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Original Article

Natural gonadal compartmentalizing of economical important *Hemiramphus far* from Thailand by visual observation and histological technique

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Abstract

In order to increase knowledge on the reproductive biology of *Hemiramphus far* as the most economical marine fish, the gonadal structure and gametogenic differentiation of this fish were clearly described by visual observation and histological technique. Based on the visual observation in female fish, the typical morphology of the mature ovary was a paired cystovarian type. It showed the yellow-orange color ova with containing the differentiating stage of oocytes. The ovary of this fish developed synchronous oocyte. At the same time, the classification of the oogenic stages was divided into three distinct phases; primary growth phase (perinucleolar and oil droplet and cortical alveolar stages), secondary growth phase (early secondary growth step, late secondary growth step, and full-grown oocyte step stages) and post-ovulatory phase. In particular, the post-ovulatory stage was found indicating the spawning season at this time. In male fish, the mature testis was histologically designed as the restricted spermatogonial type, in which the developing spermatogenetic stages could be classified into three phases; spermatogonial, spermatocyte and spermiogenetic phases. Although the gonad histological results from this study provided a comprehensive pattern of the reproduction, they are relevant to further observations (reproductive cycle and endocrinology) with *H. far* as well as with Hemiramphidae.

Keywords: germ cells, Hemiramphidae, histology, reproductive system

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1. Introduction

The most accurate information to determine the gonadal pattern and gametogenesis in teleost has been wildly approached due to the acquisition to understand the reproductive biology and to improve the fisheries management. There have been many histological investigations on the gonadal development in a variety of teleost. It was included such as *Dicentrachus labrax* (Mayer, Shackley, & Rylan, 1988) and *Centropomus undecimalis* (Neidig, Skapura, Geier, & Dennis, 2000), *Serra Spanish* (Chellappa, Lima, Araújo, & Chellappa, 2010) and *T. orientalis* (Chen, Crone, & Hsu, 2006). At the time of the differentiation of the oocytes was usually noted in female fish and occurred within the stromal compartment; subsequently, many oogenic steps including oogonia, immature oocyte, maturing oocyte and mature oocyte were classified. Uribe, Grier, and Parenti (2012) also accurately described the division of mature oocyte stages to four phases: oogonia proliferation stage, chromatin-nucleolus stage, primary growth stage, and secondary growth stage. In male fish, usually, numerous convoluted seminiferous lobules were found in the testicular structure and suggested the division of the spermatogenic developing process into three stages including spermatogonial phase, spermatocyte phase and spermiogenetic phase (Schulz *et al*., 2010).

Belonging to family Hemiramphidae, the number of the research projects and publications on the gonadal structure increased and focused on many Hemiramphids such as *Hemiramphus brasiliensis* and *He. balao* (McBride & Thurman, 2003), *Hemirhamphodon chryopunctatus, Hem. kapuasensis*, *Hem. pogonognathus* and *Hem. tengah* (Downing & Burns, 1995), but it has not yet been reported in the literature revelation of *Hemiramphus far* from Thailand. This fish is often found in the Upper Gulf of Thailand and it is one of the most commercialized marine fish. In this study, the mature gonadal structure and gametogenic stage of *H. far* were field-assessed a full description of two methods: the visual observation and routine histological technique. This first provided baseline data is essential to understand the reproductive process of this species before entering to study the reproductive cycle and the spawning season of natural *H. far*.

2. Materials and Methods

Death specimens of *H. far* with a total length 26.9 ± 0.32 cm (n = 10 individual fish, each sex = 5 samples) were donated from local fisherman at the Upper Gulf of Thailand, Samut Songkhram province (13°16’18.4” N, 100°02’13.4” E). The fish were identified to species by the Food and Agriculture Organization of the United Nations (FAO, 1983) guide. For each individual, the gonadal tissue was immediately dissected to assess its morphology using a stereoscopic microscope. Dissected gonads were fixed overnight in Davison´s fixative and processed according to standard histological techniques (Presnell & Schreibman, 1997; Suvarna, Layton, & Bancroft, 2013). All paraffin blocks were cut at 4-5 µm thickness and then stained with haematoxylin–eosin (H&E), periodic acid Schiff (PAS), Masson's Trichrome (MAT) and Verhoeff's elastic stain (Ve) (Presnell & Schreibman, 1997; Suvarna *et al*., 2013). The histological sections were examined and measured to classify the ovarian structure under the light microscope (Leica digital 750) using the description of Dietrich and Krieger (2009) and Uribe *et al*. (2012).

