RESEARCH ARTICLE

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

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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.

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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 decapeptides with a well-conserved structure during evolution (Somoza et al., 2002b). It is now well established that

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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 maturation, ovulation, and spawning are available, the brain GnRH could act on the pituitary and cause synthesis and release of gonadotropins (Follicle stimulating hormone [FSH] and Luteinizing hormone [LH]) from pituitary into the blood stream. In turn, the gonadotropins stimulate the gonad to synthesize sex steroids (estradiol-17β [E_2] 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 Gonadotropic 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), Radioimmunoassay (RIA), Enzyme-Linked Immunosorbent Assay, in-situ hybridization, and immunohistochemical methods (Senthilkumaran 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 recognized 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 (Demski 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 (BWs) of 1,300–2,200 g. A total of 60 fish (♀:♂ = 1:1) were reared in round concrete tanks (25 m^3 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

15–16°C; Photoperiod:11D:13L), January 2010 for yolk globe hydration and spawning stage (Temperature 9–10°C; Photoperiod:8D:16L), and May 2010 for postspawning stage (Temperature 13–14°C; Photoperiod:10D:14L). All experimental fish were euthanized with overdosed MS222 (260 mg/L) before organs were harvested. Organs including brain, pituitary, gonad, liver, spleen, kidney, stomach, intestine, and heart were immediately dissected out and preserved as the same way as for GnRH cloning. The spatial expression patterns of GnRH genes were investigated by quantitative real-time PCR also.

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 fish were recorded. Ovarian development stages were determined by biopsy and histological observation. 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).

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 (E_2) 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 E_2 and 22.0 pg/mL for T, respectively. The intra- and interassay coefficients of variation were 1.9 and 7.4% for E_2, 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 (Watsonbiot, Shanghai) to remove contaminated genomic DNA. cDNA synthesis was performed with SMARTTM RACE cDNA synthesis Kit (Clontech, Mountian 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 60 sec, 55°C for 60 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.

The 5′- and 3′-cDNA ends were amplified according to manufacturer's instructions for SMARTTM RACE cDNA Amplification Kit (Clontech, Mountian view, CA, USA). The gene-specific primers were designed based on the middle fragments and listed in Table 1. Nested PCR were conducted, primers and templates for the first and nested PCR are listed in Table 2. The first PCR amplification conditions were 10 touchdown cycles of 94°C for 30 sec, 68°C for 30 sec (with 1°C touchdown per cycle), 72°C for 1 min, followed by 20 cycles of 94°C for 40 sec, 58°C for 40 sec, 72°C for 60 sec, then followed by a final extension of 72°C for 7 min. The nested PCR was carried out following the conditions: 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min.

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 μmol/L), 1 μL of the 1:100 diluted cDNA template, and 9.5 μL of ddH_2O. 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

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

Primer	Nucleotide sequence (5′-3′)	Function
UPM-long	5′-CTAATACGACTCACTATAGGGCAAG CAGTGGTATCAACGCAGAGT-3′	5′-RACE PCR
UPM-short	CTAATACGACTCACTATAGGGC	5′-RACE PCR
NUP	AAGCAGTGGTATCAACGCAGAGT	3′-RACE PCR
cF1	CACCTGTGAACCTGTGAGAC	Middle fragment and 3′-RACE PCR for cGnRH-II
cR1	GGTCACCAGGTAGAAAAGCA	Middle fragment and 5′-RACE PCR for cGnRH-II
cR2	AGCTCTCTGGCCAAAGCATC	Middle fragment PCR for cGnRH-II
sbF	AAGACCACCGAATCGGAGAC	Middle fragment and 3′-RACE PCR for sbGnRH
sbR	CTGTAAATTCCGGCAAAAGG	Middle fragment and 5′-RACE PCR for sbGnRH
sF	TACCTTCCTCTGCAGCTCTG	Middle fragment PCR for sGnRH
sR	GCTCAGGAAGAGACACCACT	Middle fragment PCR for sGnRH
cS1	GTTCTGCTGCTGGGGCTGCTTCT	3′-RACE nested PCR for cGnRH-II
cA1	CTGAAATCTCTGACGTGCCAAAA	5′-RACE nested PCR for cGnRH-II
sbS1	CTTGTCAGTGTGGCTGCTCCTCG	3′-RACE nested PCR for sbGnRH
sbA1	GAGCAGCCACACTGACAAGGTTT	5′-RACE nested PCR for sbGnRH
sS1	CTGATGGAAGCGAGCAGCAGACT	3′-RACE nested PCR for sGnRH
sS2	GGATGATGGGCACAGGCGGAGTGGT	3′-RACE nested PCR for sGnRH
SA1	AGACACCACTCCGCCTGTGCCCATC	5′-RACE nested PCR for sbGnRH
cF2	TGACTGTAGGACGAGCAGAG	Real-time PCR for cGnRH-II
cR2	GCAGAACCAGCCGAGATG	Real-time PCR for cGnRH-II
SbF2	AAGGACTGAAGACCACCGAATC	Real-time PCR for sbGnRH
sbR2	CACGAGGAGCAGCCACAC	Real-time PCR for sbGnRH
sF2	CAGGTGTTGGTGTTGATG	Real-time PCR for sGnRH
sR2	AGGTCTCTCTTGGGTTTG	Real-time PCR for sGnRH
β-actin F	TTCTGGTGATGGTGTGAC	Real-time PCR for β-actin
β-actin R	GTGGTGGTGAAGGAGTAG	Real-time PCR for β-actin

(http://www.clustal.org/download/current/) in combination with MEGA 4.1 (http://www.megasoftware.net/mega41.html). The statistical significance of the differences between groups was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (SPSS v16.0). The significant value P was set as 0.05, the statistical significance was recognized when $P < 0.05$.

