RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2006.32.337

ScienceAsia 32 (2006): **337-343**

Immunocytochemical Identification of Gonadotropic Cell Types and Changes in Cell Numbers during Annual Reproductive Cycle in Pituitary Gland of Adult Male Sand Goby, *Oxyeleotris marmoratus*

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Received 6 Feb 2006
Accepted 19 May 2006

ABSTRACT: Pituitary gonadotropes were studied in the adult male sand goby (*Oxyeleotris marmoratus*) during its annual reproductive cycle. Pituitary glands were taken from each of the 5 stages of testicular development: resting, developing, mature, spawning and spent. The pituitary glands were processed for Masson's trichrome staining and immunocytochemistry using anti-chum salmon GTH Iβ and GTH IIβ antibodies. Most of the basophils in the proximal par distalis showed immunoreactivity to GTH IIβ. No cell showed immunoreactivity to GTH Iβ at any stage of testicular development. The number of GTH IIβ labeled cells was relatively low in the resting stage (22.46 ± 4.34 cell/mm^2) but significantly higher during the developing stage (49.21 ± 7.71 cell/mm^2) ($P<0.05$) compared to the resting stage. The number of immunoreactive cells decreased during the mature stage (31.29 ± 7.23 cell/mm^2) and was relatively constant in the spawning stage (30.41 ± 2.56 cell/mm^2) and spent stage (29.16 ± 2.21 cell/mm^2). Based on the well known function of GTH, the presence of only GTH IIβ but not GTH Iβ in the pituitary gonadotropes suggested the involvement of GTH IIβ in spermatogenesis of the male sand goby. Moreover, the high number of GTH IIβ immunoreactive cells during testicular developing stage correlated with the maturation of sperm further confirmed the role of GTH IIβ in controlling cell proliferation during spermatogenesis.

KEYWORDS: GTH I, GTH II, sand goby, pituitary gland, immunocytochemistry.

INTRODUCTION

The sand goby (*Oxyeleotris marmoratus*, Bleeker, 1852) is one of the most important freshwater Gobiidae for commercial aquaculture in Asia due to its tender flesh and good flavor. However, breeding and production are very limited owing to a lack of information concerning the regulation of its reproductive cycle. The pituitary gland is directly involved in controlling reproduction. It contains gonadotropes, producing gonadotropin hormones, that stimulate the growth and development of the ovary and testis. Two gonadotropic cell types, producing two chemically distinct gonadotropins, (GTH I and GTH II) in the teleost are characterized in several fish, e.g. chum salmon (*Oncorhynchus keta)*[1,2,3], coho salmon (*Oncorhynchus kisutch)*[4] and Japanese eel (*Anguilla japonica)*[5]. GTH I and GTH II are structurally homologous to the tetrapod follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively[1,2,4,6]. It has been suggested 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[6,7,8]. Although, the two distinct GTHs have been identified in several fish, there are still a number of species, such as the European eel (*Anguilla anguilla)*[9], chinook salmon (*Oncorhynchus tschawytsha*)[10], tilapia (*Orechromis mossambica)*[11], the African catfish (*Larias gariepinus)*[12,13], and female sand goby (*Oxyeleotris marmoratus*)[14] in which only GTH II has been identified. In the male sand goby (*Oxyeleotris marmoratus*), the information on the gonadotropic cell types and their function in relation to reproductive cycle is still unavailable. Therefore, this study aims to investigate the gonadotropic cell types and the changes in the cell number of gonadotropes during the annual reproductive cycle of the male sand goby (*Oxyeleotris marmoratus*). This study will provide an understanding in the role of the two

GTHs in the endocrine regulation of the reproductive cycle of the male sand goby.

