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***Full Paper***

# Histological analysis of reproductive system of *Dermogenys pusilla* (Kuhl & van Hasselt, 1823) from Thailand: Sperm existence in ovary indicates viviparous reproductive mode

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**Abstract:** Viviparity is a common mode of reproduction in hemiramphid fish. However, the precise reproductive mode of *Dermogenys pusilla* (Hemiranphidae), an important estuarine aquarium fish species in Thailand, remains unclear, limiting our ability to culture them effectively. In this study we investigate the morphology of its reproductive tissues by histological approaches. The testicular structure in male is a paired organ, and the distribution of spermatogonia limited in the distal termini lobules indicates that *D. pusilla* has the testis of restricted spermatogonial type. The ovary of female *D. pusilla* is a paired and saccular organ. No adhesive filaments are found in the zona pellucida. Interestingly, we observe numerous spermatozoa stored in the folds of the ovarian epithelium in *D. pusilla*, providing histological support for internal fertilisation by parthenogenesis associated with the viviparous reproductive mode of this species. These results will help us to comprehensively understand the unique reproductive strategy of the hemiramphid fish.

**Keywords:** *Dermogenys pusilla*, reproductive system, viviparity, Hemiramphidae, histology, Thailand

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## INTRODUCTION

Globally, less than 1,500 species of atherinomorph fish have been classified in its three main orders: Atheriniformes, Cyprinodontiformes and Beloniformes [1, 2]. Morphological and molecular information has supported the monophyly of atherinomorph fish, and two major reproductive characteristics have been documented for this group [2-6]. First, the distribution of the spermatogonia is limited in the distal end of testis lobule. This type of testis is called restricted spermatogonial type, in contrast to the unrestricted spermatogonial type, in which spermatogonia distribute along the entire length of the tubule [7]. Second, eggs of atherinomorph fish are relatively large in size with fluid rather than granular yolk [8, 9]. These characteristics are associated with their viviparous reproductive mode, and actually the internal fertilisation has often been observed in atherinomorphs. This viviparous reproductive mode has been reproducibly reported by many previous studies [10-12].

The hemiramphid fish (Family Hemiramphidae) are a group of fish commonly known as halfbeak. They belong to the order Beloniformes. The family Hemiramphidae is classified into 14 genera and sub-genera, containing more than 100 species and subspecies [5, 13]. The size of hemiramphid fish ranges from 30 to 400 mm (standard length) as found in *Hemirhamphodon tengah* (35.5 mm) and *Dermogenys pusilla* (46.2 mm), and up to *Euleptorhampus viridis* (405 mm) [13]. Most hemiramphid species can produce massive spherical eggs with attaching filaments as observed in other atherinomorph fish. They are perceived to have great economic importance and are frequently found in domestic markets in Thailand. Consequently, understanding the reproductive characteristics of hemiramphid species has become an important focus of attention, especially for the popular aquarium species such as *D. pusilla* [14].

The Malayan halfbeak *D. pusilla*, also known as wrestling halfbeak, lives in fresh and brackish water of rivers and costal realms in South-East Asia including Indonesia, Malaysia, Singapore and Thailand. This viviparous fish species is small and slender in shape, but its colours are different depending on the habitat, making *D. pusilla* a popular aquarium species [15]. Although *D. pusilla* has long been known as a viviparous fish [16], very little information is known about the morphology of their reproductive systems compared to other hemiramphid species, i.e. *Hemirhamphodon chryopunctatus*, *Hem. kapuasensis*, *Hem. Pogonognathus*, *Hem. tengah* [17], *Hemiramphus brasiliensis* and *He. balao* [18]. In this study we investigate the reproductive biology of *D. pusilla* using histological approaches. Our histological findings and their implication may bring about a better understanding of the reproductive modes of this species and other hemiramphids within atherinomorphs.

## MATERIALS AND METHODS

Healthy and mature *Dermogenys pusilla* (n = 10 for each sex), with the total length of 6.9 ± 3.73 cm, were collected in October 2016 from five stations in Pranburi River estuary, Thailand (N 12°24'16.5" / E 099°59'20.2", N 12°24'16.5" / E 099°59'20.2", N 12°24'06.3" / E 099°58'58.0", N 12°24'18.5" / E 099°58'36.0" and N 12°24'15.3" / E 099°58'28.6"). We followed the experimental protocol officially approved and granted by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1723004).

