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Histological Observations on Oocyte Development in the Fourfinger Threadfin (*Eleutheronema tetradactylum*)

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ABSTRACT

Background: Comprehensive information on oocyte growth and ovarian development patterns (synchronous or asynchronous) is lacking. Therefore, the present study aimed to study oocyte growth and ovarian development patterns in *Eleutheronema tetradactylum* collected from the Gujarat coast of India from September 2021 to August 2022.

Methods: 333 female *E. tetradactylum* were collected from the Navsari coast, Gujarat, India. Macroscopically, determined gonads were preserved in 10% neutral buffered formalin and processed for histology. Ovaries preserved in Gilson's fluid were examined for oocyte size and development pattern, with 150-400 oocytes per fish measured in a microscope.

Result: The results revealed that *E. tetradactylum* oocytes development stages were classified into five developmental stages. (i) pre-vitellogenic (ii) vitellogenic (iii) late-vitellogenic (iv) postvitellogenic and (v) follicular atresia. The oocyte attained the maturation stage at 550-650 µm. Histological observation and percentage distribution of oocyte types revealed that the *E. tetradactylum* is an asynchronous and multiple spawner fish species. In the various ovarian maturity stages, the presence of different stages of oocytes confirms asynchronous oocyte growth. Furthermore, the pattern of continuous oocyte size frequency in maturing to the ripe stage indicates a batch spawning strategy. The findings provide essential baseline data for assessing oocyte maturity and optimizing hormonal induction protocols in artificial breeding programs.

Key words: Asynchronous, Batch spawner, *Eleutheronema tetradactylum,* Oocyte development, Oocyte size.

INTRODUCTION

The four-finger threadfin, *Eleutheronema tetradactylum,* is a marine finfish species belonging to the family polynemidae. It is a commercially valuable fish with high demand in both domestic and international markets due to its exceptional meat quality. This species is distributed in the Indo-West Pacific from the Persian Gulf to Papua New Guinea and northern Australia (Motomura *et al.,* 2002). It occurs in the shallow coastal waters of the Bay of Bengal in the east and Mumbai and Gujarat in the west (Pillay and Ghosh, 1962; Kagwade, 1970). *E. tetradactylum* is considered a prioritized species for mariculture in India for its growth rate and market demand (Ranjan *et al.,* 2017).

To establish a captive broodstock of *E. tetradactylum* for artificial propagation, we need to gather relevant information on oocyte development, composition and size frequency distribution is very important for determining the reproductive and spawning strategy of this species.

Oogenesis is a dynamic process in the ovaries, during which the egg undergoes multiple developmental phases that are remarkably similar in different fish species (Yon *et al.,* 2008). The stages of oocyte development are categorized as primary growth, cortical alveolar, vitellogenesis and maturation, which prepare the oocyte for fertilization (Wallace and Selman, 1981). Studies on oocyte development are crucial for understanding the mechanisms of oocyte growth and ovulation patterns in ovaries, such as synchronous, group-synchronous and asynchronous development (Wallace and Selman, 1981). Histological examination of oocyte developmental stages is a crucial preliminary step in evaluating current methods for maturity assessment (West, 1990). Information on the oocyte development stages of *Eleutheronema tetradactylum* is scarce despite its commercial importance. Therefore, it is vital to understand the oogenesis process through microscopic and histological techniques; it would be helpful for the assessment of oocyte maturation size, which is essential for hormonal induction for breeding and seed production of *E. tetradactylum* in captivity.

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Currently, there is no information on the stages of oocyte development and the composition and size frequency of oocytes at various ovarian maturity stages in wild *Eleutheronema tetradactylum.* Thus, the objective of the present study is to investigate (i) the comprehensive histological description of oocyte development stages in *E. tetradactylum* and (ii) to investigate oocyte composition and size frequency in different maturity stages of the ovary in *E. tetradactylum.*

MATERIALS AND METHODS

The study was conducted at the Navsari Gujarat Research Centre of ICAR-CIBA, Navsari, from September 2021 to August 2022, 333 female specimens of *E. tetradactylum* were collected from artisanal and commercial gill net fishermen at Dholai port in Billimora, Navsari district, Gujarat, India.

Macroscopic examination of gonads

The individual specimens were dissected and ovaries were collected from the body cavity, ovaries were classified macroscopically into five stages (i) immature, (ii) developing, (iii) maturing, (iv) ripe and (v) spent.

