial cells in the acini of older snails is comparatively lower than those of the other developing stages of spermatogenesis (Figure 7C).

3.3.2. Sertoli cells

Few small Sertoli cells were found in the acini. The cells varied in shape and size, and were located throughout the acinar space. They were fewer in number than those of younger snails. The Sertoli cells had no distinct membranous digitations around the cell membrane (Figure 7D).

3.3.3. Oogenesis

Each acinus contained only one peripherally located well-developed oocyte; one end of which was attached to the acinar inner boundary wall, and its free end was directed towards the lumen (Figures 7A–B). The oocytes were large (60.9 μm × 31.89 μm) and elliptical in shape. A distinct follicular cell layer surrounded the oocytes and separated the oocytes from the rest of the acinar components (Figures 7A, 8A and 8B). The follicular cells were small, oval, and flat with prominent nucleus. The ooplasm contained a prominent nucleus (diameter: 23.19 μm) with

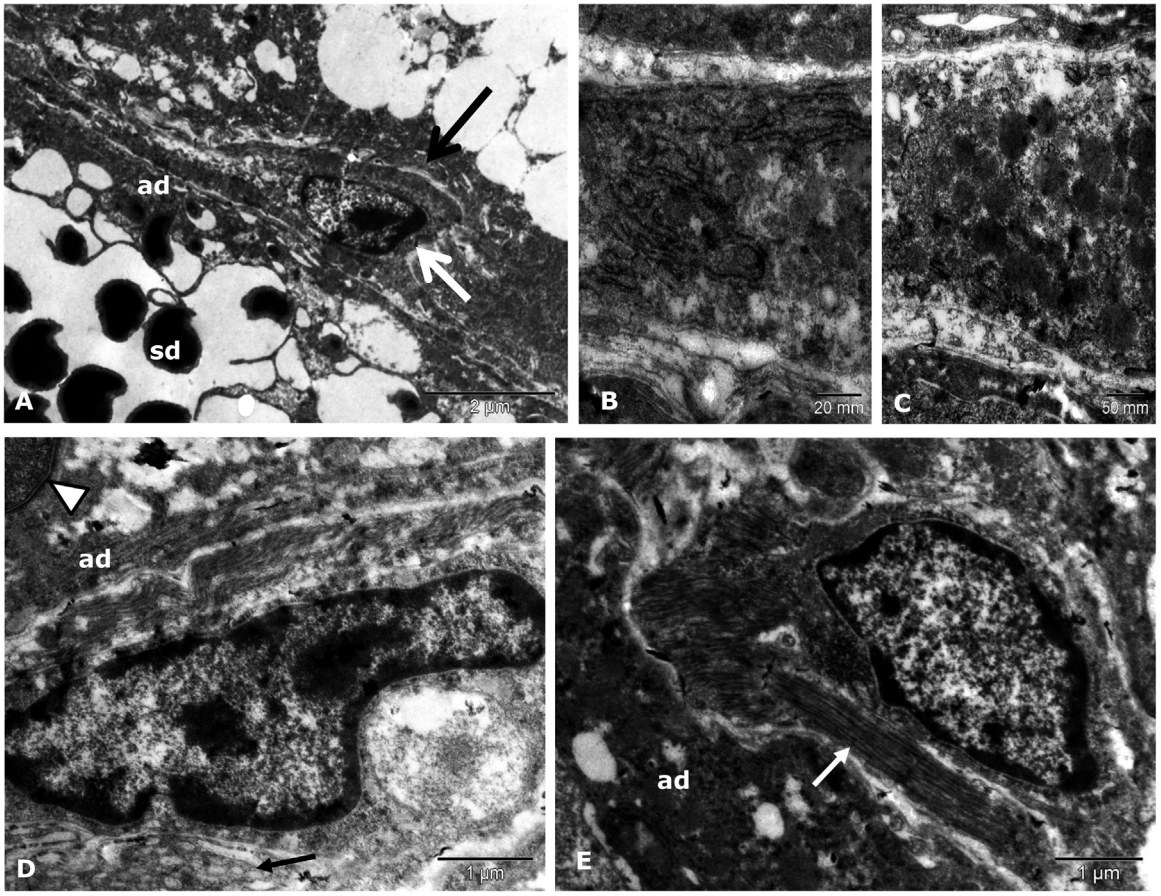

Fig. 6. Ovotestis of younger snails. (A) Thin elongated periacinar cell (white arrow), located close to the acinar outer wall (black arrow). The tip of the threads is either free or connected to the developing spermatogenic cells. (B) Enlarged view of interacinar portion showing acinar boundary and RER. (C) A part of interacinar space showing membrane-bound vesicles. (D) Cytoplasm of periacinar cell possess RER stack (arrow) and membrane-bound secretory vesicles. Note the fibrous junction between periacinar cell and acinar boundary. Arrow head indicates a portion of nucleus of a spermatogenic cell. (E) Associations of periacinar cell and its surroundings with fibrous structures (white arrow).
ad = acinar boundary; RER = rough endoplasmic reticulum; sd = spermatid.

