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Original Article

Ovarian histology of toadfish *Batrachomoeus trispinosus* from Pranburi River estuary, Thailand

Sinlapachai Senarat[1*], Jes Kettratad[2, 3], Piyakorn Boonyoung[4], Wannee Jiraungkoorskul[5], Gen Kaneko[6], Ezra Mongkolchaichana[7], and Theerakamol Pengsakul[8]

[1] *Department of Marine Science and Environment, Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang Campus, Sikao, Trang, 92150 Thailand*

[2] *Department of Marine Science, Faculty of Science, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

[3] *Marine Ecology and Marine Resources Utilization Research Unit, Aquatic Resources Research Institute, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

[4] *Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

[5] *Department of Pathobiology, Faculty of Science, Mahidol University, Ratchathewi, Bangkok, 10400 Thailand*

[6] *School of Arts and Sciences, University of Houston-Victoria, Victoria, Texas, 77901 United States of America*

[7] *Department of General Education, Faculty of Science and Health Technology, Navamindradhiraj University, Dusit, Bangkok, 10300 Thailand*

[8] *Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand*

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Abstract

Knowledge of the reproductive biology of the toadfish is still limited. The present study therefore aimed to provide basic knowledge about ovarian histology in the mangrove toadfish *Batrachomoeus trispinosus*, a potential aquaculture species in Thailand. To this end, we performed histological analysis of the ovaries (n = 20, all females, total length 15 to 20 cm) obtained from the Pranburi River estuary, Thailand, during January to April 2017. The ovary of this species was a paired sac-like organ located in parallel to the digestive tract. We described histological details for the oocytes at following phases: oogonium, primary growth phase (further classified into perinucleolar and oil droplets-cortical alveolar steps) and secondary growth phase (early secondary growth, late secondary growth, and full-grown oocyte steps). The ovary contained oocytes at different developmental phases. Most oocytes were in the secondary growth phase (60%), although those in primary growth phase (33.33%) and a few oogonia (6.66%) were also observed. The primary growth phase was associated with accumulated lipid droplets and cortical alveoli, whereas oocytes at the secondary growth phase contained larger lipid droplets, spherical yolk granules, and associated changes of the follicular cell. Overall information from this study provided accurate ovarian features, which will support further works on the reproductive biology of this species.

Keywords: Batrachoidae, lipid droplets, microanatomy, ovary, reproduction, Thailand

*Corresponding author
Email address: senarat.s@hotmail.com

1. Introduction

The female reproductive systems of fish are composed of the ovary and reproductive duct (or oviduct). The ovary contains oocytes at various differentiating stages, which are generally classified into four phases with reference to the morphological characteristics such as cell size and histological structures (Al-Daham & Bhatti, 1979; Gupta, 1975; Mayer, Shackley & Rylan, 1988): the oogonia, immature oocyte, maturing oocyte, and mature oocyte. Oocytes are further classified into previtellogenic, vitellogenic and postvitellogenic (or mature) stages according to the accumulation of yolk granules (Gupta, 1975). In a more recent classification, Uribe, Grier & Parenti (2012) categorized developing oocytes into the oogonia proliferation stage, chromatin-nucleolus stage, primary growth stage, and secondary growth stage. These classifications facilitate systematic understanding of reproductive morphology, and are especially useful for early-phase investigations of poorly studied species.

The family Batrachoidae is comprised of three sub-families containing 69 toadfish species, which have been frequently used for reproductive biology. For example, Munoz-cueto *et al.* (1996) proposed the classification of the developing oocytes (previtellogenic and vitellogenic stages) using the Lusitanian toadfish *Halobatrachus didactylus*. Four-stage classification (oogonia, previtellogenic, vitellogenic, and post-ovulatory) was also proposed from *H. didactylus* (Arias & Drake, 1990; Blanco, 1991). However, very little is known for the reproductive biology of a mangrove toadfish *Batrachomoeus trispinosus*, which is a aquaculture candidate species in Thailand due to its abundance in the Pranburi River estuary, Thailand. Therefore, in this study, we attempt to identify the ovarian histology of *B. trispinosus* from the Pranburi River estuary, Thailand, using wet-mount, histological and histochemical techniques.