Histological examination of gonads

Macroscopically identified gonads were fixed in 10% neutral buffered formalin (NBF). Three sub-samples were collected from the anterior, middle and posterior regions of each gonadal lobes and processed for histological investigation. Tissue samples were dehydrated in a series of graded isopropyl alcohol solutions (70-90%) and xylene and later embedded in paraffin wax. The embedded gonad tissues were sectioned into 3-5 µm transverse sections with a microtome (Leica RM2125 RTS). The sections were stained using hematoxylin and eosin (H and E) and subsequently mounted with DPX (Roberts, 1989). Slides representing different maturity stages were examined using a Zeiss Axio Scope A1 microscope, with high-resolution images captured using a JenoptikProg Res C3 digital camera attached to the microscope.

Ovarian follicles

All ovaries were preserved in Gilson's fluid to estimate the oocyte size. Oocytes were collected from the anterior, middle and posterior portions of the fish ovary and pooled (around 300). The oocyte development pattern was examined by analyzing the advancement of oocyte diameter at various maturity stages. A total of oocytes were measured per fish. To examine oocyte percentage distribution and size of oocytes in different stages of ovarian development in *E. tetradactylum,* 300 to 500 oocytes per fish at each developmental stage were observed. The mean oocyte size was calculated based on the method described by Shilta *et al.* (2022). The diameters of oocytes were measured under a (Zeiss Axio Lab A1) light microscope and photographs were taken using a JenoptikProg Res C3 digital camera attached to the microscope. Data presented in the figures are expressed as mean ± standard error. Bar charts were generated in Microsoft Excel to illustrate the different oocyte stages and their sizes recorded at each maturity stage of *E. tetradactylum.*

RESULTS AND DISCUSSION

Oocyte development stages in the ovary of *E. tetradactylum*

The oocytes of *Eleutheronema tetradactylum* were classified into five stages of development based on histological examination, oocyte size measurements and morphological characteristics. These stages are previtellogenic, vitellogenic, late-vitellogenic, post vitellogenic and follicular atresia (Table 1). Based on the percentage distribution of oocyte composition and size frequency, ovarian development was classified into five stages: immature, developing, maturing, mature and spent. In most teleosts, oogenesis is classified into five to eight stages (Nagahama, 1983; West, 1990). Similarly, a five-stage classification of oocyte development has been documented in other teleost species, such as *Barilius bendelisis* (Saxena *et al.,* 2018) and *Macrognathus pancalus* (Borah *et al.,* 2022), with staging determined by oocyte size, morphology and histological characteristics.

Immature stage

The immature stage of the ovary includes previtellogenic oocytes, which include the chromatin nucleolus stage, perinucleolar stage and late perinucleolar stage.

(a) Chromatin nucleolus stage

Oogonial cells undergo mitotic division, which results in the formation of the chromatin nucleolus stage. It represents the earliest phase of oocyte development in *E. tetradactylum*. Oocytes are small and compact, with a centrally located prominent nucleus (Fig 1a). The size of the oocytes ranged from 50-80 µm. The cytoplasm appears homogenous and lacks yolk inclusions. This stage indicates the initiation of oocyte growth, with the proliferation of cells inside the ovary. This phase is observed in immature ovaries and is essential for subsequent maturation (Brown-Peterson *et al.,* 2011).

(b) Early perinucleolar stage

The early perinucleolar stage is characterized by the appearance of multiple nucleoli arranged at the periphery of the nucleus (Fig 1b). The size of the oocyte ranged from 80 to 120 µm. As the oocyte increased, the number of nucleoli multiplied, accompanied by a decrease in the nucleus-to-cell ratio. Multiple nucleoli show active ribosomal gene amplification, which supports protein synthesis and is essential for oocyte growth (Vincent *et al.,* 1969; Vlad, 1976; Monaco *et al.,* 1981).

(c) Late perinucleolar stage

In the late peri-nucleolus stage, oocytes become more regularly shaped and large in size, with small lipid vacuoles beginning to appear within the cytoplasm (Fig 1c).

During the primary growth stage, the size of the oocyte increases in diameter from 50-60 µm to 120-150 µm. Similarly, a considerable increase in oocyte diameter in the primary

growth phase of rainbow trout was observed from 10-20 µm to 100-200 µm (Nagahama, 1983; Sumpter *et al.,* 1984).

