

# Histological and Ultrastructural Studies of Ovarian Development and Micropyle Structure of Zebra Sea Bream *Diplodus cervinus cervinus* (Teleostei: Sparidae)

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## Abstract

This study was designed to assess the histological and ultrastructural stages of the female sparid, zebra sea bream (*Diplodus cervinus cervinus*) sexual maturity, while publications that paid attention to the reproductive biology of this protandrous species is almost lacking. In the present study, it is suggested that oogenesis is classified into four stages: oogonia proliferation (the presence of oogonia is a typical feature of this stage), oocyte growth (is characterized by the formation of follicular layers around the oocyte. large nucleus and scattered nucleoli), vacuolization (is described by the appearance of marginal vacuoles) and final maturation (in which ovaries decreased in size, appeared with collapsed features, exhibited empty follicle and were provided with many blood vessels). *D. cervinus cervinus* is a partially ovipositing species having a multiple spawning character with asynchronous oocyte development. Transmission electron micrographs of the ripe oocyte demonstrated that it was characterized by the appearance of five ideal ovarian layers (theca, follicular epithelial, zona radiata externa, zona radiata interna and cortical alveoli). In teleost fish, eggs allow the entry of sperm from only a single site known as 'the micropyle', which is a narrow canal in the chorion of the egg. The morphology and classification of *Diplodus* micropyle were also investigated by scanning electron microscopy. During the spawning season, the full-grown *Diplodus* eggs collected from adult gravid females possess a single micropyle at the animal pole. This micropyle is a conical shaped with no pit, characterized by a tapering opening having only a deep, constricted canal. Several thickened annuli with little knobs strengthened the inside of the canal. In conclusion, this study provides a comprehension on the reproductive biology of *D. cervinus cervinus* which is important for the culture and sustainable seed production of this species.

## Introduction

Knowledge related to the dynamics of fish reproduction is significant in aquaculture and plays a vital role in assessing its exploitation level and in evaluating the management of fisheries (El-Zaeem et al. 2024). Oogenesis is a crucial process to understand the reproductive biology of species and to provide a detailed picture of the reproductive performance of females. Teleost represent a big group of vertebrates and consequently, their process of oogenesis varied among different species (Boufekane et al. 2021; Ghanem et al. 2025).

*Diplodus cervinus cervinus*, known as zebra sea bream, is a member of family Sparidae, which includes the porgies and seabreams, are mostly marine and

distributed around the Canary and Madeira Islands, in the warmer regions of the Mediterranean Sea as well as in the Indian, Pacific and the Atlantic Oceans (Nelson 2006). The genus of *D. cervinus cervinus* representing one part of Sparinae sub-family in the Egyptian coast, is considered as a great source of protein for this area, while its presence in the market is accidental (Abou Shabana 2012). Pajuelo et al. (2003) indicated that *D. cervinus* species (Lowe 1838) includes three high-valued sub-species: *D. cervinus cervinus* (Lowe 1838), *D. cervinus hottentotus* (Smith 1844) and *D. cervinus omanensis* (Bauchot and Bianchi 1984). *D. cervinus cervinus* is a species of 10 to 40 cm in total length, up to 55 cm and 757.33 g of mean weight (Alshawafi et al. 2018). Resembling all Sparids, it is characterized by one dorsal fin with thick lips, an oval raised body shape

which appears laterally compressed. Being an extensive farmed fish group, this family becomes of high economic importance (Fadel et al. 2024). Reproductive biology of fish is a crucial aspect that allows the determination of the suitable management practice to conserve fish species in their habitat. Reproduction is also the most critical stage in the life cycle of a species indicating its survival (Moslemi-aqdam et al. 2016). According to Pajuelo et al. (2008), sparids are multiple spawners with a rudimentary hermaphroditism type and a low protandry level. Fish spawning runs from spring to summer, with a peak in May and June. *D. cervinus cervinus* has received little scientific validation in the northwestern Mediterranean region as well as in the eastern one (Pajuelo et al. 2008). None of the existing publications investigated the oogenesis of *D. cervinus cervinus*. () The available data include the population ecology of the zebra sea bream from the coasts of the Canarian archipelago in North West Africa (Pajuelo et al. 2003), the sexual pattern diagnosis of another *Diplodus species* (*D. cervinus hottentotus*) from southern Angola (Winkler et al. 2014); or the somatic growth of *D. cervinus Sargussargus* in the central Algerian coast (Boufekane et al. 2021). Derbal and Hichem (2013) reported that the reproductive period of this species takes place between January and September, and its first sexual maturity occurs when reaching a total length of 25 cm. It is worth mentioning that histology offers an influential tool to study fish reproductive health (Alqahtani et al. 2025). Before maturation, histological examination of preserved gonads showed that *Diplodus species* is a rudimentary hermaphrodite having a non-functional bisexual ovotestis (Winkler et al. 2014).

In teleost fishes, the micropyle is a well-designed opening which allows the entrance of spermatozoa into the egg. Around the micropyle, eggs of all fish species own a glycoprotein tightly bound to the chorion surface. This glycoprotein called the micropylar sperm attractant (MISA) increases fertilization efficiency by directing spermatozoa into the micropylar canal in a  $\text{Ca}^{2+}$ -dependent manner (Yanagimachi et al. 2017). Before hatching, this aperture originates from the zona pellucida between the first and third late vitellogenic stages (Hart 1990). It plays vital roles for successful fertilization by allowing a path for penetration of sperm to the oocyte, attraction of spermatozoa via pheromones, avoidance of polyspermy and inflow of water during the formation of perivitelline space (Riehl 1999). Although the surface structure, number and diameter of the micropyle are species-specific. Concerning the number on the egg envelope, the majority of teleost fishes own only a single micropyle, except some species have two or numerous micropyles (Lahnsteiner 2003). By surface and proximal morphology, the structure of the micropyle was classified into four types: a long canal with a flat pit, a short canal with a deep pit, only a canal without a pit, or a short canal with two pits (Riehl and Kock 1989). Based on such variation, Breining and Britz (2000) reported

that the micropyle is used as an effective criterion for taxonomic classification. Several studies have been documented the ultrastructural aspects of oogenesis in teleost (Srijunngam et al. 2005; Abdelmeguid et al. 2024) but it is very limited in seabreams (Ramos 2003; Gülsoy and Çolak 2009).

This work is the first to provide a description and complete data on the histological and ultrastructural studies of the exploited stock of female *D. cervinus cervinus* in the central region of the Egyptian coast during oogenesis and to investigate the morphology and classification of its micropyle by scanning electron microscopy.

