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Classification stages of novel atretic structure in short mackerel

Ratrilliger brachysoma (Bleeker, 1865) from the Upper Gulf of Thailand

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Abstract: An understanding of atretic follicles in wild population is required before investigating the reproductive cycle and spawning season but these follicles have never been reported on adult short mackerel *Rattrilliger brachysoma* (Bleeker, 1865). Histology and histochemistry were used to classify the stage of atretic follicles in *R. brachysoma*, obtained from the Upper Gulf of Thailand. Microscopically, it was clear that under atretic processing this species could be successively divided into two phases: atretic follicle during previtellogenic and vitellogenic stages, in which the latter was also classified into five steps (I, II, III, IV and V). Histochemically the cortical alveoli, yolk granules and basement membrane were observed and also discussed in this study.

Key words: Atretic follicles, histology, *Rattrilliger brachysoma*, short mackerel, fish, Thailand
1. Introduction

Under the oogenic processing, one principle method in teleost fish and other vertebrates is called atretic follicles (Genten et al., 2009). In terms of these follicles being referred to as unovulated oocytes are obviously observed under the degeneration and resorption of oogenic cells (Kennedy, 2002; Santos et al., 2008) and physiological processes as well as homeostasis (Hussein, 2005). Variability degrees of this follicle are dependent upon seasons, temperature, light, food (Saidapur, 1978) and chemical compounds, particularly endocrine disrupting chemicals (Johnson et al., 2009). Therefore, investigations of follicles have been reported in several fish species and discussed in many ways including morphology and biochemistry as well as structural functions (Kennedy, 2002; Wood and van der Kraak, 2002, 2003; Santos et al., 2008).

As to the present objectives, the classification of follicles can be useful for the estimation of oocytes and the prediction of the spawning frequency in the fish population as well as reproductive health status (Hunter and Glodberg, 1980; Hunter and Macewicz, 1985; Hunter and Lo, 1997; Blazer, 2002; Galias et al., 2003; Johnson et al., 2009).

An economic marine fish, Ratrilliger brachysoma is a very popular fish due to its low price and as a source of cheap protein for Thai consumers. The catch of R. brachysoma for the year 2009 (115,400 tons) was significantly less than the annual catches of approximately 143,500 tons in 2005 to 2008 (Department of Fisheries, 2009). The reduction of the wild population of R. brachysoma may due to overfishing and/or the deterioration of their natural habitats. If the decrease of the R. brachysoma population continues at this rate, this fish might become extinct in the Gulf of Thailand and food insecurity could become a major issue in the near future. Up until now, it has
also been exclusively considered as a good candidate fish for aquaculture in Thailand. An understanding of the gonadal structure in the wild population is required before investigating the reproductive cycle and spawning seasons. Although the gonadal structure and gametogenesis in this species living in the Upper Gulf of Thailand has been primarily reported using histological and histochemical investigation (Senarat et al. in process), it was not histologically defined as to the classification schemes in the atretic follicles.

During the breeding season, histological and histochemical approaches were used to investigate the histological stage of atretic follicles in *R. brachysoma*, as obtained from the Upper Gulf of Thailand. Surely, this result will enrich the understanding regarding the gonadal histological evidences of this species. Moreover, the atresia has not yet been applied to the natural habitats of this fish so it can be used to establish the preparation for the criteria in both estimating and quantifying during the spawning season, fish health as well as sustainable management.

2. Materials and methods

2.1. Sample collection and study site

Sexual mature female, *R. brachysoma* with a weight of 90-120 g and total length of 16.5-20.0 cm were caught during the breeding season (January 2013 to February 2014; *n*=10) with bamboo strake trap from Samut Songkram province (13°16’18.4” N, 100°02’13.4” E), which were living in the Upper Gulf of Thailand. The species identification was according to the identification key of FAO (FAO, 2010). The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University (Protocol Review No. 1423003).
2.2 Histological and histochemical observations

In the laboratory, all fish were euthanized by rapidly cooling shock (Wilson et al., 2009). Afterwards, ovaries were dissected out and suddenly fixed in Davidson’s fixative (about 36 hrs.) for further histological analysis. Ovarian tissues were primary dehydrated, cleared and embedded in paraffin. Cross and longitudinal sections were cut at a thickness of 6-7 µm and then, they were stained by hematoxylin and cosin (H&E). Other sections were histochemically stained by Masson’s trichrome (MT), aniline blue pH 2.5 (AB) periodic acid-schiff (PAS) and reticulin method (RT) (modified from Puchtler and Waldrop, 1978; Humason, 1979; Vidal, 1988; Bancroft and Gamble, 2007). Microscopically, it was examined to assess the histological stage of the atretic follicles according to the guidelines of Gania et al. (2003).

