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Ovarian Development and Histological Investigation of *Arius subrostratus* (Shovelnose Sea Catfish) from Cochin Estuary, India

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Abstract

In this evaluation, both macroscopic and histological level examination of the ovary of Shovelnose Sea catfish *Arius subrostratus* were carried out to find out its exact maturity stages. During this study, complicated cellular level changes could be observed throughout the entire maturation process of the ovary. The external appearance of ovary was estimated from the fresh fish specimens collected from different parts of Cochin estuary, Kerala, India. Ovary of *A. subrostratus* were classified into five stages based on morphological examination. Subsequently, oogenesis was explained with the aid of many intricate phases like pre vitellogenic phase, vitellogenic phase and the atretic phase which were further subdivided into the chromatin nucleolar phase, the cortical alveolar phase and the yolk and mature phase and finally the spent phase which contained two types of regenerating oocytes called as post ovulatory follicle and atretic oocyte. Besides, three layers of ovarian walls such as the peritoneum, tunica albuginea and germinal epithelium were noticeable in the context of oogenesis.

Keywords: *Arius subrostratus*, histology, oogenesis, oocyte, gonads

Introduction

The majority of the fish in the globe are dioecious, and they exhibit biparental reproduction, where eggs are formed in the ovary of females and spermatozoa are formed in the testes of males. The incident of discharging fish eggs and spermatozoa and their fertilization process is commonly called as spawning. The sexes of fishes can be separated by the external examination of gonads. Gonads (testes of males and ovaries of females) are paired structures that are suspended by mesenteries across the roof of the intestinal cavity. Primordial germ cells converted into mature gametes by a variety of cytological changes are known as gametogenesis. When the process of gametogenesis takes place in male gametes it is called as spermatogenesis and in female it is oogenesis. There are several studies that unveil valuable information about the intracellular organization of the oocyte of fishes. Abdalla and da Cruz-Landim (2003) described vitellogenesis, chorion formation and atresia while studying the histology of the ovary of *Piaractus mesopotamicus.* Another experiment carried out by Kagawa (2013) explained influence of endocrine control over morphological and cellular changes that occurred in the ovary during oogenesis.

Catfishes in the family Ariidae are significant due to their mouth-brooding characteristic. They are low- fecund fishes and can tolerate wide varieties of salinity fluctuations. Sound knowledge on the reproductive characteristics of this native fish resource is of utmost importance for developing the long- term conservation and management measures. Numerous studies have explained the reproductive aspects of different species of ariid catfish across the world, such as the reproductive cycle of *Arius graeffie,* was elucidated by Rimmer (1985); Rahman, Hossain, Ullah, and Iqbal. (2020) conducted reproductive analysis in *Arius gagora* and Vo et al. (2024) studied *Arius maculatus*. Most of these studies were based on the morphological features of fish gonads

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and not on histological analysis. However, Senarat et al. (2020) made a detailed investigation on the gonadal maturation of *Arius maculatus* by histological analysis. Ambily and Nandan (2017) worked on some basic aspects of reproductive biology on *A. subrostratus,* such as gonadosomatic ratio, ova diameter analysis, fecundity estimation, etc. *Arius subrostratus* is known as shovel nose catfish due to the shape of its head and snout. *Arius subrostratus*, a non-migratory catfish, is always seen in the same areas of the estuary throughout the year. Hence, variation in the ovarian developmental stages will be an indicator of any contamination that occurs in the water (Sooraj, Vineetha, Sagar, Tejaswi, & Pillai 2023). The experimental data on microscopical-level studies of the ovary may be a prerequisite for further scientific research related to the impacts of pollution or toxicity. The high level of protein and essential amino acids in this species (Ambily & Nandan, 2018) make it a better option for aquaculture, and it can be used for the preparation of value-added products.

Unfolding the precise pattern of ovarian development in fish is vital for developing conservation strategies to some extent (Akhi, Haque, Miah, & Haque, 2024).

The objective of the study was to understand the detailed macroscopic and microscopic examination of the ovaries of this species, for which information is essential but scarce.

