Molecular Cloning and Differential Expression of Three GnRH Genes during Ovarian Maturation of Spotted Halibut, *Verasper variegatus*

YONG‐JIANG XU1,2, XUE‐ZHOU LIU2, MEI‐JIE LIAO2, HAN‐PING WANG3, AND QING‐YIN WANG2*

1College of Marine Life Sciences, Ocean University of China, Qingdao, Shandong, China
2Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, Shandong, China
3Aquaculture Genetics and Breeding Laboratory, The Ohio State University South Centers, Piketon, Ohio

ABSTRACT

In this study, the gonadotropin‐releasing hormone (GnRH) genes in spotted halibut were cloned and sequenced by isolating their cDNAs. The species expressed three molecular forms of GnRH in the brain: chicken‐type GnRH‐II (cGnRH‐II), seabream‐type GnRH (sbGnRH), and salmon‐type GnRH (sGnRH). Phylogenetic analysis divided the molecular forms of GnRHs into three branches: cGnRH‐II branch, sGnRH branch, and fish‐specific GnRH branch. The spatial expression showed that they had the highest expression levels in the brain. cGnRH‐II was exclusively detected in the brain, while sbGnRH had a global expression pattern in all examined organs. sGnRH was detected in the brain, pituitary, and ovary. The temporal changes of brain GnRH mRNA expression levels were examined during ovarian maturation and postspawning, and the serum steroid hormones and gonadosomatic index (GSI) were recorded. Amounts of sbGnRH mRNA substantially elevated \( (P < 0.05) \) during ovarian maturation, which concomitant with considerable elevation of GSI and serum steroids levels. On the contrary, neither sGnRH nor cGnRH‐II mRNA levels showed significant changes during ovarian maturation in this study. These results suggested that these three GnRH genes are the important regulators for the differential expression of GnRH in spotted halibut, and would help us better understand the reproductive endocrine mechanism of spotted halibut. *J. Exp. Zool.* 317A:434–446, 2012. © 2012 Wiley Periodicals, Inc.


The gonadotropin‐releasing hormone (GnRH) is a key endocrine hormone along the brain‐pituitary‐gonad (BPG) axis that controls reproduction in fish. It plays a very important role in the development and maintenance of reproductive function and is also involved in the regulation of sexual behavior possibly by modulating neuronal activity (Oka, 2009). To date, a total of 14 distinct GnRH peptides have been isolated in vertebrates. These different forms of GnRHs are grouped into a family because they are all decapetides with a well‐conserved structure during evolution (Somoza et al., 2002b). It is now well established that...
two to three GnRH forms are present in the brain of some teleost species (Anderson et al., 2001; Adams et al., 2002). A species-specific variant (e.g., sbGnRH, seabream-type GnRH) is mainly located in the preoptic area and plays a major role in regulation of reproduction (Servili et al., 2010). A highly preserved variant (cGnRH-II, chicken-type cGnRH-II) in the midbrain may act as a neurotransmitter and/or neuromodulator, and a recent study showed it also had a function of stimulating melatonin secretion (Servili et al., 2010). The third one, sGnRH (salmon-type GnRH), has mainly been identified in the terminal nerve ganglion and olfactory bulbs (Somoza et al., 2002a), and indicated to have neuromodulatory function (Oka, 2009). In teleost, the evaluation of the role of GnRH in relation to reproduction is complicated by the presence of more than one molecular form of the peptide in the brain. However, the detailed physiological function of each GnRH form is still not fully understood in fish species.

In teleosts, when environmental factors required for matura‐

vetization and ovulation are available, the brain GnRH could act on the pituitary and cause synthesis and release of glandotropins [Follicle stimulating hormone [FSH] and Luteiniz‐

ing hormone [LH]] from pituitary into the blood stream. In turn, the glandotropins stimulate the gonad to synthesize sex steroids (estradiol-17β [E2] and testosterone [T], etc.), which regulate gametogenesis, gonadal growth, and reproductive function (Dubois et al., 2002; Zohar et al., 2010). The sex steroids exhibit a feedback effect on GnRH and Gonadotropin hormone [GtH] secretion, which has been demonstrated in vertebrates (Kitahashi et al., 2005; Hu et al., 2008). Recent studies revealed that the kisspeptin system also acts as a regulator of GnRH and gonadotropin expression, and might be part of the sex steroids feedback pathway in teleosts (Filby et al., 2008; Grone et al., 2010; Shi et al., 2010; Servili et al., 2011). However, the detailed regulatory mechanism controlling the expression of GnRH still remains unclear, especially in flatfish. Thus, the investigation on relationships between plasma steroid level, gonadal development, and GnRH expression level would provide a direct explanation of the divergent physiological roles of the GnRH system in teleosts expressing three GnRH variants. The cloning, secretory neurons distribution, and differential expression of GnRH during the gonadal maturation cycle have been studied in many teleosts by using Rapid-amplification of cDNA ends (RACE), Radiomunno‐