Developing stage

The developing stage of the ovary consists of cortical alveolar oocytes. The cytoplasm shows the accumulation of lipid vesicles and cortical alveolus, along with presence of numerous nucleoli around the periphery of the nucleus. The follicular layers of the oocyte, zonaradiata, granulosa and theca are clearly visible at this stage (Fig 1d). The diameter of cortical alveolar oocytes ranged from 150 to 250 microns. This is the transition from primary growth to the vitellogenesis stage.The presence of cortical alveolar oocytes in the developing stage of the *E. tetradactylum* ovary has also been reported by (Pember *et al*., 2005; Shihab *et al.,* 2017; Soe *et al.,* 2023).

Maturing stage

The maturing stage of the ovary of *E. tetradactylum* consists of vitellogenic and late vitellogenic oocytes, such as the primary yolk, secondary yolk and tertiary yolk stages.

Primary yolk vesicle oocytes were characterized by yolk globules between yolk vesicles in the peripheral region of the cytoplasm (Fig 1e). This indicates the initiation of vitellogenesis. Yolk granules are small, spherical in shape and basophilic. Increase in size and numbers of oil droplets in the central region of oocytes, which distributed around the nucleus. Increase in size and number of oil droplets in the central region of oocyte, distributed around the nucleus. The thickness of the follicular layer increased to 5 µ.

Secondary yolk stage oocytes measure 330-420 µm. This stage represents the active vitellogenic phase, where the oocyte increases in size due to the uniform accumulation of yolk globules and oil droplets throughout the cytoplasm. Oil globules and yolk vesicles surround the nucleus (Fig 1f). The follicular layer of the oocyte differentiates into two distinct layers: the zonaradiata externa and the zonaradiata interna.

The tertiary yolk stage oocytes measure (420-550 µm). The increased accumulation of yolk globules in the cytoplasm results in a significant increase in oocyte size. Oil droplets in the cytoplasm attach and fuse, forming a few large oil globules (Fig 1g). Further thickening of the zonaradiata is observed. Vitellogenesis is the vital mechanism in teleosts that causes the enormous growth

Table 1: Ovarian maturity stages and oocyte development stages in *Eleutheronema tetradactylum.*

Ovarian maturity stage	Oocyte stages	Type of oocytes	Diameter (µm)	Characteristics
Immature	Pre-vitellogenic (50-250 µm)	Chromatin nucleolus stage	50-80	Oocytes are small and compact, with a single prominent nucleus located centrally.
		Perinucleolar stage	80-120	Presence of numerous nucleoli along the periphery of nuclear membrane of oocyte.
		Late perinucleolar	120-150	small lipid vacuoles beginning to appear within the cytoplasm.
Developing		Cortical alveolar	150-250	Accumulation of yolk globule and few oil droplets in cytoplasm and follicular layers are distinctly visible.
Maturing	Vitellogenic (250-420 µm)	Primary yolk stage	250-330	The oocyte size gradually increases due to the accumulation of yolk vesicles and Increase in number of lipid droplets with in cytoplasm.
		Secondary yolk stage	330-420	Yolk globules and oil droplets are uniformly distributed within the cytoplasm. Zonaradiatadifferentiates into two distinct layers: the outer and inner zonaradiata.
	Late Vitellogenic (420-510 µm)	Tertiary yolk stage	420-550	Increase in the size of yolk granules and accumulation in the cytoplasm, large oil droplets are distributed around the nucleus.
Ripe	Post Vitellogenic (550-750 µm)	GVM	550-650	Oil droplets combine into a single oil globule, which displace the germinal vesicle to the cytoplasm periphery.
		GVBD	650-720	Migration of germinal vesicle to the animal pole is evident with the presence of single large oil globule at the center of oocyte.
		Hydrated	720-750	Oocyte become translucent and enlarged due to intake of water.
Spent	Post spawning	Follicular atresia	-	Post ovulatory follicles, characterized by presence of empty ovarian follicle.

of oocytes; it involves the uptake and incorporation of vitellogenin, a liver-derived plasma precursor, into the developing yolk proteins. This process can contribute up to 95% of the final egg size (Wallace, 1978; Tyler, 1991).

Ripe stage

In ripe oocytes, the number of oil droplets increases and they gradually merge to form a single large oil globule, which displaces the germinal vesicle to the cytoplasm periphery (Fig 1h).