## Material and Methods

In the present study, 105 mature female *D. cervinus cervinus* specimens of 17–37 cm total length and 54–555 g total weight were collected by hooks from January to July 2024 from the deep seas of Kayet Bay shore in Alexandria, Egypt (Figure 1). During the sampling period, 15 fish samples were monthly collected, their total lengths were measured to the nearest cm then ovaries were dissected out to identify the maturity stage. Subsequently, fish samples ( $n=10$ ) were prepared for light examination and ultrastructural studies. The research was carried out in accordance with the Scientific Research Ethics of National Institute of Oceanography and Fisheries 'NIOF', Egypt.

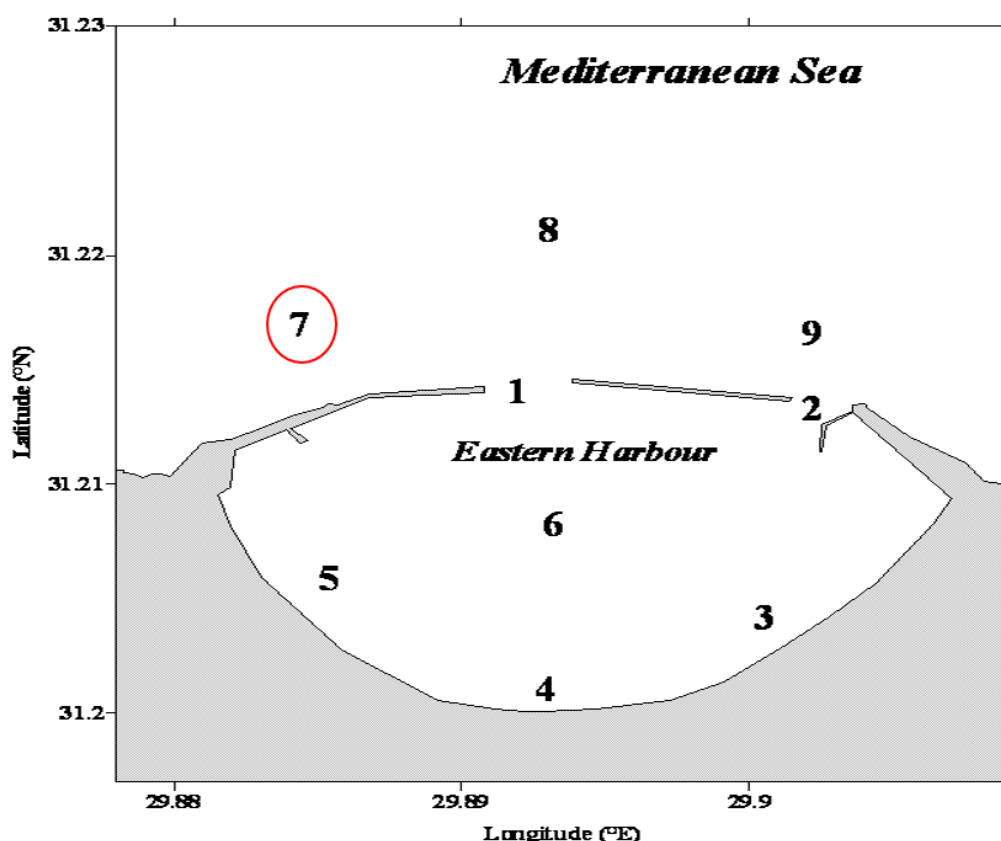
### Microscopic Investigation

#### Light Microscope Specimens' Preparations and Egg Size Measurements

For light microscopy, small pieces of ovary specimens from 10 fish in each developmental stage were fixed in 10% neutral formalin for 24 hrs, washed in distilled water, dehydrated in ascending grades of ethanol at 4°C, cleared by methyl benzoate, and embedded in paraffin. Sections 5µm were stained with Hematoxylin and Eosin and examined using a light microscope (Radwan et al. 2023). Measurements of egg diameter and oogenic structure such as zona radiate and the micropyle openings were accomplished according to Witthames and Walker (1987) protocol.

#### Electron Microscope Specimens' Preparations

For transmission electron microscopy (TEM), slices of ripe ovaries from 10 fish were fixed for 24 hrs., in  $4\text{F}_1\text{G}$ , (4 Formaldehyde: 1 Glutaraldehyde) in phosphate buffer (pH 7.4) at 4°C, post-fixed in 2%  $\text{OsO}_4$  in phosphate buffer for 2 hrs., washed, dehydrated through a series of ethanol, and embedded in Epon-Araldite mixture. LKB ultramicrotome was used to obtain semi-thin 1 µm and ultra-thin (50 nm) sections. The semi-thin sections were stained with Toluidine Blue, while the ultrathin sections were double stained with Uranyl acetate for ½ hr. and



**Figure 1.** Geographical location of Kayet Bay shore along the Eastern Harbour of Alexandria during the sampling study. 1) El-Boughaz, 2) El-Silsila (Inshore), 3) Mosque of leader Ibrahim, 4) El-Manshia, 5) Navigation Club, 6) Harbour center, 7) Kayet Bay shore (the sampling site of the study), 8) El Boughaz, 9) El Silsila II (offshore).

Lead citrate for 30 min. and then examined using Joel 100 CX TEM (Reynolds 1963).

For scanning electron microscopy (SEM) of *D. cervinus cervinus* micropyle: 5-8 fully grown eggs were collected per female fish ( $n=7$ ) during the breeding season in the laboratory. After release, unfertilized eggs were obtained as bundles of eggs and collected directly from females. These eggs were fixed in 2.5% Glutaraldehyde solution for 24 hours, placed two times in buffered solution (pH 7.4) for 30 minutes, fixed for six hours in 1% osmium tetroxide solution with 0.1 M phosphate buffer (pH 7.4), and placed in buffered solution (pH 7.4). They were dehydrated with a graded ethanol series and stored in pure terta-butyl alcohol. Subsequently, they were freeze-dried under vacuum by evaporation (VFD-21S, Vacuum Device Co., Ltd., Ibaragi, Japan). The dried samples were then coated with osmium tetroxide by ion sputtering (HPC-1SW, Vacuum Device Inc., Tokyo, Japan) and examined under a scanning electron microscope (Carl Zeiss, SUPRA40VP, Germany) following Kim and Park (2021) method.

#### Analytical Statistics

Data are presented as mean $\pm$ standard deviation (SD). All calculations of egg dimensions were performed on microscopic scale then data were calculated on Excel sheet for mean and standard deviation ( $n=10$ ).