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

	Target product	Template	The first PCR primers	The nested PCR primers
cGnRH-II	5′ partial cDNA	5′-RACE-Ready cDNA	cR1 and UPM	cA1 and NUP
	3′ partial cDNA	3′-RACE-Ready cDNA	cF1 and UPM	cS1 and NUP
sbGnRH	5′ partial cDNA	5′-RACE-Ready cDNA	sbR and UPM	sbA1 and NUP
	3′ partial cDNA	3′-RACE-Ready cDNA	sbF and UPM	sbS1 and NUP
sGnRH	5′ partial cDNA	5′-RACE-Ready cDNA	sA1 and UPM	sA1 and NUP
	3′ partial cDNA	3′-RACE-Ready cDNA	sS1 and UPM	sS2 and NUP

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 9.59 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%), pjGnRH (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 Pleuronetiformes 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.

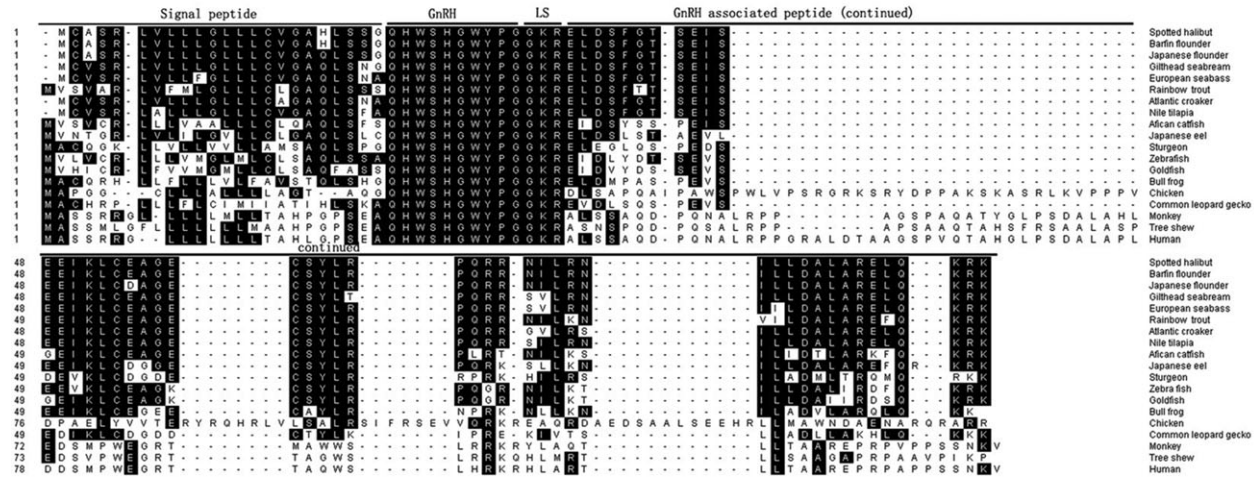

Figure 1. Comparison of cGnRH-II precursors among spotted halibut, mammals, birds, amphibians, reptiles, and other fish species. The single-letter code is used to designate amino acids. The three domains, signal peptide, GnRH, and GnRH-associated peptide, together with the cleavage site (LS) are indicated. Gaps (indicated by hyphens) were introduced optionally to achieve maximum similarity, taking into account conservative amino acid substitutions. GenBank accession numbers of the GnRH sequences from mammals, birds, amphibians, reptiles, and other fish species were used in this comparison: Spotted halibut (HM131601); Barfin flounder (Q8UW81); Japanese flounder (AAY28981); Gilthead seabream (P51925); European sea bass (AAF62900); Rainbow trout (AAF08687); Atlantic croaker (AAQ16502); Nile tilapia (BAC56850); African catfish (CAA54969); Japanese eel (BAA82609); Sturgeon (BQ01979); Zebrafish (NP_852104); Goldfish (AAC59858); Bull frog (AAL05971); Chicken (BAE80724); Monkey (NP_001029374); Tree shrew (AAB16838); Human (AAC02980).