All fish were euthanised by rapid cooling shock [19]. The gonadal tissue of each individual fish was carefully dissected and fixed overnight in Davidson´s fixative. Taxonomic confirmation of all fish was done according to the taxonomical key from Rainboth [20]. Then these tissues were

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transferred to 70% ethanol to assess the morphology under the stereomicroscopy. The fixed tissues were processed according to standard histological protocols [21]. The embedded tissues were cut at a thickness of 4 μm, and the sections were stained with Harris haematoxylin-eosin [21]. Additionally, fresh ovarian tissues were cut at 20 μm and stained with oil red O to observe the lipid distribution [22]. The reproductive structure and gametogenesis of *D. pusilla* were examined from the histological sections and photographed under a light microscope (Leica digital 750; Leica, Germany). Gonadal maturation stages were evaluated according to Uribe et al. [23].

## RESULTS AND DISCUSSION

A comprehensive feature of the reproductive morphology of fish is a rich source of their reproductive mode [2, 3, 5, 6]. In monophyletic atherinoporphs, two major observations of morphological characteristics, the restricted spermatogonia and the relatively large egg size, have implicated the viviparous reproductive mode and internal fertilisation [2-6, 8, 9]. This is in agreement with several investigators [10, 11, 24]. In this study we clearly show the gonadal structure and gametogenesis of *D. pusilla*, which provides detailed information about their precise reproductive mode.

### Male Reproductive System and Spermatogenic Stages

The male reproductive system of *D. pusilla* is composed of the testis and testicular duct (Figure 1A). The testis was found to be a paired organ that is located in the centre of the body cavity. This feature is basically similar to some viviparous zenarchopterids [17, 24], but inconsistent with other fish species such as *Tomeurus gracilis* and *Cnesterodon decemmaculatus*, which have a single testicular organ [25]. The testis is divided into three parts: proximal, middle and distal. The proximal part is a short convoluted tube, and the middle part is the major structure consisting of enlarged tubes (Figure 1A). The large convoluted tube of the distal part is also observed (Figure 1A). Histologically, the testis of *D. pusilla* is surrounded by a thin layer of tunica albuginea (Figure 1B). The inner part of testis is separated into testicular tissue and vasa efferentia (Figure 1B).

The section of the testicular tissue shows that the oval-shaped spermatogonia are only located in the distal termini lobules, indicating that *D. pusilla* testis has the restricted spermatogonial type (Figures 2A, 2B), as in the case of several other hemiramphid species including *He. chryopunctatus*, *He. kapuasensis*, *He.pogonognathus* and *He. tengah* [9, 17, 26, 27], as well as those in other genera (*Tomeurus gracilis* and *Cnesterodon decemmaculatus*) [25]. These results obviously indicate that *D. pusilla* has the reproductive characteristics of atherinomorph species [9, 26].

During spermatogenesis, spermatogonia differentiate into primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. The primary spermatocyte of *D. pusilla* has a spherical shape with moderately basophilic nuclei (Figures 2C, 2D). The spermatid also has the basophilic nuclei due to high chromatin condensation (Figure 2D), whereas nuclei with oval-elongate shape and acidophilic elongated tail are observed in the spermatozoa (Figure 2E). Upon spermiation, each spermatocyst releases the spermatozoa into the central lumen of the vasa efferentia (Figure 2F), and thus packed spermatozoa are observed in the histological section (Figure 2G). The elongated sperm nuclei are found in the periphery and flagella toward the central part of the vasa efferentia (Figure 2G). This observation is consistent with previous reports of the subfamily Poeciliinae [28].

