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Changes in brain seabream GnRH mRNA and pituitary seabream GnRH peptide levels during ovarian maturation in female barfin flounder

Masafumi Amano [a,*], Ky Xuan Pham [a], Noriko Amiya [a], Takeshi Yamanome [b], Kunio Yamamori [a]

[a] School of Marine Biosciences, Kitasato University, Sanriku, Ofunato, Iwate 022-0101, Japan
[b] Iwate Fisheries Technology Center, Kamaishi, Iwate 026-0001, Japan

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ABSTRACT

The pleuronectid barfin flounder *Verasper moseri* expresses three forms of gonadotropin-releasing hormones (GnRHs), i.e., seabream GnRH (sbGnRH), salmon GnRH, and chicken GnRH-II. Among these, sbGnRH is the dominant form in the pituitary, indicating that sbGnRH regulates gonadal maturation. In order to clarify the physiological roles of sbGnRH during ovarian maturation in reared female barfin flounder, the changes in brain sbGnRH mRNA levels and pituitary sbGnRH peptide levels were examined by real-time quantitative PCR and time-resolved fluoroimmunoassay, respectively. The fish hatched in April 2002. The gonadosomatic index remained low until August 2004 and increased thereafter until April 2005 when the fish began to ovulate. The sbGnRH mRNA levels per brain increased significantly from April 2004 to April 2005. Pituitary sbGnRH peptide levels also increased significantly during this period. These results indicate that sbGnRH is involved in ovarian maturation and ovulation in the barfin flounder.

1. Introduction

Gonadal maturation in teleost fish is primarily regulated by the brain-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) stimulates the synthesis and release of pituitary gonadotropins (GTHs; follicle stimulating hormone [FSH] and luteinizing hormone [LH]), and GTHs stimulate the secretion of steroid hormones from the gonads (King and Millar, 1992; Sherwood et al., 1993). To date, 14 forms of GnRHs have been identified based on their primary structure or complementary DNAs (cDNAs) in vertebrates (see Kah et al., 2007). Studies on the involvement of GnRH in various reproductive processes have been complicated by the discovery of multiple GnRH forms in the brain of a single species (Holland et al., 2001).

The barfin flounder *Verasper moseri* is a large, multiple-spawning flatfish inhabiting the cold sea basins around eastern Hokkaido, Japan. This is a promising species for aquaculture and resource enhancement in northern Japan due to its high commercial value. Barfin flounder first matures in early spring at the age of 2 years (male) and 3 years (female). This species has three forms of GnRHs, i.e., salmon GnRH (sGnRH), chicken GnRH-II (cGnRH-II), and seabream GnRH (sbGnRH) (Amano et al., 2002a). We have demonstrated previously that only sbGnRH is involved in gonadal maturation through its action of stimulating GTH secretion; sbGnRH-immunoreactive (ir) cell bodies that are located in the preoptic area were observed to send fibers into the pituitary, and the pituitary levels of sbGnRH were considerably higher than those of sGnRH and cGnRH-II (Amano et al., 2002b). In addition, the brain sbGnRH mRNA levels and the pituitary sbGnRH peptide levels increased in accordance with testicular maturation in male barfin flounder (Amano et al., 2004).

It is necessary to measure both GnRH mRNA levels in the brain and GnRH peptide levels in the pituitary gland in order to clarify the physiological roles of GnRH in teleost reproduction. However, such studies have not been reported in female teleost fish. In the present study, in order to clarify the physiological roles of sbGnRH in ovarian maturation in female barfin flounder, changes in the brain sbGnRH mRNA levels and the pituitary sbGnRH peptide levels were examined by real-time polymerase chain reaction (PCR) and time-resolved fluoroimmunoassay (TR-FIA), respectively. We also examined the changes in plasma levels of testosterone (T), estradiol-17β (E_2), and 17α,20β-dihydroxy-4-pregnen-3-one (DHP).

2. Materials and methods

2.1. Fish

Barfin flounder that hatched in April 2002 at the Iwate Fisheries Technology Center (Iwate Prefecture, Japan) were used for the

* Corresponding author. Fax: +81 192 44 1904.
E-mail address: amanoma@kitasato-u.ac.jp (M. Amano).

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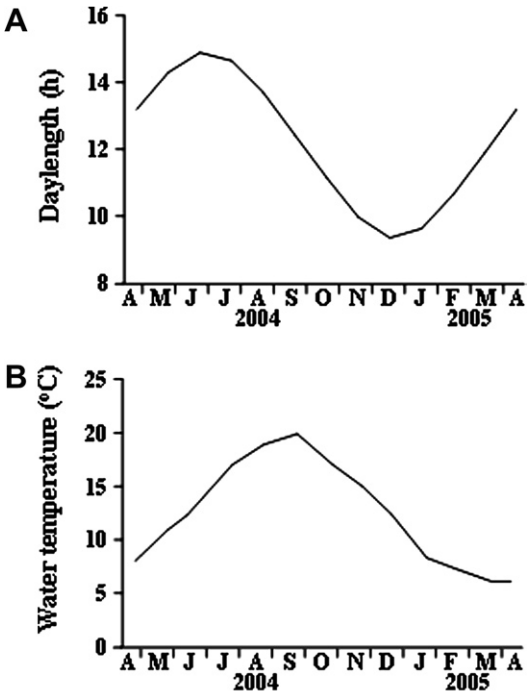

Fig. 1. Changes in (A) photoperiod (hr) and (B) water temperature (°C) during the experiment.

study. The fish were reared under natural photoperiod conditions in seawater at natural temperature (Fig. 1A and B).