## Results

### Ovarian Histology and Sexual Maturity Stages of *D. cervinus cervinus*

Microscopically, the process of oogenesis can be divided into four stages: oogonia proliferation, oocyte growth, vacuolization and final maturation.

The following are the histological and ultrastructural representations of the most essential features for each developmental stage.

#### Oogonia Proliferation Stage

The presence of oogonia cells is the most characteristic feature of the immature stage (Figure 1A). Histological Preparations indicated that oogonia are found singly or in clusters forming nests inside the lamellae, indicating their original from the germinal epithelium (Figure 2D). Each oogonium usually exhibits spherical outline which appears very small in size having a diameter of  $13\pm0.67\ \mu\text{m}$  L X  $15\pm0.88\ \mu\text{m}$  W.

Light micrographs of primary oocytes appeared mostly polygonal in shape, having a large circular nucleus with either one or two nucleoli (Figures 2 D-F). They have dimensions of  $28\pm4.07\ \mu\text{m}$  in width and  $32\pm9.95\ \mu\text{m}$  in length. In this stage, light preparations also indicated the presence of secondary oocyte with

rounded nucleus exhibiting detached nucleoli. These nucleoli with a diameter of  $41 \pm 2.71 \mu\text{m}$  are arranged in the periphery of the nucleus (Table1).

### Oocyte Growth Stage

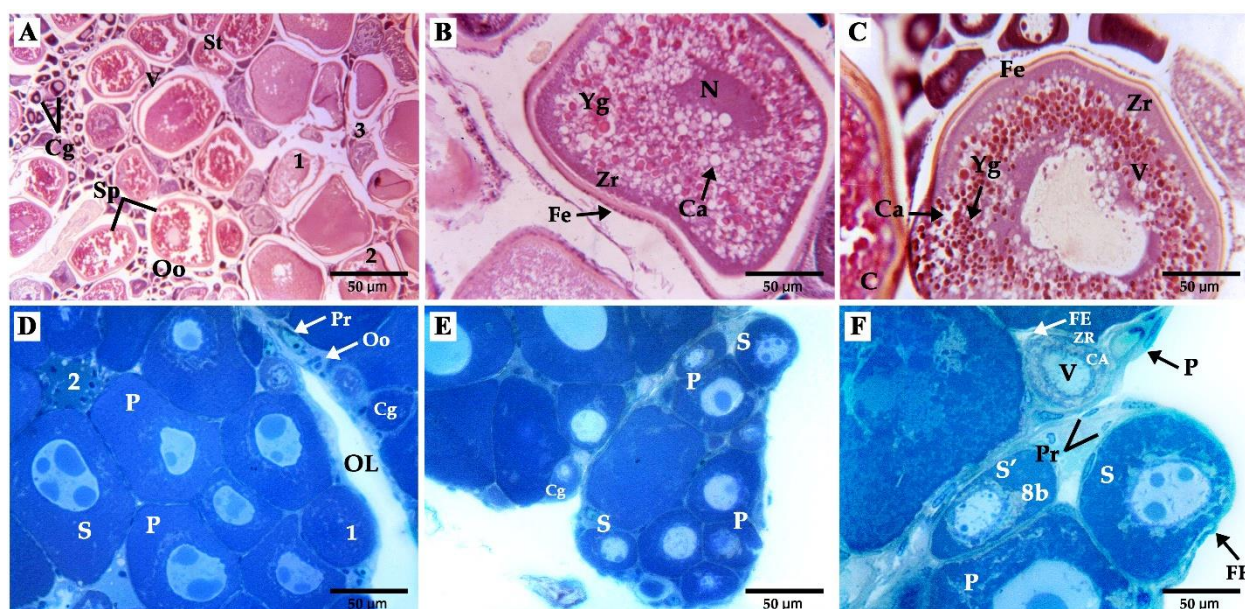
In this oocyte growth stage (maturation phase), light micrographs demonstrated that oogonia still present, as single cells locating the center of ovigerous lamellae. TEM preparations demonstrated that *early previtellogenic cells* are characterized by the electron-dense "Nuages" which appear irregular in shape in the form of clouds or electron dense spots in certain regions of the cytoplasm (Figure 3 A, B). Primary oocytes appear rounded with diameters of  $101 \pm 3.65 \mu\text{m}$  and provided with spherical nuclei. The nucleoli distributed in the nucleus increase in their number (reaching 25 nucleoli) if compared to their number in immature stages, indicating an increase in the nuclear activity.

In the maturation stage also secondary oocytes house spherical shaped nuclei with diameter of  $123 \pm 5.26 \mu\text{m}$  and include 19-28 nucleoli (Figures 2 D-F). It was also noticed that the cytoplasm of *late previtellogenic oocytes* appeared homogenously and strongly basophilic next to the nucleus, indicating the existence of the "yolk nucleus". Balbiani body or 'yolk nucleus' appears as a small bright corpuscle with a new cytoplasmic inclusion in the region from the nucleus to the follicle (Figure 2F). The oocytes at this stage become isolated and surrounded by a follicular layer consisting

of a row of simple squamous epithelial cells arranged around the cell boundary limiting the whole cell (Figure 2B).

### Vacuolization Stage

Examination of light micrographs of oocytes in the vacuolization stage in the current study with diameters of  $148 \pm 9.19 \mu\text{m}$  revealed their characterization by the appearance of marginal vacuoles. These vacuoles ranged in diameter from  $12 \mu\text{m}$  minimally to  $28 \mu\text{m}$  maximally. However, the additive follicular layer became more obvious and the formation of zona radiata indicated the beginning of vacuolization period (Figure 2 B, C). Some oocytes, which appeared with cytoplasm lacking its basophilic property, revealed the formation of a narrow zone of small cortical alveoli marks (yolk vesicles) in the periphery of the cytoplasm indicating the beginning of the formation of a vitelline envelope. During this phase, the nucleus was enlarged, and the nucleoli were dissociated from the nuclear envelope. These oocytes revealed the presence of several vacuoles which appeared in the basophilic layer of the cytoplasm around the nucleus. Number of these vacuoles also increased since their diameters reach  $32 \pm 1.18 \mu\text{m}$  to form several irregular rows filling cortical region of the cytoplasm, forming a sponge- looking area (Figure 2C). Features of these vacuoles indicated a late vacuolized stage. Furthermore, light micrographs of semi-thin sections of *D. cervinus cervinus* in the

