ORIGINAL ARTICLE

Alteration of gonadotrophs in the pituitary gland during the annual reproductive cycle of the adult female sand goby (*Oxyeleotris marmoratus*)

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Abstract

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Pituitary gonadotrophs were studied in the adult female sand goby (*Oxyeleotris marmoratus*) during its annual reproductive cycle, aiming at investigating the alteration of gonadotropic cell types and their functions. The glands were divided into 3 groups according to maturity stages of the ovary: immature, mature and gravid stages. All of the ovarian stages were found throughout the year except in November, when only the gravid stage was identified. By using anti-chum salmon GTH Iβ and anti-chum salmon GTH IIβ antibodies for immunohistochemistry, strong anti-GTH IIβ reaction were observed in the proximal pars distalis (PPD) of the pituitary gland in all stages and the number of cells was significantly increased in the gravid stage (60.1±3.5 cell/mm^2) compared to that of the immature (35.5±4.4 cell/mm^2) and mature (48.3±7.2 cell/

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mm^2) stages (P<0.05). In the immature and mature ovarian stages, a great number of vacuoles was observed in areas of the PPD normally occupied by the gonadotrophs. The anti-GTH IIβ labeling gonadotrophs exhibited patterns of activity, correlated with the ovarian maturity. Anti-GTH Iβ labeling gonadotrophs were not observed in any stage, suggesting that GTH IIβ may be the only hormone regulating ovarian function of the sand goby.

Key words : gonadotroph, reproductive cycle, sand goby

บทคัดย่อ

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การเปลี่ยนแปลงของเซลล์โกนาโดโทรปภายในต่อมใต้สมอง ในช่วงวงจรการสืบพันธุ์ในรอบปีของปลาบู่ทรายเพศเมียตัวเต็มวัย

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การศึกษาการเปลี่ยนแปลงของเซลล์โกนาโดโทรปภายในต่อมใต้สมอง ในช่วงวงจรการสืบพันธุ์ในรอบปีของปลาบู่ทราย (*Oxyeleotris marmoratus*) เพศเมียตัวเต็มวัย เพื่อดูลักษณะและหน้าที่ของเซลล์โกนาโดโทรป ต่อมใต้สมองจะถูกจัดเป็น 3 กลุ่ม ตามระยะความสมบูรณ์ของรังไข่ คือ ระยะรังไข่เริ่มเจริญ (immature) ระยะรังไข่เจริญเต็มที่ (mature) และระยะรังไข่สุก (gravid) โดยการเก็บตัวอย่างปลาในรอบ 1 ปี พบว่าเดือนพฤศจิกายนจะพบเฉพาะระยะรังไข่สุกเท่านั้น ในขณะที่เดือนอื่น ๆ จะพบระยะความสมบูรณ์ของรังไข่ทั้ง 3 ระยะ จากการย้อมต่อมใต้สมองด้วย anti-chum salmon GTH Iβ และ anti-chum salmon GTH IIβ โดยวิธีอิมโมโนฮิสโตเคมมิสตรี้ พบเซลล์โกนาโดโทรปที่ย้อมติดสีเข้มของ anti-GTH IIβ ที่บริเวณพรอกซิมัลฟาร์ดิสทัลสิลของต่อมใต้สมองในทุกกลุ่ม โดยมีจำนวนเพิ่มขึ้นอย่างมีนัยสำคัญในระยะรังไข่สุก (60.1±3.5 cell/mm^2) ที่ P<0.05 เมื่อเทียบกับระยะรังไข่เริ่มเจริญ (35.5±4.4 cell/mm^2) และระยะรังไข่เจริญเต็มที่ (48.3±7.2 cell/mm^2) ซึ่งพบน้อยกว่า และพบแวคคูโอ (vacuole) ภายในเซลล์เป็นจำนวนมาก ขณะที่เซลล์โกนาโดโทรปที่ย้อมติด anti-GTH Iβ ไม่พบในระยะใดเลย บ่งชี้ว่าปลาบู่ทราย อาจจะมี GTH IIβ เพียงฮอร์โมนเดียวที่ทำหน้าที่เกี่ยวข้องกับการทำงานของรังไข่