Materials and Methods

Tissue Preparation and Ordinary Histology

Six male, 20-28 cm long, sand gobies (*Oxyeleotris marmoratus*) were collected each month from natural freshwater marshes at Pattani Provice, Southern Thailand between March 2003 and March 2004. The pituitary glands were removed, fixed in 10% formalin for preservation and processed for paraffin embedding. The glands were divided into 5 groups according to the stage of testicular development: resting, developing, mature, spawning and spent[15]. Deparaffinized mid sagittal pituitary sections were stained with Masson's trichrome staining which has been shown to stain gonadotropes and thyrotropes with aniline blue[16].

Antibodies and Immunocytochemistry

The antisera used in these studies were anti-chum salmon GTH Iβ and GTH IIβ, which were kindly given by Professor H. Kawaushi (School of Fisheries Science, Kitasato University, Iwate, Japan). The specificity of these antisera were proven for immunochemical detection of the GTH I and GTH II gonadotropes in several fish, e. g. Pejerrey (*Odontestes bonariensis*)[17] and Nile Tilapia (*Oreochromis niloticus*)[18]. The origin and characteristics of these antisera have been described previously[1,2].

The sections were deparaffinized, 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, Burlingame, USA) in PBS (60 min), and finally with the anti-chum salmon GTH-Iβ or anti-chum salmon GTH-IIβ at dilutions 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. The sections were then rinsed with PBS and incubated with the biotinylated secondary 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–peroxidase complexes were constructed using ABC reagent (Vector laboratories) and visualized using the chromogen-based system, diaminobenzidene (DAB). A negative control was performed by omitting the primary antibodies. A positive control was performed under the same staining condition with Wistar rat's pituitary gland sections. Pars distalis of rat's pituitary gland is known to contain both FSH and LH gonadotrophs[19,20,21]. Finally, the sections were counterstained with hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene and mounted 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 group was calculated as followed: six pituitary glands from each testicular stage (total 30 glands) were randomly selected. Ten sections of each gland were systematically selected[22]. Pictures of the proximal par distalis (PPD) of each section were taken by using an Olympus DP11 digital camera. The number of immunostained cells were counted and the area of sections examined was estimated by Microimage analysis software (Olympus). The results were expressed as mean± S.E of immunostained cells per mm^2.

Data Analysis

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 glands of the five different testicular stages. Statistical significance was determined at a value of $P<0.05$.

Results

Gross Morphology of the Male Sand Gobies (*Oxyeleotris marmoratus*) Pituitary Gland

The pituitary gland of sand gobies 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 circumferential constriction (Fig. 1a and b). A considerable increase in the size of the PPD was observed in the developing stage compared with the resting stage (Fig. 1a and b). The PPD size then gradually reduced through the mature, spawning and spent stages. However, the average weight of the pituitary gland in each stage of reproductive cycle was not significantly different (resting: 0.72± 0.26 mg, developing: 0.75±0.46 mg, mature: 0.71±0.34 mg, spawning: 0.67±0.40 mg and spent: 0.65±0.42 mg). All stages were found throughout the year, except for November, when only the spawning stage was identified.

Identification of Cell Types in Pituitary Gland of Male Sand Gobies

The overall histological structure of the pituitary gland was shown in Fig. 2. In the RPD, acidophils formed the major component, whereas the PPD consisted of two cell types: acidophils and basophils (Fig. 2b and c). Basophils which appeared to be homologous to the