2. Materials and Methods

2.1 Fish collection, study area, and gravimetric analysis

Dead female specimens of *Batrachomoeus trispinosus* (n=20) used for this study were donated by a local fisherman during January to April 2017. The sampling season was determined based on our preliminary observation of maturation stages in this and other toadfish species. To our knowledge, there is no report regarding the breeding season of *B. trispinosus*. These specimens were caught in the Pranburi River estuary, Thailand (12° 24.314' N, 099° 58.597' E). Because we only used dead specimens, this study did not require the protocol approval by the Faculty of Science, Chulalongkorn University. The authors have no conflict of interests relevant to the sampling method. All fish (n = 20) were used for the measurement of total length and body weight, followed by abdominal incisions starting from the cloacal opening toward the anterior region. Their reproductive systems (ovary and reproductive ducts) were dissected out and weighted for calculating the gonadosomatic indices (GSI) using the following formula (GSI = [ovarian weight/total weight x 100]). Subsequently, the ovaries were placed in Ringer's solution for the morphological observation. A small ovarian area was also used to study oogenesis under stereomicroscopy.

2.2 Histological and histochemical observation

To confirm the histological structure of the reproductive system, *B. trispinosus* tissue specimens were processed according to standard histological procedures (Presnell and Schreibman, 1997; Suvarna, Layton & Bancroft, 2013). Paraffin sections at 4 µm thickness were stained with haematoxylin–eosin (H&E), as well as Masson's trichrome (MAT) and periodic acid Schiff (PAS) to identify structural and chemical characteristics (Presnell & Schreibman, 1997; Suvarna *et al.*, 2013). Sections were observed under a light microscope, and ovarian developmental stages were classified according to Uribe *et al.* (2012) and Dietrich & Krieger (2009) with minor modifications. Photographs were taken with a Leica DM750 light microscope (Boston Industries, Inc.; U.S.A.).

2.3 Oocyte diameter and oocyte proportion

Oocyte diameters were measured for 15–30 oocytes in three randomly selected regions of three ovarian sections (anterior, middle, and posterior) following the guidelines under light microscopy (10x and 40x) (Blazer, 2002). The length of each section was about 1 cm. The proportion of oocytes in each developmental stage (oogonia, primary growth phase and secondary growth phase) was determined from each histological section (30 oocytes per section).

2.4 Lipid distribution

Lipid distribution was investigated through observation of wet mount slides stained with oil red O (ORO) (Central Drug House (P) Ltd., New Delhi, India). We used ten representative ovarian areas in the secondary growth stage, and images were illustrated by Adobe PS6 software.

3. Results and Discussion

3.1 Gross morphology of the reproductive system

In this study, our *B. trispinosus* specimens had 18.72 ± 1.45 cm of total length and 77.48 ± 1.98 g of mean body weight (Table 1; n = 20, mean ± standard deviation). The total length ranged from 15.00 to 20.07 cm, and the average GSI was 3.59 ± 2.90%. There was a tendency that large individuals have higher GSI. The highest GSI was recorded in a large individual (4.67%, 20.07 cm of total length), whereas the lowest GSI in a small individual (2.30%, 15.00 cm of total length).

All specimens analyzed in this study showed similar anatomical features in the female reproductive system. We observed a typical paired ovary situated in fat bodies, which was located dorsally to the digestive tract. The ovary had a sac-like oval shape (Figure 1A) and was anchored to the kidney by the mesovarium. All external morphological feature of the ovary showed as a yellow-orange color with differential stages of oocyte. It is suggested that all samples were collected during the breeding season. The posterior part of the ovary was connected to the short oviduct, which was further

Table 1. Mean total length (TL), mean total weight (TW), gonadosomatic index (GSI) and ovarian development (OD) of *Batrachomoeus trispinosus.*

Characterizations of fish	TL (cm)	TW (g)	GSI	OD
B. trispinosus	18.72±1.45	114.15±2.41	3.59±2.90	Most of secondary growth phase**,** primary growth phase and a few oogonia

connected separately to the cloaca (Figure 1A).