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Sand goby (*Oxyeleotris marmoratus,* Bleeker) is known as the largest fish in the group of gobiidae that inhabits natural freshwater marshes. It is one of the most important cultured fish in Asia. However, the number of sand gobies is declining due to deterioration of their habitat. In addition, breeding and fingerling hatcheries are very few, owing to a lack of information concerning its reproductive physiology and endocrinology. Studies on its reproductive physiology and endocrinology would help to provide an understanding of the fundamental mechanisms involved in sand goby reproduction, which could lead to an improved sand goby aquaculture in the future.

In several species, gonadotrophs of the pituitary gland play an important role in stimulating gonadal maturation. In a number of teleost fishes, e.g. chum salmon (*Oncorhynchus keta*) (Suzuki *et al*., 1988 a,b; Kawauchi *et al*, 1989), coho salmon (*Oncorhynchus kisutch*) (Swanson *et al*., 1991) and Japanese eel (*Anguilla japonica*) (Yoshiura *et al*., 1999) it is known that the pituitary glands contain two distinct gonadotropic cell types, which produce two chemically distinct gonadotropins: GTH I (FSH-like) and GTH II (LH-like). In rainbow trout, the GTH I gonadotrophs are found in greater number in immature fish, whereas in mature fish the GTH II gonadotrophs dominate

(Nozaki *et al*., 1990a; Naito *et al*., 1991). Therefore, it has been suggested in salmonid fishes that GTH I and GTH II possess different functions; GTH I contributes to early spermatogenesis and follicular growth, whereas GTH II encourages the maturation of gametes and is implicated in spermiation and ovulation (Suzuki *et al*., 1988c; Tyler *et al*., 1991; Planas *et al*., 1993). Although the two distinct GTHs have been identified in several fishes, there are still a number of species, such as the European eel (*Anguilla anguilla*) (Querat *et al*.,1990), chinook salmon (*Oncorhynchus tschawytsha*) (Breton *et al*, 1978), tilapia, (*Orechromis mossambica*) (Farmer & Parkoff, 1977), and the African catfish (*Larias gariepinus*) (Koide *et al*., 1992; Schulz *et al*., 1997), in which LH but not FSH has been found. A single GTH is referred as maturational GTH.

The present study focuses on the alteration of the gonadotrophs throughout the annual reproductive cycle of *O. marmoratus*, by identifying gonadotropic cell types using immunohistochemistry. In samonids, GTHs are composed of α and β subunits; the α subunit is the same as the α subunit of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH), while the β subunit confers specificity to each hormone (Pierce & Pearson, 1981). The antisera used in this studies were anti-chum salmon GTH Iβ and GTH IIβ, which were supposedly FSH and LH, respectively. These antisera have been used to identified GTH Iβ (FSH) and GTH IIβ (LH) gonadotrophs in several fishes, e.g. Pejerrey (*Odontestes bonariensis*) (Miranda *et al*, 2001) and Nile Tilapia (*Oreochromis niloticus*) (Mousa & Mousa, 1999).

Materials and Methods

Animals

Six female sand gobies (*Oxyeleotris marmoratus*) over 15 cm long were collected each month from natural freshwater marshes at Pattani Province, Southern Thailand, between March 2003 and March 2004.

Gross examination of paired ovaries and maturity stage was identified by a method modified from Mayer *et al*. (1988). The pituitary glands with the attached brain were removed immediately after decapitation and fixed in 10% formalin for about 24 h. Following dehydration and embedding in paraffin, serial 5 μm sections were cut in the saggital plane and mounted on TESPA coated slides. Random sections were histologically stained with Masson's Trichrome for identification of the pituitary cell types.