2.2. Sampling

The fish were sampled in April, June, August, October, and December 2004, and in February and April 2005. They were anesthetized by the administration of 2-phenoxyethanol (0.05%), and their total length (TL) and body weight (BW) was measured. Blood from the caudal vessels was collected to measure the plasma levels of T, E_2, and DHP levels. Blood samples were centrifuged at 2500*g* for 15 min and plasma was stored at −35 °C until analysis. The brain and the pituitary gland were sampled. To measure the sbGnRH mRNA levels, the brain without the cerebellum and the medulla oblongata was quickly dissected out and frozen in liquid nitrogen. To measure the sbGnRH, sGnRH and cGnRH-II levels, the pituitary gland was also dissected and frozen in liquid nitrogen. The gonads were dissected, and their weights were measured to calculate the gonadosomatic index (GSI=[gonadal weight/BW] × 100). Subsequently, the gonads were embedded in Paraplast Plus (Monoject, Sherwood Medical, St. Louis, MO, USA) and 5 μm sections were obtained. The sections were stained with hematoxylin and eosin for histological observation.

2.3. Real-time PCR for the measurement of GnRH mRNA

We performed real-time quantitative PCR using the method of Amano et al. (2004) with a slight modification in order to measure the brain sbGnRH mRNA levels. The final output was expressed as the copy number of the target mRNA per brain.

2.4. Measurements of plasma steroid hormone and pituitary sbGnRH peptide levels

Plasma T, E_2, and DHP were extracted and their levels were measured by TR-FIA (Yamada et al., 1997, 2002). The pituitary sbGnRH, sGnRH and cGnRH-II peptide levels were also measured by TR-FIA (Amano et al., 2004).

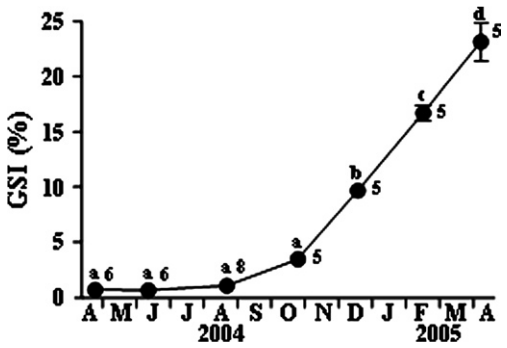

Fig. 2. Changes in GSI (%). Each value is expressed as the mean and standard error (bar). The numbers beside each symbol indicate the number of fish employed. Means with different letters differ significantly ($P<0.05$).

2.5. Statistics

Changes in the GSI, plasma levels of T, E_2 and DHP, brain sbGnRH mRNA levels, and pituitary sbGnRH, sGnRH and cGnRH-II peptide levels were analyzed by the one-way analysis of variance followed by Bonferroni's multiple comparison test.

3. Results

3.1. GSI and ovarian development

Changes in GSI are shown in Fig. 2. At the beginning of the experiment in April 2004 (mean TL: 37.8 cm, mean BW: 866 g) the GSI was low (0.68%). The GSI remained low until August 2004 ($\leqslant$1.0%) and increased thereafter until April 2005 when the fish began to ovulate (mean TL: 48.2 cm, mean BW: 1837*g*). Oocytes at the perinucleolus stage were observed in all the fish in April and June 2004 (Fig. 3A). Ovaries at the previtellogenic stage (the most advanced oocytes had developed into cortical alveoli) were observed in all the fish in August 2004 (Fig. 3B). Ovaries at the early yolk globule stage (the most advanced oocytes were in the primary yolk stage) were observed in October 2004 (Fig. 3C), and those at the late yolk globule stage (the most advanced oocytes were in the tertiary yolk stage without any signs of spawning) were observed in December 2004 (Fig. 3D). The early spawning stage (oocytes were in the migratory nucleus stage or germinal vesicle breakdown) was observed in February and April 2005 (Fig. 3E).

3.2. Plasma steroid hormone levels

The plasma T levels began to increase from August 2004, remained high until February 2005, and decreased in April 2005 (Fig. 4A). The plasma E_2 levels also began to increase from October 2004, remained high until February 2005, and decreased in April 2005 (Fig. 4B). The plasma DHP levels remained low (<0.2 ng/ml) during the experimental period and did not change significantly (Fig. 4C).

3.3. The brain GnRH mRNA levels and pituitary GnRH peptide levels

The brain sbGnRH mRNA levels (copies/brain) tended to decrease from April to August 2004, but subsequently the levels increased significantly until April 2005 when ovulation was observed (Fig. 5). The pituitary sbGnRH peptide levels also tended to decrease from April to August 2004, and it significantly increased from August 2004 to April 2005 (Fig. 6). Pituitary sGnRH peptide and cGnRH-II peptide levels were considerably lower than the sbGnRH peptide levels and there were no significant changes (data not shown).