3. Results and Discussion

3.1 Ovarian structure and oogenesis

Morphologically, two unequal lobes of the mature ovary of individual *H. far* were elongated along the medial-caudal region (Figure 1A), which was designated as the cystovarian type. All ovaries of this fish showed a yellow-orange color and it was surrounded by the ovarian wall (Figures 1A-1B). The different sizes of oocytes and post-ovulatory follicles were clearly identified to undergo the morphological observation (Figure 1B). In the histological sections, a prominent component of the mature ovary revealed that the tunica albuginea protruded continuously into the central region, as also called the ovigerous fold and. This result was similarly reported in other teleost (Selman & Walllace, 1986; Selman, Wallace, Sarka, 1993; Wallace & Selman, 1990). Although the ovigerous fold had a large area and was also distinctive in some fishes (Senarat, Kettretad, & Jiraungkoorskul, 2017), it was difficult to identify during the ovarian maturation of *H. far*. Two compartments in the ovigerous fold (germinal and stromal compartments) were recognized during the ovarian development.

Apart from the germinal compartment was composed of the germinal epithelium covering the ovigerous fold (Figure 2B), primordial germ cell and oogonial cysts (Data not shown). It did not differ from that in other teleost (Selman & Wallace, 1986; Wallace & Selman, 1990; Grier, 2000). The proliferation of the oocytes in teleost is accepted to raise from the germinal epithelium, suggesting the differentiation of the epithelium from the lumen of the ovary (Wallace & Selman 1990).

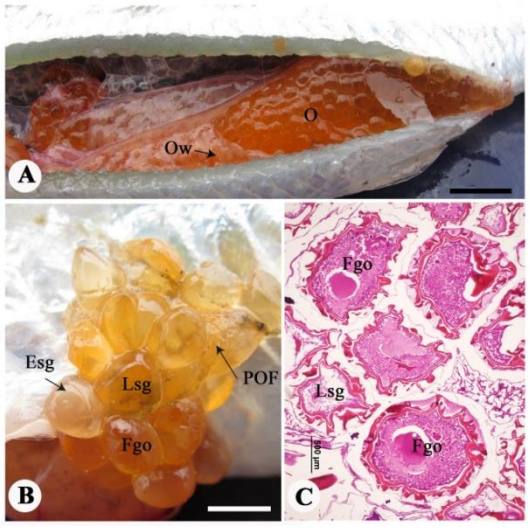

Figure 1. Morphology and Light photomicrograph of the ovarian structure (O) with surrounding the ovarian wall (Ow) and oogenic process in *Hemirahamphus far*. Esg = early secondary growth step, Fgo = Full-grown oocyte step, Lsg = late secondary growth step, POF = post-ovulatory follicle.