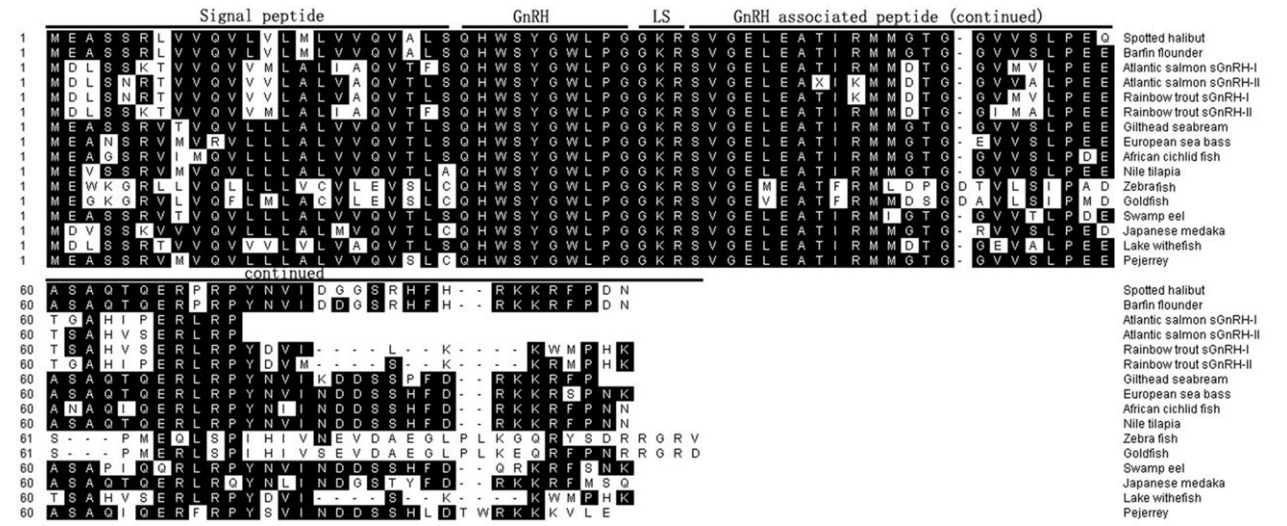

Figure 2. Comparison of sGnRH precursors between spotted halibut and other fish species. For details, see legend of Fig. 1. Accession number of the GnRH sequences from fish species in Genbank: Spotted halibut (HM131602); Barfin flounder (Q8UW82); Japanese flounder (ABD97879); Atlantic salmon (AAR20401; AAR20404); Rainbow trout (AAF91281, AAF91280); Gilthead seabream (AAD02425); European sea bass (Q9IA09); African cichlid fish (AAC27718); Nile tilapia (AAV74403); Zebrafish (CAC18539); Goldfish (BAF98577); Swamp eel (AAW51120); Japanese medaka (BAD02405); Lake whitefish (AAP57220); Pejerrey (AAU94308).

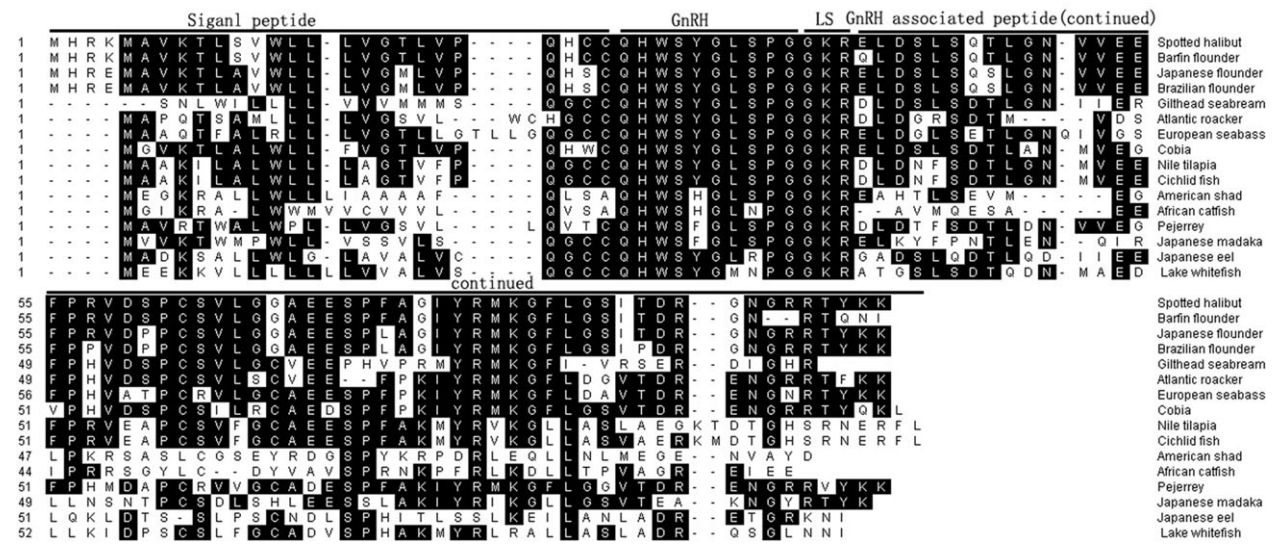

Figure 3. Comparison of sbGnRH precursors between spotted halibut and other fish species. For details, see the legend to Fig. 1. GenBank accession numbers of the GnRH sequences from fish species used in this comparison: Spotted halibut (HM131600); Barfin flounder (BAB83984); Brazilian flounder (ACS88343); Japanese flounder (AAY83273); Gilthead seabream (AAD02427); European sea bass (AAF62898); Cobia (AAT80334); Nile tilapia (BAC56849); Cichlid fish (AAC59691); American shad (AAN04492); African catfish (CAA54971), Pejerrey (AAU94309); Japanese medaka (BAB16303); Japanese eel (BAA82608); 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-IImRNA level and sGnRH mRNA level, respectively.