a small round nucleolus and was filled with electron-lucent and electron-dense yolk granules, as well as lipid droplets of various sizes. These granules were distributed throughout the ooplasm in a mosaic manner. Numerous large lipid droplets, membrane-bound vesicles and mitochondria were present in the ooplasm (Figures 8A–C). The nuclear membranes had prominent nuclear pores. The nucleoplasm contained scattered chromatin materials (Figure 8D).

3.3.4. Acinar boundary

Unlike younger snails, the thread like extensions of the acinar boundary in older snails were either small or absent (Figure 7C).

3.3.5. Interacinar component

The interacinar space was occupied by oval interstitial cells (9.43 μm × 3.62 μm in size) and periacinar cells (7.97 μm × 3.62 μm in size) that were also closely adhered to the acinar outer boundary wall as in younger snails (Figure 7B).

4. Discussion

Pulmonates are usually protandrous hermaphrodites. There is a positive relationship between body size and production of male–female gametes [6]. The smaller younger snails produce mainly male germ cells, while larger, older snails produce oocytes simultaneously with spermatogenic cells in *Helisoma trivolvis* [6,7]. It was stated that the older individuals act as females and younger individuals act as males, and the body size is related to the production of female ova [6]. Similarly, in *M. indica*, there is an apparent relationship between body size and production of different types of gametes that corroborates the above findings.

It is reported that, the acinus of hermaphroditic snails (*Papillifera papillaris*) is subdivided into peripheral and central zones [25]. In the younger *M. indica*, all zones of each acinus are filled with only developing spermatogenic cells and Sertoli cells. However, in older snails, a part of the peripheral zone contains only one oocyte, whereas the rest of this zone as well as the central zone of each acinus are filled with some spermatogenic and Sertoli cells.