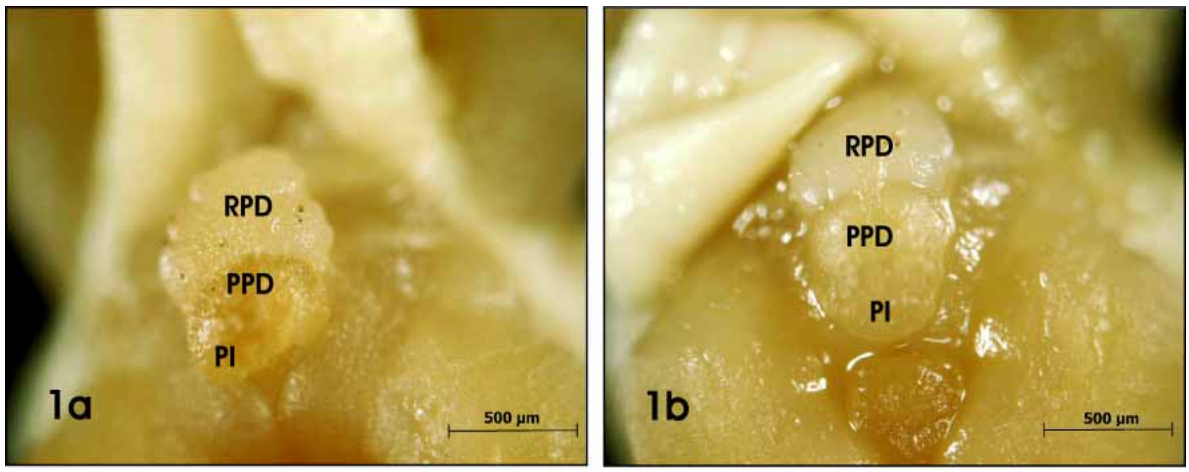

Fig 1. Pituitary gland of male sand gobies, showing a considerable increase in size of the PPD in the developing stage (b) compared with the resting stage (a).
RPD: Rostral pars distalis; PPD: Proximal pars distalis; PI: Pars intermedia.