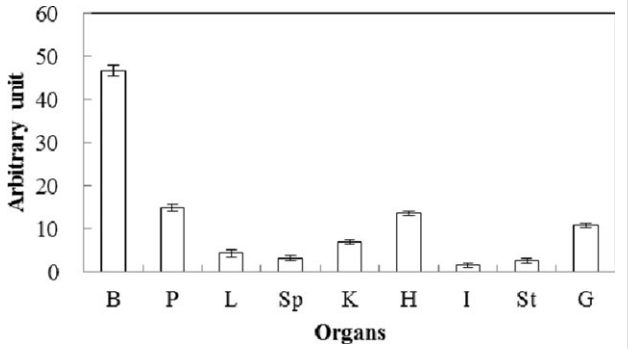

Figure 5. sbGnRH mRNA expression in various organs determined by quantitative RT-PCR.B, brain; P, pituitary; G, gonad; L, liver; Sp, spleen; K, kidney; H, heart; I, intestine; St, stomach.

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),

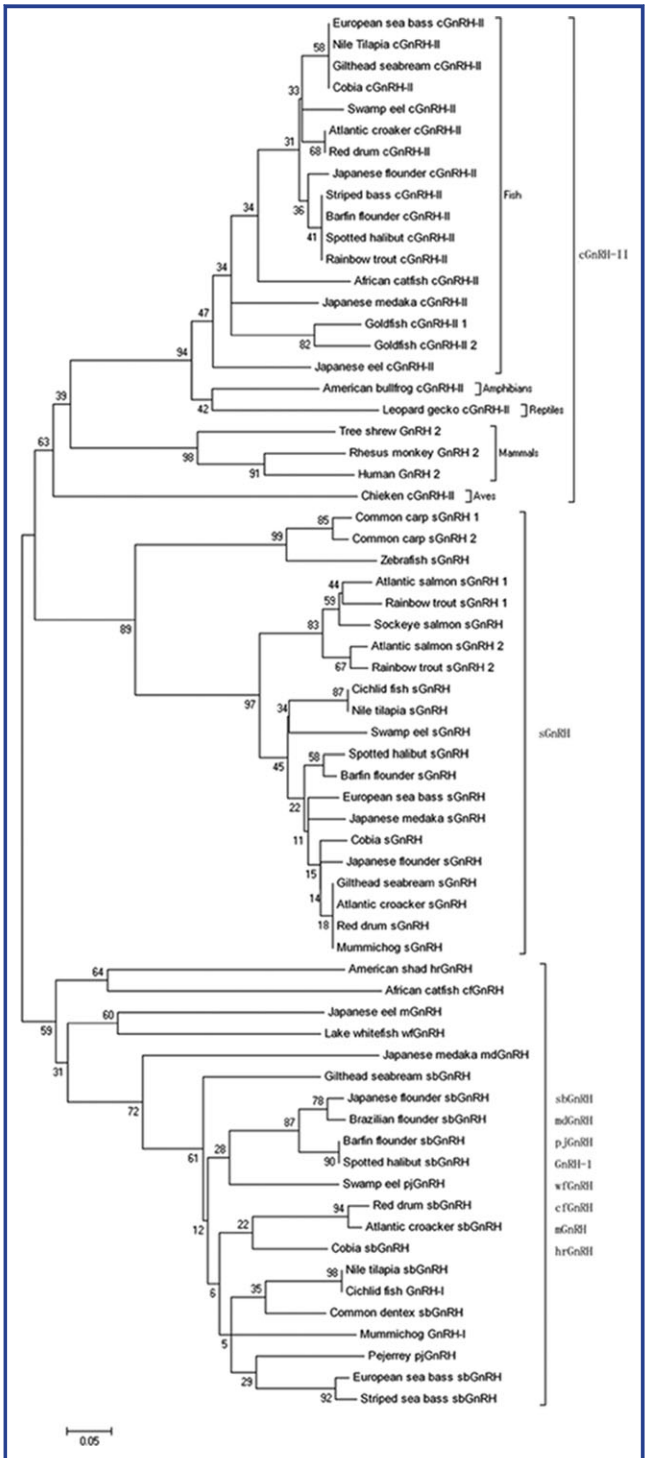

Figure 4. Continued.

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 (BAC99084); sGnRH group: Common carp (AAO39752; AAO39976), Sockeye salmon (BAA06666), Mummichog (BAF95685), Red drum (AAT80329), Atlantic croacker (AAQ16503), Cobia (AAT80332), Nile tilapia (BAC56851); the third group: Swamp eel (AAW51121), Red drum (AAT80331), Atlantic croacker (AAQ16501), Common dentex (BAC07229), Mummichog (BAF57234), European sea bass (AF224279), Striped bass (AAD03817). For the rest GenBank accession numbers of GnRH precursors, see legends of Figures 1, 2, and 3.

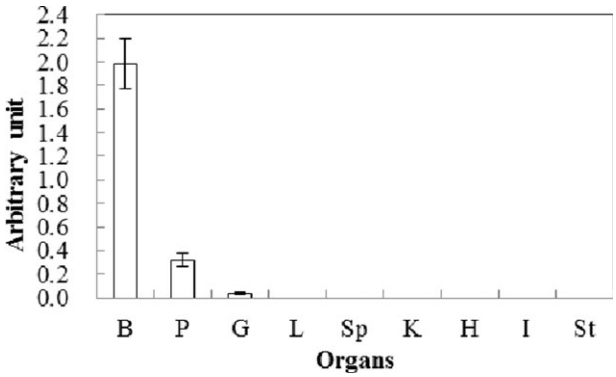

Figure 6. sGnRH mRNA expression in various organs determined by quantitative RT-PCR.B, brain; P, pituitary; G, gonad; L, liver; Sp, spleen; K, kidney; H, heart; I, intestine; St, stomach.