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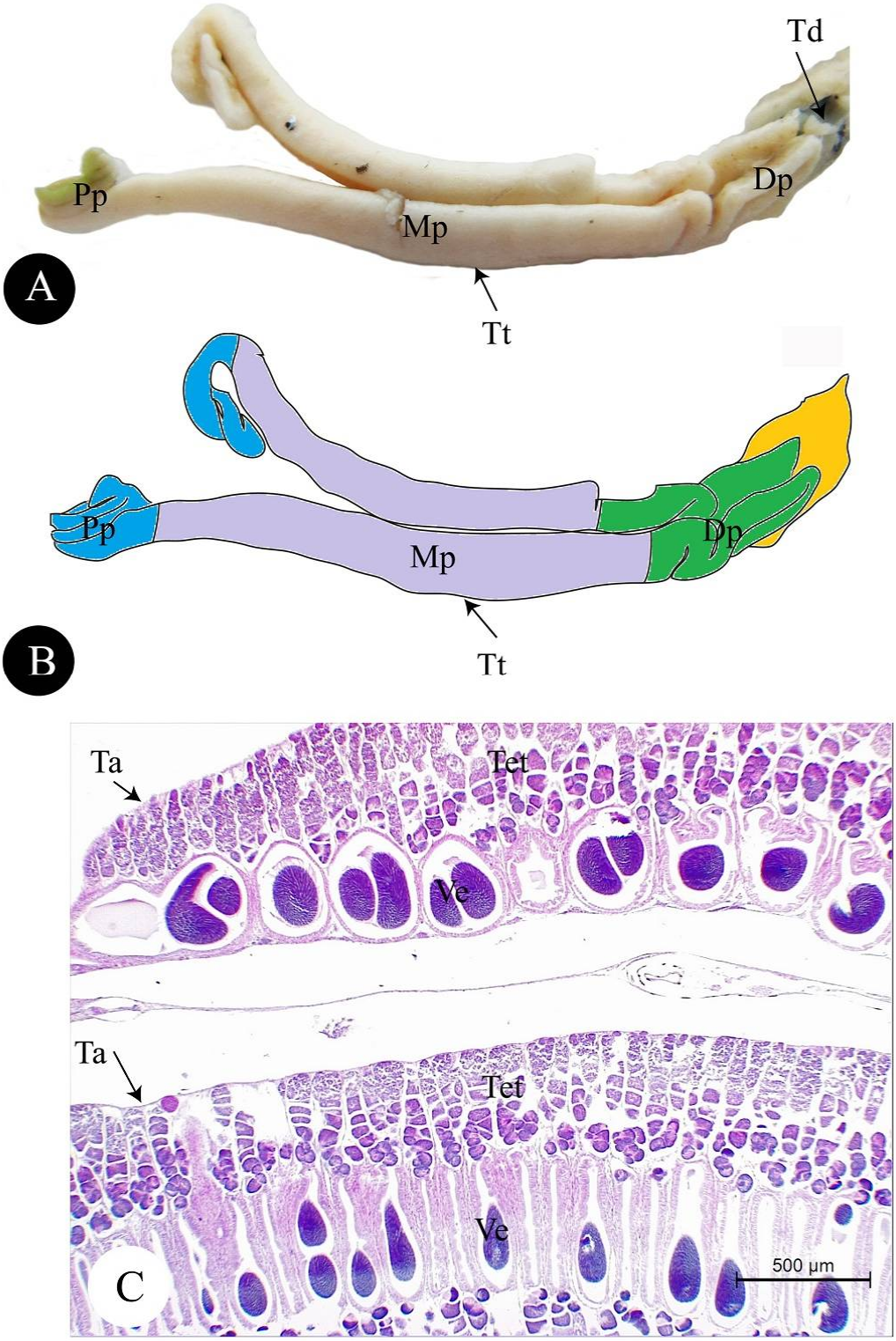


**Figure 1.** Overall morphology of male reproductive system of *Dermogenys pusilla*: (A, B) photograph and schematic diagram of male reproductive system consisting of two regions [testis (Tt) and testicular duct (Td)]; (C) Light photomicrographs showing testicular structure (Tet) of *D. pusilla* surrounded by a thin layer of tunica albuginea (Ta). (Dp = distal part, Mp = middle part, Pp = proximal part, Ve = vasa efferentia)