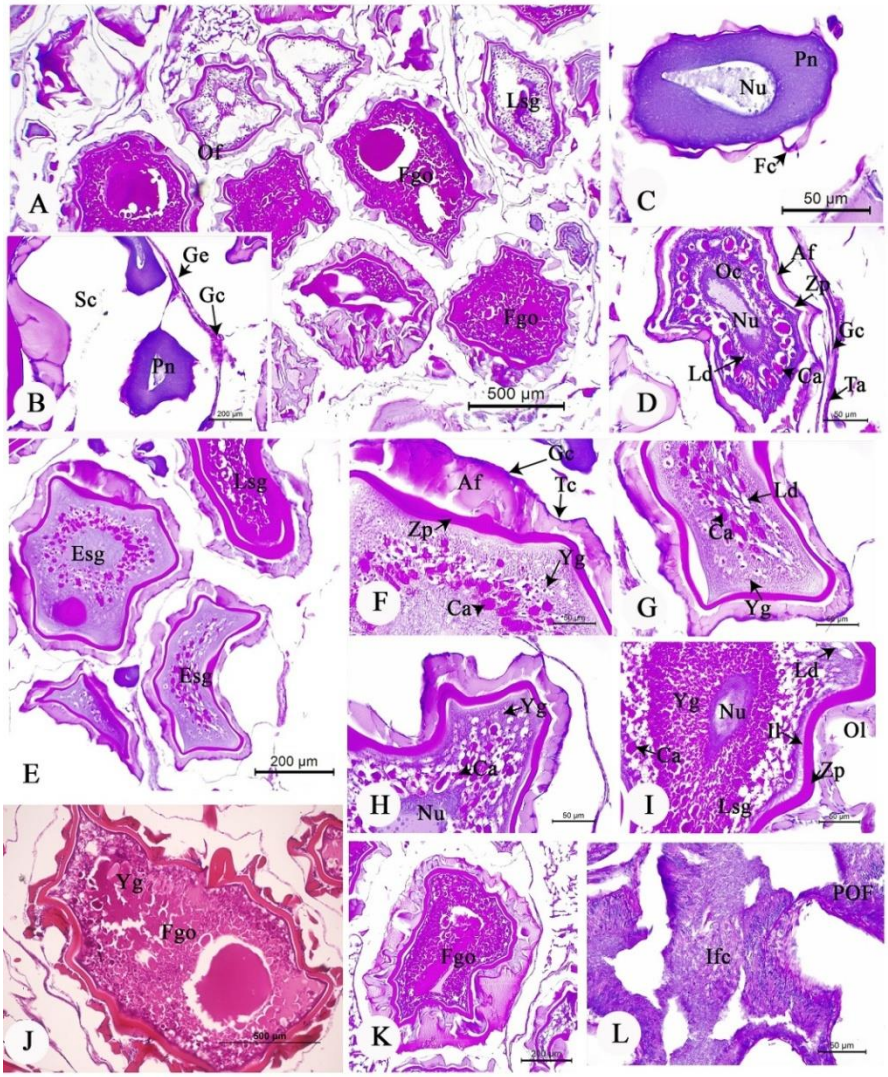

Figure 2. Light photomicrograph of the ovarian structure and oogenesis in *Hemirahamphus far*. Af = acidophilic fibrils, Ca = cortical alveoli, Esg = early secondary growth step, Fc = follicular cell, Fgo = Full-grown oocyte step, Gc = granulosa cell, Ge = germinal epithelium, Ifc = Irregular shape of the stratified follicle cell layer, Il=interstitial cell of tissue, Ld = lipid droplet, Lsg = late secondary growth step,Nu = nucleus,Oc = oil droplets and cortical alveolar step, Of = ovigernous fold, Ol = ovarian fold, POF = post-ovulatory follicle, Pn = perinucleolar stage, Sc = stromal compartment, Tc = theca cell, Yg = yolk granules, Zp = zona pellucida.

The stromal compartment was separated by a thin basement membrane from the germinal compartment. This compartment was embedded by differential stages of oocytes, which was also considered as an asynchronous developmental type (Figures 2A-2B). This phenomenon was consistent to *Hyporhamphus regularis ardelio* (Nattall, Stewart, & Hughes, 2012) and other fish (Chellappa *et al*., 2010) with the result that the fishes implied to multiple spawning seasons. Therefore, *H. far* samples from the study site may be considered as protracted for spawning period, the reproductive cycle or spawning season is needed to give a more accurate hypothesize in further study. Three phages of oogenic steps though primary growth phase, secondary growth phase and post-ovulatory phases of fish were distinctly classified according to staining of sections, homogeneity and histological aspects of sex cells. It is likely seen in other hemiramphids such as *He. brasiliensis*, and *He. balao* (McBride & Thurman, 2003) and *H. melanochir* (Ling, 1958). However, some characteristics in *H. far* such as irregular shape and shrinkage of oocytes occurred due to fixation or the embedding medium from the histological processes, as suggested by Uribe *et al*. (2012).

3.2 Primary growth phase

Following the last mitotic division of the oogonia, two steps of the oocyte in the mature ovaries perinucleolar (Pn) and oil droplets and cortical alveolar step (Oc) were observed as follows:

The oocyte of Pn increased cell size reaching to 105 µm and was transformed under the primary oocytes. The first

sign of Pn showed that the large central nucleus about 40 µm in diameter with multiple basophilic nucleoli (Figure 2C). The sizes of basophilic nucleoli were about 5 µm that detected near the nuclear membrane. A similar phenomenon was reported in several teleost species (Blazer, 2002; Patiño *et al*., 2003; Patiño & Sullivan, 2002; Selman & Wallace, 1986). The ooplasm was a strongly basophilic stain due to its high affinity for haematoxylin (Figure 2C). This was to support an active synthesis of RNAs and the abundance of ribosomes and mitochondria in the cytoplasm (Wallace & Selman, 1990). The elongated follicle cells were completely defined under during folliculogenesis along the oocyte.