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 hydration and spawning stage (Fig. 7D), and May 2010 was postspawning stage (Fig. 7E).

GSI was relatively low (0.86 ± 0.09) during perinucleolus stage, and then significantly increased during cortical alveoli stage (7.01 ± 1.18) ($P < 0.05$). It reached 21.67 ± 2.86 during vitellogenic stage and peaked during spawning stage (33.09 ± 5.57) ($P < 0.05$), then dramatically dropped to 8.65 ± 0.87 during postspawning stage ($P < 0.05$).

The average E_2 level was 34.80 ± 1.22 pg/mL during perinucleolus stage, and then gradually increased with the gonadal development. It reached 44.10 ± 7.70 pg/mL during cortical alveoli stage and 71.70 ± 7.20 pg/mL during vitellogenic stage. Plasma E_2 level peaked during spawning stage (97.10 ± 6.70 pg/mL) ($P < 0.05$) and then remarkably fell to 31.60 ± 5.20 pg/mL during postspawning stage ($P < 0.05$). The plasma T level did not show significant change during the gonadal maturation. The T level was 206.10 ± 10.50 pg/mL during perinucleolus stage, 189.10 ± 23.30 pg/mL during cortical alveoli stage, 293.60 ± 20.30 pg/mL during vitellogenic stage ($P < 0.05$), and 244.50 ± 6.90 pg/mL during spawning stage ($P < 0.05$), respectively. But it dramatically fell to 113.80 ± 10.90 pg/mL during postspawning stage ($P < 0.05$).

Brain GnRH mRNA Levels during Ovary Maturation Cycle

Changes in brain sbGnRH mRNA levels were closely correlated with variation of GSI, E_2, and T expression levels during ovary maturation. sbGnRH expression continuously rises throughout the ovarian development. It significantly increased since the cortical alveoli stage and peaked during spawning stage ($P < 0.05$), and dropped remarkably during postspawning stage ($P < 0.05$) (Fig. 8). sGnRH expression level increased with the gonadal development and reached maximum during vitellogenic stage. Thereafter, it dropped to a relatively low level ($P > 0.05$) (Fig. 9). Meanwhile, cGnRH-II expression levels showed no significant changes ($P > 0.05$) except slightly elevated at the vitellogenic stage during the ovary maturation (Fig. 8).

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 of spotted halibut 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, *Scoohthalmus 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-IIpeptide 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 sebream, *Sparus auratus* (Holland et al., '98), red seabream, *Pagrus 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