Hydrated stage

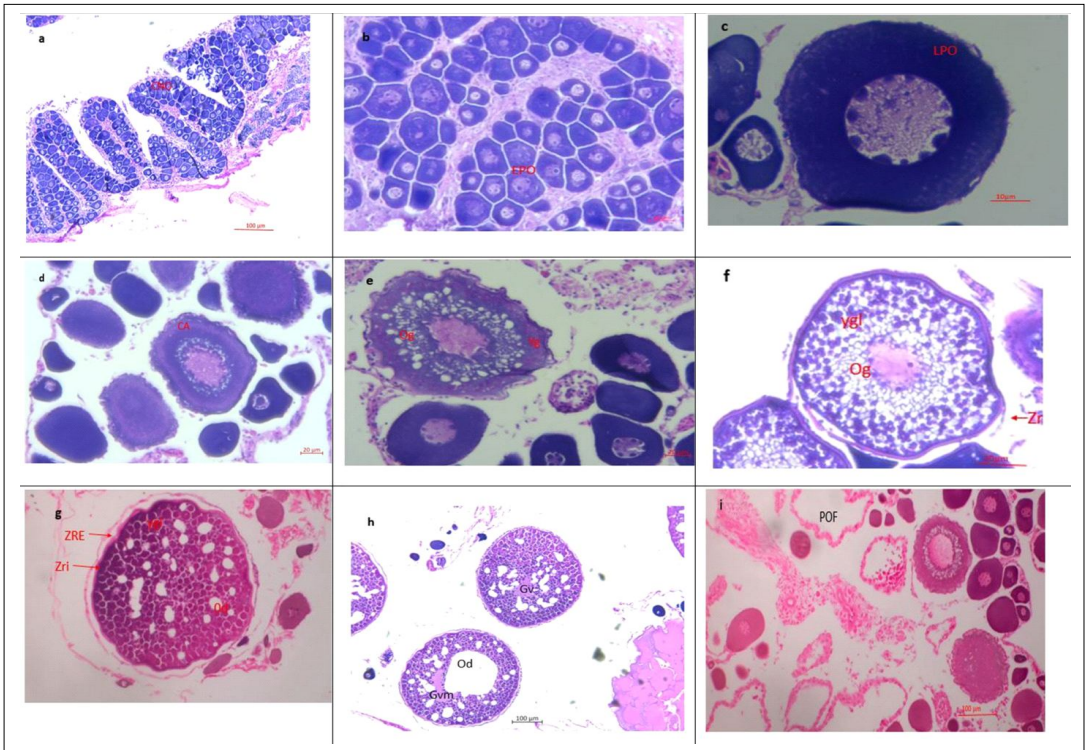
The size of the oocyte increases to 720-750 µm due to excessive water intake and becomes translucent. Hydration is essential for buoyancy and fertilization. In many marine finfish, hydration facilitates the size of the oocyte during maturation (Fulton, 1898; Clemens and Grant, 1964; Hirose and Ishida, 1974; Hirose *et al.,* 1976). The hydration of oocytes during the final maturation is crucial to producing buoyant pelagic eggs in species like sea bass, mummichog, cod and halibut (Carnevali *et al.,* 1991, 1992).

Spent stage

The spent stage of the ovary consists of post-ovulatory follicles, characterized by the presence of empty ovarian follicle and, the presence of primary oocytes like chromatin nucleolus stage, perinucleolar stages, cortical alveolar and primary stage oocytes can be seen (Fig 1i).