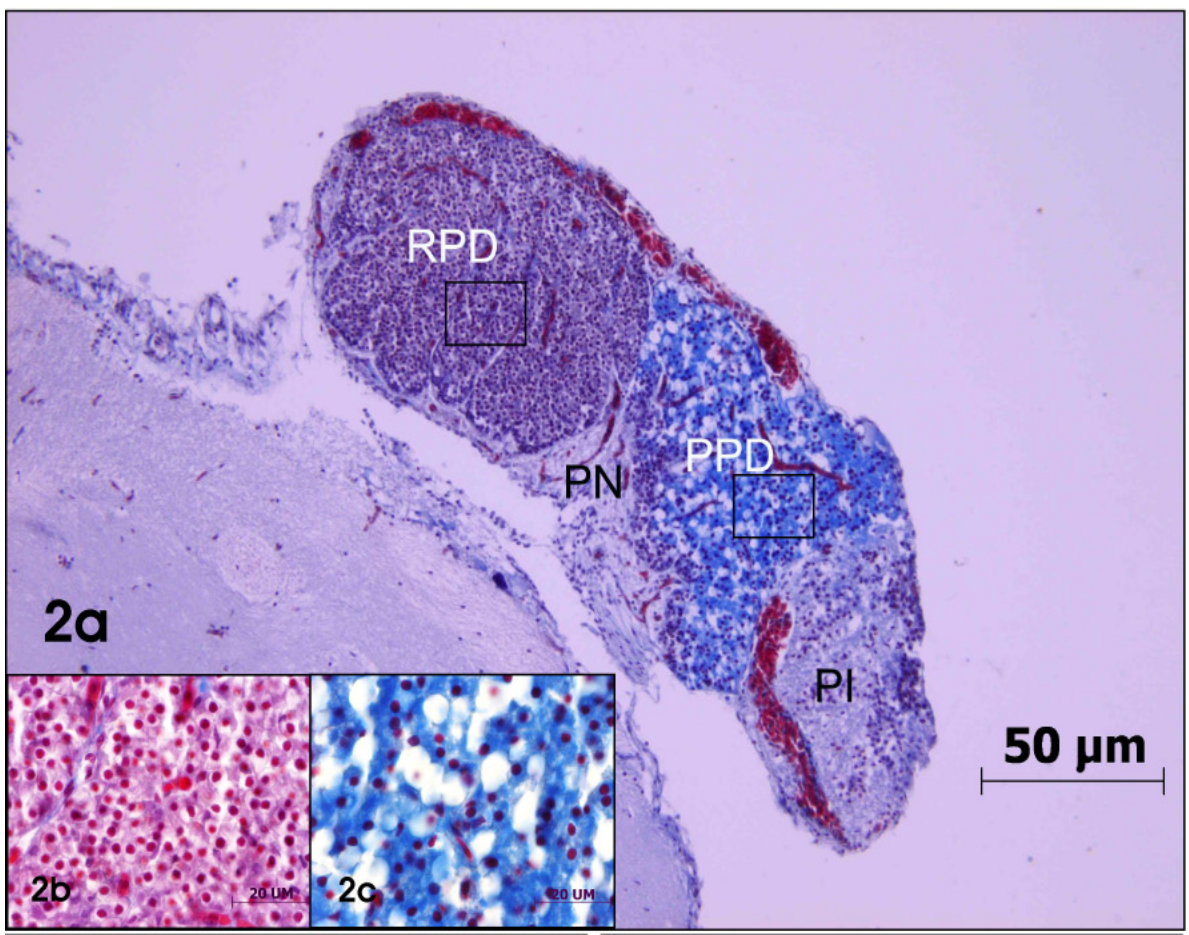

Fig 2. (a) Mid sagittal sections of the male sand goby's pituitary gland, stained with Masson's trichrome, showing the cellular composition of the pituitary gland in the developing stage. Insets of higher magnification of acidophils (b) and basophils (c). RPD: Rostral pars distalis; PPD: Proximal pars distalis; PN: Pars nervosa, PI: Pars intermedia.

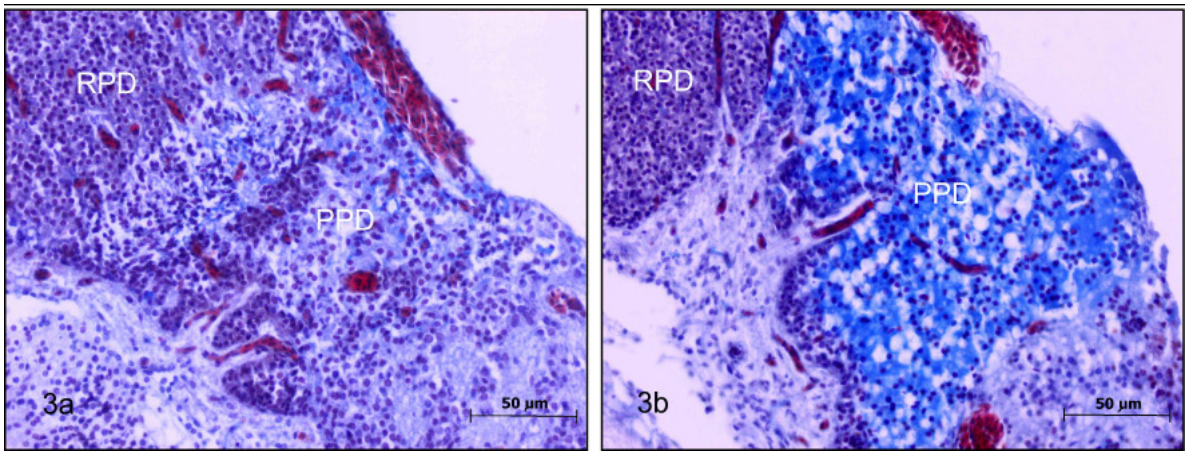

Fig 3. Mid sagittal sections of the sand goby's pituitary gland, stained with Masson's trichrome, showing a considerable increase in number of basophils (blue cytoplasm) in the PPD in the developing stage (b) compared with resting stage (a). RPD: Rostral pars distalis; PPD: Proximal pars distalis.