3. Results and discussion

The gross anatomy of ovaries in the *R. brachysoma* during the breeding season had paired and elongated structures showing yellow color (before fixation) and white color (after fixation) within the peritoneal cavity (Figure 1A-1B). At the light microscopic level, the analysis of ovaries of *R. brachysoma* obviously exhibited during breeding season as containing the differential stages of oocyte because it was considered as asynchronous developmental oocytes according to MT staining (Figure 1C-1D). In this study, it could be classified into two phases based on size, histological structure and staining properties as follows:

Phase I the classification of the atretic follicle during previtellogenic stage
The characterization of atretic follicle in the previtellogenic stage was similarly seen with normal stage, but the ooplasm shown as basophilic cytoplasm with a surrounding thin follicular layer (PAS staining) (Figure 2A-2B).

Phase II the classification stage of the atretic follicles during vitellogenic stage

In general, the characterization of normal vitellogenic stage based on histology and histochemistry was 250-300 µm in diameter with numerous small yolk granules. Among its yolk granules, the oil droplets and cortical alveoli were distinctly detected. Also, this stage was surrounded by follicular complex which was clearly divided into three well-developed layers: (i) zona pellucida composing of two layers; inner and outer zona pellucida, (ii) granulosa and (iii) theca cells, respectively. Among normal vitellogenic stage, histological details of several atretic follicles during vitellogenic stage in this species were detected. It could be completely divided into four steps according to shape, characterizations of nucleus and follicle complex (Figure 2D-2I).

Stage I, the histological appearance in this stage was quite similar to that of normal vitellogenic stage, but some microstructures were initially seen including nucleic disintegration with irregular shapes. Some areas of yolk granules were digested, especially in the periphery of the ooplasm. Irregularity in shape and degeneration with separation between inner and outer layers of the zona pellucida were initially and continuously detected. Additionally, hypertrophies together with pyknosis of some granulosa and theca cells were detected (Figure 2C, 2K). Based on histochemistry, the cortical alveoli were still positive with MT as reddish and AB as bluish. Other characterizations, yolk granules with slight positively stain with PAS reaction were specially surrounded by basement membrane (black line with RT) (Figure 2L-2O).
Stage II, the early stage of stage II was irregular in shape. A sequence of event characterization as follows: the degradation and regression of yolk granules in some area located in the peripheral ooplasm were detected more than in the previous stage, as indicated by the fusing granules. According to H&E, the inner zona pellucida became broken down and fragmented from the degenerating of oocyte, whereas the outer zona pellucida highly increased, containing several fragments as well as islets greater than the inner zona pellucida. Also, the hypertrophy of granulosa cells were continuously proliferated, which each cell had a spherical nucleus with surrounding the eosinophilic cytoplasm. Externally, the thecal cell layer was exhibited among a few blood vessels (Figure 2J, 2P). AB, MT and PAS reactions conformed to previous stage but RT reaction distinctly showed the degeneration due to the fragment of basement membrane (Figure 2Q-2T).

Stage III, the disorganizations both irregular in shape and shrinkage were obviously seen when compared with the prior stage. The nucleus in this stage was seen and slightly observed in some oocytes among the degeneration and digestion of yolk granules. The highly increasing fragmentations of zona pellucida gradually continued more than in the previous stage. Exclusively, it was also confirmed that inner layers of zona pellucida were first degenerated and continuously observed in its outer layer. The granulosa and theca were no separated and rarely seen. Surprisingly, in this stage, the most leucocytes were found and continued to come near oocyte (Figure 2U, 2V). Histochemically, the cortical alveoli were slightly seen and began to degenerate (AB and MT stains) whereas RT reaction confirmed that no basement membrane was seen during this stage (Figure 2W-2Z).
Stage IV, the cell size was intermediately decreased when compared with former stage. Unlike the previous stage, the zona pellucida was greatly degenerated among liquefaction of yolk granules. During reabsorption, some area in the ooplasm was shown to contain the vacuoles, referred as empty space. Then, the follicular cells were become and entered to phagocytize degenerating materials because several leucocytes were presented among a few blood vessels (Figure 2Z1, 2a). No mucopolysaccharide was observed according to AB and MT, indicating the complete degeneration of cortical alveoli (Figure 2b-2e).