The size of Oc dramatically increased to 155 µm in diameter. In parallel to the decreased nucleus in size, attaining 50 µm showed a slight folding nucleus in shape and was well detected. Many nucleoli were still observed. The basophilic ooplasm lost the affinity for haematoxylin due to oil droplets and cortical alveoli. The first prominent of the small/ a few oil droplets was an empty-vacuolar structure, whereas the cortical alveoli were detectable close to the follicular complex, which was also positively reacted with PAS method (Figure 2D). It was similar to previous reports in other teleosts (Abascal & Medina, 2005; Patiño & Sullivan, 2002; Selman & Wallace, 1986). The presence and distribution of cortical alveoli suggested that the function of this inclusion involved to physiological response especially the prevention of polyspermy after ovulation (Abascal & Medina, 2005; Nagahama, 1983; Selman & Wallace, 1986; Selman, Wallace, & Barr, 1988). In *C. humboldtianum*, the cortical alveoli were produced from RER cisternae (Selman *et al.*, 1988; Selman & Wallace, 1986). Concomitantly, the folliculogenic process was well defined for the first time and clearly consisted of three layers including zona pellucida, granulosa cell, and theca cell, surrounding the ovarian surface. A thin layer of the acidophilic zona pellucida, about 16 µm thickness was clearly striated and a thick layer found between the oocyte and follicular cells. The formation of the acidophilic fibrils was locally seen between single layers of granulose cells and theca cells in many positions (Figure 2D). At the end of this phage, oil droplets were increased in both number and size and randomly dispersed in the peripheral region of the ooplasm.

3.3 Secondary growth phase

The secondary growth phase composed three steps of oocyte: early secondary growth step (Esg), late secondary growth step (Lsg) and full-grown oocyte step (Fgo) stages.

In visual observation, the Esg had a slightly yellowish color with a diameter of 500 µm (Figure 1B). The nucleus was still the centrally-located nucleus, but the obvious fold of this structure was detected. As the prominent characterization of this stage began to form the small yolk granules in the periphery of the ooplasm (Figures 2F-2G). Each yolk granule was of small, spherical shape and showed deeply acidophilic stain in the ooplasm based on H&E and PAS methods (Figure 2F). This result correlates with many fish species (Chen, Crone, & Hsu, 2006). As was observed previously, the principle component of yolk granule is the vitallogenin. The vitellogenin was produced from the hepatic origin and then it secreted to the blood plasma. As following, the vitellogenin uptake passed the microvilli extending through the pores of the vitelline membrane (Selman & Wallace, 1986). The oil droplets and cortical alveoli still detected, however, these inclusions progressively increased in both number and size. The zona pellucida not only increased in constant thickness, attaining 10 µm (Figure 2F-2H) but also well-became the striated layer. The acidophilic fibrils were well defined and increased in number and size. The granulosa cell was observed, and the cellular shape changed from squamous epithelium to low columnar epithelium. A layer of the vascularized theca cells was similarly exhibited at prior stages.

The yellowish color of Lsg was grossly seen and increased up to 680 µm in diameter of *H. far* (Figure 1B). The irregular nucleus was observed and it moved to the animal pole, also called as the germinal vesicle migration (GVM). Several coalescence yolk granules progressively increased by number and cell size especially the middle-periphery of acidophilic ooplasm, as similarly reported to *He. brasiliensis* and *He. balao* (McBride & Thurman, 2003). Some areas of ooplasm were completely filled, as acidophilic yolk plates. The presence of the oil droplets and cortical alveoli remained, but they were progressively evidenced in size throughout the ooplasm. Although the good structure of the zona pellucida was still visible, it differentiated into a thin striated outer layer and thick homogeneous inner layer. The layers of the granulosa and theca cells showed the same structure as at the previous stage (Figures 2F, 2L, 2P).

The Fgo was found at the end of the oogenic process in *H. far*. The largest oocyte was a yellow-orange color, reaching 730 µm in diameter (Figure 1B), when it compared to Esg and Lsg. Germinal vesicle breakdown (GVB) was detectable. The more compact appearance of the yolk globules was seen.