The ovary contained oocytes at different developmental phases. Most oocytes were in the secondary growth phase (60%), although those in primary growth phase (33.33%) and a few oogonia (6.66%) were also observed. These results indicate that *B. trispinosus* in the size range of 15–20 cm generally has reached sexual maturation in the Pranburi River estuary, facilitating the future determination of the length at first maturity of this species (must be below 15 cm) with a larger sample size. *Halobatrachus didactylus* females are reported to reach sexual maturity at 19.1 cm in total length, although the length at first maturity may be changed by nutritional and environmental conditions (Palazón-Fernández, Arias & Sarasquete, 2001).

3.2 Histological and histochemical observations of the ovary

Cross-sections revealed that the ovary of *B. trispinosus* is clearly separated to cortex and medulla, and covered by a thick layer of tunica albuginea (Figures 1B, 1C). The major component of the ovary was loose connective tissue (Figures 1B, 1C). The germinal epithelium contained a layer of tall cells (Figure 1D). Higher magnification images confirmed that the tunica albuginea was composed of three layers: peritoneum, stroma and germinal epithelium (Figures 1C, 1D).

Oocytes at different developmental stages were observed in the cortex region throughout the ovary (Figures 1E-1F). This result suggests that this species undergo asynchronous oocyte development, which is commonly observed in iteroparous species. This possibility should be validated by additional sampling to determine the reproductive cycle and spawning season. This hypothesis needs to be done in further study. The medulla region was composed of a connective tissue [with pink color in H&E staining (Figure 1G) and greenish color in MAT staining (Figures 1H, 1I)] containing several blood vessels. Blood vessels were also observed in cortex.

3.3 Histological and histochemical observations of oocytes

In this study, developmental phases of *B. trispinosus* oocytes were classified into four phases including oogonia proliferation, primary growth phase, secondary growth phase, and post-ovulatory phases based on histological features. This classification is similar to those of *H. didactylus* and other fishes (Lahaye, 1981; Wallace & Selman, 1981; Selman & Wallace, 1989).

