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Research article

The ovarian structure and oogenesis of the pea crab *Pinnotheres cyclinus* Gordon, 1932: A histological investigation

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Abstract

The pea crab *Pinnotheres cyclinus* is a parasite of cultured mollusks. However, the information on reproductive histology of this crab has remained limited. Therefore, the present study investigated the ovarian structure and oogenesis of *Pinnotheres cyclinus* during maturation using histological techniques. Histologically, the ovary of this crab was found to be surrounded by a thin epithelium and connective tissue of the ovarian wall. Different phases of oogenesis were observed in the germinal area and could be classified into four phases: oogonial proliferation, the primary growth phase, the secondary growth phase, and the atretic oocyte phase. An oogonium was located in the ovarian cyst of the ovarian lobe, which was surrounded by a layer of pre-follicular cells. During the primary growth phase, oogonia continued to develop in the ovarian cyst, accumulating lipids and cortical alveoli. The appearance of spherical yolk granules related to changes in follicular cells during the secondary growth phase was also observed. These yolk granules reacted positively to Masson's trichrome and Mollary's trichrome staining, implying the presence of mucopolysaccharides and glycoproteins. Atretic oocytes were also found. Stages of embryonic development were also observed, including the formation of egg membranes covering embryos. Consequently, a fertilized egg was then filled with yolk granules, which all gradually combine to become one within the egg.

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Introduction

Pinnotheres cyclinus, locally known as the pea crab, is a symbiont and parasite of bivalves such as scallops and mussels (Silas and Alagarswawi, 1967; Majchacheep, 1989). This species is broadly known in China, Japan, Korea, and other Southeast Asian countries (Miyake et al., 1962; Silas and Alagarswawi, 1967; Morton and Morton, 1983). This symbiotic/parasitic crab is of interest in mariculture as it positively or negatively affects the life cycle of its bivalve host, which is cultivated and/or harvested from wild populations for commercial exploits (Majchacheep, 1989; Sun et al., 2006; Science Learning Hub, 2019). To date, *P. cyclinus* controlling in several areas has shown little success (Majchacheep, 1989; Science Learning Hub, 2019).

Comprehensive data concerning the ovarian and oocyte structure development of *P. cyclinus* is an important knowledge for further understanding on reproductive biology, reproductive cycle and spawning season of the crab (Castiglioni et al., 2007; Stewart et al., 2007). During oocyte development within crabs, oogonia are produced in the perennial germinal zone and divide mitotically to form primary oocytes (Stewart et al., 2007). Multiple stages of oocyte differentiation can be classified according to morphological and histological characteristics. Five stages of oocytes differentiation were found in *Scylla serrata*, *S. paramamosain,* and *Portunus pelagicus* (Stewart et al., 2007; Islam et al., 2010). Subsequently, Castiglioni et al. (2007) proposed that the oogenetic process of the crab *Uca rapax* consists of six stages. In *Travancoriana schirnerae*, oocyte development was categorized into ten stages (Smija and Devi, 2015).

The knowledge of *P. cyclinus* reproductive cycles of is necessary to control the population of this crab and maintain the economic reserves of economically valuable mollusks. However, information regarding the reproductive histology of this crab is lacking. In the present work, we describe in detail the ovarian structure and oogenesis process of *P. cyclinus* using histological and histochemical techniques. The dynamics of these oogenetic processes were also schematically drawn. Data drawn from our observations can help to increase understanding of the female reproductive biology of *P. cyclinus.* This knowledge can be used to respond to the infective effects of this crab on bivalve mariculture and other aquatic organisms both in Thailand and elsewhere.

Materials and Methods

Ten female *P. cyclinus* with a mean carapace width of 30.50 ± 0.45 mm (CW range: 28.0–31.0 mm) were collected from infected *Perna viridis* from the Upper Gulf of Thailand in Chonburi Province, near Si Racha District (13°10’28.4”N, 100°55’10.1”E), from December 2015 to February 2016. Following capture, all live crabs were air-lifted and transported to a laboratory. The crabs were euthanized by rapid cooling shock (Wilson et al., 2009), and then the dorsal parts of their carapaces were opened. Then, the whole body of each crab, without the carapace, was fixed in Davidson’s fixative (48 hr) and processed into thin sections according to standard histological techniques (Presnell and Schreibman, 1997; Suvarna et al., 2018). These sections were dehydrated using a series of alcohols from 95% ethyl alcohol to absolute alcohol and embedded in paraffin. Each serial section was cut to a thickness of 4 μm and then stained with hematoxylin-eosin (H&E) to study the basic structure of the ovarian tissue. For the detection of chemical details, some sections were histochemically stained with Masson’s trichrome (MT), periodic acid-Schiff (PAS), or cresyl violet (CV) (Presnell and Schreibman, 1997; Suvarna et al., 2018). The ovarian structure and oogenesis processes of *P. cyclinus* were examined and photographed with a Leica TE2000-U, following guidelines described by Mota and Tomé (1965), Kulkarni et al. (1991), Becker et al. (2011), and Ravi et al. (2013). Oocyte and follicular layer diameters were measured using a calibrated micrometer with a Leica TE2000-U. A schematic diagram of the ovarian structure and the development stages of oocytes in this species was made using Adobe Illustrator (version CS6).