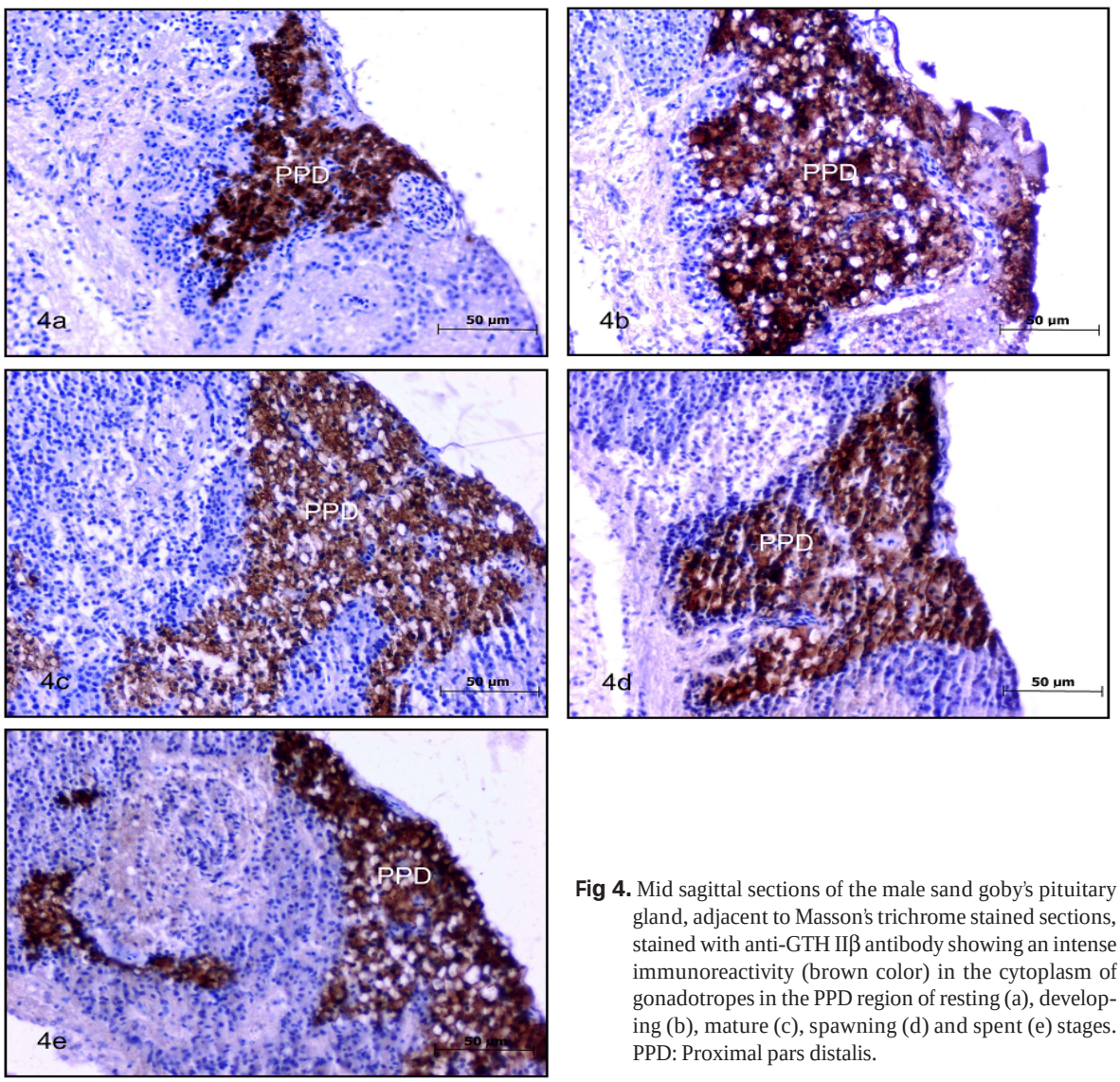

Fig 4. Mid sagittal sections of the male sand goby's pituitary gland, adjacent to Masson's trichrome stained sections, stained with anti-GTH IIβ antibody showing an intense immunoreactivity (brown color) in the cytoplasm of gonadotropes in the PPD region of resting (a), developing (b), mature (c), spawning (d) and spent (e) stages. PPD: Proximal pars distalis.

somatotrope and gonadotrope described in other teleostes[16, 23] were observed mainly in the PPD. Most of the cells in PI showed clear cytoplasm. A considerable increase in the number of basophils in the PPD was observed in the developing stage compared with the resting stage (Fig. 3a and b) whereas the number of basophils gradually reduced through the mature, spawning and spent stages. It was noticed that vacuoles appeared in the basophils of all stages. However, these vacuoles appeared more in the developing and mature stages than in the resting, spawning and spent stages. The neurohypophysis, pars nervosa (PN) had an intrusive structure into the adenohypophysis (Fig. 2a).