assay (RIA), Enzyme-Linked Immunosorbent Assay, in-situ hybridization, and immunohistochemical methods (Senthilkumar‐
maran et al., ’99; Collins et al., 2001; Shahjahan et al., 2010). However, the information on the differential physiological role of GnRH in flatfish is limited (Amano et al., 2002, 2004). Spotted halibut, *Verasper variegatus*, has been widely recog‐

ized as a promising candidate for aquaculture and fisheries enhancement in Asia due to its high market value. However, spontaneous maturation and ovulation of broodfish have not occurred in captivity. A potential explanation for the inhibition of final gonadal maturation in spotted halibut may be the depletion or impairment of the dominant GnRH form under artificial conditions. This would cause inhibited secretion of gonadotropins from the pituitary and consequently the gonadal steroidogenesis and final maturation processes are arrested. This has been demonstrated in cartilaginous fish (Denski et al., ’97) and teleost fish (Holland et al., 2001). Evidently, GnRH is the main regulator controlling the reproductive cycle in teleost (Zohar et al., 2010). However, there are no data available to elucidate the distribution of GnRH and other related neuroendocrine factors controlling the reproductive process of this species to date.

In the present study, we cloned the GnRH genes from the brain of a large flatfish species, spotted halibut, and examined their spatial and temporal expression pattern. The possible physiological roles of each GnRH form during gonadal maturation were also identified by elucidating the relationships between GnRH expression levels, ovarian maturation, and plasma sex steroid levels. This information is essential for us to understand the reproductive mechanisms and develop aquaculture in this commercially important species.

**MATERIALS AND METHODS**

**Fish Culture and Maturation Regulation**

Experimental fish were obtained from Qingdao Zhonghai Fishery Co., Ltd in March 2009 (Qingdao, China). They were all above 3 years of age with body lengths of 22–31 cm and body weights (BW) of 1,300–2,200 g. A total of 60 fish (♂:♀ = 1:1) were reared in round concrete tanks (25 m³ in volume) supplied with flow‐through seawater (water exchange rate: 400% per day; salinity: 30–33 ppt; pH: 7.7–8.1). The seawater was sand filtered and decanted prior to tank supply. Fish were fed to satiation twice a day with a commercial available dry diet (Shengsuo, Shengsuo Aquafeed Co., Ltd, Yantai, China) at a rate of 2–3% of BW.

All the fish exposed to natural photothermal regimes until August 1, 2009, artificial photothermal regime was applied to the experimental fish with water temperature changed from 18°C to 9°C and photoperiod changed from 10.5D:13.5L to 8D:16L. The experimental fish experienced the ovarian maturation process after exposure to photothermal conditioning treatment. The artificial photothermal treatment ended postspawning on April 2010.

**Samples for GnRH Cloning and Expression**

Three female spotted halibut were sampled in August 2009 for GnRH cloning. Brains excluding the pituitary were collected and flash frozen in liquid nitrogen, and then stored at −80°C until use. The temporal changes in brain GnRH mRNA levels during ovary maturation were investigated using quantitative real-time PCR. For this purpose, three females were sampled at each of the following points, respectively: June 2009 for perinucleolus stage (Temperature 15–16°C; Photoperiod:10:5D:13:5L), August 2009 for cortical alveoli stage (Temperature 17–18°C; Photoperiod:11D:13L), October 2009 for vitellogenic stage (Temperature...
Circulating levels of plasma testosterone (T) and estradiol plasma were obtained by centrifugation (3000–4000 g, 4°C, 2222 (60 mg/L). Ovary and viscera weights of the sampling nearest 0.1 mm and 0.01 g after anesthetization with MS 222 (1.5 mL) were collected from the caudal vessels of Steroid Radioimmunoassay (260 mg/L). Ovary and viscera weights of the sampling environment. A small portion of the ovary from each fish (n = 3) was fixed in Bouin’s fixative, washed in 50% ethanol, and stored in 70% ethanol until histological processing using conventional histological procedures. The gonadosomatic index (GSI) was calculated as: GSI = [gonad weight/(body weight – viscera weight)] × 100 (Chen et al., 2010).