Immunohistochemistry

The sections were dewaxed, rehydrated and incubated sequentially with 0.3% Triton X-100 in phosphate buffered saline (PBS: 0.14 M NaCl, 0.01 phosphate buffer) pH 7.4 (30 min), 3% H_2O_2 in methanol (30 min), 10% normal goat serum (Vector Laboratories) in PBS (60 min), and finally with the anti-chum salmon GTH Iβ or anti-chum salmon GTH IIβ at dilution of 1:500, 1:1000, 1:2000, 1:4000, 1:6000, 1:8000, 1:10000, 1:15000, 1:20000 in PBS overnight at 4°C. Antisera to chum salmon GTH Iβ and GTH IIβ were obtained from Dr H. Kawaushi (School of Fisheries Science, Kita-sato University, Iwate, Japan). The sections were then rinsed with PBS and incubated with the biotinylated secondary anti-rabbit antibody (anti-rabbit IgG, Vector Laboratories), at a dilution of 1:200 in PBS for 2 hours at room temperature. After three rinses, the avidin-biotin-peroxides complex was constructed using ABC reagent (Vector laboratories) and visualized using the chromogen-based system, DAB (Vector laboratories). A negative control was performed by omitting the primary antibodies. Finally, the sections were counter-stained with hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene and cover-slipped with DPX. Images were captured with an Olympus DP11 digital camera and image files were processed using Microimage software (Olympus).

Counting of immunostained cells

The number of immunostained cells per mm^2 in each fish was calculated as follows. Six pituitary glands from each maturity stage (total 18 glands) were randomly selected. Ten sections of each gland were systematically selected (Mayhew, 1991). Two

pictures of proximal par distalis (PPD) of each section were randomly taken by using an Olympus DP11 digital camera (objective lens = 40 and camera lens = 3.3). The number of immunostained cell was counted and the area of sections examined was estimated by Microimage analysis software (Olympus). The results were expressed as number of immunostained cells per mm^2

Data analysis

Data are reported as mean±S.D. Statistical analysis was performed by one way ANOVA and Least-Significant Difference (LSD) for post hoc analyses, to compare the number of immunostained cell/mm^2 in the pituitary gland of three different maturity stages of ovary. Statistical significance was accepted at a value of $P<0.05$.

Results

Maturity stages

Gross examination of the paired ovaries revealed that the ovarian development of *O. marmoratus* could be classified into immature, mature and gravid stages. In the immature stage, the ovaries were small, thread-like, pale yellow-orange in color and occupied one-third of the ventral cavity. In the mature stage, the ovaries were orange in color, 1.5-4 mm in diameter and occupied one-half of ventral cavity. In the gravid stage, the ovaries were swollen, yellow in color, 2.5-6.5 mm in diameter and fully occupied ventral cavity. All stages were found throughout the year, except in November, when only the gravid stage was identified.

General morphology of the pituitary gland

The pituitary gland of *O. marmoratus* consisted of the adenohypophysis and the neurohypophysis. The adenohypophysis was divided into three regions: the rostral pars distalis (RPD), the proximal par distalis (PPD) and the pars intermedia (PI). The rostral pars distalis was separated from the rest of the pituitary gland by a distinct circum-ferential constriction (Figures 1A, B and C). The pituitary glands were divided into 3 groups according to the stages of the ovaries. Size of the PPD was increased in the mature stage and reached maximum at the gravid stage (Figures 1B and C).

Cellular composition of the pituitary gland

Saggital sections of *O. marmoratus* pituitary gland stained with Masson's Trichrome showed various cell types, segregated into three zones of the adenohypophysis. In the RPD, acidophils formed the major component, whereas the PPD consisted mainly of two cell types: basophils and acidophils (Figures 2A, 3A and 4A). Basophils appeared to be homologous with the somatotroph and gonadotroph described in other teleosts. In the