Results and Discussion

Histological observation of the ovarian structure

The results showed that all specimens had attained ovarian maturation, at which point the ovarian structures were located among the digestive organs (Figs. 1A) and surrounded by digestive glands (Fig. 1B). The ovarian wall of *P. cyclinus* was histologically composed of a thin layer of epithelium and connective tissue (Figs. 1C–D). However, it differed from the ovarian walls of other decapods, which consist of multiple layers: an outermost thin pavement epithelium, a middle layer of connective tissue, and an innermost layer of germinal epithelium, as was reported in *Portunus pelagicus* (Ravi et al., 2013), *Litopenaeus setiferus* (King, 1948), *Monodon monoceros* (Abraham, 2005), and *Ranina ranina* (Minagawa et al., 1993). In *P. cyclinus,* the layers of the ovarian wall were filled with interstitial tissue, which had separated into several ovarian lobes (Figs. 1C–D). Each lobe composed of several ovarian cysts (Figs. 1D and 2). The different stages of oocyte development occurred in the ovarian cysts (Fig. 2). The following four phases of oocyte development were classified based on cell size, nucleus characterization, cytoplasmic properties, and degree of yolk granules: oogonia proliferation, the primary growth phase (PGP), the secondary growth (SG) phase, and the atretic oocyte phase. These criteria have been commonly used to classify oogenesis in *P. pisum* (Becker et al., 2011) and other decapods (Mota and Tomé, 1965; Kulkarni et al., 1991; Ravi et al., 2013).

Histological observation of oogenesis

Oogonia proliferation (Op)

The initial stage of oogonial (Og) maturation was initiated in the cell nest of the internal germinal area (Figs. 3A–B). Each cell nest was separated from other oocyte stages by the basement membrane (Fig. 3A). This observation is consistent with previous observations (e.g., Ryan, 1967; Eurenius, 1973), such as observations of *Libinia emarginata* (Hinsch and Cone, 1969) and *R. ranina* (Minagawa et al., 1993). Each individual oogonium had an oval to spherical shape.

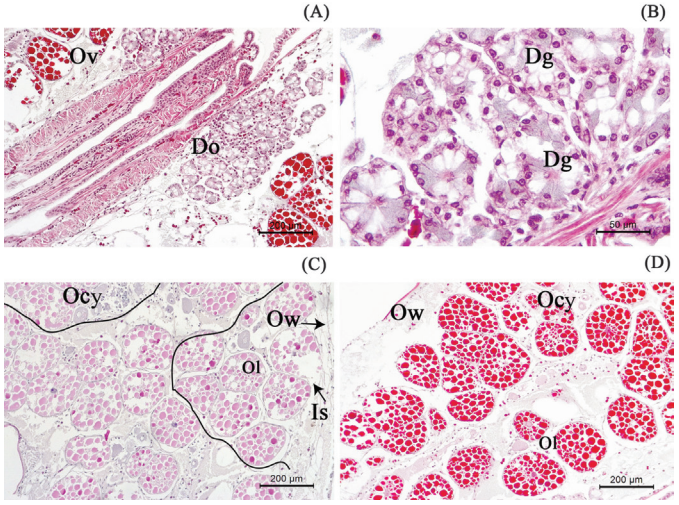

Fig. 1. Light photomicrograph of the ovarian parenchyma of *Pinnotheres cyclinus*. A-B: The localization of the ovarian structure (Ov) among the digestive organ (Do) consisting of several digestive glands (Dg) was observed. C-D: The ovarian structure was surrouned with ovarian wall (Ow); the ovary comprised of several ovarian lobes (Ol). Note: Is = interstitial tissue, Ocy = Ovarian cysts. A-B, D = Masson's Trichrome (MT), C = haematoxylin–eosin (H&E).