Evaluation of Gonadal Development

The total length (TL) and BW of each fish were measured to the nearest 0.1 mm and 0.01 g after anesthetization with MS-222 (260 mg/L). Ovary and viscera weights of the sampling were recorded. Ovarian development stages were determined by biopsy and histological observation. The plasma were obtained by centrifugation (3000 × g, 10 min, 4°C), and stored at −40°C until steroid extraction and analysis. Circulating levels of plasma testosterone (T) and estradiol-17β (E2) were measured by RIA according to the methods optimized by Chen et al. (2010) and He et al. (2008). The lower limit of the detection level for all assays was set at 1.8 pg/mL for E2 and 22.0 pg/mL for T, respectively. The intra- and interassay coefficients of variation were 1.9 and 7.4% for E2, 2.1 and 7.7% for T, respectively.

Steroid Radioimmunoassay

Blood samples (1.5 mL) were collected from the caudal vessels of five experimental fish at each developmental stage according to the histological observation using cold heparinized syringes. The plasma were obtained by centrifugation (3000 × g, 10 min, 4°C), and stored at −40°C until steroid extraction and analysis. Circulating levels of plasma testosterone (T) and estradiol-17β (E2) were measured by RIA according to the methods optimized by Chen et al. (2010) and He et al. (2008). The lower limit of the detection level for all assays was set at 1.8 pg/mL for E2 and 22.0 pg/mL for T, respectively. The intra- and interassay coefficients of variation were 1.9 and 7.4% for E2, 2.1 and 7.7% for T, respectively.

RNA Extraction and cDNA Synthesis

Total RNA was extracted according to the protocol of Trizol reagent (Invitrogen, New York, USA). Total RNA was treated with RNase-Free DNase I (Watsonbiet, Shanghai) to remove contaminated genomic DNA. cDNA synthesis was performed with SMARTTM RACE cDNA synthesis Kit (Clontech, Mountain view, CA, USA) according to the manufacturer’s instructions. The first strand cDNA was synthesized using a fixed amount of treated total RNA (1 μg) with oligo (dT) primer and Moloney murine leukemia virus reverse transcriptase. Thereafter, the second strand was synthesized.

Rapid Amplification of cDNA ends (5′- and 3′-RACE)

The primers for the PCR amplification were synthesized based on highly conserved cDNA sequences of flatfish GnRHs (Table 1). The middle fragments of GnRH cDNAs were amplified from 1 μL of 3′-RACE-Ready cDNA solution using TakaRa Ex TaqTM (Takara Biotechnology, Dalian, China). The amplification conditions were: 94°C for 5 min, 30 cycles of 94°C for 40 sec, 55°C for 40 sec, and 72°C for 90 sec, followed by extension of 72°C for 5 min. The product was rapidly cooled to 4°C and separated on 1% agarose gel. The target PCR bands were recovered from the gel and then purified with a DNA purification kit (Sangon, Shanghai, China). The purified PCR products were cloned into pEASY-T1 vector (Transgene Biotechnology, Beijing, China) separately and then transformed into competent Escherichia coli JM109. After overnight culture, the positive clones were picked out, PCR detected, and sequenced.

Nested PCR were conducted, primers and templates for the first and nested PCR are listed in Table 1. The 5′- and 3′-cDNA ends were amplified according to manufacturer’s instructions for SMARTTM RACE cDNA Amplification Kit (Clontech, Mountain view, CA, USA). The gene-specific primers were designed based on the middle fragments and listed in Table 1. The lower limit of the detection level for all assays was set at 1.8 pg/mL for E2 and 22.0 pg/mL for T, respectively. The intra- and interassay coefficients of variation were 1.9 and 7.4% for E2, 2.1 and 7.7% for T, respectively.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed with realplex real-time PCR system (Eppendorf, Germany). The reaction mixture in a total volume of 25 μL contained 12.5 μL 2×SYBR® Premix Ex TaqTM II, 1 μL each of specific sense and antisense primers (1 pmol/L), 1 μL of the 1:100 diluted cDNA template, and 9.5 μL of ddH2O. Beta-actin was used as the internal standard. The primers used for quantitative real-time PCR amplification were designed with Beacon Designer 7.0 software (Premier BioSoft, Palo Alto, CA, USA) (Table 1). The real-time PCR amplification conditions were 94°C for 30 sec, 40 cycles of 94°C for 5 sec, 60°C for 30 sec. Following PCR amplification, products were verified by direct sequencing. The specific quantities were normalized against the amount of beta-actin amplified. The quantitative expression level of GnRH mRNA was calculated and analyzed according to protocol described by Schmittgen and Livak (2008).