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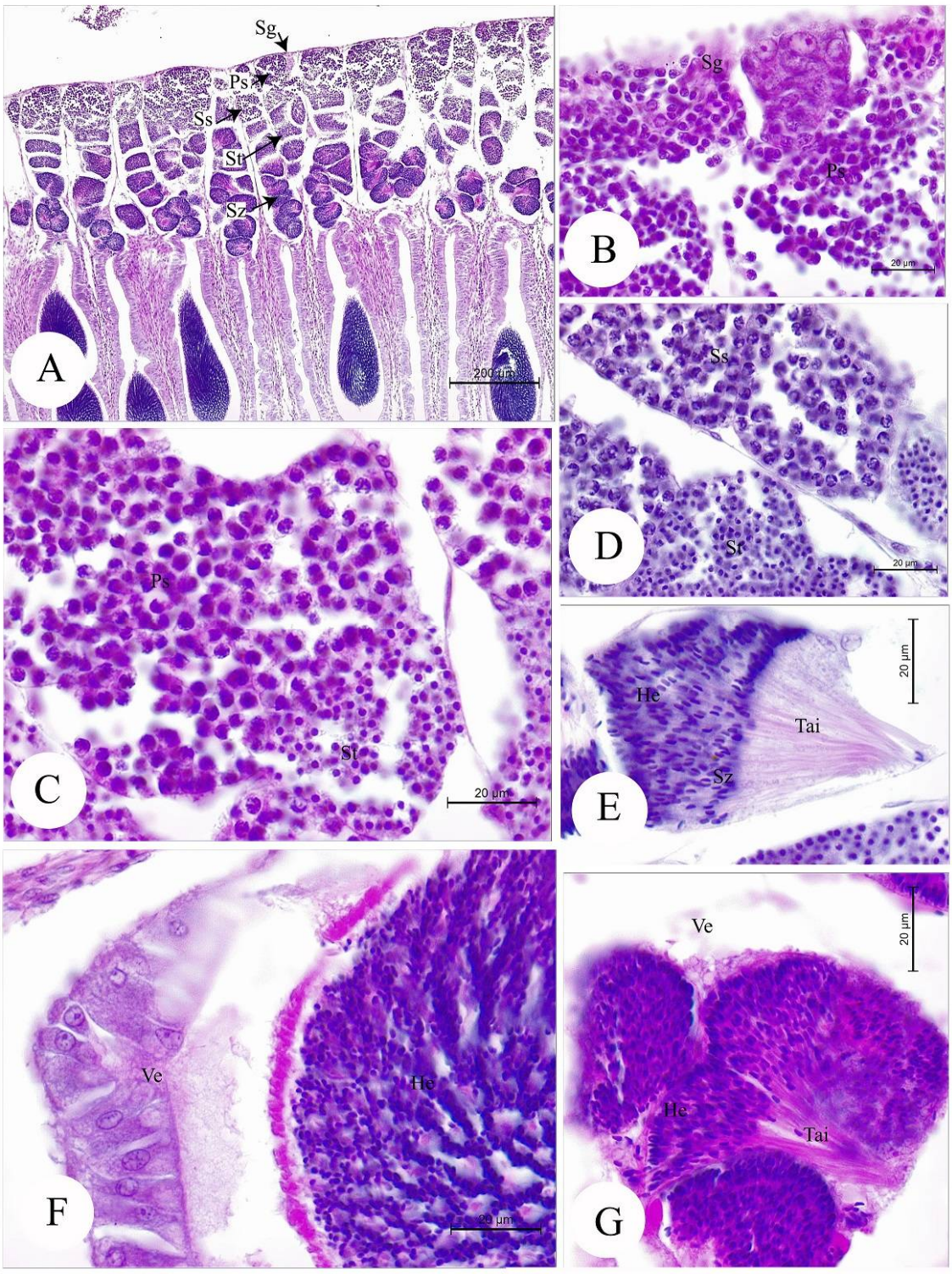


**Figure 2.** Light photomicrographs of spermatogenesis of *D. pusilla*: (A, B) *D. pusilla* testes classified as restricted spermatogonial type as spermatogonia are found only at distal part of the testis. The spermatogenesis is divided into spermatogonia (Sg) [B], primary spermatocyte (Ps) [B-C], secondary spermatocyte (Ss) [D] and spermatid (St) [C-D]. (E) Spermatozoa (Sz), composed of head (He) and tail of sperm (Tai); (F-G) Packed head (He) and tail (Tai) of spermatozoa found in vasa efferentia (Ve).

