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

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ABSTRACT: Pituitary gonadotropes were studied in the adult male sand goby (\textit{Oxyleotris 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 I\textbeta and GTH II\textbeta antibodies. Most of the basophils in the proximal par distalis showed immunoreactivity to GTH II\textbeta. No cell showed immunoreactivity to GTH I\textbeta at any stage of testicular development. The number of GTH II\textbeta labeled cells was relatively low in the resting stage (22.46 ± 4.34 cell/mm\textsuperscript{2}) but significantly higher during the developing stage (49.21 ± 7.71 cell/mm\textsuperscript{2}) (\textit{P}<0.05) compared to the resting stage. The number of immunoreactive cells decreased during the mature stage (31.29 ± 7.23 cell/mm\textsuperscript{2}) and was relatively constant in the spawning stage (30.41 ± 2.56 cell/mm\textsuperscript{2}) and spent stage (29.16 ± 2.21 cell/mm\textsuperscript{2}). Based on the well known function of GTH, the presence of only GTH II\textbeta but not GTH I\textbeta in the pituitary gonadotropes suggested the involvement of GTH II\textbeta in spermatogenesis of the male sand goby. Moreover, the high number of GTH II\textbeta immunoreactive cells during testicular developing stage correlated with the maturation of sperm further confirmed the role of GTH II\textbeta in controlling cell proliferation during spermatogenesis.

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

INTRODUCTION

The sand goby (\textit{Oxyleotris 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 (\textit{Oncorhynchus keta})\textsuperscript{1,2,3}, coho salmon (\textit{Oncorhynchus kisutch})\textsuperscript{4} and Japanese eel (\textit{Anguilla japonica})\textsuperscript{5}. GTH I and GTH II are structurally homologous to the tetrapod follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively\textsuperscript{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\textsuperscript{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 (\textit{Anguilla anguilla})\textsuperscript{9}, chinook salmon (\textit{Oncorhynchus tschawytscha})\textsuperscript{10}, tilapia (\textit{Orechromis mossambica})\textsuperscript{11}, the African catfish (\textit{Larias guerkepinus})\textsuperscript{12,13}, and female sand goby (\textit{Oxyleotris marmoratus})\textsuperscript{14} in which only GTH II has been identified. In the males sand goby (\textit{Oxyleotris marmoratus}), the information on the gonadotropic cell types and their function in relation to reproductive cycle is still unavaiable. 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 (\textit{Oxyleotris 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 Province, Southern Thailand between March 2003 and March 2004. The pituitary glands were removed, fixed in 10% formalin overnight at 4°C. The sections were then rinsed with phosphate buffered saline (PBS: 0.14 M NaCl, 0.01 phosphate buffer) pH 7.4 (30 min), 3% H2O2 in methanol (30 min), 10% normal goat serum (Vector Laboratories, Burlingame, USA) in PBS (60 min), and finally with the anti-chum salmon GTH-I β or anti-chum salmon GTH-II β at dilutions of 1: 500, 1: 1000, 1: 2000, 1: 4000, 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, diaminobenzidine (DAB). A negative control was performed by omitting the primary antibodies. A positive control was performed under the same staining condition with Wistar rat pituitary gland sections. Pars distalis of rat’s pituitary gland is known to contain both FSH and LH gonadotropes18,20,21. 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 mm² in each group was calculated as follows: six pituitary glands from each testicular stage (total 30 glands) were randomly selected. Ten sections of each gland were systematically selected22. 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 mm².

**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/ mm² 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 pars intermedia (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. 2a and b). 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 teleosts 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).

**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-
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. 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), chinook salmon (Oncorhynchus keta), coho salmon (Oncorhynchus kisutch) and Japanese eel (Anguilla japonica) as well as female sand goby (Oxyeleotris marmoratus). 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. 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, 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 Iβ 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. This steroid is required for spermatogenesis. While GTH II has been identified in some fish, e.g., chum salmon (Oncorhynchus keta), coho salmon (Oncorhynchus kisutch) and Japanese eel (Anguilla japonica) and its role in regulating reproduction has been well known, a specific role for GTH I in fish spermatogenesis has not been established. 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...
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.

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