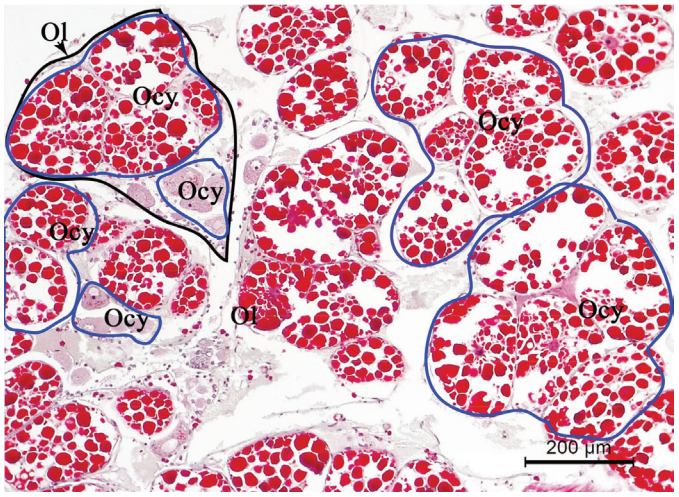

Fig. 2. Light photomicrograph of the ovarian structure of *Pinnotheres cyclinus*. Each ovarian lobe (Ol) contained various ovarian cysts (Ocy) which were consisted of different oocytes. A = Masson's Trichrome (MT).

The oogonium had a mean diameter of 10±0.98 µm, whereas the average nuclear diameter was approximately 3.4±1.03 µm (Figs. 3A and C). A slightly oval nucleus occurred and contained moderately condensed heterochromatin, which was arranged as a clock-face feature (Figs. 3A–B). A spherical nucleolus was also observed (Fig. 3A). The nucleus was partially surrounded by slightly basophilic cytoplasm (Fig. 3A). During the process of folliculogenesis, which was observed using MT and CV staining methods, each cell was associated with a few squamous-shaped prefollicular cells (Figs. 3A–B).

Primary growth phase (PGP)

In this phase, we classified two stages of oocyte development: (1) perinucleolar (Pn) oocytes and (2) oil droplets and cortical alveolar (Oc) oocytes (Figs. 3B-E), which were still present in germinal cysts as follows. The Pn oocytes had spherical shapes and were larger than oogonia, approximately 40 µm in diameter (Fig. 3B). Each Pn oocyte had a central nucleus with a mean diameter of 20.00±0.56 µm containing euchromatin and small blocks of heterochromatin, which were pale-stained and distributed throughout the nucleoplasm (Fig. 3B). At the same time, multiple nucleoli with a mean diameter of 5.00±0.92 µm were located along the nuclear membrane (Fig. 3D). The ooplasm exhibited strong basophilic staining (Figs. 3B and C), and the basophilic characterization of each oocyte was previously related to an accumulation of ribosomes and, for a period, an intense vesicular nucleus (Beninger et al., 1993) with three to four chromatin clumps (Weitzman, 1966; Okumura and Sakiyama, 2004). Similar to those of two other crab species - *Cambarus virilis* (Beams and Kessel, 1962) and *Cancer pagurus* (Eurenius, 1973) - each Pn oocyte contained an enrichment of ribosomes and the development of rough endoplasmic reticulum as revealed by ultrastructural examination. During the process of folliculogenesis, follicular cells became completely surrounded by Pn oocytes (Fig. 3D).

The oil droplets and Oc oocytes had ovoid shapes and had slightly increased in size to a diameter of approximately 70 µm, but a decrease in nuclear diameter (of approximately 25 µm) was recorded as well. The ooplasm of this type of oocyte was deeply stained with hematoxylin (Fig. 3B). Interestingly, Oc oocytes increased in cell size due to an accumulation of vacuolated globules, such as oil droplets and cortical alveoli (Fig. 3E). The appearance of oil droplets was visible as clear vacuoles because the oil droplets dissolved during histological preparation (Figs. 3D–E). This pattern of oil droplet presentation has been commonly recorded in decapods (Chen et al., 2004; Walker et al., 2006). Lipid accumulations have been reported as nutrient reservoirs for nutritionally dependent zoeae (Chen et al., 2004; Walker et al., 2006). The presently observed cortical alveoli were slightly colored by Haemotoxylin and Eosin (H&E staining) (Fig. 3E) and positively colored with PAS staining (data not shown), indicating the presence of glycoproteins. This finding could indicate a build-up of cortical rods, which function in forming a jelly layer to cover an oocyte (Clark et al., 1990). Supporting follicular cells were well-defined and approximately 2 µm in diameter (Fig. 3D). Comparable situations have been investigated in several decapods, such as *Penaeus semisulcatus, P. monodon* (Ongvarrasopone et al., 2006), *Marsupenaeus japonicus,* and other penaeoid shrimps (Okumura et al., 2006).