Sequence Analysis and Statistics

The cDNA sequences analysis, amino acid sequences deduction, molecular weights, and isoelectric point calculation for three GnRHs were performed using DNAStar software 5.0.1. The Signal sequences of three GnRHs were determined by SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/). In addition, amino acid sequences alignments, phylogenetic and evolutionary analysis was carried out with Clustalx 2.0.12.
RESULTS

cDNA Sequences for GnRHs

Three cDNA sequences encoding cGnRH-II, sGnRH, and sbGnRH were isolated from spotted halibut. All the cDNA sequences consisted of a 5’ UTR, signal peptide, GnRH decapeptide, GnRH Associated Peptide, and a 3’ UTR. The cGnRH-II consists of 85

Table 1. Primers used for PCR amplification of spotted halibut GnRH cDNAs.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPM-long</td>
<td>5’-CTAATACGACTCACTATAGGGCAAG CAGTGTTACAAGCAGAGT-3’</td>
<td>5’-RACE PCR</td>
</tr>
<tr>
<td>UPM-short</td>
<td>CTAATACGACTCACTATAGGGC</td>
<td>5’-RACE PCR</td>
</tr>
<tr>
<td>NUP</td>
<td>AAACAGTGTTATCAACGACAGGT</td>
<td>3’-RACE PCR</td>
</tr>
<tr>
<td>cf1</td>
<td>CACGTGCCACGTGGAAGAC</td>
<td>Middle fragment and 3’-RACE PCR for cGnRH-II</td>
</tr>
<tr>
<td>cr1</td>
<td>GTAGCACTAGTGGCTGCCCAA</td>
<td>Middle fragment and 5’-RACE PCR for cGnRH-II</td>
</tr>
<tr>
<td>cr2</td>
<td>AGCTCTGCGCAACAAGCATC</td>
<td>Middle fragment PCR for cGnRH-II</td>
</tr>
<tr>
<td>sbF</td>
<td>AAGACCCACGATCATGGAGAC</td>
<td>Middle fragment and 3’-RACE PCR for sbGnRH</td>
</tr>
<tr>
<td>sbR</td>
<td>CTGTAATTCGGCAAAAAGG</td>
<td>Middle fragment and 5’-RACE PCR for sbGnRH</td>
</tr>
<tr>
<td>sF</td>
<td>TACTCCCTGTCAGCTCTG</td>
<td>Middle fragment PCR for sGnRH</td>
</tr>
<tr>
<td>sR</td>
<td>GCCTGAGAGAGAGACACCA</td>
<td>Middle fragment PCR for sGnRH</td>
</tr>
<tr>
<td>cS1</td>
<td>GTTCTGCTGCTGGGCTGCTGC</td>
<td>3’-RACE nested PCR for cGnRH-II</td>
</tr>
<tr>
<td>cA1</td>
<td>CTGAAATCTCTGACGTGCCAAA</td>
<td>5’-RACE nested PCR for cGnRH-II</td>
</tr>
<tr>
<td>sbS1</td>
<td>CTGTCACTGTGGCTGCCCTCTG</td>
<td>3’-RACE nested PCR for sbGnRH</td>
</tr>
<tr>
<td>sbA1</td>
<td>GAGCGCCTGCACTGCGAGGGTTT</td>
<td>5’-RACE nested PCR for sbGnRH</td>
</tr>
<tr>
<td>sS1</td>
<td>CTGGAAGCCACGCAAGCAGACTG</td>
<td>3’-RACE nested PCR for sGnRH</td>
</tr>
<tr>
<td>sS2</td>
<td>GAGGGTGAGGGCCACCGAGGAGGTGTG</td>
<td>3’-RACE nested PCR for sGnRH</td>
</tr>
<tr>
<td>SA1</td>
<td>AGACACCACTCCGCTGTGCGCTC</td>
<td>5’-RACE nested PCR for sbGnRH</td>
</tr>
<tr>
<td>cf2</td>
<td>TGACTTGAGAGAGCAAGAG</td>
<td>Real-time PCR for cGnRH-II</td>
</tr>
<tr>
<td>cr2</td>
<td>GCACAGGACCAGCAGAGGTG</td>
<td>Real-time PCR for cGnRH-II</td>
</tr>
<tr>
<td>sbF2</td>
<td>AAGGACTGAGACCCAACATTCC</td>
<td>Real-time PCR for sbGnRH</td>
</tr>
<tr>
<td>sbR2</td>
<td>CACGGAGGAGACCCAACAC</td>
<td>Real-time PCR for sbGnRH</td>
</tr>
<tr>
<td>sF2</td>
<td>CAGGTGTGTTGTGAT</td>
<td>Real-time PCR for sGnRH</td>
</tr>
<tr>
<td>sR2</td>
<td>AGCTCTGCTGTGGATGT</td>
<td>Real-time PCR for sGnRH</td>
</tr>
<tr>
<td>β-actin F</td>
<td>TGTGGAGATGATG</td>
<td>Real-time PCR for β-actin</td>
</tr>
<tr>
<td>β-actin R</td>
<td>GTGTCAGGAGAGTAG</td>
<td>Real-time PCR for β-actin</td>
</tr>
</tbody>
</table>

Table 2. Primers and templates used for PCR to amplify 5’- and 3’-cDNA ends.