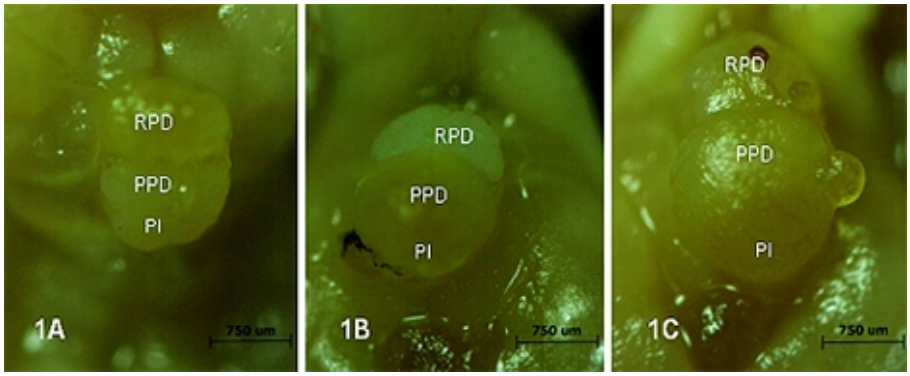
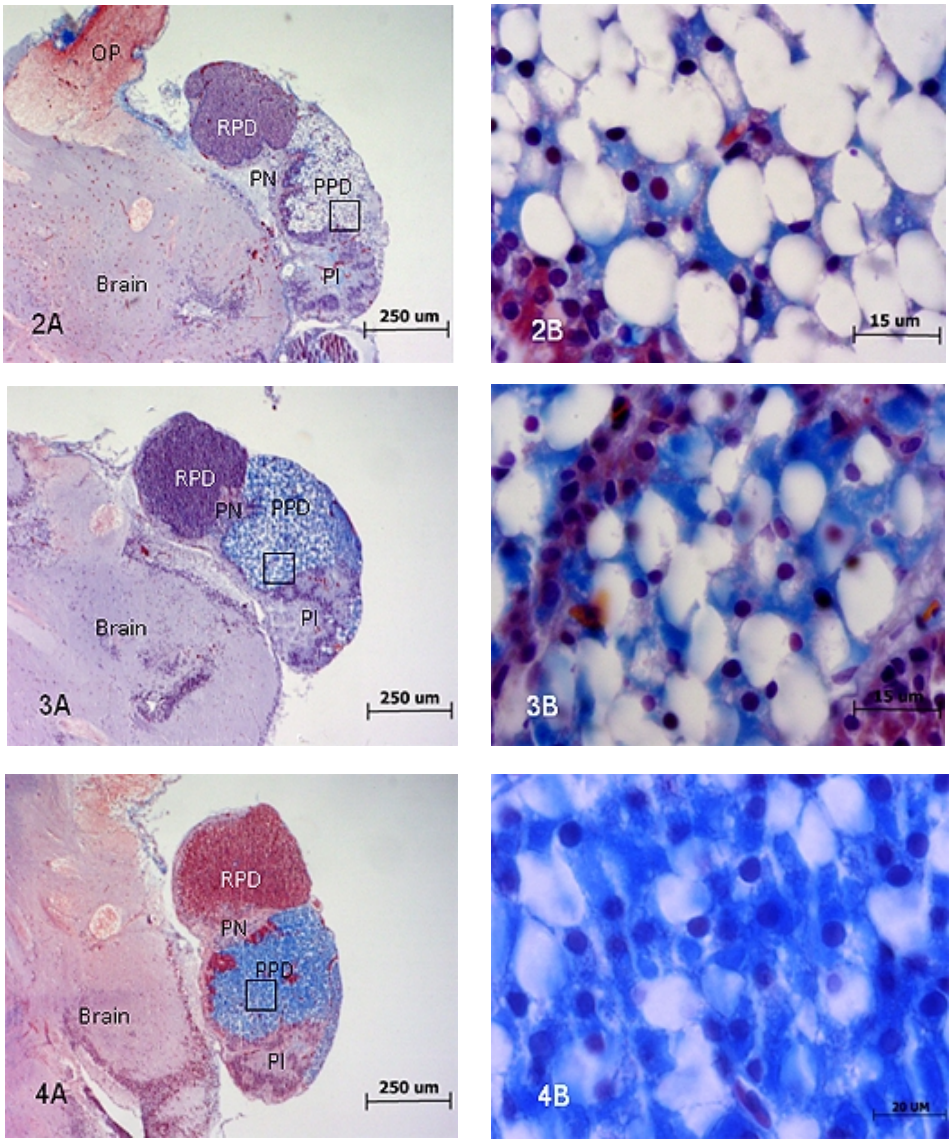