Materials and Methods

Fish specimens for this experiment were collected monthly from Cochin estuary, Kerala, India (9^0 91′ N and 76^0 32' E to 9^0 51′N and 76^0 15' E) from April 2011 to March 2013. Monthly sampling of two years was done to get all maturity stages, and a total of 88 specimens of *Arius subrostratus* ranging in size from 12.5 to 34.5 cm in total length were used for estimating maturity stages and histochemical analysis. The fresh specimens were taken to the laboratory for investigation. Subsequently, their gonads were dissected out and external examination of gonads were noted down macroscopically and preserved in 4% formalin for histological analysis. During this study, fish were grouped into different gonadal stages of development according to Nikolsky (1963) and Sang, Lam, and Hai (2019). Although the macroscopic level staging of ovaries is inexpensive, it has some limitations in discriminating between certain stages of maturity. In order to quantify maturity stages by histological analysis, formalin-preserved ovaries were washed thoroughly in distilled water and then subjected to dehydration in an ascending concentration of alcohol as followed by Uribe, Grier, and Parenti (2012). Afterward, slices of tissues were embedded in paraffin wax and the sections were cut at 3 -6 μm in the LEICA RM 2125 RTS microtome. Then staining was done with haematoxylin, followed by eosin and observed under a research microscope (LEICA DM 500) (Suvarna, Layton & Bancroft, 2013). For transmission electron microscopy, tissue samples were fixed in glutaraldehyde solution and phosphate buffer and dehydrated with a series of alcohols. Finally, the sections were electromycrographed under TESCAN VEGA 3 SB.

Results and Discussion

During the investigation of macroscopic staging of ovary, the gonads of *Arius subrostratus* have been classified into five stages based on their external appearance. They are immature (I), maturing (II), matured (III), ripe or spawning (IV), and spent (V)

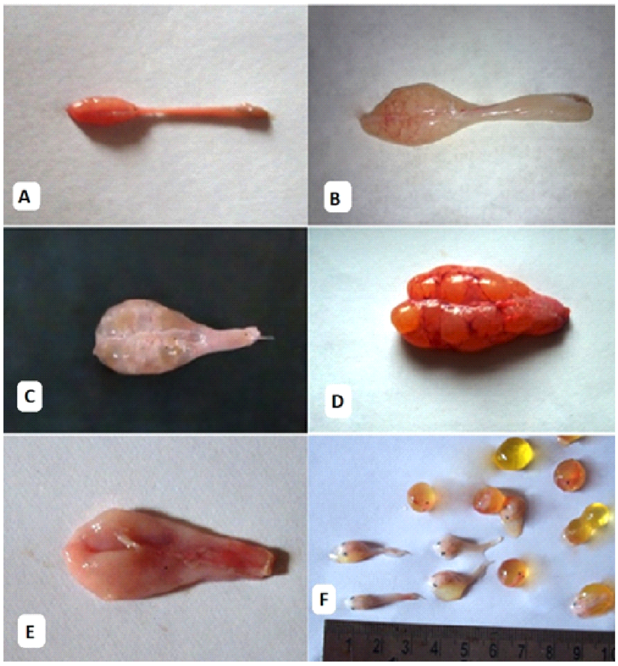

Fig. 1. Ovary of *A. subrostratus* based on different maturity stages from Cochin Estuary

A, Immature stage of ovary; B-Maturing stage of ovary; C- Matured stage of ovary; D-Ripe/Spawning stage of ovary; E-Spent stage of ovary; F- hatchlings of *A. subrostratus* from Cochin Estuary

Table 1. Morphological features of different stages of ovary of *Arius subrostratus* from Cochin estuary