<table>
<thead>
<tr>
<th>Target product</th>
<th>Template</th>
<th>The first PCR primers</th>
<th>The nested PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGnRH-II</td>
<td>5’ partial cDNA</td>
<td>5’-RACE-Ready cDNA</td>
<td>cr1 and UPM</td>
</tr>
<tr>
<td></td>
<td>3’ partial cDNA</td>
<td>3’-RACE-Ready cDNA</td>
<td>cf1 and UPM</td>
</tr>
<tr>
<td>sbGnRH</td>
<td>5’ partial cDNA</td>
<td>5’-RACE-Ready cDNA</td>
<td>sbF and UPM</td>
</tr>
<tr>
<td></td>
<td>3’ partial cDNA</td>
<td>3’-RACE-Ready cDNA</td>
<td>sbR and UPM</td>
</tr>
<tr>
<td>sGnRH</td>
<td>5’ partial cDNA</td>
<td>5’-RACE-Ready cDNA</td>
<td>sA1 and UPM</td>
</tr>
<tr>
<td></td>
<td>3’ partial cDNA</td>
<td>3’-RACE-Ready cDNA</td>
<td>sS1 and UPM</td>
</tr>
</tbody>
</table>
amino acid residues and the cGnRH-II cDNA (GenBank accession no. HM131601) is 568 bp in length, excluding a poly (A) tail, and consists of a 141-bp 5'UTR, a 255-bp open reading frame (ORF), a stop codon (TGA), and a 169-bp 3'UTR. The predicted molecular weight was 9.99 kD and the isoelectric point was 8.53. The sGnRH (GenBank accession number HM131602) consists of 90 amino acid residues and is 457 bp in length. It consists of a 41-bp 5'UTR, excluding a poly (A) tail, a 270-bp ORF, a stop codon (TGA), and a 143-bp 3'UTR. The predicted molecular weight was 10 kD and isoelectric point was 10.18. The sbGnRH (GenBank accession no. HM131600) precursor consists of 98 amino acid residues and the cDNA is 381 bp in length, and consists of a 48-bp 5'UTR, a 294-bp ORF, a stop codon (TGA), and a 36-bp 3'UTR with the predicted molecular weight of 10.84 kD and isoelectric point at 9.31.

Comparison of GnRHs Amino Acid Sequence of Spotted Halibut with Other Species

The deduced amino acid sequence of the cGnRH-II precursor was compared with its homologs of mammals, birds, amphibians, reptiles, and other fish species. The precursor of cGnRH-II had 56.5–100% identity to its fish counterparts. Similarity dropped to 25.9–61.2% when compared with cGnRH-II precursors of mammals, birds, amphibians, and reptiles (Fig. 1). The sGnRH precursor of spotted halibut showed the highest identity with barfin flounder sGnRH (97.8%), and high homologies (78.9–85.2%) when compared with sGnRH precursors of Perciformes (Fig. 2). The sbGnRH precursor shared the highest amino acid identity with its homologs from Pleuronectiformes (90.8–93.8%). The similarity dropped to 55.2–77.9% when compared with sbGnRH precursors of Perciformes while relatively low percentage identities were found when compared with its homologs of hrGnRH (33.7%), pjiGnRH (68.1%), mdGnRH (51.6%), mGnRH (44%), and wfGnRH (46.2%) (Fig. 3).

Phylogenetic Analysis

Multiple molecular forms of GnRH were divided into three major branches by phylogenetic analysis supported by high bootstrap values: cGnRH-II branch, sGnRH branch, and the fish-specific GnRH branch (Fig. 4). The cGnRH-II branch was further divided into two additional sub-branches, where all fish cGnRH-II form one branch and tetrapods form another general branch. The sGnRH identified in this study clustered into one clade with Pleuronectiformes and Perciformes. The spotted halibut sbGnRH clustered into one additional clade with its Pleuronectiform counterparts and swamp eel. In addition, the sbGnRH clustered into one branch with its homologs of mdGnRH, sbGnRH, mGnRH, cfGnRH-I, wfgnRH, and pjGnRH.
Figure 2. Comparison of sGnRH precursors between spotted halibut and other fish species. For details, see legend of Fig. 1. Accession number of the GnRH sequences from fish species in Genbank: Spotted halibut (HM131602); Barfin flounder (Q8UW82); Japanese flounder (ABD97879); Atlantic salmon (AAR20401; AAR20404); Rainbow trout (AAF91281, AAF91280); Gilthead seabream (AAD02425); European sea bass (Q8IA09); African cichlid fish (AAC27718); Nile tilapia (AAV74403); Zebrafish (CAC18539); Goldfish (BAF98577); Swamp eel (AAW51120); Japanese medaka (BAD02405); Lake whitefish (AAP57220); Pejerrey (AAU94308).