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**Female Reproductive System and Oogenic Stages**

The ovary of *D. pusilla* is a paired and saccular organ that is located in the middle area of the body cavity (Figures 3A, 3B). A synchronous development of oocytes in the stages of primary growth (the perinucleolar step and oil droplets-cortical alveolar step) and secondary growth (full-grown oocyte step) can be observed simultaneously (Figure 3C). A similar pattern of oogenesis was observed in *Crenichthys baileyi* and *Empetrichthys latos* [23].

Longitudinal sections show that the oocytes in the perinucleolar step contain multiple nucleolus along the nuclear membrane. Basophilic ooplasm is also observed (Figure 3D). Oil droplets positively reacting with ORO was observed throughout the ooplasm during this stage (data not shown).

The oocytes in the secondary growth stage are characterised by the deposition of yolk granule (Figures 4A, 4B). The fusion of the yolk granules results in the formation of fluid yolk in the full-grown oocyte step (Figures 4C, 4D). Many oil droplets are also found in the peripheral region of these oocytes. Our histological analysis also identifies the zona pellucida (chorion or vitelline envelope) in *D. pusilla* oocytes during the secondary growth stage (Figure. 4B). Interestingly, the thickness of zona pellucida (11.50 ± 0.54 μm) dramatically increases during oogenesis: 3 μm in the oil droplet-cortical alveolar step, 10 μm in the early secondary growth step (Figures 4A, 4B), and about 45 μm in the full-grown oocyte step (Figure 4D). It is possible that these features may be associated with the uptake of yolk granules.

Moreover, no adhesive filaments on the oocytes were found in the zona pellucida, indicating that *D. pusilla* is a viviparous atherinomorph, as proposed by Rosen and Bailey [29]. It is believed that the adhesive filament has been lost or reduced during the evolution of viviparity [25]. Parenti et al. [25] speculated that the adhesive filament was present in the zona pellicida of *T. gracilis* and *C. decemmaculatus* and this is probably a characteristic of oviparous reproductive mode.

Interestingly, numerous spermatozoa are observed in the folds of the ovarian lumen of oocytes during the multiple stages which include perinucleolar step (Figure 4E), early secondary growth step (Figure 4F) and full-grown oocyte step (Figures 4G, 4H), as reported previously in other species [30, 31]. The position of sperm heads is close to the apical end of the epithelial cells, whereas sperm tails extend into the ovarian lumen. We also observe the free spermatozoa in the ovary (Figures 4G-4H). Although the physiological significance of these sperms needs to be determined more precisely, it is conceivable that the female fish might create the functional sperm storage in their ovary, as reported by Parenti et al. [31] in *P. formosa*. The location of spermatozoa might be convenient for their interaction with the ovary [32, 33] as well as for their long-term storage in the ovary. The overall feature of sperms in the ovary is similar to those previously reported in *T. gracilis* and *C. decemmaculatus* [25] and other internally fertilised atherinomorphs such as and *P. reticulata* [30] and *Xiphophorus maculatus* [31, 32]. Taken together, our histological observations support the previous idea that *D. pusilla* has the viviparous reproductive mode, and further suggest that the viviparous reproduction is achieved by parthenogenes due to sperm storage in the ovary. Hemirhamphids are known to undergo internal fertilisation, but there is no evidence to differentiate the parthenogenesis [33, 34].