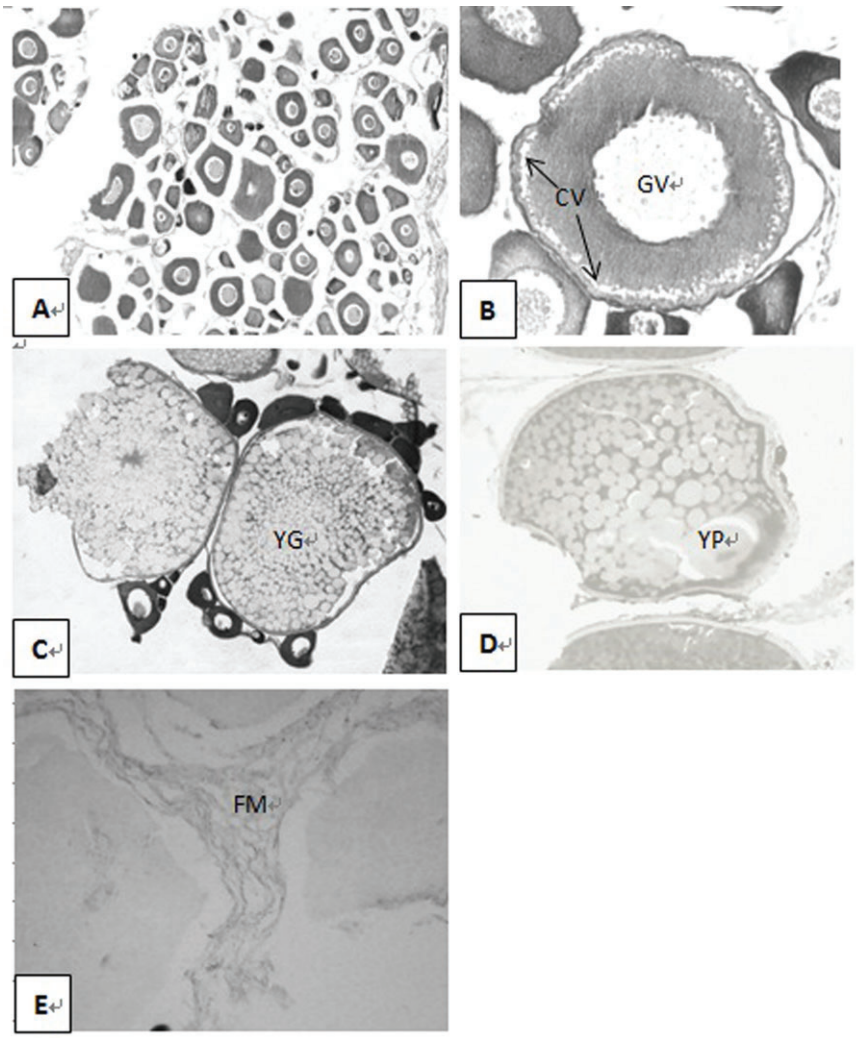

Figure 7. Photomicrographs of gonadal maturation stages of spotted halibut. (A) Oocyte of phaseII (perinucleolus stage). (B) Oocyte of phase III (cortical alveoli stage). (C) Oocyte of phase IV(vitellogenic stage). (D) Oocyte of phase V(hydration stage, spawning stage). (E) Rudimental follicle membrane (FM) (post spawning stage). GV, geminal vesicle; CA, cortical alveoli; YG, yolk globules; YP, yolk plate; FM, follicle membrane. Magnification 40× (A, C–D); 100× (B, E).

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

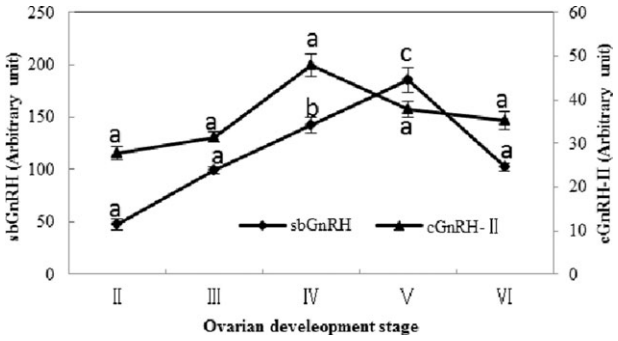

Figure 8. Temporal changes of brain cGnRH-IImRNA and sbGnRH mRNA levels during ovarian maturation. Beta actin is used as an internal control. Values are expressed as mean ± SEM. Different letters indicate significant difference ($P < 0.05$).

and Pleuronectiformes expressing three GnRH forms (Holland et al., '98; Senthilkumaran et al., '99; Anderson et al., 2001; Amano et al., 2002). In some special cases, the secretion neurons of sGnRH were also found widely distributed in the brain and even overlapped in their specific localization with sbGnRH neurons (Gonzalez-Martinez et al., 2002a;, Soga et al., 2005; Kah et al., 2007). Several studies in masu salmon found the sGnRH-ir fibers 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.

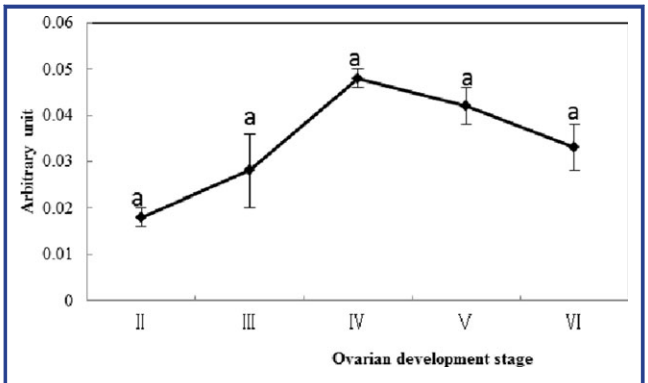

Figure 9. Temporal changes of sGnRH mRNA level during ovary maturation. Values are expressed as mean ± SEM. Different letters indicate significant difference ($P < 0.05$).

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 elevated in spawning fish and showed strong correlation with GSI and elevation in plasma E_2 and T levels (Shahjahan et al., 2010). In turbot, *Scophthalmus maximus*, brain and pituitary sbGnRH levels showed significant variation and correlated with increase in oocyte diameter during the spawning season (Anderson et al., 2001). It should be noticed that sbGnRH significantly elevated during ovarian maturation compared with sGnRH and cGnRH-II in 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 (Okuzawa et al., 2003). Miranda et al. (2009) also reported a high correlation between pjGnRH, GSI, and plasma sex steroids levels in female *Odontesthes bonatiensis*. It is known that sex steroids exerted a positive feedback on the GnRH system in fish brain (Okuzawa, 2002). This fact has been verified not only in mammals (Ng et al., 2009) but also in fish. For example, the exogenous T increased the terminal nerve sGnRH mRNA levels and decreased the preoptic sbGnRH mRNA level in castrated male tilapia (Soga et al., '98). In grass fish, the E_2 might stimulate sbGnRH gene expression through binding to an ER (estradiol receptor) responsive element and/or two composite binding sites for ER, COUP (chick ovalbumin upstream promoter transcription factor), and RAR (retinoic acid receptor) (Shahjahan et al., 2010). These potential binding sites for steroid receptor in GnRH promotors have previously been described in other teleosts (Kitahashi et al., 2005; Hu et al., 2008). In barfin flounder, the sex steroids were believed to have a positive feedback on sbGnRH neurons in the brain and secretion of sbGnRH to the pituitary (Amano et al., 2004). In present study, the GSI and sex steroids level increased with the ovary maturation and peaked at the spawning stage, the sbGnRH expression level was found to be consistent with the GSI and serum sex steroids level, which peaked during spawning stage and dramatically dropped during postspawning stage. These evidences suggested sex steroids were candidates for regulators of sbGnRH synthesis in teleosts including spotted halibut. FSH and LH are critical hormones connecting GnRH and the sex steroid hormone, the detailed mechanism of sex steroids regulating GnRH expression depends on the further investigation of FSH and LH gene expression pattern during gonadal maturation in the future.