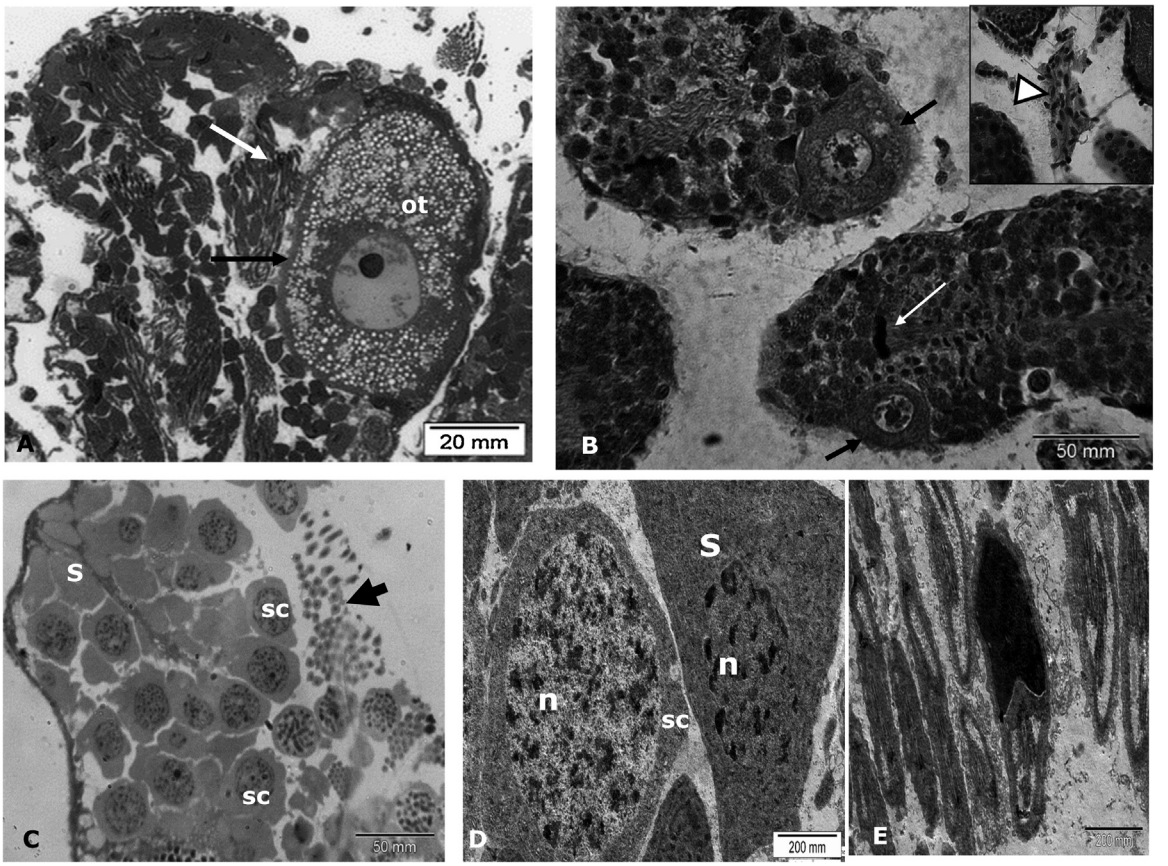

Fig. 7. Semithin section of ovotestis of older *Macrochlamys indica*. (A) Large oocyte is surrounded by a thin follicular cell layer (black arrow) with nucleus and nucleolus. White arrow indicates spermatids. (B) An acinus showing one large oocyte (black arrow). White arrow indicates a bunch of sperm. A magnified view of interacinar space with many interstitial cells (white arrow head) is shown in box. (C) An acinar part showing spermatocytes, spermatids (arrow) and there is no Sertoli cell pocket formation between these cells. (D) Transmission electron microscopy of the ovotestis of older snails, showing the associations of spermatocytes with adjacent Sertoli cell. Sertoli cell has no membranous digitations around the cell membrane. (E) Developed spermatids of older snails. n = nucleus; ot = oocyte; S = Sertoli cell; sc = spermatocyte.

A controversy prevails with the presence of several oocytes per acinus in different pulmonates. The acinus of aquatic hermaphrodite *L. stagnalis* consists of more than one oocyte [21–23] whereas terrestrial hermaphrodite *A. fulica* possesses only one oocyte in the acinus [5]. The number of oocytes in each acinus probably depends on individual species [6], type of habitat, as well as the maturity of individual snails. In the present work, we observed that the older *M. indica* possessed only one oocyte in the acini, whereas the acini of the younger snails lacked oocytes. The ooplasm contained numerous yolk granules. In molluscs as well as other invertebrates, it is suggested that the important characteristic of developing oocytes is the formation of yolk droplets by the accumulation of yolk granules in the ooplasm ([26–30]; Roy et al., unpublished observation). It is reported also that female cells are generated by autodifferentiation of undifferentiated gonadal tissues [31]. The present study reveals that some acini of younger snails contain undifferentiated cells that are frequently distributed near to and on the acinar inner boundary wall. These cells are supposed to be the precursor cells of future oogenic cells.