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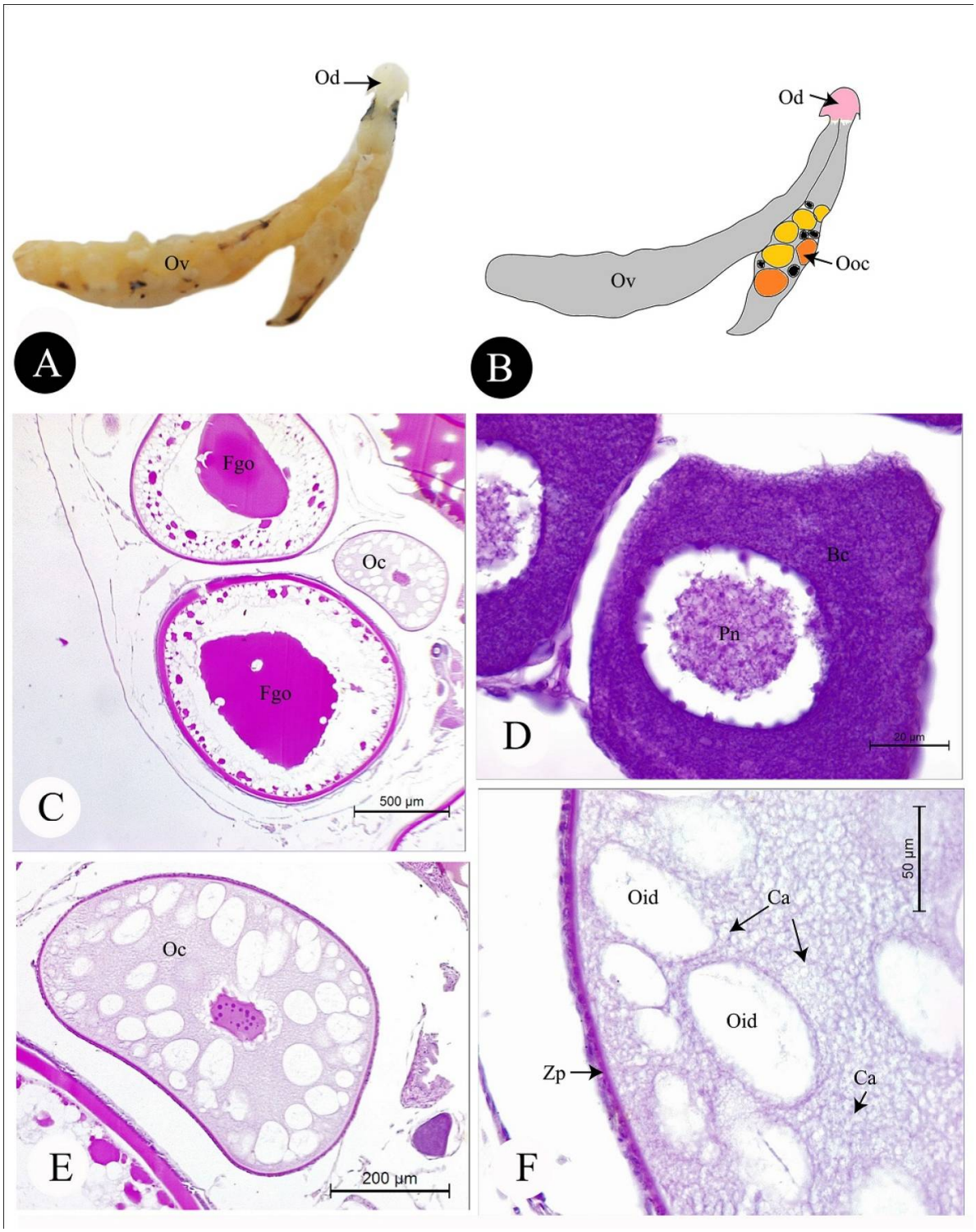


**Figure 3.** Overall morphology of female reproductive system of *D. pusilla*: (A, B) photograph and schematic diagram of female reproductive system composing of two regions [ovary (Ov) and ovarian duct (Od]; light photomicrographs show developing stages of oocyte (C): perinucleolar step (Pn) [D] and oil droplet-cortical alvelolar step (Oc) [E-F]. (Bc = basophilic cytoplasm, Ca = caltical alveoli, Oid = oil droplet, Ooc = oocyte, Zp = zona pellucida)