3.4 Post-ovulatory phase

The convoluted postovulatory follicles (POF) or ruptured empty oocytes occurred in the mature ovary. The irregular shape of the stratified follicle cell layer was observed (Figure 2L). The layers of the granulosa were closely adherent to the theca cell, which contained in blood capillaries. From the data on the occurrence of this stage indicated that it developed in the ovary after ovulation and that the fish had spawned. We suggest that this time may be advantageous for particular spawning time. In a further study, the precise reproductive cycle and spawning season will be investigated

3.5 Testicular structure and spermatogenic stages

As the morphology of the mature testis, it was a paired, elongated organs and creamy white along the body cavity. Under light microscopic level, the testicular histology was surrounded by tunica albuginea, which its parenchyma could be classified into two regions including testicular structure and vasa efferent (Figures 3A-3C). In the testicular structure was also composed of two compartments, including germinal and interstitial compartments (Figure 3D). However, the formation of the thin interstitial compartment during the testicular maturation was a thin layer and protruded from the tunica albuginea and accommodated in many cellular components including Leydig cells (Figure 3D), myeloid cells, fibroblast and blood vessels. The regular branching seminiferous tubules and Sertolic cells occurred in the germinal

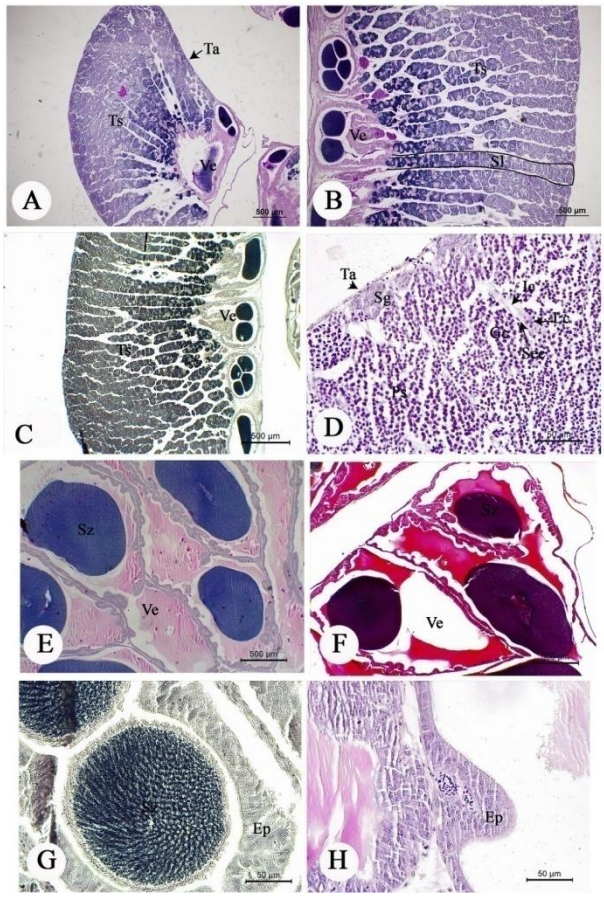

Figure 3. Light photomicrograph of the testicular structure (Ts) with jointing the vasa effect (Ve) in *Hemirahamphus far*. Ep = epithelial layer, Gc = germinal compartment, Ic = interstiatial compartment, Lc = Leydig cell, Ps = primary spermatocyte, Sec = Sertoli cell, Sg = spermatogonium, Sl = seminiferous lobule, Sz = spermatozoa, Ta = tunica albuginea.

compartment (Figure 3D). At a higher magnification, thin cytoplasmic processes of the Sertoli cells usually around the germ cell extended and packed a germinal cyst-liked structure, as likely recommended by several authors (Billard, 1992; Dietrich & Krieger, 2009; Nagahama, 1983).

The testicular type of *H. far* was defined as restricted-spermatogonial type (Figures 3A-3C), which the spermatogonia distributed in the only distal end of the seminiferous lobule (Figure 3D). This finding confirms previous reports in hemiramphids such as *Hemirhamphodon chryopunctatus*, *Hemirhamphodon kapuasensis*, *Hemirhamphodon pogonognathus* and *Hemirhamphodon tengah* (Downing & Burns, 1995; Grier, Linton, Leatherland, & de Vlaming, 1980; Nagahama, 1983; Parenti & Grier, 2004). Each seminiferous lobule jointed with the vasa efferent (Figures 3B-3C). The vasa efferent was lined by a columnar epithelium and embedded in a homogenous eosinophilic matrix (H&E method) among sperm mass. This substance also stained the reddish with MT and pinkish with PAS methods (Figures 3E-3H), indicating to deposit the glycoprotein. It is possible that this chemical composition may be secreted from the epithelium to support the sperm maturation.