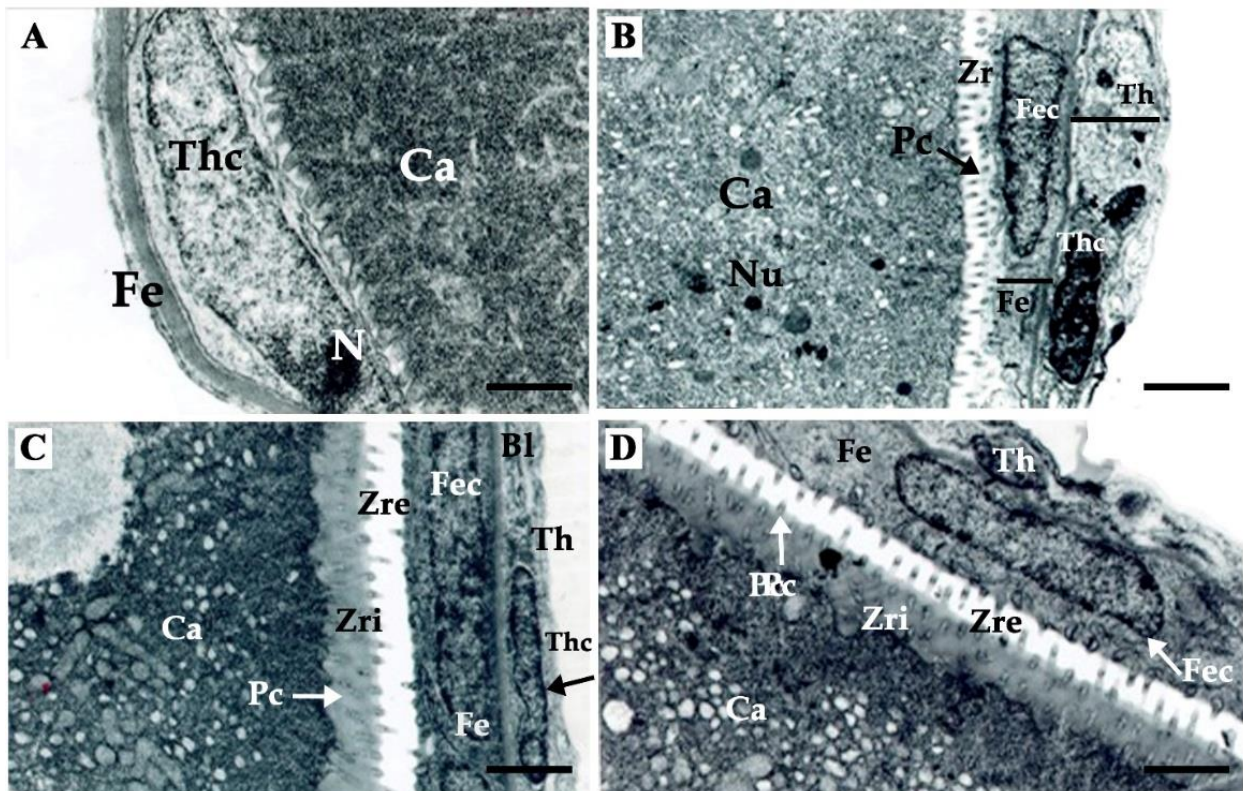


**Figure 2.** (A-C): Light micrographs of Cross section through ovary of *Diplodus cervinus cervinus* at the different developmental stages. A) oogonia (Oo) cytoplasmic growth (Cg), vacuolized, (V) oocytes, spawning (Sp) oocytes, spent (St) oocytes and various stages of atretic oocytes (1,2,3). B) Illustrating the vacuolization stage showing, normal ripe oocytes at the tertiary yolk stage, the oocyte appears with a large, rounded nucleus (N), surrounded by an outer follicular epithelial layer (Fe) and inner zona radiata layer (Zr), cortical alveoli (Ca) and yolk globules (Yg), C) Revealing, parts of ripe oocytes exhibited well manifested Fe followed by Zr, then Ca layer. Yg interfered with some vacuoles (V). [stained with H & E]. Light micrographs, Semi-thin cross section through ovary of *D. cervinus cervinus* showing oocyte growth stage illustrating (D-F). D) Primary oocytes: P, Secondary oocytes: S, Presynaptic oocytes: Pr, Atretic oocytes: 1, 2, 3, Oogonia: Oo, Cytoplasmic growth: Cg, ovigerous lamellae: OL. E) Showing oocytes-sac-like structure. F) Demonstrating the mature oocytes, more advanced secondary oocytes: So', nucleus: N; nucleolus: Nu, chromatin material: Ch, Balbiani body with cytoplasmic inclusion: Bb. [toluidine blue stain].

**Table 1.** The histological and ultrastructural representations of the most essential features for each ovarian developmental stage of *Diplodus cervinus cervinus*

Ovarian Stage	Stage Period	Oocytes	Histological and Ultrastructural Features
1- Oogonia proliferation	Present in Most months before and after spawning period.	-well-spaced ovigerous folds oriented towards the center of ovary, enclosing oogonia, primary and secondary oocytes.	<ul style="list-style-type: none"> <li>- The oogonium has a relatively large nucleus containing prominent nucleolus, and a thin layer of chromophobic ooplasm.</li> <li>-Primary oocyte: polygonal in shape, having a large circular nucleus with either one or two nucleoli.</li> <li>-Secondary oocyte with rounded nucleus exhibiting detached nucleoli arranged in the periphery of the nucleus.</li> </ul>
2- Oocyte growth stage	February to March	Oogonia, early and late previtellogenic cells	<ul style="list-style-type: none"> <li>-Oogonia still present, as single cells locating the center of ovigerous lamellae.</li> <li>-Early previtellogenic cells are characterized by the electron-dense "Nuages".</li> <li>a- This stage is characterized by the appearance of a follicular epithelium layer surrounded by squamous theca layer.</li> <li>b-Primary oocytes appear rounded with spherical nuclei. The nucleoli are distributed in the nucleus and increase in their number reaching 25 nucleoli.</li> <li>c-Secondary oocytes house spherical shaped nuclei with 19-28 nucleoli.</li> <li>- Late previtellogenic oocytes include 'yolk nucleus' in the cytoplasm.</li> <li>a-The oocytes become isolated and surrounded by a follicular layer which became thicker.</li> <li>b- Formation of the vitelline envelope took place and became visible.</li> <li>c-Invaginations of the nuclear envelope are accompanied by the irregular outline of the nucleus.</li> </ul>
3- Vacuolization stage	March to Beginning of May		<ul style="list-style-type: none"> <li>-Characterized by the appearance of marginal vacuoles.</li> <li>-These vacuoles are small, few in number (about 5), and located in the periphery of the cytoplasm.</li> <li>- The additive follicular layer became more obvious and the formation of zona radiata indicated the beginning of the vacuolization period.</li> <li>-Balbiani body migrates towards the peripheral part of cytoplasm.</li> <li>-Some oocytes revealed the formation of a narrow zone of small cortical alveoli marks (yolk vesicles) in the periphery of the cytoplasm indicating the beginning of the formation of a vitelline envelope.</li> <li>-During this phase, the nucleus was enlarged, and the nucleoli were dissociated from the nuclear envelope.</li> <li>-These oocytes revealed the presence of several vacuoles which appeared in the cytoplasm around the nucleus and increased in number to form several irregular rows filling cortical region of the cytoplasm, forming a sponge- looking area.</li> <li>- All the atretic oocytes were found at the vacuolized, 1<sup>ry</sup>, 2<sup>ry</sup> and 3<sup>ry</sup> yolk stages possessing thick outline and deformed wall with altered nucleus and cytoplasm.</li> </ul>
4- Spent stage	End of May to July	Ovaries decreased in size, exhibited empty follicle and provided with many blood vessels.	The unovulated oocytes undergo atresia accompanied with resorption of all oocyte stages.