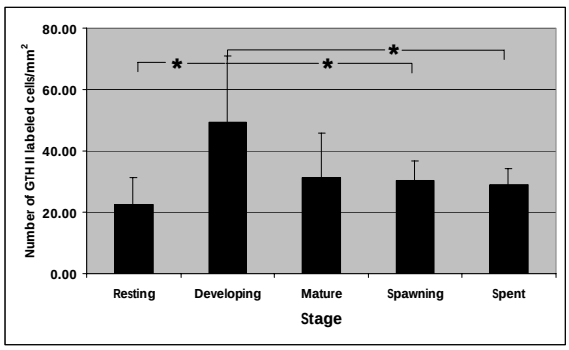

Fig 5. The number of anti-GTH IIβ labeling gonadotropes/mm² in the PPD of the male sand goby pituitary gland at different maturity stages of the testis. N=6, one way ANOVA and Least-Significant Difference (LSD) for *post hoc* analyses, *denotes significant difference between two groups.

Immunocytochemistry

Immunoreactivity of anti–GTH IIβ antibody was detected on most of the basophils. The optimal dilution of GTH IIβ antiserum was 1:8,000. Gonadotropes intensely reactive with anti-GTH IIβ antibody were found in the PPD of the pituitary gland in all stages (Fig. 4a-e). In the resting stage, the number of anti-GTH IIβ labeled gonadotropes was relatively lower than in other testicular stages (22.46 ± 4.34 cell/mm²). In the developing stage, the number of labeled gonadotropes significantly increased (49.21 ± 7.71 cell/mm²) ($P<0.05$), whereas in the mature stage (31.29± 7.23 cell/mm²), they decreased. The number of immunoreactive gonadotropes in the spawning (30.41 ± 2.56 cell/mm²) and spent (29.16 ± 2.21 cell/mm²) stages significantly reduced compared with the developing stage ($P<0.05$) (Fig. 5). Treatment with anti–GTH Iβ antibody revealed minimal staining in any stage of testicular development and at any dilution of anti–GTH Iβ antiserum (Fig. 6a-