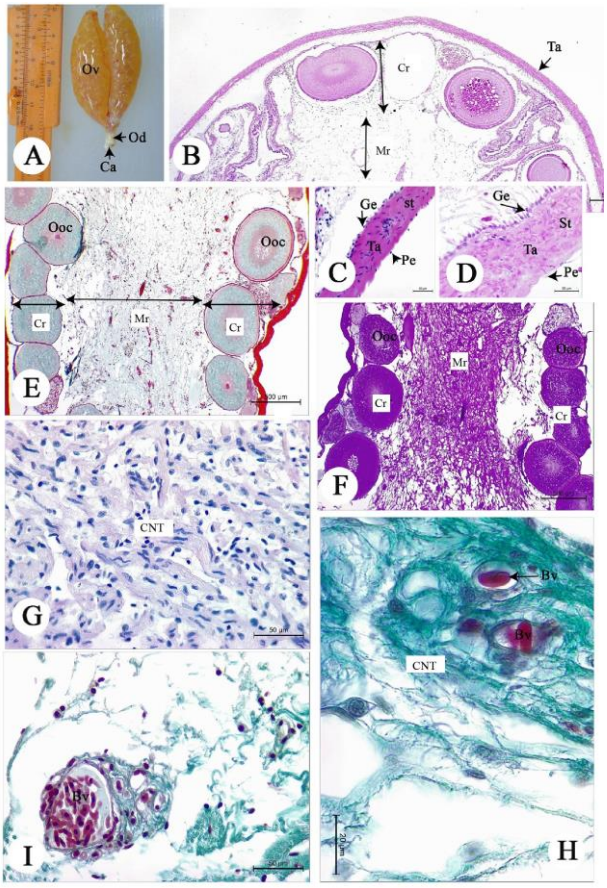

Figure 1. Morphology of the female reproductive system with consisting of two regions [Ovary (Ov) and oviduct (Od)] of *Batrachomoeus trispinosus*. A: Morphology of the paired ovary structure. B: Light photomicrographs of the ovarian structure. C-F: The oocyte was clearly separated into cortex region (Cr) and medullar region (Mr), which are enclosed by the tunica albuginea (Ta). G-I: The medullar region contained the connective tissue (CNT) and several blood vessels (Bv). Note: Ca = cloaca, Ge = germinal epithelium, Ooc = oocytes, Pe = peritoneum, St = stoma. B, C, D, G = Harris's haematoxylin and eosin (H&E), F = periodic acid-Schiff (PAS), E, H, I = Masson's trichrome (MT). Scale bars = 500 µm (E, F), 50 µm (C, D, G, I), 20 µm (H).

3.3.1 Oogonia proliferation (Op)

The oogonia (Og) were localized in the germinal compartment of the ovary. The smallest Og had 20 ± 0.87 µm diameter and an oval-rounded shape in the germinal

epithelium (Figure 2A). A spherical nucleus contained a prominent nucleolus, which was surrounded by eosinophilic cytoplasm (stained by the H&E method) (Figure 2A). Some oogonia formed in the nest as an oogonial cyst (Figure 2A). A few squamous-shaped prefollicular cells were observed, but they did not completely cover the oogonia. Such oogonial structures are commonly observed in teleosts (Selman, Wallace, & Sarka, 1993; Grier, 2000). Occasionally, the primordial germ cells were also observed in this study (Figure 2A).

3.3.2 Primary growth phase (PGP)

According to Uribe *et al.* (2012), the PGP is further classified into four steps: one nucleolus step, multiple nucleoli step, perinucleolar step, and oil droplets-cortical alveoli step. In our specimens, we observed oocytes at perinucleolar (Pn) and oil droplets-cortical alveolar steps (Oc), but not one nucleolus and multiple nucleoli steps. These results suggest that the developmental transformation from the oogonia into the perinucleolar stage is rapid in this species. This feature is quite similar to those reported in some teleosts including *Epinephelus marginatus* (Mandich *et al.*, 2002), *Thunnus orientalis* (Chen, Crone & Hsu, 2006) and *Thunnus thynnus* (Sarasquete *et al.*, 2002).

The size of oocytes in the Pn step was bigger than that of the oogonium, being about 160 ± 0.98 µm in diameter (Figures 2B, 2C) with a central nucleus of about 35 µm. The ooplasm reacted strongly in basophilic staining (Figure 2C), indicating that the oocyte was in a period of intense RNA synthesis coupled with ribosome production to support oocyte development (Wallace & Selman, 1990). It is also possible that the basophilic structure is the Balbiani body, a large organelle aggregate found in developing oocytes of many species, as reported in a previous investigation on *Oryzias latipes* (Mayer *et al.*, 1988; Wallace & Selmann, 1981). During this step, the elongated follicle cells completely surrounded the oocyte (Figure 2C).