The spermatogenic and Sertoli cells are almost the same in the acini of younger and older *M. indica*. There is an opinion that spermatogenesis is a secondary process in the gonads of older snails that act as females [32–34]. It may be due to the lack of sufficient male gonadal hormones in the acini of older snails.

There is a conflicting view about the arrangement of Sertoli cells within the acini. Many authors have suggested that Sertoli cells are arranged in a definite layer and act as a barrier between spermatogenesis and oocytes [22,23,35–39]. The acini of younger *M. indica* are composed of different stages of spermatogenesis and the acinar space is subdivided into some definite pockets due to intimate association of columnar Sertoli cells. A group of cells of specific developing stage of spermatogenesis occupies one of such kind of pockets. In some acini of younger *M. indica*, the cellular junctions are prominent [40,41]. The spermatogenesis in the ovotestis of younger snails is an exclusively active process and Sertoli cells are mainly involved in providing nutrition and nourishment to the spermatogenic cells [42]. In the ovotestis of older *M. indica*, there is no compartment between these gametes. It may be due to either regression

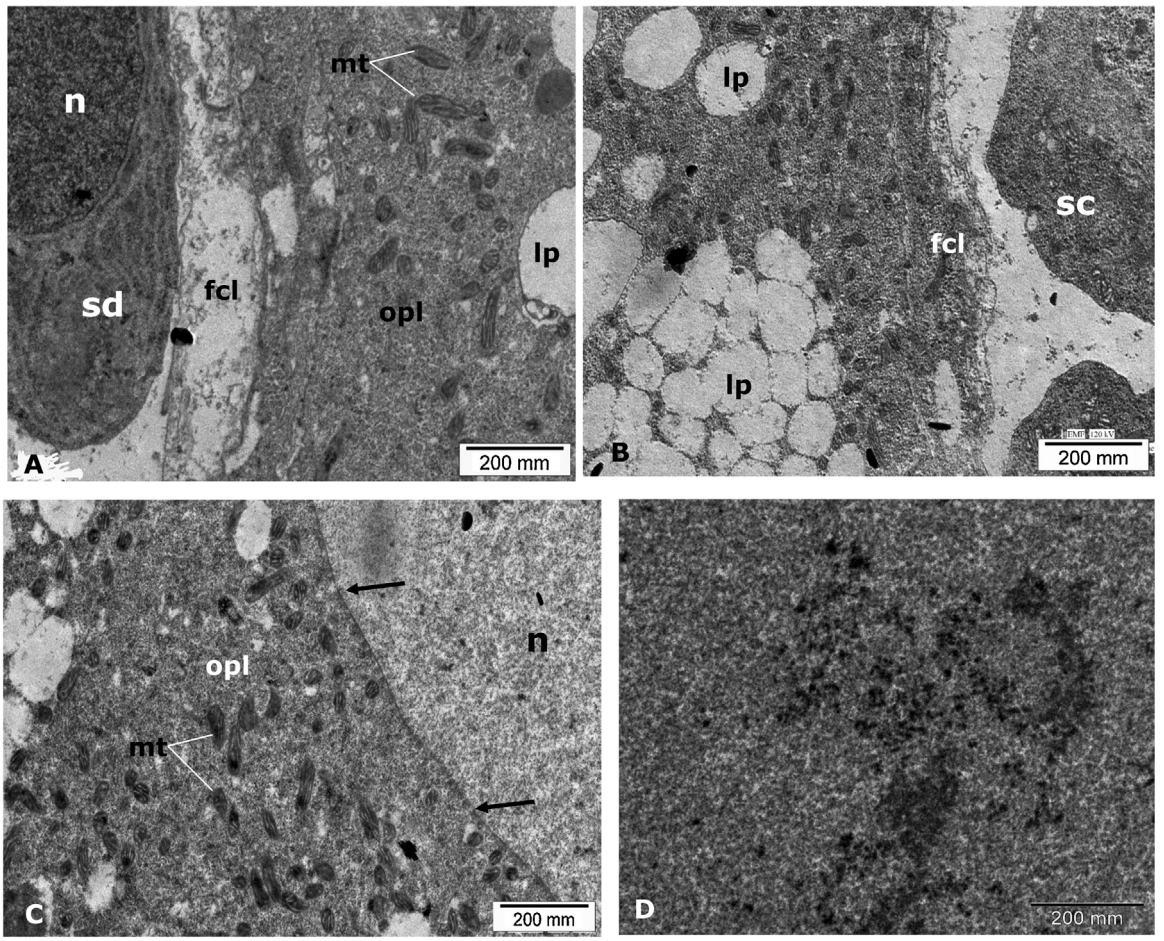

Fig. 8. Transmission electron microscopy of ovotestis of older snails. (A) Association between spermatid and follicular cell layer of oocyte. (B) A portion of follicular cell layer that separates oocyte from rest of the acinar structure. Ooplasm possesses numerous mitochondria and lipid droplets. (C) Ooplasm and nucleoplasm are separated by nuclear membrane. Note the nuclear pore (arrow). (D) Chromatin materials in the nucleoplasm of the oocyte.
fcl = follicular cell layer; lp = lipid droplet; mt = mitochondria; n = nucleus; opl = ooplasm; sc = spermatocyte; sd = spermatid.

or residual stage of Sertoli cell proliferation and spermatogenesis in the gonads of older snails [43]. As a result, the Sertoli cell number is variable in the acini of younger and older snails. It is reported that the follicular cell layer acts as a barrier between oocytes and the rest of the acinar cells [5]. In older *M. indica*, the observations about the follicular cell layer corroborate the above findings. The typical microtubular arrangement in the spermatozoon tail is surrounded by a beaded layer. It is presumed that these beads might be the mitochondrial helices. This layer probably helps with energy supply to the tail for their motility [44,45].