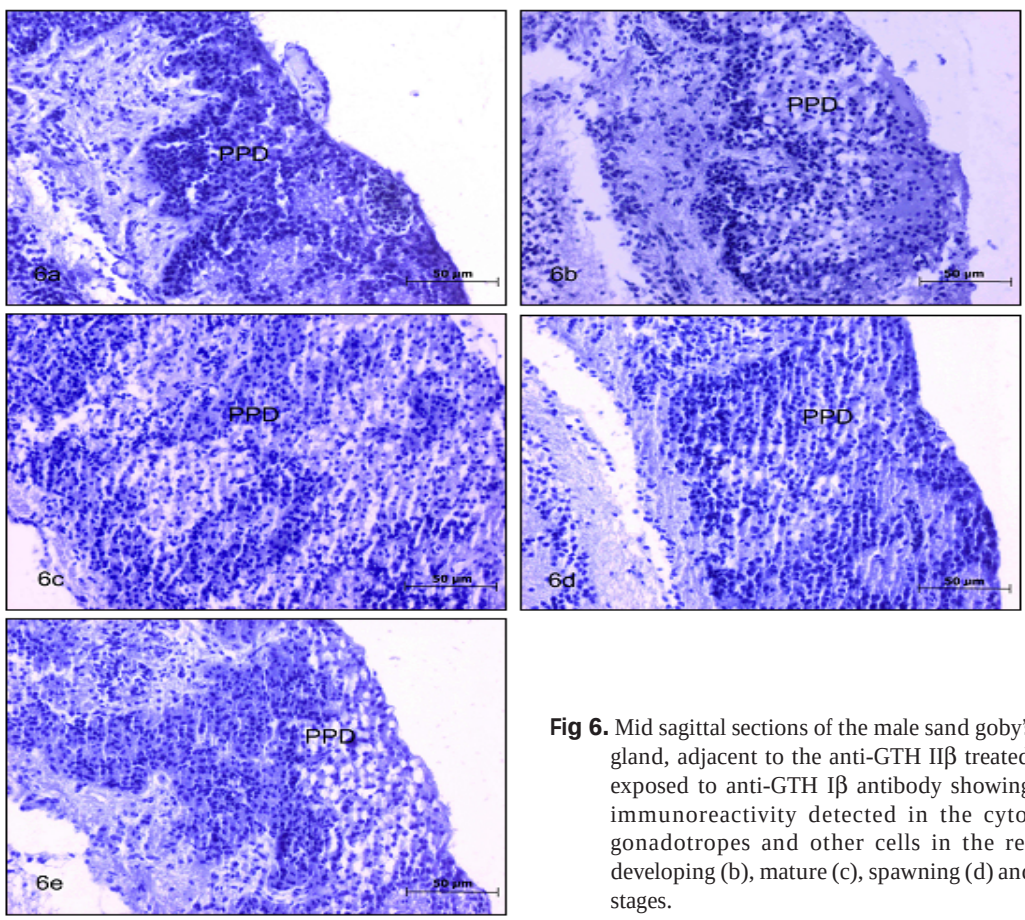

Fig 6. Mid sagittal sections of the male sand goby's pituitary gland, adjacent to the anti-GTH IIβ treated sections, exposed to anti-GTH Iβ antibody showing minimal immunoreactivity detected in the cytoplasm of gonadotropes and other cells in the resting (a), developing (b), mature (c), spawning (d) and spent (e) stages.

e). No immunoreactivity was found in the negative control sections (data not shown). Negative staining of anti-GTH Iβ was not a false negative result, as this antibody was still able to label gonadotropes in the rat pituitary gland (positive control sections) (Fig. 7 a and b).

Discussion

This study showed that the change of PPD at each testicular stage correlated with the testicular development in which a change of basophils in the PPD was clearly found (Fig. 3a and b). In a teleost (*Rhamdia hilarii Val.*) and indian freshwater major carp (*Cirrhinus mrigala*), basophilic cells, considered to be gonadotropes, discharge their contents and become increasingly vacuolated during the mature gonadal stage[24,25]. A similar characteristic was also found in the sand goby in which more vacuoles appeared in the developing and mature stages than in the resting, spawning and spent stages. After treating with anti-GTH IIβ antibody, intensely labeled gonadotropes were found and formed the major component in the PPD of the pituitary gland at all stages. Treating with anti-GTH Iβ antibody, on the other hand, showed no labeled gonadotropes at any stage of testicular development. Similar results have also been found in other teleosts such as European eel (*Anguilla anguilla)*[9], chinook salmon (*Oncorhynchus tschawytsha*)[10], tilapia, (*Orechromis mossambica)*[11], African catfish (*Larias gariepinus)*[13] as well as female sand goby (*Oxyeleotris marmoratus)*[14]. This indicates that only GTH IIβ but not GTH Iβ may be involved in testicular development in many teleost species including sand gobies. The changes observed in the number of anti-GTH IIβ labeled gonadotropes correlated with the work previously reported by Suwanjarat *et al.*[15] on testicular development in male sand goby (*Oxyeleotris marmoratus)*. In the resting stage, only early developing germ cells were found in the testis corresponding with the low number of the anti-GTH IIβ labeled gonadotropes found in pituitary gland, as reported herein (Fig. 5). In the developing stage, the number of anti-GTH IIβ labeled gonadotropes greatly increased and there was also a considerable increase in mature sperm. The number of GTH IIβ labeled gonadotropes reduced in the mature stage when the quantities of mature sperm were high. This may be due to the anti-GTH IIβ labeled gonadotropes exerting their activity on immature sperm, which were abundant in the developing stage, to become mature sperm. The number of anti-GTH IIβ labeled gonadotropes was relatively constant in the spawning and spent stage where a considerable reduction in the number of mature sperm was observed. Thus, these findings suggest that GTH II plays an important role in mediating sperm maturation. It has been shown that GHT II regulates spermatogenesis by activating receptors expressed by Leydig cells and the main biological activity of GTH II is to regulate Leydig-cell steroid production. The steroid is required for spermatogenesis[26]. While GTH II has been identified in some fish, e.g., chum salmon (*Oncorhynchus keta)*[1,2,3], coho salmon (*Oncorhynchus kisutch)*[4] and Japanese eel (*Anguilla japonica)*[5] and its role in regulating reproduction has been well known, a specific role for GTH I in fish spermatogenesis has not been established[27]. For further studies, in order to confirm the findings on gonadotropic cell types and their activity during reproductive cycle, the immunogold labeling method for electron microscopy should provide better qualitative data.