Secondary growth (SG) phase

In this phase, the following three stages of SG were classified: the early SG stage (Esg), the late SG stage (Lsg), and the fully grown oocyte stage (Fgo), which remained developed in the ovarian cysts (Figs. 3C–E and 4).

During the Esg step, the oocytes suddenly increased in size to approximately 70 µm in diameter due to an accumulation of yolk granules (Figs. 3C–D). Spherical small yolk granules were detected by their deep reddish stains following their positive reactions to MT staining, implying the presence of glycoproteins and mucopolysaccharides (Figs. 3C–D); similar results were previously observed in Malacostraca (Charniaux-Cotton, 1975), *Gecarcinus lateralis* (Weitzman, 1966), *Chionoecetes opilio* (Beninger et al., 1993), *Acanthomysis robusta* (Okumura, 2003), and *Portunus trituberculatas* (Hamasaki et al., 2004). During oocyte differentiation, an irregular outline of the center nuclear structure was slightly displaced toward the animal pole (Stewart et al., 2007). During Esg, the oocyte was more visible and still surrounded by a single layer of supporting follicular cells; in contrast, this layer also exhibited increasing size/density/width (Figs. 3D–C). These changes in oocyte structure resulted from follicular changes during this stage and were considered to have been responsible for heterosynthetic yolk deposition in our specimens, as has been described previously by Islam et al. (2010) and Yano (1988) in brachyurans and other crustaceans.

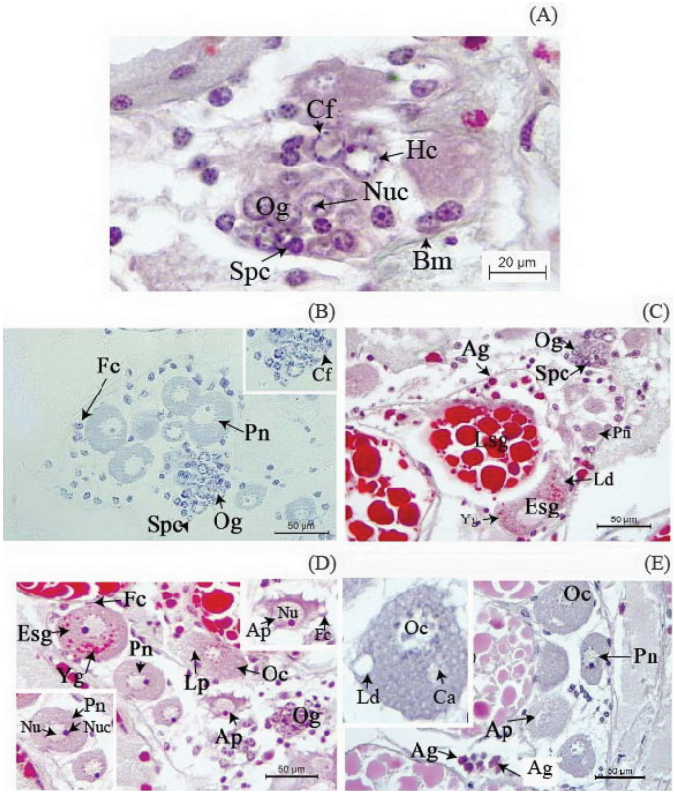

Fig. 3. Light photomicrograph of the oogenesis of *Pinnotheres cyclinus*. A: Oogonial proliferation containing the oogonium (Og). B-E: Different stages of oocytes including perinucleolar oocyte (Pn), oil droplets and cortical alveolar oocyte (Oc), Full-grown oocyte step (Fgo) and late secondary growth step (Lsg) throughout atretic oocyte of previtellogenic stage (Ap). Note: Ag = acidophilic granulocytes, Bm = basement membrane, Ca = cortical alveoli, Cf = clock-face feature, Esg = early secondary growth step, Fc = follicular cell, He = heterochromatin, Ld = lipid droplet, Nu = nucleus, Nuc = nucleolus, Spc = simple pre-follicular cell, Yg = yolk granules. A, C-D = Masson's Trichrome (MT), B = cresyl violet (CV), E = haematoxylin–eosin (H&E).