The size of oocytes at the Oc step was about 180 ± 0.63 µm in diameter. The increase in size resulted from an accumulation of oil droplets and cortical alveoli. Spherical oil droplets were observed near the nuclear membrane (Figures 2D, 2E) as reported in other fish species such as *Dicentrachus labrax* (Mayer *et al.*, 1988), *H. didactylus* (Munoz-cueto *et al.*, 1996), *C. undecimalis* (Neidig, Skapura, Grier, & Dennis 2000) and *R. brachysoma* (Senarat, Kettretad, & Jiraungkoorskul, 2017a, 2017b). The oil droplets contain several forms of lipids including neutral lipids, phospholipids and glycolipids, which provide the energy for embryonic development (Guraya, 1986; Nagahama, 1983; Wiegand, 1996). In the Oc step the cortical alveoli were spherical in shape and varied in size between 10 - 20 µm (Figure 2E; negatively stained with PAS). The function of cortical alveoli is the prevention of polyspermy after ovulation as proposed by Nagahama (1983), and the existence of cortical alveoli at this step is also reported in other fishes (Selman, Wallace, & Barr, 1988; Wallace & Selman, 1990). During this step, multiple nucleoli were often detected at the periphery of the nucleus (Figure 2D). Also, at this step we firstly observed the follicular complex consisted of three layers: zona pellucida, granulosa cells, and theca cells (Figure 2D). The acidophilic zona pellucida was a thin layer of about 2-3 µm thickness

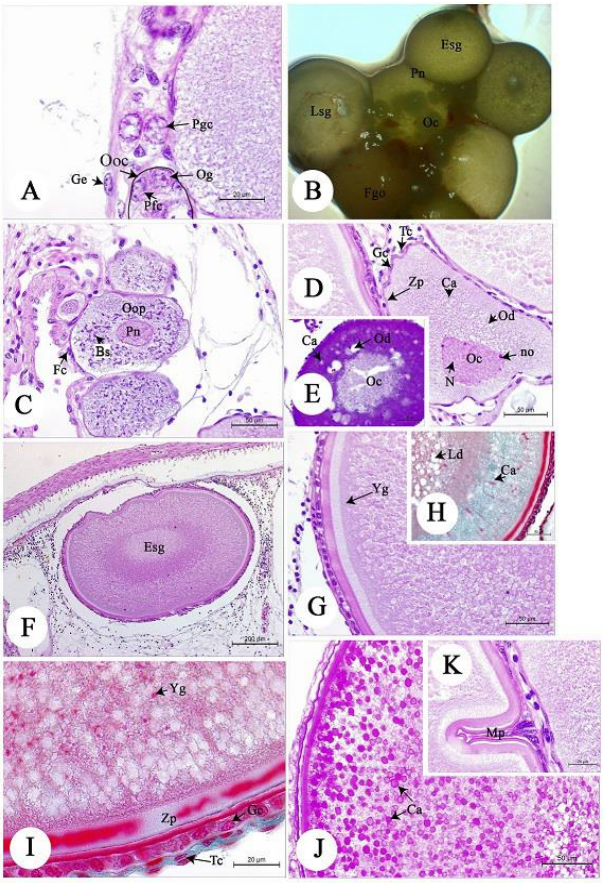

Figure 2. Light photomicrographs of the oogenesis of *Batrachomoeus trispinosus*. A: The germinal compartment consisting of primordial germ cell (Pgc) and oogonium (Og) in the oogonial cyst (Ooc) of *B. trispinosus*. B-J: Morphology and light photomicrographs showed that perinucleolar (Pn), oil droplets and cortical alveolar step (Oc) and early secondary growth step (Esg). Bs = basophilic structure, Ca = cortical alveoli, Fc = follicular cell, Fgo = full-growth oocyte, Gc = granulosa cell, Ge = germinal epithelium cell, Ld = lipid distribution, Lsg = late secondary growth, Mp = micropyle, N = nucleus, no = nucleolus, Od = oil droplets, Oop = ooplasm, Pfc = pre-follicular cell (Pfc), Pgc = primordial germ cell, Tc = theca cell, Yg = yolk granules, Zp = zona pellucida. A, C-D, F-G = Harris's haematoxylin and eosin (H&E), G = Masson's trichrome (MT), J-K = periodic acid-Schiff (PAS). Scale bars = 200 µm (F), 50 µm (C, D, E, G, J), 20 µm (A, I, K).

(Figure 2D). At the final step of this phase, two inclusions (oil droplets and cortical alveoli) progressively increased in number and size in the ooplasm; they varied in size (Figure 2E).