Stage V, in the final step of this stage was an amoeboid-shape and intermediately decreased in size. It was continuously completed and digested both yolk granules and zona pellucida as referred the flocculent materials. Certainly, the large vacuole associated with yellow-brownish pigments within the ooplasm was accumulated (Figure 2Z2, 2f). Also, some follicular cells were presented. Moreover, this stage was surrounded by fibroblast-like cells, as strongly positive with MT and PAS stained (Figure 2g-2h). Interestingly, it was observed that this stage was possible to be the apoptosis under RT reaction (Figure 2j). The leucocytes were found around the apoptotic follicles (based on RT stained).

Until now, the characterization of atretic follicle has been basically reported in several fishes under regulation of reproductive hormone and reproductive physiology (Ganias et al., 2003; Nagahama, 1983). Also in this study, its feather has been applied to determine the fecundity and spawning season (Jans and van der Kraak, 1997) and reproductive health (Blazer, 2002). The previous research of the classification stages of atretic follicle has been exclusively investigated; especially Grier et al. (2009) which established and basically classified the atretic follicles during secondary growth stage.
into two successive events in teleost fish. In comparison, these characterizations of atretic follicles were also found in *R. brachysoma* too. The first process the degeneration and resorption of oocyte and zona pellucida together with hypertrophy of the granulosa cells which were found in stage I-V. Of additional importance, the second process is called as phagocytosis. The accumulations of yellow-brownish pigments, the greater degeneration and reabsorption of follicular cells occurred. This characterization was similar to stage V of this fish.

It was clearly concluded that the processing of ovarian follicles in this fish could be successive divided into two phases: atretic follicle during previtellogenic and vitellogenic stages (I, II, III, IV and V). Herein, the present results are the first report provided the basic information and the charges of chemical details during the ovarian follicle atretic denegation that could be applied for further studies including spawning and reproductive seasons in natural habitat and culture of *R. brachysoma*.

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**Conflict of Interest**
The authors declare that there are no conflicts of interest

References


Figure 1 Micrograph of gross anatomy and histology of ovaries in *Ratrilliger brachysoma* during breeding season; (A) ovarian morphology (pre-fixation) = 1 cm; (B) ovarian morphology (post-fixation) = 1 cm; (C-D) ovarian histology = 200 µm. *I* = intestine, *L* = liver, *O* = ovarain tissue; *Of* = ovigerous fold; *Ol* = ovarian lumen. (*MT* = Masson's trichrome stain)
Figure 2 Scheming diagram, micrograph of histology and histochemistry of atretic follicles in Rattriliger brachysoma; (A-B) atretic follicle of previtellogenic stage (Ap), A = 100 µm, B = 50 µm; (D-I) Normal vitellogenic stage (V) = 70 µm; (C, K-O) Stage Is = 50 µm; (J, P-T) Stage II = 50 µm; (U-Z) Stage III = 50 µm; (Z1, a-e) Stage IV = 50 µm; (Z2, f-j) Stage V = 50 µm; Apt = apoptosis, B = basement membrane, Bv = blood vessel, Ca = Cortical alveoli, G = granulosa cell, Hg = hypertrophy of granulosa cell, Lc = lymphocytes, Ly = liquefaction of yolk granules, N = nucleus, P = previtellogenic stage, Pt = pynotic nuclei, T = theca cell, VC = vacuole, Wbc = white blood cells, Y = yolk granules, Zi = inner layer of zona pellucida, Zo = outer layer of zona pellucida, Zp = zona pellucida, * = degeneration of yolk granule, ** = degeneration of cortical alveoli. (MT = Masson’s trichrome, PAS = periodic acid-schiff, AB = aniline blue, Rt = reticulin method)