**Figure 3.** (A-D): Transmission electron micrographs (TEM). Cross section through ovary of *D. cervinus* in: growing mature oocytes (A, B) and oocyte at the vacuolization stage (C, D). Magnified part of oocyte wall illustrating the normal structure of the oocyte wall and the steps of ovarian wall layers formation, outer theca layer (Th) containing flattened squamous theca cells (Thc) provided with nucleus (N) followed by thin basal lamina (Bl), then appear the second follicular epithelial layer (Fe) with oval follicular epithelial cells (Fec), zona radiata layer was differentiated into third zona radiata externa (Zre) and fourth zona radiata interna (Zri) layers containing pore canals (Pc) followed by cortical alveoli layer (Ca) containing yolk globules (Yg) intermingled with vacuoles (V). Note also the nuage (Nu) structure. (A: X, 2500; B: X, 7500, C&D: 10000) [Uranyl acetate and lead citrate stain].

vacuolized stage demonstrated that the oocyte wall consists of three layers; cortical alveoli followed by zona radiata (Zr) layer followed by follicular epithelial layer (Figures 2 C, F). The cell boundary of this oocyte appeared full of less dense yolk granules, which exhibited various diameters and interfered with numerous vacuoles.

It is worth to mention that according to the size of nuclei and yolk granules in the cytoplasm, the oocyte at the ripe stage could be classified as 1<sup>ry</sup>, 2<sup>ry</sup> and 3<sup>ry</sup> yolk stage with diameters corresponding to  $384 \pm 8.01 \mu\text{m L} \times 362 \pm 12.09 \mu\text{m W}$ ,  $470 \pm 6.14 \mu\text{m L} \times 591 \pm 99.4 \mu\text{m W}$  and  $707 \pm 55.19 \mu\text{m L} \times 604 \pm 51.8 \mu\text{m W}$ , respectively (Figures 2 B, C). This stage is characterized by liquification of yolk. Light micrographs of ripe oocytes at the tertiary yolk stage of *D. cervinus cervinus* fixed in Bouin's fluid and stained with H&E demonstrated that these oocytes exhibited an elongated shape with a cellular boundary consisting of zona radiata layer surrounded with follicular epithelial layer. Each oocyte possesses small size nuclei exhibited in an eccentric position near the animal pole. These nuclei contain a small number of nucleoli arranged at the periphery and inside the nucleus. A large number of yolk granules interfered with several vacuoles of different diameters appeared in the cytoplasm (Figure 2C).

At the onset of spawning stage, light micrographs of ovary revealed early cytoplasmic growth, ripe ova with different stages of yolk deposition, large number of postovulatory follicles (empty follicles), as well as atretic oocytes due to the discharge of considerable number of ripe oocytes (Figures 2 A, D).

Due to the high resolving power of *transmission electron microscope*, many more details than those observed with light microscope were noticed in ripe oocytes of *Diplodus*. Ultrastructural preparations demonstrated that the ripe oocyte is surrounded by five ideal ovarian layers nominated as; theca, follicular epithelial, zona radiata externa, zona radiata interna and cortical alveoli. The theca is the outermost layer, which holds squamous flattened theca cells. Theca cells contained flattened nuclei with a relatively large amount of cytoplasm, appeared less electron-dense, indicating less heterochromatin content which exhibited normal distribution. Preparations also revealed that this layer of cells aligned with a dense basal lamina (Figures 3 C, D). The second layer is made up of one row of follicular epithelial cells. These cells appeared oval in shape and were provided with oval centrally located nuclei. One prominent nucleolus was observed in each cell with a regular shape outline. In the nuclei, heterochromatin clumps were seen opposed to the

nuclear envelope (Figures 3 C, D). Besides, zona radiata externa, which is the third layer, and the fourth one called zona radiata interna assuming a fine striated appearance interrupted with pore canals. Figures 4 A-D demonstrate the different stages of zona radiata formation. In addition, the fifth layer known as cortical alveoli was also noticed (Figures 3 C, D).

### Final Maturation Stage

Since a large number of ripe ova were discharged, the ovaries came to the end of breeding season during the spent stage. Light preparations of specimens stained with H & E at this stage demonstrated that the cytoplasmic yolk vesicles undergo phagocytosis as shown by the presence of ruminants of oocyte contents. In addition, the ovary included a new generation of cytoplasmic growth cells and was provided with many empty follicles in between stroma (Figure 2 A).