3.3.3 Secondary growth phase (SG)

The SG is divided into early secondary growth step (Esg), late secondary growth step (Lsg), and full-grown oocyte step (Fgo) (Uribe *et al.*, 2012). Oocytes increased their size in the Esg up to about 450 ± 0.91 µm in diameter (Figure 2F). Deeply acidophilic small yolk granules were first detected at this step (Figure 2G). These features were similar

to those observed in other teleosts (Guraya, 1986; Wallace & Selman, 1990); indeed, Wallace & Selman (1990) reported the occurrence of the yolk granules to mark the initiation of the secondary growth stage (Wallace & Selman 1990; Chen *et al.*, 2006). The small yolk granules slightly reacted with PAS, implying the presence of glycoprotein (Figures 2G, 2I). It is well known that yolk granules contain vitellogenin, a glycolipophosphoprotein transported by blood vessels from the liver to the ovarian follicle, which is eventually taken up by developing oocytes. Vitellogenin is cleaved into smaller molecular weight polypeptides: the yolk proteins lipovitellin, phosvitin, and ß-component (LaFleur *et al.*, 2005; Grier, Uribe-Aranzábal, & Patiño, 2009; Yoon *et al.*, 2008;).

The oil droplets and cortical alveoli progressively increased the number and size in the Esg. Cortical alveoli had strong reactions with MAT and PAS displaying a greenish (Figure 2H) and pink colors (Figures. 2I, 2J), respectively, demonstrating the presence of polysaccharide and mucopolysaccharides. The zona pellucida was 10 µm in thickness and distinctly striated by the MAT method (Figure. 2I). A single layer of the granulosa cell still remained, although it became thin during this step (Figure 2I). A layer of the theca cells was also seen (Figure 2I). A well-developed micropyle, containing the follicular complex, was appeared in this step (Figure 2K). It is well known that this structure allows the spermatozoa to directly reach the oocyte surface without acrosomal reaction (Bartsch & Britz, 1997).

In the Lsg, oocytes increased their diameter to 856 ± 1.22 µm because of the extensive accumulation of yolk granules (Figures 3A, 3B). The fusion of the yolk granules was observed in ooplasm (Figure 3B). The oil droplets and cortical alveoli were still observed, but they moved toward the follicular complex (Figure 3C). This comprehensive feature was commonly observed in other fishes, for example *Fundulus heteroclitus* (Kuchnow and Scott, 1977), *T. orientalis* (Chen *et al.*, 2006) and *R. brachysoma* oocyte (Senarat *et al.*, 2017b). The striated zona pellucida was firstly classified into the thin external layer and thick internal layer at this step (Figure 3C). The layers of the granulosa had columnar epithelium cells (Figure 3C). The theca cells were similar to that seen in the prior step (Figure 3C).

We observed the largest oocyte, attaining 1,200 ± 1.82 µm, in the Fgo, which was the final step of the oogenic process (Figure 3D). No nucleus was observed, which suggests that the oocyte is under the first meiotic cell division. This feature was widely observed at the final maturation step in the teleost (West, 1990). The yolk globules were fused completely and considered as demersal eggs as shown by H&E (Figures 3D-3E) and MAT methods (Figure 3G). These results are in agreement with the previous characterization of yolk granule in *Gasterosteus aculeatus* and *Apeltes quadracus* (Selman & Wallace, 1989). In contrast, the yolk granule of *Scomberomorus brasiliensis* (Chellappa, Lima & Araújo, 2010) and *R. brachysoma* (Senarat *et al.*, 2017b) do not fuse until the mature stage. Note that the single layer of granulosa cells changed into simple squamous epithelium cells (Figures 3F, 3G), whereas the vasculazized theca cells were firstly seen in this step (Figures 3F, 3G).