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

Adams BA, Vickers ED, Warby C, Park M, Minkyu Park, Fische WH, Grey Craig A, Rivier JE, Sherwood NM. 2002. Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, *Coregonus clupeaformis*. Biol Reprod 67:232–239.

Amano M, Oka Y, Aida K, Okumoto K, Kawashima S, Hasegawa Y. 1991. Immunocytochemical demonstration of salmon GnRH and chicken GnRH-II in the brain of masu salmon, *Oncorhynchus masou*. J Comp Neurol 314:587–597.

Amano M, Okubo K, Yamanome T, Yamada H, Aida K, Yamamori K. 2004. Changes in brain GnRH mRNA and pituitary GnRH peptide during testicular maturation in barfin flounder. Comp Biochem Phys B 138:435–443.

Amano M, Takahashi A, Yamanome T, Okubo K, Aida K, Yamamori K. 2002. Molecular cloning of three cDNAs encoding different GnRHs in the brain of barfin flounder. Gen Comp Endocrinol 126:325–333.

Anderson E, Fjelldal PG, Klenke U, Vikingstad E, Taranger GL, Zohar Y, Stefansson SO. 2001. Three forms of GnRH in the brain and pituitary of the turbot, *Scophthalmus maximus*: immunological characterization and seasonal variation. Comp Biochem Physiol B 129:551–558.

Chen CF, Wen HS, Wang ZP, He F, Zhang JR, Chen XY, Jin GX, Shi B, Shi D, Yang YP, Li JF, Qi BX, Li N. 2010. Cloning and expression of P450c17-I (17a-hydroxylase/17,20-lyase) in brain and ovary during gonad development in *Cynoglossus semilaevis*. Fish Physiol Biochem 36:1001–1012.

Collins PM, Neill DFO, Barron BR, Moore RK, Sherwood NM. 2001. Gonadotropin-releasing hormone content in the brain and pituitary of male and female grass rockfish (*Sebastes rastrelliger*) in relation to seasonal changes in reproductive status. Biol Reprod 65:173–179.

Demski LS, Beaver JA, Sudberry JJ, Custis JR. 1997. GnRH systems in cartilaginous fishes. In: Parhar IS, Sakuma Y, editors. GnRH neurons: gene to behavior. Tokyo: Brain Shuppan. p 131–152.

Dubois EA, Zandbergen MA, Peute J, GoosTh HJ. 2002. Evolutionary development of three gonadotropin-releasing hormone (GnRH) systems in vertebrates. Brain Res Bull 57:413–418.

Fang BH, Sun XQ, Qu LY, Zhang ZW. 2006. Molecular cloning and expression analysis of two types of GnRH gene in Japanese flounder (*Paralichthys olivaceus*) [in Chinese with abstract]. High Technol Lett 16:529–534.

Filby AL, Aerle RV, Duitman J, Tyler CR. 2008. The kisspeptin/gonadotropin-releasing hormone pathway and molecular signaling of puberty in fish. Biol Reprod 78:278–289.

Gonzalez-Martinez D, Zmora N, Mananos E, Saligaut D, Zanuy S, Zohar Y, Elizur A, Kah O, Munoz-Cueto JA. 2002b. Immunohistochemical localization of three different prepro-GnRHs in the brain and pituitary of the European sea bass (*Dicentrarchus labrax*) using antibodies to the corresponding GnRH-associated peptides. J Comp Neurol 446:95–113.

Gonzalez-Martinez D, Zmora N, Zanuy S, Sarasquete C, Elizur A, Kah O, Munoz-Cueto JA. 2002a. Developmental expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). J Chem Neuroanat 23:255–267.

Grone BP, Maruska KP, Korzan WJ, Fernald RD. 2010. Social status regulates kisspeptin receptor mRNA in the brain of *Astatotilapia burtoni*. Gen Comp Endocrinol 169:98–107.

He F, Wen HS, Dong SL, Wang LS, Chen CF, Shi B, Mu XJ, Yao J, Zhou YG. 2008. Identification of estrogen receptor a gene polymorphisms by PCR-SSCP and its effect on reproductive traits in Japanese flounder (*Paralichthys olivaceus*). Comp Physiol Biochem B 150:278–283.

Holland MC, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A, Zohar Y. 1998. Levels of the native forms of GnRH in the pituitary of the gilthead seabream, *Sparus aurata*, at several characteristic stages of the gonadal cycle. Gen Comp Endocrinol 112:394–405.

Holland MC, Hassin Y, Zohar Y. 2001. Seasonal fluctuations in pituitary levels of the three forms of gonadotropin-releasing hormone in striped bass, *Morone saxatilis* (Teleostei), during juvenile and pubertal development. J Endocrinol 169:527–538.