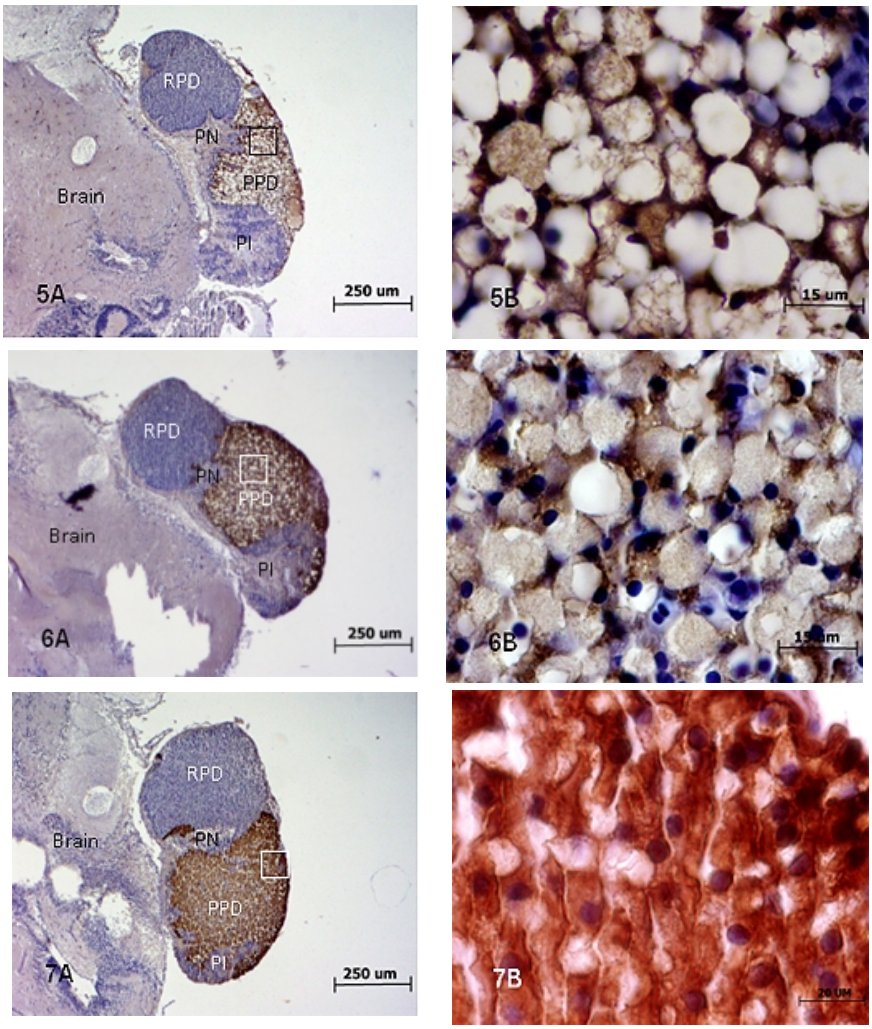