Maturity stages	Identifying characteristics
Stage I immature	Very small sexual organs close to the vertebral column. Ovaries were transparent, colourless to grey and eggs were invisible to the naked eye. Under microscope irregular ova with a clear nucleus are visible (Fig. 1A).
Stage II maturing	In this stage ovaries were translucent with a length of half of the ventral cavity. Single eggs can be seen with magnifying glasses. Ovary became yellowish in colour. Each ovum was independently not detectable with naked eye. (Fig .1B).
Stage III matured	Here, ovary occupied about two thirds of ventral cavity and appeared as opaque. Each ovum was round in shape and easily recognizable with the naked eye. Under microscope each ovum could be seen as spherical in shape and opaque in appearance. The peripheral regions are transparent (Fig 1C).
Stage IV ripe/spawning	Sexual organ was filling the ventral cavity. Eggs oozed out with an application of slight pressure. Orange yellow large ova measuring about 1 cm diameter were visible to the naked eye. Most eggs were translucent with some opaque ovum. Cloudiness of ovum is attributed to the large amount of yolk. Each ovum became slight oval in shape in this stage (Fig. 1D).
Stage V spent	Gonads were flaccid and shrinking. Ventral cavity seems not fully empty. Ovaries were pale white in colour. No opaque eggs left in ovary but rarely one or two ova visible in the ovary. Under microscope ova appeared with irregular and small in size (Fig. 1E).

(Table 1 & Fig. 1: A, B, C, D, E and F). Some other investigators also classified the gonads of different species of fish into five stages of maturity while studying their reproductive characteristics, (Mitu, 2017; Sang et al., 2019). The immature ovaries of *A. subrostratus* observed were thin and transparent. Immature ovaries are generally obtained from fish under the size at first maturity (Sang et al., 2019). In maturing stages, each ovum was individually not recognizable with the bare eye, but under a microscope, spherical ova appeared. This opaque appearance of the ovary is due to the commencement of the yolk during the secondary growth phase (Roy & Mandal, 2015). During the ripe stage, each ovum became translucent and large. Hatchlings were obtained from the mouths of ripe fish during the current study due to the mouth-brooding behavior of catfish. Most of the ariidae species exhibit mouth-brooding behavior as a part of parental care (Vo et al., 2024). Hatchling of *A. subrostratus* appeared very large in diameter, i.e., more than one 10 mm and it hatches out from a big ovum (Fig. 1F). There are three types of ovaries detected in fish: gymnovarian, secondary gymnovarian and cystovarian. In gymnovarian type the oocytes are expelled into the coelomic cavity, enter the ostium, then come out through the oviduct, whereas in secondary gymnovarian type, the oocytes are conveyed directly into the oviduct (Ganguly, 2013; Porcu et al., 2022). *A. subrostratus* exhibited cystovarian type ovary, where the elimination of oocytes occurs only through the oviduct.

Different maturity stages of the ovary of *A. subrostratus* were classified according to the cytological changes occurred in the ovary during the developmental phases. The differentiation of ovum known as oogenesis has been well explained with the help of histological analysis during the study of microscopic staging of ovaries.

The organization of the cellular constituents of the ovary undergoes structural, functional and biochemical level changes during the process of oogenesis (Guzman, Luckenbach, Yamamoto, & Swanson, 2014). The nucleus, cortical alveoli, follicular wall, cytoplasm and cytoplasmic content are the major components that endure extreme changes during an incident of oogenesis. The progression of oogenesis in *A. subrostratus* has been classified into different phases based on the position and size of the oocyte, pattern of staining, nucleoli, occurrence of the follicular layer, and appearance of yolk granules. These phases are known as pre - vitellogenic phase, vitellogenic phase and atretic phase. Similar types of classification of ovarian maturation were explained by other researchers, like Shabanipour and Heidari (2004); Siddiqua, Ahmed, and Hussain