We had classified the developmental stages of the germ cells in the seminiferous tubule according to histological structures and chromatin condensation. There were threedistinct phases: spermatogonial, spermatocyte and spermiogenetic phases (Figure 4H). The different generations of spermatogonia with undergoing mitotic divisions were divided into two types: type A and type B spermatogonia (Figures 4A-4B). A cluster of type A spermatogonium was the largest germ cells with a mean cell diameter of 12 µm and located in the distal region of the seminiferous tubule. It had a large nucleus with a few heterochromatin as well as a single nucleolus surrounded by a thin eosinophilic cytoplasm. Type B spermatogonium was noted similar to the previous stage, but it was slightly decreased in size (about 11-12 µm) and proceeded in synchrony.

When the meiotic division the spermatocyte phase was developed from the Type B spermatogonium (Schulz *et al.*, 2010), which divided into two cell cycles (primary and secondary spermatocytes) also occurred in the cysts (Figures 4C-4D). The primary spermatocyte was of spherical cells and

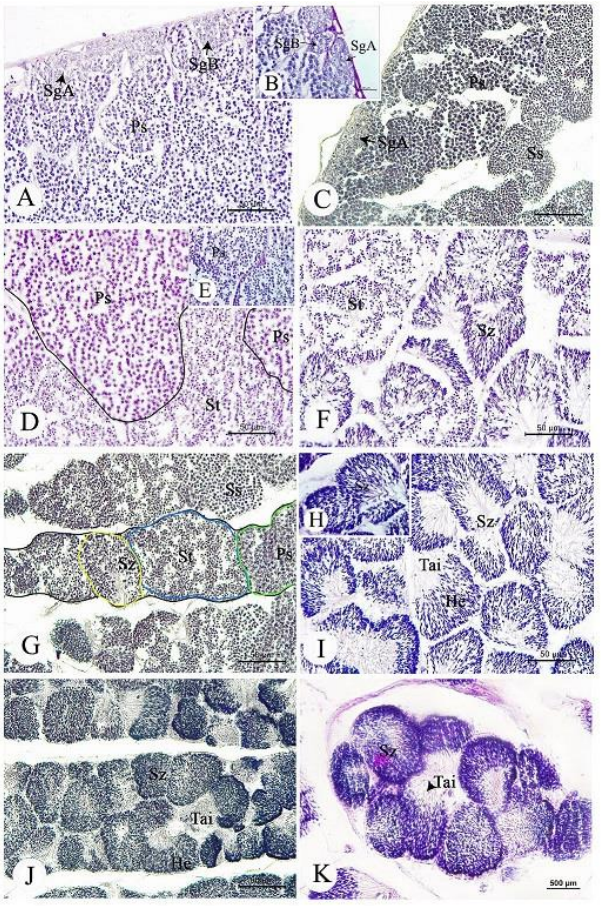

Figure 4. Light photomicrograph of the spermatogenic steps in *Hemirahamphus far*. He = head of spermatozoa, Ps = primary spermatocyte, SgA = spermatogonium type A, SgB = spermatogonium type B, Sl = seminiferous lobule, Ss = secondary spermatocytes, St = spermatids, Sz = spermatozoa, Tai = tail of spermatozoa.

smaller than the spermatogonium, approximately 10 µm. The organization of heterochromatin blocks (nuclear chromatins) were prominently identified due to their dramatical condensations in this stage. They were also scatted in the nucleoplasm. The secondary spermatocyte was smaller than the primary spermatocytes and was about approximately 6 µm undergoing the first meiotic division from the primary spermatocyte. Most of the heterochromatin blocks clump in the nucleus were noted; however, this stage was very rarely seen in the testicular maturation.

The spermiogenic phase was differentiated from spermatids into spermatozoa. The spermatid was differentiated from the secondary spermatocyte undergoing the second meiotic division. A high nuclear condensation was observed in the spermatid (Figures 4F-4G). Very thin eosinophilic cytoplasm also occurred. The smallest cell of spermatozoan is noted. The oval head and elongated tail of the spermatozoa were clumped together (Figures 4H-4K).

4. Conclusions

The mature ovarian structure of *H. far* was identified for the first time. It was a synchronous development oocyte consisting of three phases: primary growth phase (perinucleolar and oil droplet and cortical alveolar stages), secondary growth phase (early secondary growth step, late secondary growth step, and full-grown oocyte step stages) and post-ovulatory phase. Three phases including spermatogonial, spermatocyte and spermiogenetic phases were seen in the testis of male fish. The results of our study hopefully will contribute knowledge either to understanding the gametogenic process or to support further information in Hemiramphids.

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