Figure 1. General morphology of the sand gobies (*Oxyeleotris marmoratus*) pituitary gland at different maturity stages of the ovary: (A) immature, (B) mature, and (C) gravid stage. RPD, Rostral pars distalis; PPD, Proximal pars distalis; PI, Pars intermedia.



Saggital sections of the sand goby's pituitary gland, stained with Masson's Trichrome, shows the cellular composition of the pituitary gland in three stages:
Figure 2 (A) Immature stage
Figure 3 (A) Mature stage
Figure 4 (A) Gravid stage
In each of these, the enlarged box shows details of the cellular structure:
Figure 2 (B) A great number of vacuoles and comparatively few basophils (blue cytoplasm), and a few acidophil (red cytoplasm) in PPD.
Figure 3 (B) A reduction of vacuoles and an increase of basophils in PPD.
Figure 4 (B) A marked numerical increasing of basophils in PPD.
RPD, Rostral pars distalis; PPD, Proximal pars distalis; PI, Pars intermedia; PN, Pars nervosa; OP, Optic nerve.



Saggital sections of the sand goby's pituitary gland, stained with anti- GTH II β (LH) at a dilution of 1:8000, shows immunoreactivity (brown color) in the cytoplasm of gonadotrophs in PPD in three stages:

Figure 5 (A) Immature stage

Figure 6 (A) Mature stage

Figure 7 (A) Gravid stage

In each of these, the enlarged box shows details of the cellular structure:

Figure 5 (B) A great number of vacuoles and comparatively few anti-GTH II β (LH) labeling gonadotrophs (brown cytoplasm) in PPD. The GTH II β (LH) immunoreactivity is found inside some vacuoles.

Figure 6 (B) An increase number of anti-GTH II β (LH) labeling gonadotrophs and the GTH II β (LH) immunoreactivity is found inside most vacuoles.

Figure 7 (B) A marked numerical increasing of anti- GTH II β (LH) labeling gonadotrophs in PPD and an increase of anti- GTH II β (LH) immunoreactivity in the cytoplasm of gonadotrophs.

RPD, Rostral pars distalis; PPD, Proximal pars distalis; PI, Pars intermedia; PN, Pars nervosa.

gravid stage, the number of basophils was markedly increased, whereas in the immature and mature stages, the number was comparatively few and a great number of vacuoles was observed in areas of the PPD normally occupied by the basophils (Figures 2B, 3B and 4B). The neurohypophysial processes originating in hypothalamic regions interdigitate with all the three zones through the pituitary stalk. The neurohypophysis, establishing the pars nervosa (PN), was especially deep and elaborate with the PI.

Immunohistochemistry

The anti-GTH IIβ stained most of basophils and the optimal dilution of GTH IIβ antiserum was 1:8000. The strong anti-GTH IIβ labeling gonadotrophs were found in the PPD of the pituitary gland in all stages (Figures 5A, 6A and 7A). In the gravid stage, the number of GTH II β-positive cells was markedly increased whereas in the immature and mature stages, the number was comparatively low and several vacuoles were observed in PPD areas normally occupied by the gonadotrophs (Figures 5B, 6B and 7B). In the immature and mature stages, the GTH II β immunoreactivity was located inside some vacuoles (Figures 5B and 6B). In the gravid stage, the number of vacuoles was markedly reduced since most of the vacuoles were filled with the GTH II β (Fig 7B). The number of GTH IIβ-positive cells in the mature stage (35.5±4.4 cell/mm^2) was significantly higher than that of the immature stage (48.3±7.2 cell/mm^2) ($P<0.05$). In addition, the number of GTH II β-positive cells in the gravid stage (60.1±3.5 cell/mm^2) was significantly higher than that of the immature ($P<0.05$) and mature stages ($P<0.05$) (Figure 8). Interestingly, anti-GTH Iβ labeling gonadotrophs were not found in any stage of ovarian maturation, at any dilution of anti-GTH Iβ anti-serum. No immunoreactivity was found in the negative control sections.