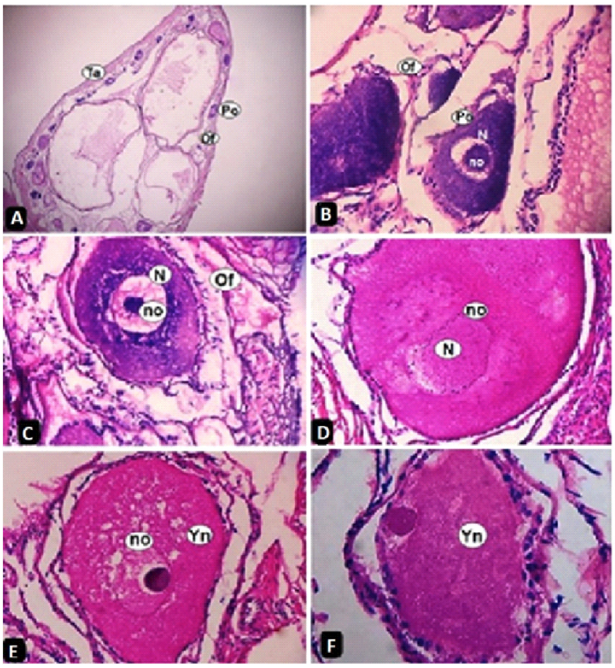

Fig. 2. Pre vitellogenic phase in the ovary of *A. subrostratus*

A- Cross section of ovary containing ovigerous lamellae or ovigerous fold (Of) from the *tunica albuginea* (Ta) and primary oocytes are visible (Po) (40x); B-Primary oocyte (Po)with well diagnosed lamellae (of)(400X); C- Chromatin nucleolous stage showing large nucleus (N) and nucleolous (no) eventually distributed in the nucleus. (Hematoxylin& eosin. 400 X); D- Perinuclear stage of primary oocyte having several nucleoli (no) appearing the periphery of the nucleus (N) (Hematoxylin& eosin. 400 X); E- perinuclear stage with nucleoli (no) and yolk nucleus (Yn) (400 X);F- Late perinuclear stage showing migration of yolk nucleus (Yn)(400X).

(2011); and Cek and Yilmaz (2007). The ovarian wall of *A. subrostratus* in the present study exhibited three layers, such as the outermost peritoneum, middle *tunica albuginea* (connective tissue layer) and innermost germinal epithelium which projected into the ovocoel and developed into lamellae. The maturation of oocytes took place in the ovigerous lamellae. Furthermore, the oocyte wall also consisted of three layers: outer follicular layer, middle *zona radiata* (ZR) and innermost plasma membrane. The follicular layer is composed of two layers, such as the outer theca layer and the inner granulose layer (Fig. 3).

Present study denotes an upsurge in number of nuclei as well as a huge quantity of cytoplasm (ooplasm) during the pre vitellogenic phase. This stage has been divided into two phases according to the development of cellular materials.