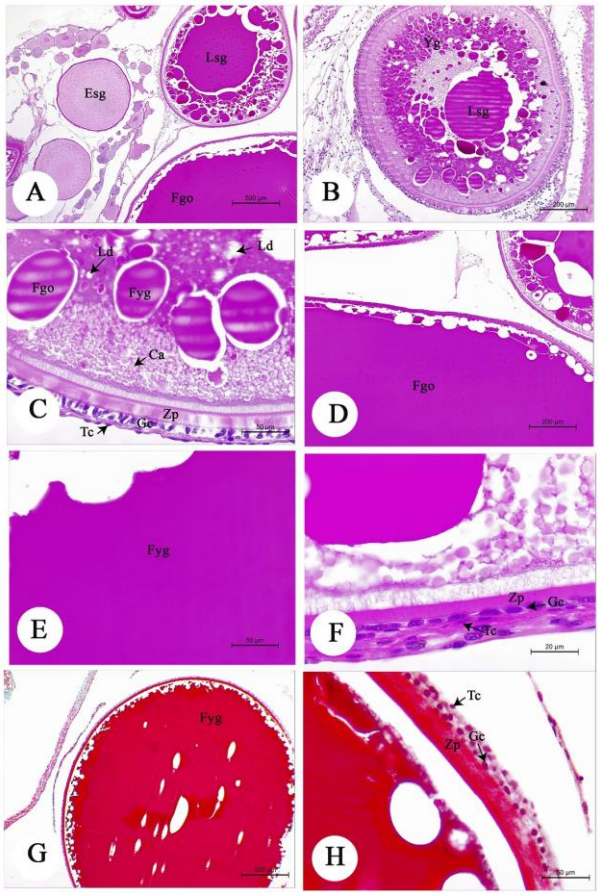

Figure 3. Light photomicrographs showed the oogenesis of *Batrachomoeus trispinosus*. A-B: The differentiating stages of oocytes including the late secondary growth step (Lsg), and full-grown oocyte step (Fgo) of *Batrachus trispinosus*. C-H: The yolk globules of the full-grown oocyte step (Fgo) were fused completely and surrounded by a well-development of the follicular complex (granulosa cell (Gc), theca cell (Tc) and zona pellucida (Zp)). Esg = early secondary growth step, Ca = cortical alveoli, Fyg = filled yolk granules, Ld = lipid distribution, Yg = yolk granules. A-F = Harris's haematoxylin and eosin (H&E), G-H = Masson's trichrome (MT). Scale bars = 500 µm (C, G), 200 µm (B, D), 50 µm (C, E, H), 20 µm (F).

3.3.4 Post-ovulatory phase (POP)

The oocytes at the post-ovulatory phase had a stratified follicle cell layer of the irregular shape (Figures 4A–4C). Several blood capillaries and melanomacrophage centers were also observed (Figure 4D). It is speculated from the presence of POP oocytes that our *B. trispinosus* specimens were in a spawning period. The mangrove reproductive cycle and spawning season of this fish will be determined in further study.

3.3.5 Lipid distribution in the oocytes

According to Mansour *et al.* (2007), the lipid distribution in the oocytes can be classified into four categories, termed category I, II, III, and IV. These categories

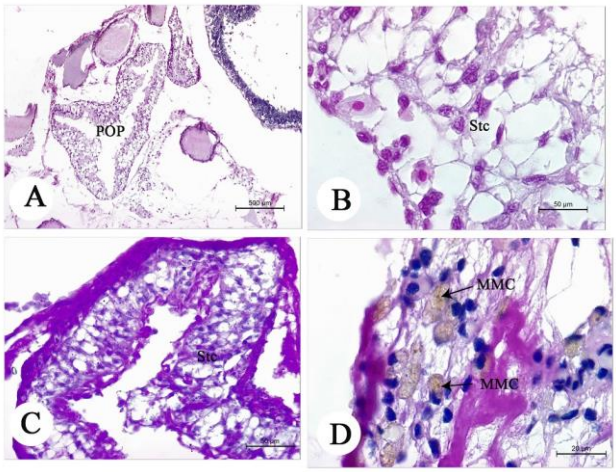

Figure 4. Light photomicrographs of the post-ovulatory phase (POP) composing of the post-ovulatory follicle (A-C) and melanomacrophage center (MMC) (D) of *Batrachomoeus trispinosus*. Stc = stratified follicular cell. A-D = Harris's haematoxylin and eosin (H&E). Scale bars = 500 µm (A), 50 µm (B-C), 20 µm (D).