In summary, the present results showed that only a single type of gonadotrope was present in the male sand goby (*Oxyeleotris marmoratus*), namely the anti-GTH IIβ labeling gonadotrope. This gonadotrope increased in cell number that correlated with sperm maturity, suggesting that GTH II may regulate

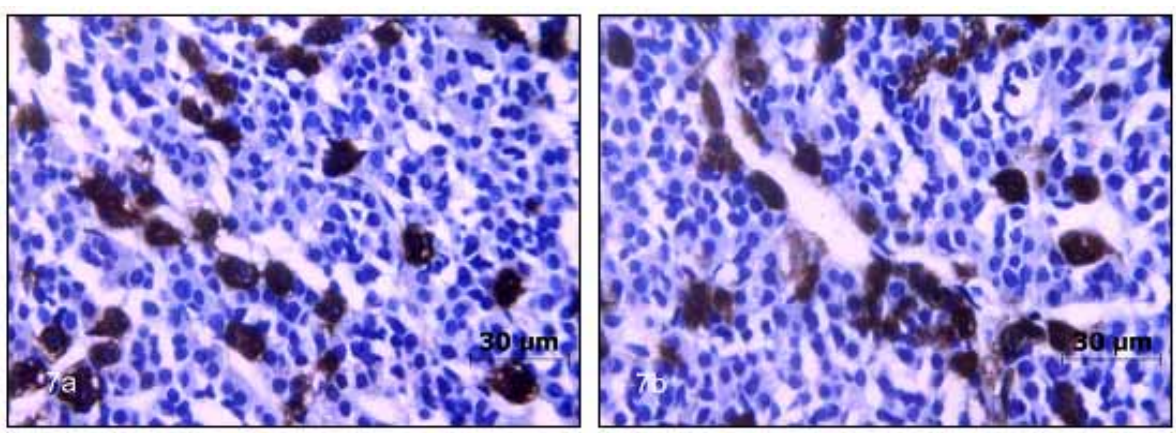

Fig 7. Sagittal sections of the Wistar rat's pituitary glands (a) stained with anti-GTH Iβ antibody and (b) stained with anti-GTH IIβ antibody showing immunoreactivity (brown color) in the cytoplasm of gonadotropes in the PPD region.

downstream hormonal functions required for spermatogenesis. Further investigation should be performed to gain more knowledge about GTH function, prior to the hormonal application for increasing breeding and production.

ACKNOWLEDGEMENTS

We thank Prof. H. Kawauchi (Kitasato University, Japan) for the donation of anti-chum salmon GTH Iβ and GTH IIβ antisera and Prof. Brian Hodgson editing the English language. This study was supported by the Department of Anatomy, Faculty of Science, Prince of Songkla University.

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