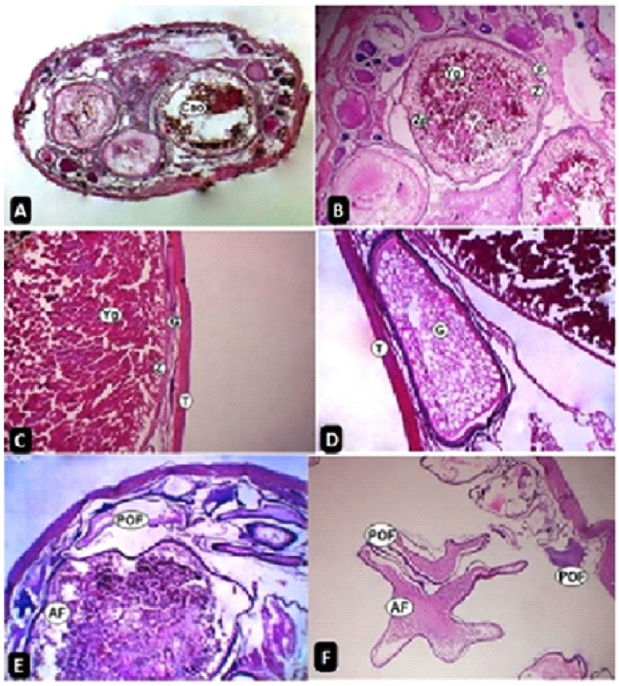

Fig. 3. Vitellogenic phase and Atretic phase

A- Ovary with cortical alveolar oocytes (Cao) (40X);B- Alveoli fill the oocyte and contained Yolk granules (Yg), *Zona radiata* (Z) and follicular cell layer (F)(400X);C- Mature oocyte with yolk granules (Yg) surrounded by inner *Zona radiata* (Z), outermost theca (T) granulocyte (G) also visible(40X);D-Mature oocyte with development of granulocyte (G) with outer theca layer (T)(400X); E- Atresia occurred in the spent phase(100X;F-Oocyte showing atretic follicle (AF) and post ovulatory follicle (POF) at the spent phase(400X).

Chromatin nucleolar phase (primary oocyte, chromatin nucleolar oocyte) is the immature phase or primary growth phase where numerous oogonia got scattered in the ooplasm with well diagnosed lamellae. Oogonia are known as the precursor cells of oocytes (Fig. 2A). The chromatin nucleolus oocyte contains large spherical nucleus with a number of nucleoli. There are both primary and secondary oogonia in which primary oogonia is characterised by a large nucleus (Fig. 2B), and the nucleus of secondary oogonia are filled with basophilic chromatin threads, hence the chromatin nucleolar stage (Fig. 2C). In this phase the nucleus is centrally located and the *zona radiata* around the follicles occurred as thin and invisible. Typically, chromatin-nucleolus oocytes of teleost fishes contained large nucleolus and perinucleolar oocytes with a large number of small nucleoli (Lee, Huang, & Chang, 2008).

During cytoplasmic growth phase (early peri – nucleolar stage, late peri nuclear stage), the quantity of cytoplasm increased along with a surge in the number of nuclei, which were arranged at the periphery when the oocytes transferred to the early peri – nucleolar stage. However, the increase in number and size of nucleoli became stable only during the later peri – nuclear stage (Fig. 2D). During the late peri nuclear stage, a dark and spherical body appeared just outside the nuclear membrane called the 'yolk nucleus' or Balbiani body (Fig. 2E) and then it migrated to the cortical ooplasm and vanished (Fig. 2F). In the cortical alveolar phase of Zebrafish, *Danio rerio* showed beginning of the formation of *zona radiata* and an uneven nucleus (Yon, Aytekin, & Yuce, 2008). Granular structures in the ooplasm increases throughout the cortical alveolar phase of pre vitellogenesis during the present study.

The vitellogenic phase occurred at the end of the cytoplasmic growth phase. The enlargement of oocyte is observed during this phase and is divided into three stages: the cortical alveolar phase, the mature or yolky phase and the ripe or migratory nucleus stage.

In the cortical alveolar phase, peri – nuclear oocytes develop in cortical alveolar oocyte, which is characterised by spherical cortical granules (Fig. 3A). A cellular membrane, *zona radiata* or vitelline membrane became noticeable and thicker around the periphery of the oocyte, hence the name vitellogenic phase. Besides, the oocyte was surrounded by two follicle layers known as theca and granulosa (Fig. 3B). The cellular membranes such as the *zona radiata* and follicular layers are observable during the vitellogenic phase. Condensation of yolk granules also took place during this phase. Accumulation of yolk in the ooplasm during the vitellogenesis has been reported by many researchers, (Dadzie, 1974; Lee et al., 2008; Rao, Krishnan, Sanil, 2014; Sharma & Bhat, 2014; Schilling, Loziuk, Muddiman, Daniels, & Reading, 2015). During the mature or yolky phase, the cortical alveoli generated at the periphery of the oocyte increased in number in order to fill the whole cytoplasm. Accordingly, the oocyte reached at the early vesicular stage. There are two stages during this phase: the early yolk vesicle stage or the yolk granular stage in which spherical yolk proteins deposited around the nucleus and cortical alveolar layer increase. Then, the *zona radiata* and follicular layer also became condensed, number of nucleoli increased and the nuclear membrane disappeared to form the late yolk vesicular stage or yolk globular stage. Consequently, yolk granules became thicker and more globular. Afterward, oocyte enlargement occurred due to the increase in yolk deposition and *zona radiata* became very prominent (Fig. 3 C, D).