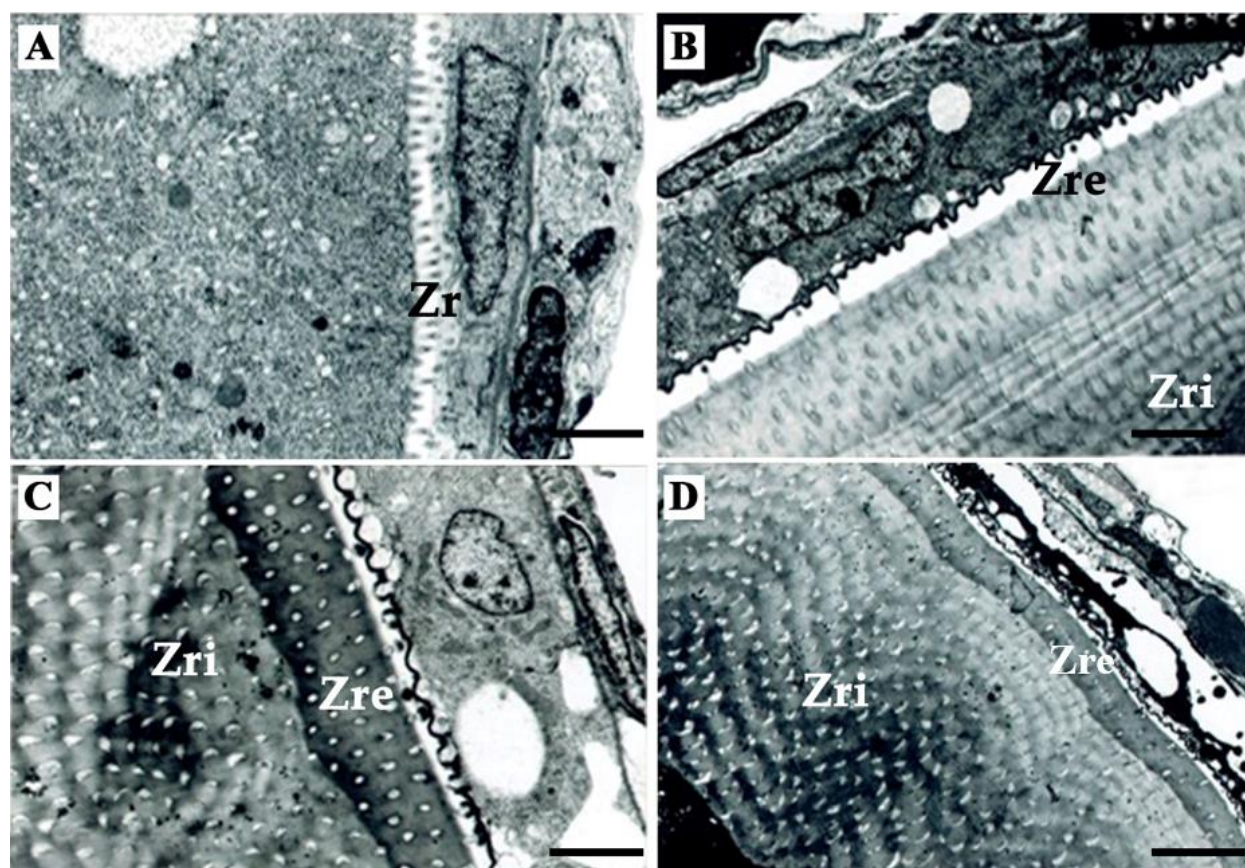
### Ultrastructural Features of *D. cervinus cervinus* Micropyle

Current results revealed that the egg diameter of *D. cervinus cervinus* was  $748 \pm 2.62 \mu\text{m}$  with approximately  $73 \pm 0.51 \mu\text{m}$  variation before fertilization. SEM preparations illustrated that the micropyle of *D.*

*cervinus cervinus* has a conical shape with only a deep canal and no outer pit (Figures 5 A, B). The micropylar canal opening of this species is apparently approximately  $5.58 \pm 0.22 \mu\text{m}$  in diameter. A number of thickened annuli strengthened the inside of the canal on the sides provided with several little knobs. Usually, the outward canal opening is closely directed into the center. The arrangement of these thickened annuli exhibited a clockwise spiral pattern from the base to the external opening in the micropylar canal forming over ten layers (Figure 5 C). The micropyle is also surrounded by a number of helical ridges. At the animal pole, the micropyle is located and enclosed with non-attaching filaments, contrasting with the vegetal pole, which is provided with elongated attaching filaments. Several pore canals are located in the boundary region of the micropyle, protruding upward from its surface (Figures 5 A-C).

### Discussion

Histology is the most reliable and common technique used in the assessment of the reproductive studies of teleost involving awareness of the gonadal development period (El-Sayed et al. 2025). Hence, egg development course is separated into stages to differentiate the changes in egg peculiarities gradually



**Figure 4.** (A-D) Transmission electron micrographs (TEM). Cross section through ovary of *D. cervinus* in the vacuolization stage. Magnified part of normal ripe oocyte wall demonstrating the different stages of zona radiata (Zr) formation and its differentiation into zona radiata externa (Zre) and zona radiata interna (Zri) (X, 10,000). [Uranyl acetate and lead citrate stain].



(Yön et al. 2008). The gross appearance of the ovaries judged from their light and electron photographs within the stages was used to identify the criteria of the diverse macroscopic maturity stages. In the present study, it was observed that *D. cervinus cervinus* displays in their reproductive activity a seasonal rhythm in which ovaries change morphologically in color, shape and size all over the year in the different maturity stages increasing in magnitude towards the spawning stage. The present results agree with that reported by Ahmadi and Ghanem (2025), who revealed that the ovarian structure differs during the various stages of the reproductive cycle.

Throughout the annual reproductive cycle, we proposed that this *Diplodus* species belongs to the fish category of fractional and long spawning season with asynchronous oocyte development due to the existence of oocytes at various developmental stages. These results indicate that fish may spawn several times during the spawning period. Similar observations were reported by Ismail et al. (2016) revealing that *Dentex dentex* had a prolonged spawning period since all the oocytes don't undergo at any time the same developmental stage.

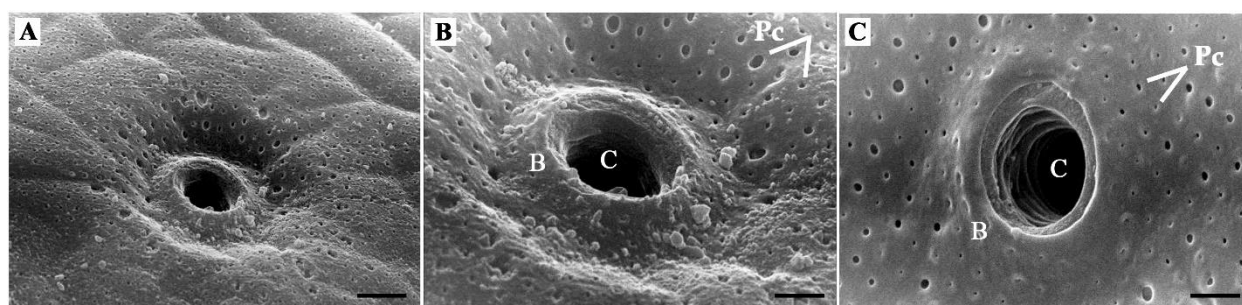
According to the present morphological and histological features, it is proposed that the oogenesis process in *D. cervinus cervinus* could be classified into four stages. This indicates that the growth and development of oocytes of zebra sea bream appear to be approximately similar to that of other teleost fishes. In the current study, changes in developmental stages in the ovaries of *D. cervinus cervinus* were similar to those observed in *Acanthopagrus latus* females (Karimi et al. 2014).