Lsg was accompanied by a rapid increase in cell volume, up to 200 µm in diameter, due to accumulated yolk granules (Figs. 4A–C). The fusion of the yolk granules or yolk plate, with a maximum diameter of 15 µm, was initially observed in eosinophilic ooplasm (H&E staining; Fig. 4A) and reddish ooplasm (MT staining; Fig. 4B), but these yolk granules did not react to the CV staining method (Fig. 4C). Some yolk granules were also hydrated and coalesced. At this stage, the irregular nuclei were reduced in size and number and had moved to the animal pole of the oocyte through germinal vesicle migration (GVM). At the same time, the nucleoli were not visible. A reduced follicular cell was observed (Figs. 4A–D), and at the end of this stage, small nuclei were observed (Figs. 4A–D).

Fgo consisted of fully mature oocytes. Oocytes were largest at this stage, with maximum diameters ranging from 250–300 µm. Due to membrane breakdown, also called germinal vesicle breakdown, no nucleus was detected during this stage (Figs. 4E–F). The oocyte still exhibited an increased abundance of yolk plates in the ooplasm.

Atretic oocyte phase

Although most post-ovulatory phases were not observed throughout the present study, the atretic oocyte phase was commonly found among the oocytes. Atretic oocytes during the PGP, the irregular nucleic shapes were surrounded with the detachment of the thin follicular

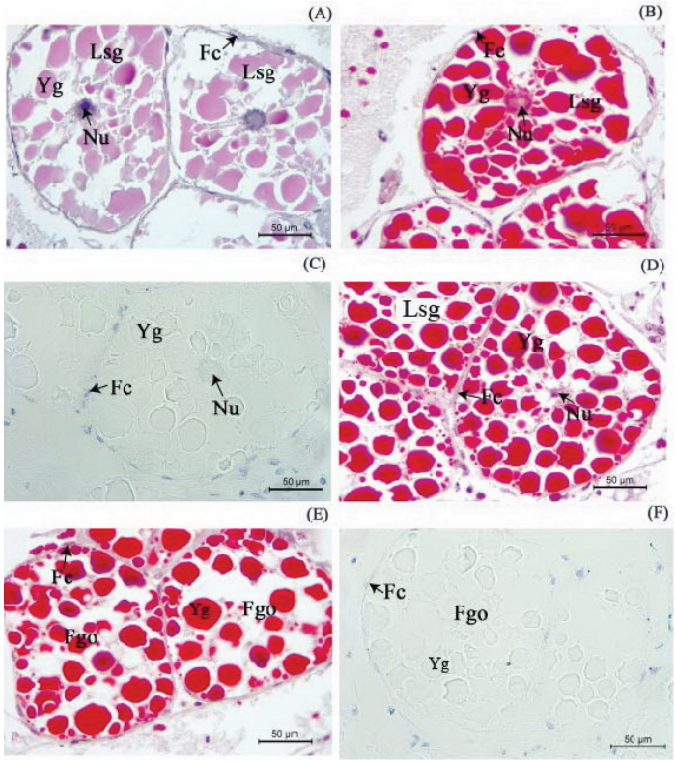

Fig. 4. Light photomicrograph of the oogenesis of *Pinnotheres cyclinus*. A-D: Late secondary growth step (Lsg) containing the large yolk granules (Yg). E-F:The absence of nucleus was found in full-grown oocyte step (Fgo). Note: Fc = follicular cell, Nu = nucleus. A = haematoxylin–eosin (H&E), B, D, E = Masson's Trichrome (MT), C, F = cresyl violet (CV).

layer (MT staining; Fig. 3D). It could be argued that the cause of pronounced atresia in aquatic organisms is related to a wide range of environmental stressors such as heavy metals (Pierron et al., 2008), endocrine-disrupting chemicals (Pollino et al., 2007), starvation, and lipid-poor diets (Hunter and Macewicz, 1985). Additionally, it should be noted that in crabs and crustacea, oocytes and ovarian lobes begin to degrade at the end of ovarian development and, subsequently, germinal areas appear for the next cycle (Subramoniam, 2016). Alternatively, this unusual progression of atresia may be associated with the unusual habitat/lifestyle of this crab and/or its unusually high fecundity.