Figure 3. Comparison of sbGnRH precursors between spotted halibut and other fish species. For details, see the legend to Fig. 1. GenBank accession numbers of the GnRH sequences from fish species used in this comparison: Spotted halibut (HM131600); Barfin flounder (BAB83984); Brazilian flounder (ACS88343); Japanese flounder (AAY83273); Gilthead seabream (AAD02427); European sea bass (AAF62898); Cobia (AAT80334); Nile tilapia (BAC56849); Cichlid fish (AAC59691); American shad (AAN04492); African catfish (CAAS4571); Pejerrey (AAU94309); Japanese medaka (BAB168303); Japanese eel (BAAS2608); Lake whitefish (AAP57221).
Spatial Distribution of Tissue and Sex-Specific Expression of GnRH Genes

sbGnRH, cGnRH-II, and sGnRH mRNA were all at the highest expression levels in the brain. cGnRH-II was exclusively detected in the brain while sbGnRH had a global expression pattern in all organs (Fig. 5). sGnRH was detected in the brain, pituitary, and ovary (Fig. 6). The dominant form in the brain was sbGnRH, with mRNA level being 30- to 2500-fold higher than cGnRH-II and sGnRH mRNA level, respectively.

Relationship Between GSI, Serum Steroids Levels, and the Ovarian Developmental Phase

The criteria used for staging ovary maturation of spotted halibut was according to Merson et al. (2000). The histological observation results (Fig. 7) revealed that spotted halibut features asynchronous oocyte development and batch spawnings. Ovaries sampled in June 2009 were full of oocytes at perinucleolus stage (Fig. 7A),

Figure 4. Phylogenetic tree of prepro-GnRHs in vertebrates. This unrooted tree was made by Neighbor-Joining method using Mega 4.1 software based on the alignments of the amino acid sequences of whole prepro-GnRHs using ClustalX. Boot strap values are based on 1000 repeats. GenBank accession numbers: cGnRH-II group: Cobia (AAT80333), Goldfish cGnRH-II 2 (AAB86989), Swamp eel (AAV41875), Striped bass (AAD03816), Red drum (AAT80330), Japanese medaka (BAC06423), Leopard gecko (BAC091084); sGnRH group: Common carp (AA039752; AA039976), Sockeye salmon (BAA056966), Mummichog (BAF95685), Red drum (AAT80329), Atlantic croacker (AAQ16503), Cobia (AAP80332), Nile tilapia (BAC56851); the third group: Swamp eel (AA41121), Red drum (AAT80331), Atlantic croacker (AAO16501), Common dentex (BAC07229), Mummichog (BAF57234), European sea bass (AF224279), Striped bass (AAO3817). For the rest GenBank accession numbers of GnRH precursors, see legends of Figures 1, 2, and 3.

Figure 5. sbGnRH mRNA expression in various organs determined by quantitative RT-PCR. B, brain; P, pituitary; G, gonad; L, liver; Sp, spleen; K, kidney; H, heart; I, intestine; St, stomach.
ovaries sampled in August 2009 were at cortical alveoli stage (Fig. 7B), ovaries sampled in October 2009 were at vitellogenic stage (Fig. 7C), ovaries sampled in January 2010 were at yolk globe stage. Plasma E2 level peaked during spawning stage (97.10 pg/mL) and dropped remarkably during postspawning stage (20.30 pg/mL during vitellogenic stage and 71.70 pg/mL during postspawning stage (Fig. 7E).

**DISCUSSION**

Three GnRH forms in the brain of spotted halibut were cloned by successfully isolating and sequencing the cDNAs encoding sGnRH, sbGnRH, and cGnRH-II precursors. The results provided further evidence to the fact that three GnRH variants coexisted in the brain of Pleuronectiformes (Anderson et al., 2001; Amano et al., 2002; Fang et al., 2006; Pham et al., 2007). Results from our study supported the hypothesis of three lineages of vertebrate GnRHs (Leonardo et al., 2007). Each prepro-GnRH could cluster into a separate clade with its homologs from fish, mammalian, birds, and amphibian counterparts within each of the following groups: sGnRH group, cGnRH-IIgroup, and the fish-specific group (sbGnRH, mdGnRH, cfGnRH-I, etc.).