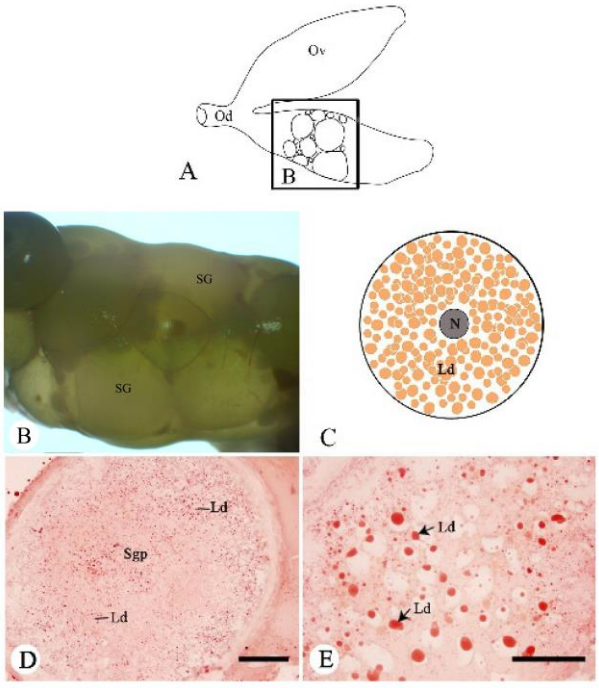

Figure 5. Overall characterizations showed the lipid distribution (Ld) of secondary growth phase (Sgp) of *Batrachomoeus trispinosus*. A: schematic diagram of the female reproductive system [Ovary (Ov) and oviduct (Od)]. B-E: Morphology and light photomicrographs the lipid distribution of secondary growth phase (SG) of Batrachus trispinosus, using oil red O method. Scale bars = 500 µm (B, D), 200 µm (E).

of the lipid distribution have been used to assess the egg quality of fish. When the lipid is uniformly distributed throughout the oocyte, the egg is classified as Category I. In Category II eggs, some lipid droplets are coalesced in one pole of the oocyte, whereas lipid droplets are still observed throughout the oocyte. In Category III eggs, most lipids are concentrated in two pole of the oocyte. Category IV eggs contains a large lipid droplet in one pole of the oocyte, and very few lipid droplets can be observed in other parts.

In this study, we mainly used oocytes in the early secondary growth phase for the observation of lipid distribution. Lipid droplets were clearly visualized by oil red O staining (n = 10) beneath the oolemma on the surface of the oocytes (Supplementary Figures 1A-1C). These lipid droplets were mostly evenly distributed throughout the oocytes (Supplementary Figures 1D-1E), and thus these oocytes were considered to be the Category I (Mansour *et al.*, 2007). Therefore, this category had been associated with high quality egg of *B. trispinosus*. We also observed eggs of Category II (data not shown). These results raise the possibility that lipid distribution pattern can be used to assess egg quality, although several techniques (ovarian fluid composition, egg physiology and egg metabolism) to associate lipid distribution with egg quality need to be applied further.

4. Conclusions

In conclusion, the present study demonstrated the reproductive histology of a mangrove toadfish *B. trispinosus* captured between January and April, 2017. Because all specimens had mature oocytes, total length at first maturity of the fish was estimated to be below 15 cm. The female reproductive system was composed of ovaries and oviducts similar to other fishes. Based on the basic information obtained in this study, further investigation of the reproductive cycle and reproductive strategies of *B. trispinosus* will lead to appropriate estimation of recruitment and effective fishery management.

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