Histological observation of embryonic development

During our observation of the ovarian structure of the pea crab, its embryonic stages were also detected (Figs. 5A–F). First, the embryonic surface was covered by the egg membrane, referred to as the vitelline envelope. The embryonic surface also reacted positively to MT and CV staining. Subsequently, the fertilized egg was filled with yolk granules, which were homogeneously distributed within the egg. This stage might have occurred approximately three days after fertilization, as was previously observed in other decapod species (Habashy et al., 2012).

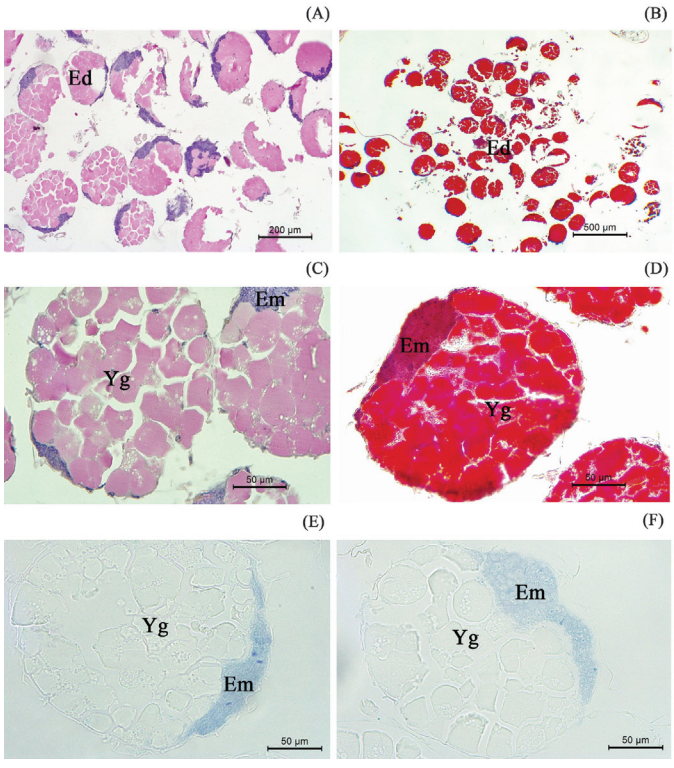

Fig. 5. Light photomicrograph of embryonic development of *Pinnotheres cyclinus*. A-B: Overall of the embryonic development (Ed). C-F: Each embryonic development with a well developed-egg membrane (Em) and large structure of yolk granule (Yg). A,C = haematoxylin–eosin (H&E), B, D = Masson's Trichrome (MT), E, F = cresyl Violet (CV).

In the present study, the application of histological techniques clearly revealed the ovarian structure and oogenctic processes of *P. cyclinus* for the first time, as summarized in Fig. 6. This study provides basic information that may help in the study of the reproductive cycle and reproductive physiology of *P. cyclinus*.

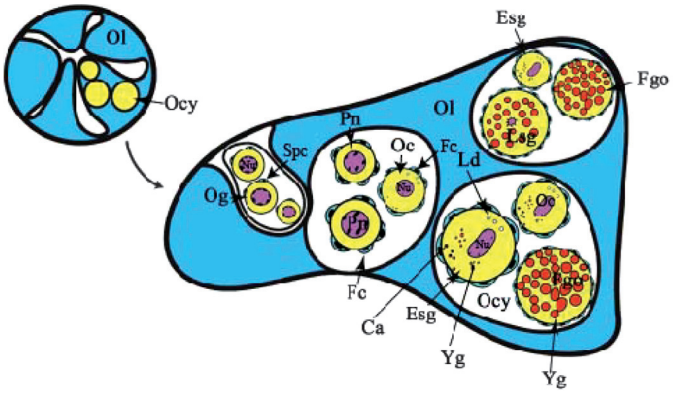

Fig. 6. Schematic diagram of the ovarian structure and oogenesis of *Pinnotheres cyclinus*. The ovarian lobe (Ol) was contained with several ovarian cysts (ocy), which each ocarian cyst consisted of the differentiating stages of oocyte including oogonium (Og), perinucleolar stage (Pn), oil droplets and cortical alveolar step (Oc), early secondary growth step (Esg), late secondary growth step (Lsg) and full-grown oocyte step (Fgo). Note: Ca = cortical alveoli, Fc = follicular cell, Ld = lipid droplet, Nu = nucleus, Ocy = ovarian cyst, Ol = ovarian lobe, Yg = yolk granule

Conflict of Interest

There is no conflict of interest.

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