Discussion

The study shows that strong anti-GTH IIβ labeling gonadotrophs were found in *O. marmoratus*'s pituitary gland in all stages by means of specific staining with anti-GTH II β. The number of GTH II β-positive gonadotrophs increases towards mature ovarian stage and reaches its maximum in the gravid stage; this finding correlates well with the large size of PPD in the gravid stage seen in gross observations. Similar immunocytochemical observations were demonstrated in *Salmo gairdneri irideus* (Nozaki *et al*., 1990b). In

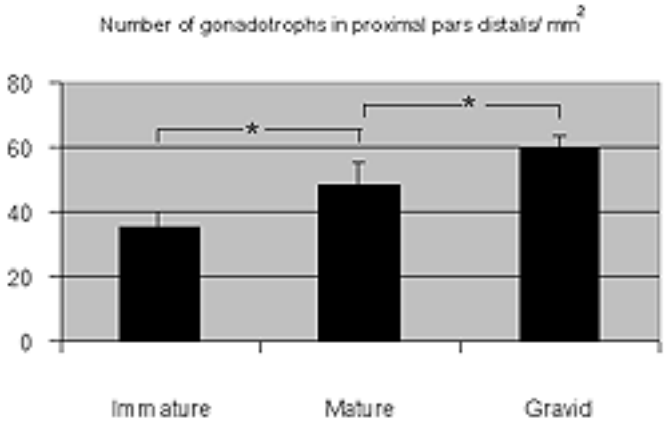


Figure 8. The number of anti- GTH II β (LH) labeling gonadotrophs/mm^2 in PPD of the sand goby pituitary gland at different maturity stages of ovary. N= 6, one way ANOVA and Least-Significant Difference LSD for post hoc analyses,
*** = significant difference between two groups**

the immature and mature stages, the GTH II β immunoreactivity is found inside some vacuoles. In the gravid stage, the number of vacuoles was markedly reduced, because most of the vacuoles were filled with the GTH II, suggesting that the gonadotrophs began to produce GTH II hormone in the immature stage and the hormone fully occupied most vacuoles in the gravid stage. This was confirmed by histology. In every stage, strongly immunoreactive gonadotrophs often showed cytoplasmic extension that contacted other gonadotrophs. The similar feature was also found in the gonadotrophs of African catfish, *Clarias ganepinus*, possibly revealing that a later stage of the gonadotroph differentiation implicates transient, homologous cell-to-cell contacts (Zandgergen *et al*.,1993). The anti-GTH II β labeling gonadotrophs exhibited patterns of activity correlated to ovarian maturity. In-terestingly, the anti-GTH Iβ labeling gonadotroph was not found in any ovarian stage, suggesting that GTH II may cover all ovarian regulation functions in the sand goby. This result is also found in the primitive teleosts such as the European eel (*Anguilla anguilla*) (Querat *et al*., 1990), chinook salmon (*Oncorhynchus tschawytsha*) (Breton *et al*., 1978), tilapia, (*Orechromis mossambica*) (Farmer & Papkoff, 1977), and the African catfish (*Larias gariepinus*) (Koide *et al*., 1992; Schulz *et al*., 1997), in which only a single gonadotroph is present. This contrasts with the situation in relatively modern species e.g chum salmon (*Oncorhynchus keta*) (Suzuki *et al*., 1988 a,b; Kawauchi *et al*, 1989), coho salmon (*Oncorhynchus kisutch*) (Swanson *et al*., 1991) and Japanese eel (*Anguilla japonica*) (Yoshiura *et al*., 1999), in which two gonadotrophs: GTH I and GTH II are identified. It would be interesting to study the gonadotropic cell type in the male sand goby, as GTH I may be present at early stages of gonadal development. Our data show that all stages of ovary were found throughout the year, but the gravid stage was only found in November, which is the rainy seasons and wettest month in Southern Thailand. The results correspond to the work of Boonyoung *et al*. (2003), who demonstrated that the gonadosomatic index and spawning period of the female *O. marmoratus* are highest in November. Environmental factors may thus affect reproductive physiology and endocrinology.

In summary, the present results show that only a single type of gonadotroph is present in female sand goby (*Oxyeleotris marmoratus*), namely anti-GTH II β labeling gonadotrophs. Their gonadotrophs exhibited patterns of activity correlating with ovarian maturity, thus it is likely that GTH II or LH in higher species may regulate all functions required for ovarian maturation.

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