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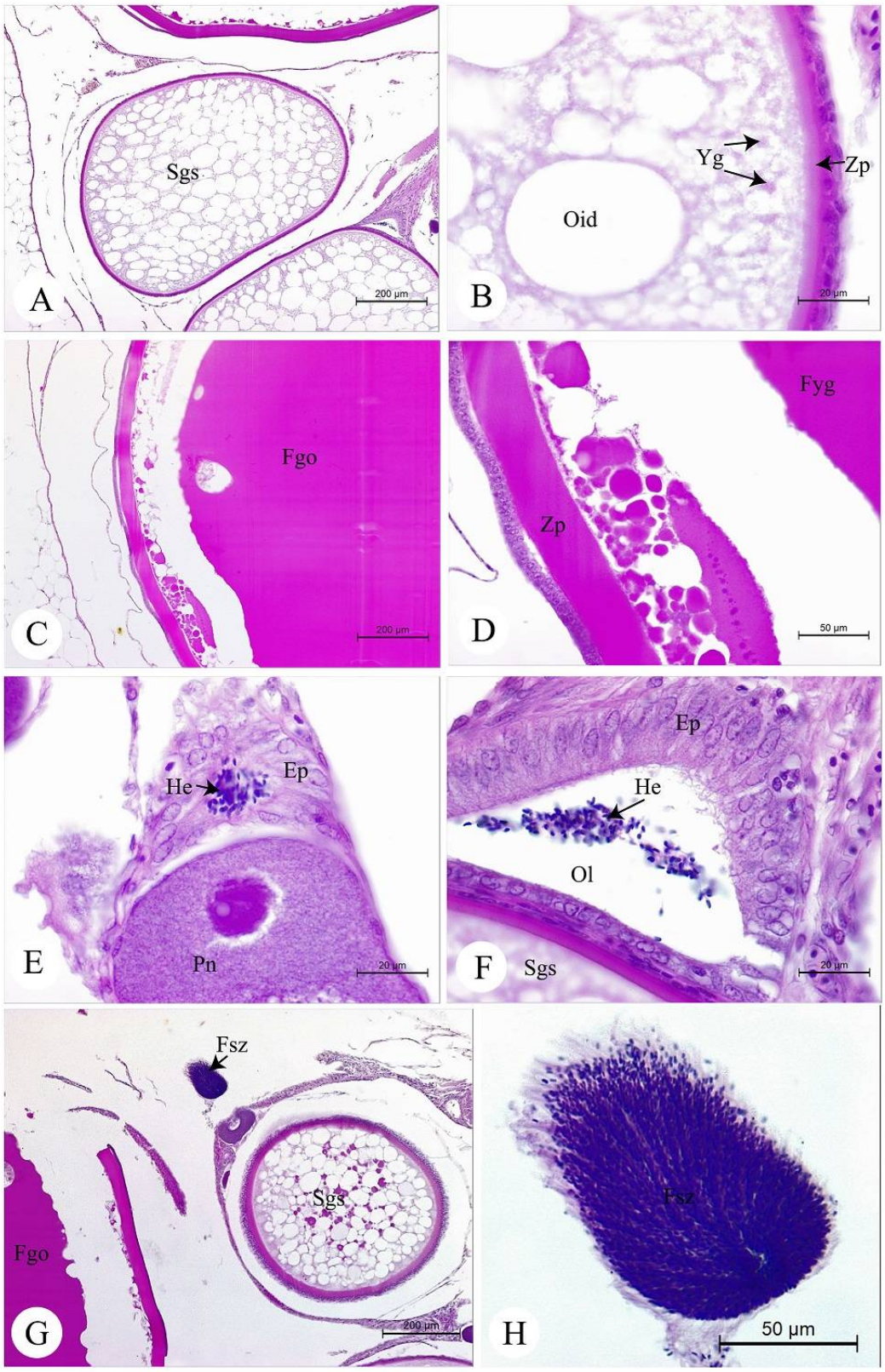


**Figure 4.** Light photomicrographs of *D. pusilla* oocytes: (A, B) oocytes in secondary growth step (Sgs); (C, D) oocytes in full-growth oocyte step (Fgo)*;* (E–F) occurrence of prominent head (He) of sperm within ovarian lumen (Ol) being nearly located in the ovary; (G-H) free spermatozoa (Fsz) present. (Ep = epithelium, Fyg = fusion of yolk granule, Oid = oil droplet, Yg = yolk granules, Zp = zona pellucida)

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## CONCLUSIONS

The viviparous reproductive mode of *D. pusilla* has been supported by our histological data including spermatogonial distribution, feature of zona pellucida and presence of sperm storage in the ovary. Future molecular biological analysis will facilitate our understanding of the evolution of this interesting reproductive system in the family Hemiramphidae.

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