It is presumed that the functions of interacinar cells might be similar to those of mammalian Leydig cells. The function of the periacinar cells is supposed to be similar to that of mammalian peritubular myoid cells of seminiferous tubules, and probably produces peristaltic waves to release the mature sperm into the acinar lumen [46–48]. The ovotestes of younger and older snails possess the interstitial and periacinar cells but their number is high in the ovotestes of younger snails. This kind of bias of the periacinar cell production in the ovotestis is due to the requirement for more peristaltic waves in younger snails to support the release of numerous mature sperms into the acinar lumen and to perform as a potential male in copulation.

It is proposed that the secretion of the nutritive cells of the periacinar boundary might help in the development of all kinds of acinar cells. It is also assumed that the thin inward extensions of the acinar boundary probably maintain the cellular communications with gametogenesis and exert some influence on cellular maturation and differentiation.

The size of the albumen gland is a marker of male/female discrimination [19,20]. It is suggested that the assessment of comparative body size by crawling between two hermaphroditic snails might help to determine their male/female partner during copulation. In *M. indica*, the premating behavior, including repeated crawling over mate partner for a certain time, corroborates the above observations in different hermaphroditic individuals. It is also advocated that the spermatogenesis predominates in the acini of younger snails and act as male whereas oogenesis is highly active in that of the older snails that perform

as female during copulation and approve the hypothesis of this work.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

The authors express their thanks to the Principal of City College, Kolkata, India and laboratory staff of this college. The authors also thank the staff of Transmission Electron Microscopy Unit, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmau.2016.03.001.

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