In the ripe or migratory nucleus stage, as a result of membrane degradation, the nucleus got shifted towards the animal pole and the ooplasm became homogeneous. The cortical alveolar layer at the peripheral region got reduced with no change in *zona radiata*. The vitelline membrane began to collapse, and some follicle epithelium cells with nuclei were generated outside the membrane. Dadzie (1974) and Lee et al. (2008) observed large number of lipid droplets in the ooplasm during the vitellogenic phase of different fish species. Whereas, Rao et al. (2014) also experimentally unveiled many dramatic changes that happened during the vitellogenic phase, like aggregation of mitochondria, formation of lipid droplets, development of *zona radiata* and the dense endoplasmic reticulum. Eventually, the oil droplets fused together to form large drops. However, studies by Sharma and Bhat (2014)

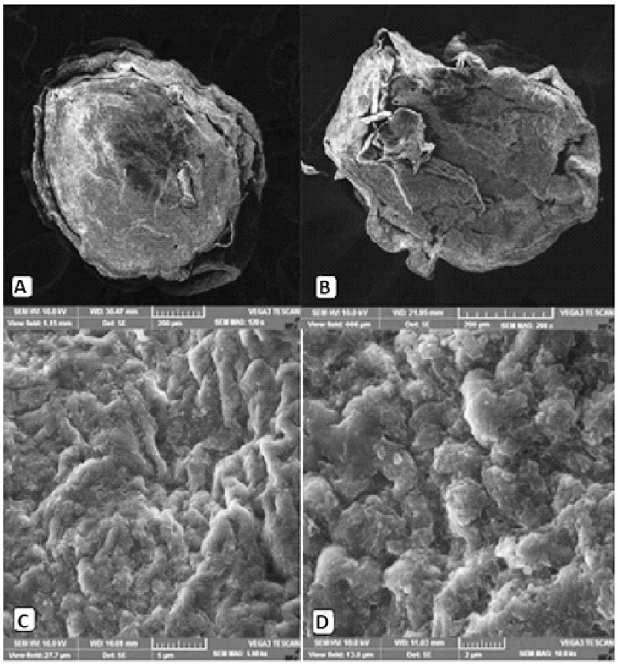

Fig. 4. Scanning Electron microscopic image of unfertilized egg of *A. subrostratus*

A, B, A whole view (by SEM) of unfertilized egg; C, D. Higher magnification of unfertilized egg surface showing polygonal projections.

showed that the thickness of *zona pellucida* increased at the stage of vitellogenesis of rainbow trout.

After spawning, the ovary of *A. subrostratus* remained almost empty and contained two types of follicles, i.e., post- ovulatory follicles and atretic follicles (Fig. 3 E, F). Non-ovulated ovum generated either from perinuclear or from vitellogenic phase was also visible in the spent ovary. In the post ovulatory follicle, the follicle layer remained in the ovary after spawning. Due to the lack of endocrine regulation, it got reabsorbed by the follicle cells and this process is known as apoptosis. Whereas, in atretic oocytes the *zona radiata* gets ruptured, followed by a disintegrated cytoplasm and the oocyte is attacked by phagocytic cells and cannot undergo maturation. This process mainly occurred at the post- vitellogenic phase (Fig. 3 E, F).