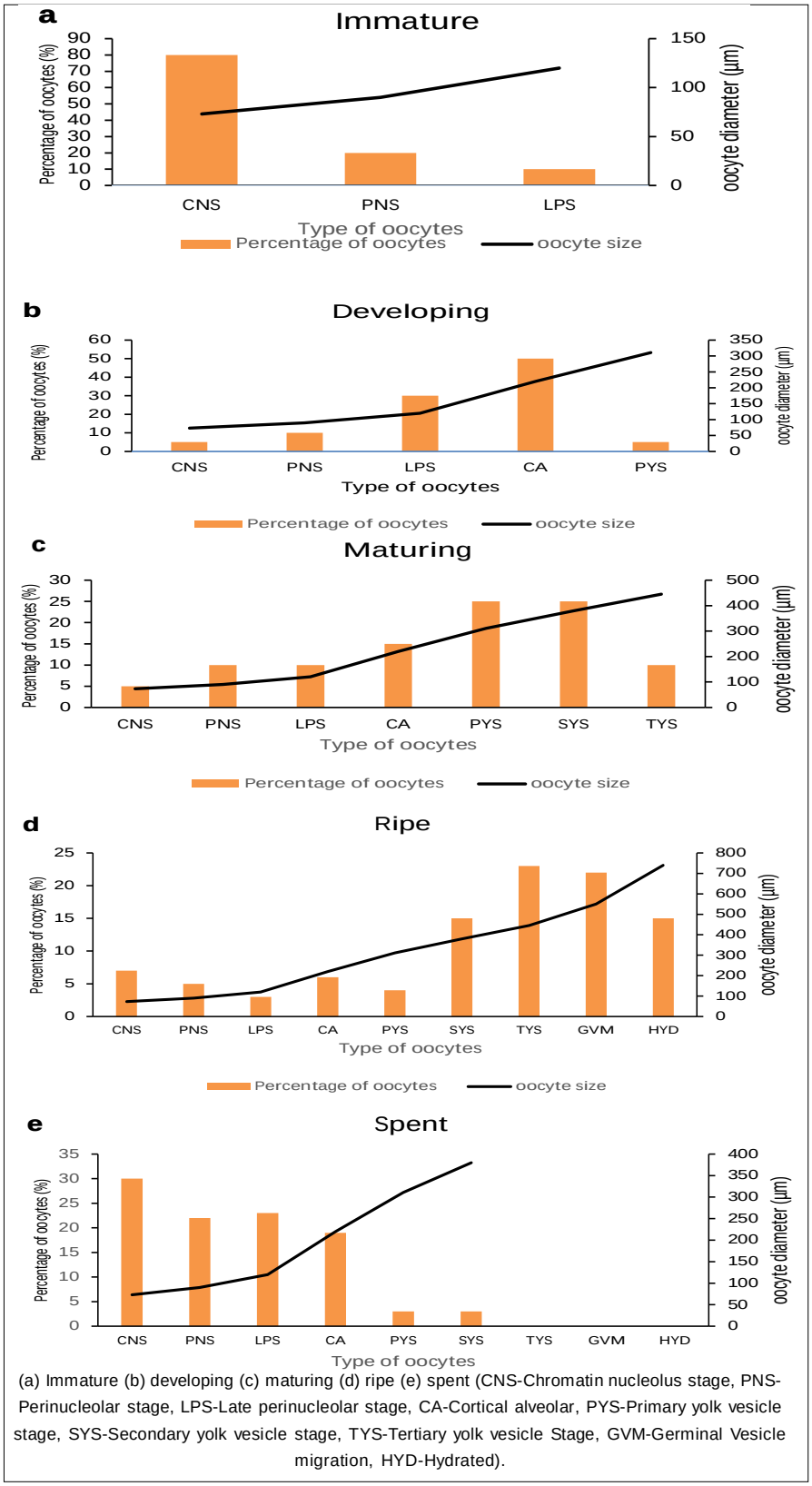
Percentage distribution and size of oocytes in different stages of ovarian development in *Eleutheronema tetradactylum*

The percentage frequency distribution of oocytes in different stages of ovarian development is depicted in (Fig 2 a to 2 e). In the Immature stage of the ovary, 80% of the oocytes were in the chromatin nucleolus (50-80 µm) stage, 20% were early-perinucleolar stage (80-100 µm) and 10 % were late-perinucleolar stage (120-150 µm) (Fig 2a). In developing stage of the ovary, indicated the presence of different stages of the ovary, dominated by the presence of a higher percentage of cortical alveolar stage (50 %) followed by late perinucleolar stages, early perinucleolar and chromatin nucleolus stage (Fig 2b). The maturing overy contains oocytes of all developmental stages, previtellogenic to vitellogenic. The ovary consists mainly of primary yolk stage oocytes, which comprise 25% with a diameter of 311 µm and secondary yolk stage oocytes, which constitute 25% with a diameter of 380 µm (Fig 2c). The ripe ovary consists of oocytes of all developmental phases, ranging from



(a) Clutches of oocytes in chromatin nucleolus stage; (b) Early perinucleolar stage; (c) Late perinucleolar stage (d) Cortical alveolar stage; (e) Primary yolk granule oocyte (f) Secondary yolk granule oocyte (g) Tertiary yolk granule oocyte (h) Germinal vesicle migration oocyte (i) Hydrated oocyte (j) Post ovulatory follicles (CNO-chromatin nucleolus stage, Yg-Yolk granule, Ygl-Yolk globule, Og-Oil globule, Od-Oil droplet, Zr-Zona radiata, Zri-Zonaradiata interna, Zre-Zonaradiataexterna, POF-Post ovulatory follicle.