During the *oogonia proliferation period*, the presence of oogonia is a typical feature of this stage. The early oogonium in the ovary of *Diplodus* in the present study is small in size with a spherical outline, characterized by a pale nucleus and a thin, indistinct peripheral zone of cytoplasm. The current observations are in the same line with the results of Marutirao (2013) in the ovaries of *Puntius ticto*.

Besides, light preparations illustrated that the early previtellogenic oocytes appeared commonly polygonal in shape, possessing a large spherical nucleus with one

or two nucleoli and characterized by a cytoplasm with strong staining affinity. This is consistent with the results of Yön et al. (2008) who described the primary oocyte with 2-4 nucleoli located in the center of the nucleoplasm. Ultrastructure aspects of the previtellogenic oocytes of zebra seabream appear significantly similar to those described previously for *D. cervinus cervinus puntazzo* (Gülsoy and Çolak 2009). TEM preparations illustrated that oogonia are small, house large nuclei with a single nucleolus and their morphological features are very common during oogenesis, as reported in *Sparus aurata* (Ramos 2003). The most characteristic features of this stage are the electron-dense "Nuages," which appear irregular in shape in the form of clouds or dense spots in certain regions of the cytoplasm. Nuage is considered as a universal component of germ cells formed of ribonucleoproteins. Hence, it is concerned with the constitution of germ plasm (Abdel-Aziz et al. 2012).

Besides, preparations of the present study indicated that *oocyte growth stage* is characterized by the formation of follicular layers around the oocyte, large nucleus and scattered nucleoli. Yön et al. (2008) indicated that the follicular epithelium in *Danio rerio* differed among the different stages, and it was formed by the multiplication of follicular cells consisting of a continuous layer as the oocytes grow. At the beginning of maturation period, the cytoplasm of oocyte is represented by the appearance of Yolky nucleus (Balbiani body) as a new cytoplasmic inclusion. In most teleost fish, El Saba et al. (2013) described the Balbiani body as a conspicuous characteristic of previtellogenic oocytes, while it has a variable morphology between different fish species. It is typically comprised of endoplasmic reticulum, Golgi complexes, mitochondria and multivesicular bodies. Guraya (1979) explained that this yolky nucleus plays a key role in organelles formation of the mature oocytes. The current histological results agree with Ismail et al. (2016), who observed the yolky nucleus in primary oocyte's cytoplasm and the presence of a clear follicular epithelium appeared between these primary growth phase oocytes.



**Figure 5.** (A-C): Scanning electron micrographs of *Diplodus cervinus cervinus* egg illustrating a) the micropyle was surrounded by centrifugally arranged ridges in the chorion. (X, 5000), b) (X, 7500) &c) showing micropyle of *D. cervinus cervinus* (conical shape having canal without a pit), boundary part: B, micropyle canal: C, pore canal: Pc, helical ridges: R. All bars indicate 1µm, X, 15000. [Uranyl acetate and lead citrate stain].



The oocyte at the *vacuolization period* in the current work is defined by the appearance of marginal vacuoles. First, these vacuoles were small in size, few in number and peripherally situated in the cytoplasm. The present findings also coincide with that observed in the vacuolization stage of *D. dentex* concerning marginal vacuoles, cortical alveoli, conspicuous follicular and zona radiata (Zr) layers formation (Ismail et al. 2016). Investigators believed that Zr in the vacuolized oocytes is considered as secretory product from the follicular cells (El Saba et al. 2013). Within oocyte development, thickness of zona radiata increases towards the ripe and spawning season (El Saba et al. 2013). In the present study, vacuolized oocyte membrane consisted of zona radiata surrounded by a follicular epithelial layer and then cortical alveoli layer. Likewise, Unal et al. (2005) have entitled vacuolized stage in *Chalcalburnus tarichias* the cortical alveoli phase due to the presence of cortical alveoli and the accumulation of lipid droplets in the cytoplasm. They added that the late vitellogenic phase was characterized by the appearance of yolk globules between lipid droplets in the periphery of the cytoplasm. Similar reports were described in *Bagrus bayad*, *Bagrus docmac* (Gaber 2000) and hybrid red tilapia (Eissa et al. 2024; El-Sayed et al. 2025). Bawazeer et al. (2006) pointed out that cortical granules play a vital role in the chorionic materials formation.

Similar to the present data, Rao et al. (2014) revealed an increase in development of Zr thickness, movement of germinal vesicle towards the periphery and yolk globules formation in vitellogenic oocytes of female *Epinephelus diacanthus*. In accordance with the results of Gaber (2000), light micrographs of the present study showed that the oocytes at the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> yolk stages were characterized by vacuoles fusion. Moreover, at this stage oocytes increased in diameter, house amoeboid nucleus, its zona radiata increased in thickness and surrounded with follicular epithelium layer. Reaching the final yolk stage, oocytes characterized by the liquefaction of yolk droplets and coalesced vacuoles. It is known that yolk globule phase is the main stage of oocyte development.

The present ultrastructural data concerning theca layer agrees with the findings of El Saba et al. (2013) in *O. niloticus*. In addition, the follicular epithelial layer differentiates from a simple flattened cell layer in the oocyte growth stage to a cuboidal cell layer in the vitellogenic stage. The current ultrastructural observations are similar to the data reported by Abascal and Medina (2005), who described a continuous follicular layer formed by flattened cells provided with elongated follicle cells, mitochondria, free ribosomes, rER cisternae and small vesicles. Moreover, theca and follicular epithelial layers in the ripe *D. cervinus cervinus* oocytes appeared similar to that observed in *Chalcalburnus tarichi Pallas* (Ünal et al. 2005), *Rusty Parrotfish* and *Scarus ferrugineus* (Abdel-Aziz et al. 2012). According to Ismail et al. (2016) theca and follicular cells are concerned with synthesis of sex

steroids throughout the oocyte maturation and the synthesis of vitellogenin in fish ovary. Consequently, this vitellogenin was deposited in the oocyte as a yolk (Eissa et al. 2024). The theca layer followed by a distinct, thick basal lamina which separates the follicular cell layer from the overlying theca cells. Cruz-Landim and Cruz-Hofling (2001) described the basal lamina as a thick, amorphous layer possessing electron-dense material deposited in its external surface. Our observations concur with that of Rao et al. (2014) concerning basal lamina and theca cells in the oocytes of grouper *Epinephelus diacanthus*.