Numerous evidences showed cGnRH-II only expressed in the brain and acted as a neurotransmitter and/or neuromodulator in teleosts (Powell et al., '95; Rissman, '96; Penlington et al., '98; Servili et al., 2010). For example, in masu salmon, Oncorhynchus masou, and barfin flounder, V. moseri, cGnRH-II–ir cell bodies were detected only in the mid brain, and cGnRH-II functioned only as a neuromodulator (Holland et al., '98; Okuzawa and Kobayashi, '99; Amano et al., 2002). In turbot, Scoolithalmus maximus, the cGnRH–II level in brain extracts elevated from May to July, concomitant with an increase in oocyte diameter. However, the absence of cGnRH–II in the pituitary revealed that cGnRH–II was not directly involved into the reproduction control (Anderson et al., 2001). Amano et al. (2004) reported that brain cGnRH–II level did not show significant changes and pituitary cGnRH–II peptide contents were extremely low during testicular maturation of barfin flounder. In grass puffer, Takifugu niphobles, the brain cGnRH–II mRNA level showed no noticeable changes except for postspawning females (Shahjahan et al., 2010). Moreover, in some Perciformes, such as gilthead seabream, Sparus auratus (Holland et al., '98), red seabream, P. major (Senthilkumaran et al., '99), and striped bass, Morone saxatilis (Holland et al., 2001), the absence or low levels of cGnRH–II in the pituitary almost ruled out its involvement in the regulation of GtH or other pituitary hormones. However, its wide distribution pattern in the brain showed that it is involved in the neurotransmission and/or neuromodulation. In the present study, cGnRH–II was exclusively detected in the brain and its expression levels showed no significant changes (P > 0.05) during the ovary maturation.

Therefore, we believe that cGnRH–II may only play a role as a neurotransmitter and/or neuromodulator in spotted halibut.

sGnRH–like forms have been detected in the brain of mammals besides fish using HPLC and RIA methods, thus the sGnRH–like
form may also be a universal form in all vertebrates (Montaner et al., '98, '99; Yahalom et al., '99; Okubo et al., 2000). Numerous data demonstrated the presence of GnRH mRNA in reproductive organs of fish, such as goldfish, Carassius auratus (Lin and Peter, '96), salmon (Von Schalburg and Sherwood, '99), and seabream (Nabissi et al., 2000). In rainbow trout, gonadal sGnRH mRNA expression level decreased during the period of active spermatogonial proliferation in testis and increased during meiosis occurrence in the testis and ovary, indicating an antiproliferative and meiosis-stimulating effect of sGnRH during gametogenesis (Uzbekova et al., 2001, 2002). These results suggested that GnRH is involved in paracrine/autocrine regulation in gonads, acting in processes of steroidogenesis (Pati and Habibi, '98) and meiosis regulation (Nabissi et al., 2000; Pati and Habibi, 2000). In the present study, sGnRH was detected in the brain, pituitary, and ovary. The wide distribution hinted its multiple physiological functions besides its GtH secretion function in salmonids. Furthermore, sGnRH expression levels increased with the gonadal development, reached maximum during vitellogenic stage, decreased during spawning stage in our study. This phenomenon was also observed in grass puffer during ovarian maturation as well as other teleosts including Perciformes.

Figure 7. Photomicrographs of gonadal maturation stages of spotted halibut. (A) Oocyte of phase II (perinucleolus stage). (B) Oocyte of phase III (cortical alveoli stage). (C) Oocyte of phase IV (vitellogenic stage). (D) Oocyte of phase V (hydration stage, spawning stage). (E) Rudimental follicle membrane (FM) (post spawning stage). GV, germinative vesicle; CA, cortical alveoli; YG, yolk globules; YP, yolk plate; FM, follicle membrane. Magnification 40× (A, C–D); 100× (B, E).
speculated that sGnRH is unlikely involved in GtH secretion in mRNA level and GSI and sex steroids level (indicated that there is no signi cant that of sbGnRH even cGnRH sGnRH expression level was substantially lower (et al., '97; Okuzawa and Kobayashi, '99). In the present study, the of GtH but also functioned as a neuromodulator (Kobayashi hybridization, indicating that sGnRH not only regulated secretion fi bers in both the brain and pituitary through in-situ hybridization, indicating that sGnRH not only regulated secretion of GtH but also functioned as a neuromodulator (Kobayashi et al., '97; Okuzawa and Kobayashi, '99). In the present study, the sGnRH expression level was substantially lower \( P < 0.05 \) than that of sbGnRH even cGnRH-II. Furthermore, statistical analysis indicated that there is no significant relationship between sGnRH mRNA level and GSI and sex steroids level \( P > 0.05 \). Thus, we speculated that sGnRH is unlikely involved in GtH secretion in spotted halibut based on the significantly low level during ovarian maturation and the statistical analysis, but this deduction needs more support from gene knockdown and immunohistochemical studies on sGnRH in the future.