Fig 1: Histological representation of oocyte development stages of *E. tetradactylum*.



(a) Immature (b) developing (c) maturing (d) ripe (e) spent (CNS-Chromatin nucleolus stage, PNS-Perinucleolar stage, LPS-Late perinucleolar stage, CA-Cortical alveolar, PYS-Primary yolk vesicle stage, SYS-Secondary yolk vesicle stage, TYS-Tertiary yolk vesicle Stage, GVM-Germinal Vesicle migration, HYD-Hydrated).

Fig 2: Percentage distribution and sizes of oocytes in different stages of ovarian development in *Eleutheronema tetradactylum.*

previtellogenic to hydrated (Fig 2d). The tertiary yolk stage oocytes comprise 23% and have a diameter of 445 µm. Oocytes in the germinal vesicle migration stage constitute 22% with a diameter of 550 µm, while hydrated oocytes constitute 15% with a diameter of 740 µm. The spent ovary primarily contains oocytes in the early stages of development (Fig 2e). Cortical alveolar stage oocytes account for 19% with a diameter of 220 µm, late perinucleolar stage oocytes represent 23% with a diameter of 120 µm and primary perinucleolar stage oocytes comprise 22% with a diameter of 90 µm. Cortical alveolar stage oocytes comprise 30% of the population, exhibiting a diameter of 73.12 µm. Oocytes at the primary and secondary yolk stages are diminished, representing 3%, with diameters measuring 311 µm and 380 µm, respectively. The absence of the tertiary yolk stage, germinal vesicle migration stage and hydrated oocytes was noted.

Based on histological sections, percentage of type and size of oocyte distributions in the ovary, we describe *E. tetradactylum* is asynchronous multiple spawners with indeterminate fecundity.

The various ovarian maturity stages of many stages of oocytes confirm asynchronous oocyte growth and further, the pattern of continuous oocyte size frequency in all maturity stages from maturing to ripe stage, batch spawning strategy, where, oocytes are continuously recruited, matured and ovulated in batches during the spawning season. Similarly, Pember *et al.* (2005) reported indeterminate fecundity in *E. tetradactylum,* characterized by sustained oocyte recruitment and maturation during the spawning season in Australian waters.In ovaries characterized by asynchronous development, oocytes of various stages of development are present, with no single stage dominating the population (Murua and Saborido-Rey, 2003; Lubzens *et al.,* 2010; Lowerre-Barbieri *et al.,* 2011). Batch spawners with indeterminate fecundity show unrestricted secondary growth (SG) and continuously recruit oocytes for spawning throughout the season (Ganias *et al.,* 2017). In line with our observations, previous studies reported ova are released in batches, suggesting continuous spawning in *Eleutheronema tetradactylum*, supported by the consistent presence of larvae and fry stages in the waters of Bombay (Karandikar and Palekar, 1950) and the Hooghly estuary (Sarojini and Malhotra, 1952; Ravish, 1962). Similarly, Soe *et al.* (2023) reported asynchronous pattern of ovarian maturation in *E. tetradactylum* from the waters of Thailand.

CONCLUSION

In this study, we described the oocyte developmental stages of *Eleutheronema tetradactylum* into five distinct stages: previtellogenic, vitellogenic, late-vitellogenic, post-vitellogenic and follicular atresia. Further, through histological, microscopic and percentage type and size distribution of oocytes analysis. We confirmed *E. tetradactylum* is an asynchronous multiple spawner through the presence of continuous oocyte size frequency in maturing to the ripe stage, which indicates a batch spawning strategy. The findings provide essential baseline data for assessing oocyte maturity and optimizing hormonal induction protocols in artificial breeding programs.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided but do not accept any liability for any direct or indirect losses resulting from the application of this content.

Ethics statement

This study did not involve the use of live animals. All data collection and analyses were conducted using non-invasive methods and ethically obtained samples in compliance with relevant guidelines and regulations.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the study, data collection, analysis, or decision to publish or write the manuscript.

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