TEM preparations of the present work also revealed the presence of the following: 3<sup>rd</sup> Zona radiata externa (Zre), 4<sup>th</sup> Zona radiata interna (Zri) and 5<sup>th</sup> cortical alveoli layers in the ripe oocytes. Similar to the present findings, the well-developed oocytes in early to mid-vitellogenesis of female goby were ultrastructurally described with intact follicular epithelium and Zr (Louiz et al. 2009). During maturation, Guiliano and Ferrero (2000) also indicated that follicular cells play a role in Zre building in the ovary of the grass goby (*Zosterisessorocephalus*). According to the observations of Moghaddam et al. (2013), Zr is made up of two distinct striated layers known as zona radiata interna and zona radiata externa (named vitelline envelope). The vitelline envelope acts as a microbial and mechanical protector, permits species-specific fertilization and avoids polyspermy. Unal et al. (2005) explained that the development of Zr externa occurs when cortical alveoli start to appear. At ripe stage, the striated appearance of Zr observed ultrastructurally in the present study concur with that detected in the ripening and spawning stage of *Dentex dentex* (Ismail et al. 2016). Current ultrastructural observations also revealed that the stripes of the Zr are pathways or canals that extend from the external surface of Zr to its internal surface. These canals are described as passageways of microvilli originating from oolemma and follicular cells toward each other. Each striated line in the vitelline envelope of *Liza aurata* was described as a canal containing microvilli and provided with pores opened at both edges (Shabanipour and Heidari 2004). Furthermore, Gülsoy and Çolak (2009) explained that pore canals on the Zr surfaces act in the transport of essential materials for yolk synthesis to the oocyte and the properties of these canals reveal a type of selective transport. These microvilli that appeared in the form of finger like projections passing the pore canals and associated with growth of Zr thickness were developed. Similar findings were also reported in *Hoplias malabaricus* and *Hoplerthrinus unitaeniatus* ovaries (Gomes et al. 2007). Besides, Perazzolo et al. (1999) mentioned that the synthesis of the cortical alveoli layer occurs in the oocyte wall of teleosts when yolk begins to accumulate in the oocyte.

At the onset of spawning season, the ovary in the current work exhibited numerous peculiarities with a large number of empty follicles and diverse yolk

deposition stages. During the studied spawning season, the discharge of a great number of ripe ova was accompanied by a decrease in the ova quantity, presence of empty follicle and appearance of a new generation of cytoplasmic growth as a form of preparation for the following breeding season. The present findings also agree with the observed descriptions of *Dentex dentex ovaries* in spawning season (Ismail et al. 2016).

At the final maturation stage, the unovulated eggs in the present results were observed to undergo atresia. Follicular atresia is a well-known phenomenon comprising degeneration of the oocyte and disintegration of the follicular wall (Abdel-Aziz et al. 2012). The present histological preparations revealed that the ovaries reach the end of breeding season during this stage. At this spent stage, the cytoplasmic yolk vesicles were undergoing phagocytosis for atretic oocytes and gradually the yolk vesicles were transformed into empty vacuoles with a new generation of cytoplasmic growth cells. Similar to our findings, El Saba et al. (2013) indicated that the follicles undergoing atresia existed all over the year and were generally seen at any ovarian stage of *O. niloticus*, but they were plentiful during spent stage. Based on the histological and ultrastructural examination, degenerated oocytes were described as corrugated and characterized by pyknotic nuclei, cytoplasmic vacuolation and organelles clumping revealing atresia (Abdel-Aziz et al. 2012; Radwan et al. 2023).

It was reported that the micropyle structure varies between different species and appears as a long canal with a flat pit, a short canal with a deep pit, only a canal without a pit, or a short canal with two pits (Riehl and Kock 1989). In the present ultrastructural observations, the micropyle of *D. cervinus cervinus* has only a deep canal with no outer pit. Our preparations also demonstrated that the upper end of the micropylar canal on the external egg membrane was broader than that existed internally at the bottom of egg membrane. Following SEM observations, Chen et al. (1999) studied the structure of micropyles of four Sparidae species and classified it as only a canal with no pit. They recorded that the micropylar canals and the helical ridges which reinforced their sides were the most characteristic features used in the identification of different Sparidae species. They also explained that the reported differences in micropylar structure could be used as taxonomic features to classify the closely related species and considered as morphological adaptations with the shape of sperm to facilitate its rapid entrance into the micropyle. In accordance with the findings of Chen et al. (1999), micropylar canals of *D. cervinus cervinus* in the current work are strengthened by several annuli provided with slim knobs. In our results, the micropyle of *D. cervinus cervinus* also appears conical in shape, located on the animal pole of an unfertilized egg having a single spherical aperture similar to that observed in *Hypophthalmichthys molitrix* (Esmaeili and Johal 2005)

and *Oryzias sinensis* (Kim and Park 2021). On the contrary, a micropyle like a funnel is obvious in *Sparus sarba*, *Acanthopagrus latus*, *Pagrus major* (Chen et al. 1999) and *Oryzias latipes* (Kim and Park 2021). Moreover, small pore canals are evident on the surface near the micropyle of *D. cervinus cervinus*. This finding concurs with that found in *Oryzias sinensis* (Kim and Park 2021). No publication concerning histological or ultrastructural investigation of pore canals near the micropyle of *D. cervinus cervinus* subspecies has been previously studied. Although Park and Kim (2024) explained the role of pore canal in fish eggs indicating that it is concerned with water absorption into the eggs to reduce the physical environmental impact.

## Conclusion

In conclusion, *Diplodus cervinus cervinus* was described as a partially ovipositing species with asynchronous oocyte development. Oogenesis process in *D. cervinus cervinus* could be classified into four stages: oogonia proliferation, oocyte growth, vacuolization and final maturation. The micropyle of zebra sea bream is a conical shaped with no pit having only a deep constricted canal. More attention would be rewarded to this economic fish species in aquaculture by establishing a protocol of induced spawning for fish farming since popular sparids had been previously well farmed.

## Ethical Statement

The research was carried out in accordance with the Scientific Research Ethics of National Institute of Oceanography and Fisheries 'NIOF', Egypt.

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## Author Contribution

Both authors contributed equally to conceiving, designing the experiment, writing, reading and approving the final manuscript.

## Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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