The universal expression pattern and possible physiological role of sbGnRH have been widely demonstrated in teleost species such as turbot, Japanese flounder, Paralichthys olivaceus, striped bass, Grass rockfish, Sebastes rastrelliger, etc. (Anderson et al., 2001; Collins et al., 2001; Holland et al., 2001; Amano et al., 2004; Fang et al., 2006). In Pleuronectiformes, a universal distribution of sbGnRH in different organs including brain, pituitary, gonad, intestine, etc. was reported in Japanese flounder (Fang et al., 2006). The universal distribution pattern of sbGnRH was observed in our study. This phenomenon indicated that sbGnRH is likely to have more diversified functions, in addition to gonadotropin secretion in different systems including the reproductive system, digestive system, and immune system (Amano et al., '91; Suzuki et al., '92; Kim et al., '95; Adams et al., 2002). To date, the existing evidences support the fact that sbGnRH is the main hypophysiotropic GnRH form in the majority of teleosts expressing three GnRH forms. In our study, the significant fluctuation of brain sbGnRH expression level throughout the ovarian development was observed. It significantly increased since cortical alveoli stage and peaked during hydration stage \( P < 0.05 \), and dropped remarkably during postspawning stage \( P < 0.05 \). This trend was also found in numerous other teleosts. Collins et al. (2001) reported that sbGnRH fluctuated during the reproductive cycle and the largest accumulations were detected in the brains and pituitaries in Grass Rockfish. In another study, levels of brain and pituitary sbGnRH increased from immature phase to spawning stage in red seabream (Senthilkumaran et al., '99; Okuzawa et al., 2003). In grass puffer, the amounts of sbGnRH mRNA were substantially accumulated in the brains and pituitaries in spawning phase (Collins et al., 2001). These results clearly indicated that sbGnRH was the main hypophysiotropic GnRH form in the brain and pituitary of spotted halibut. Meanwhile, sbGnRH was the most abundant GnRH form in the brain and pituitary of spotted halibut based on the quantitative PCR analysis. This was in accordance with the findings in striped bass (Holland et al., 2001) and grass rockfish (Collins et al., 2001). These results clearly indicated that synthesis and release of sbGnRH are temporally activated to induce massive secretion of GtH during the spawning stage, and it possibly was the pivotal hypophysiotropic hormone controlling reproductive cycle in spotted halibut.

The GSI and sex steroids levels were recorded in this study. The GSI is a good index for evaluating gonadal maturation in teleosts, it is closely related with the sex steroid levels during the
reproductive season (Methven et al., ’92; Merson et al., 2000). Sex steroids are required for germ cell development and maturation, and exert feedback effects in the pituitary and brain to regulate gonadotropin and GnRH secretion (Nagahama et al., ’95). Thus, GSI is indirectly related with the GnRH expression in teleost species. The GSI and the circulating levels of sex steroids are useful tools to assess the reproductive status of fish (Merson et al., 2000). Interestingly, GSI values were very high for spotted halibut in this study. For example, the GSI reached 21.67 ± 2.86 during vitellogenic stage and reached 33.09 ± 5.57 during spawning stage, this is different from that of other flatfish species, usually with the GSI never exceeding 10 (Methven et al., ’92). However, the similar high female GSI values were also observed in barfin flounder (Takaaki, 2005), another teleost species of the genus Verasper. Thus, the high GSI values of spotted halibut may be related with its reproductive characteristics as well as the calculation method for GSI used in the present study. The relationship between brain and/or pituitary GnRH levels and gonadal maturation in fish has been demonstrated in several species. In most cases, sbGnRH level in maturing fish correlated with changes in oocyte diameter and GSI and LH levels in pituitary (Holland et al., 2001). The increase of the sbGnRH mRNA level was correlated with plasma steroids levels in female red seabream (Holland et al., 2001). The increase of the sbGnRH mRNA level was correlated with changes in oocyte diameter and GSI and LH levels in pituitary species. In most cases, sbGnRH level in maturing

Based on the results from the present study, we proposed that sbGnRH is physiologically the most important hypophysiotropic hormone in the regulation of reproduction of spotted halibut. More physiological and biochemical evidences are needed for confirming this. Results from this study revealed that the three GnRH genes differentially regulated ovarian development and sex steroids and they were the important regulators for the differential expression of GnRH in spotted halibut. The findings would help us better understand the reproductive endocrine mechanism of spotted halibut. It is worth noting that all the data in this study were obtained from the fish cultured under artificial conditions. The natural environmental factors are largely different from the artificial photothermal conditioning, thus further study is needed on wild individuals to compare and verify the results obtained from this study.

LITERATURE CITED


CLONING AND EXPRESSION OF GnRHs IN SPOTTED HALIBUT


J. Exp. Zool.
Okuzawa K, Kobayashi M. 1999. Gonadotropin-releasing hormone neuronal systems in the teleostean brain and functional signifi-


