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 the subsequent maturity stage was performed followed by classification of the pituitary glands, according to the maturity stages of the ovary. The classification of the maturity stages of the ovary was a modification of the method used by Mayer *et al.* (1988) and the gonadosomatic index (GSI) was calculated according to the formula (Elorduy-Garaya and Ramirez-Luna, 1994):

$$\text{Gonadosomatic index (GSI)} = \frac{\text{ovarian weight}}{\text{body weight}} \times 100$$

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. The 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 I β (FSH) or anti-chum salmon GTH II β (LH) 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 I β (FSH) and GTH II β (LH) were obtained from Dr H. Kawaushi (School of Fisheries Science, Kitasato 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 counterstained 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 cell per mm^2

DATA ANALYSIS

Data are reported as mean $\pm$ S.D. Statistical analysis was performed by one way ANOVA and Least-Significant Different (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 $P<0.05$.