The remnant of oocytes seen in the spent ovary either come from the perinucleolar phase or the cortical alveoli phase. Normally, in fish oogenesis, five types of oogonia occur: chromatin nucleolus oocytes, perinucleolar oocytes, cortical alveolar oocytes, vitellogenic oocytes, and maturing oocytes (Lee et al., 2008). In addition, another type of oocyte known as atretic oocyte. Generally, the formation of follicular atresia takes place throughout the ovarian cycle of a fish (Sharma & Bhat, 2013). Follicular atresia normally commences with an increase in the gap between zona pellucida and ooplasmic contents. The occurrence of atresia in the breeding season allows the female fishes to reabsorb the nutrients, thereby diminishing the number of eggs and consequently improving the reproductive potential of the fishes in hostile situations like pollution, deficit of nutrients etc. Nevertheless, some other authors illustrated follicular atresia as a representation of pollution, climate change and so on (Corriero, Zupa, Mylonas, & Passantino, 2021). According to El-Zoghby, Bakry, Ghallab, and Emam (2009), season plays a vital role in the cytological organization of the testes and ovary, particularly winter season, which is the resting season for the gonads of catfish species. They confirmed resting or spend stages by showing predominance of previtellogenic stages in the ovaries and degeneration of the testes. Also, Cassel, Mehanna, Mateus, and Ferreira (2013) mentioned the importance of season in the gonadal maturation of fish during the analysis of reproductive characteristics of *Melanorivulus aff. Punctatus*.

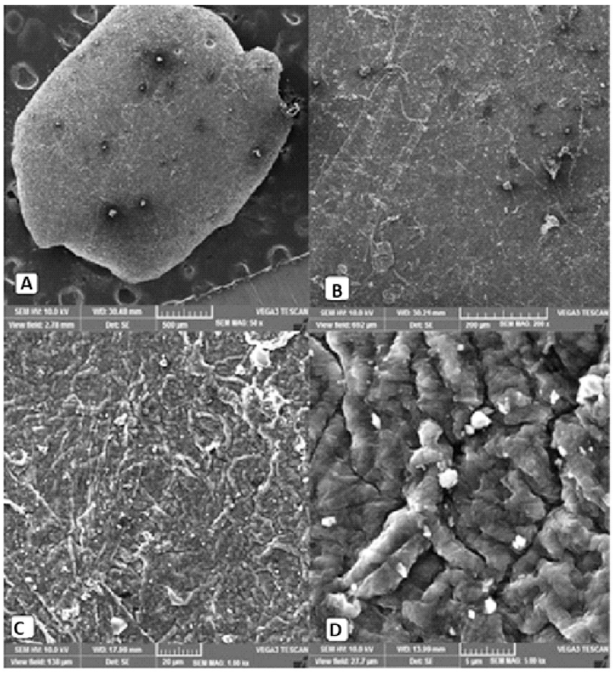

Fig. 5. Scanning Electron microscopic image of fertilized egg of *A. subrostratus*

A, B, An external view (by SEM) of fertilized egg; C, D. SEM view of matured egg without polygonal protuberances

Follicular atresia, a degenerative process of germ cells and their associated somatic cells, is a complex process involving apoptosis, a programmed form of cell death whose mechanisms are highly conserved in vertebrates and invertebrates, and are characterized by biochemical and structural changes, including chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Corriero, Zupa, Mylonas, & Passantino, 2021).

The immature egg of *A. subrostratus* was round in shape and enveloped by a thick *zona radiata.* It appeared as rough and regular arrangement of polygonal protuberances (Fig. 4). After fertilization the protuberances became disappeared and attained rough and irregular surface (Fig. 5). These polygonal protuberances are extracellular materials and pore canals filled with bud-like bulges or microvilli (Rizzo, Sato, Barreto, & Godinho, 2002). Shabanipour and Heidari (2004) emphasized the role of *zona radiata,* and the bud-like projections that appeared in the outer layer of *zona radiata* increased the surface area for the transportation of materials for the synthesis of yolk. During the later stages thickness of *zona radiata* reduced and loose around the oocyte.

This study provides a comprehensive macroscopic and histological examination of the ovaries of the Shovelnose Sea catfish, and details the cellular processes of oogenesis, covering the pre-vitellogenic, vitellogenic, and atretic phases, thus filling a significant gap in the literature. The findings offer valuable insights into reproductive biology of this species, and are crucial for developing effective conservation strategies for *A. subrostratus* by identifying critical spawning periods and implementing protective management measures. Additionally, detailed knowledge of reproductive biology can help optimize breeding programs in aquaculture, enhancing yield and contributing to food security.

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