Hu SY, Chen MH, Lin YC, Lin GH, Gong HY, Yang TH, Wu JL. 2008. Cloning and functional analysis of the proximal promoter region of the three GnRH genes from the silver sea bream (*Sparus sarba*). Comp Biochem Physiol B 151:373–380.

Kah O, Lethimonier C, Somoza G, Guilgur LG, Vaillant C, Lareyre JJ. 2007. GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. Gen Comp Endocrinol 153:346–364.

Kim MH, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashima S, Suzuki Y, Aida K. 1995. Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. J Comp Neurol 356:72–82.

Kitahashi T, Sato H, Sakuma Y, Parhar IS. 2005. Cloning and functional analysis of promoters of three GnRH genes in a cichlid. Biochem Biophys Res Commun 336:536–543.

Kobayashi M, Amano M, Kim MH, Yoshiura Y, Sohn YC, Suetake H, Aida K. 1997. Gonadotropin-releasing hormone and gonadotropin in goldfish and masou salmon. Fish Physiol Biochem 17:1–8.

Leonardo G, Guilgur GO, Pablo HSM, Fernandino JI, Miranda LA, Somoza GM. 2007. Characterization of the cDNAs encoding three GnRH forms in the pejerrey fish *Odontesthes bonariensis* (Atheriniformes) and the evolution of GnRH precursors. J Mol Evol 64:614–627.

Lin XW, Peter RE. 1996. Expression of salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II precursor messenger ribonucleic acids in the brain and ovary of goldfish. Gen Comp Endocrinol 101:282–296.

Merson RR, Casey CS, Martinez C, Soffientino B, Chandlee M, Specker JL. 2000. Oocyte development in summer flounder: seasonal changes and steroid correlates. J Fish Biol 57:182–196.

Methven DA, Crim LW, Norberg B, Brown JA, Goff GP, Huse I. 1992. Seasonal reproduction and plasma levels of sex steroids and vitellogenin in Atlantic halibut (*Hippoglossus hippoglossus*). Can J Fish Aquat Sci 49:754–759.

Miranda LA, Strüssman CA, Somoza GM. 2009. Effects of light and temperature conditions on the expression of GnRH and GtH genes and levels of plasma steroids in *Odontesthes bonariensis* females. Fish Physiol Biochem 35:101–108.

Montaner AD, Affanni JM, King JA, Bianchini JJ, Tonarelli G, Somoza GM. 1999. Differential distribution of gonadotropin-releasing hormone variants in the brain of *Hydrochaeris hydrochaeris* (Mammalia, Rodentia). Cell Mol Neurobiol 19:635–651.

Montaner AD, Somoza GM, King JA, Bianchini JJ, Bolis CG, Affanni JM. 1998. Chromatographic and immunological identification of GnRH (gonadotropin-releasing hormone) variants. Occurrence of mammalian and a salmon-like GnRH in the forebrain of an eutherian mammal: *Hydrochaeris hydrochaeris* (Mammalia, Rodentia). Regul Pept 73:197–204.

Nabissi M, Soverchia L, Polzonetti-Magni AM, Habibi HR. 2000. Differential splicing of three gonadotropin-releasing hormone transcript in the ovary of the sea bream (*Sparus aurata*). Biol Reprod 62:1329–1334.

Nagahama Y, Yoshikuni M, Yamashita M, Tokumoto T, Katsu Y. 1995. Regulation of oocyte growth and maturation in fish. Curr Top Dev Biol 30:103–145.

Ng Y, Wolfe A, Novaira HJ, Radovick S. 2009. Estrogen regulation of gene expression in GnRH neurons. Mol Cell Endocrinol 303:25–33.

Oka Y. 2009. Three types of gonadotropin-releasing hormone neurons and steroidsensitive sexually dimorphic kisspeptin neurons in teleosts. J Neuroendocrinol 21:334–338.

Okubo K, Amano M, Yoshiura Y, Suetake H, Aida K. 2000. A novel form of gonadotropin-releasing hormone in the Medaka, *Oryzias latipes*. Biochem Bioph Res Commun 276:298–303.

Okuzawa K, Kobayashi M. 1999. Gonadotropin-releasing hormone neuronal systems in the teleostean brain and functional significance. In: Prasada R, Peter RE, editors. Neural regulation in the vertebrate endocrine system. New York: Kluwer Academic Publisher/ Plenum Press. p 85–100.

Okuzawa K, Gen K, Bruysters M, Bogerd J, Gothilf Y, Zohar Y, Kagawa H. 2003. Seasonal variation of the three native gonadotropin-releasing hormone messenger ribonucleic acids levels in the brain of female red sea bream. Gen Comp Endocrinol 130:324–332.

Okuzawa K. 2002. Puberty in teleosts. Fish Physiol Biochem 26: 31–41.

Pati D, Habibi HR. 1998. Presence of salmon GnRH and compounds with GnRH-like activity in the ovary of goldfish. Endocrinology 139:2015–2024.

Pati D, Habibi HR. 2000. Direct action of GnRH variants on goldfish oocyte meiosis and follicular steroidogenesis. Mol Cell Endocrinol 160:75–88.

Penlington MC, Harden N, Garside J, Hoole D, Arme C. 1998. Isolation and characterization of mRNA encoding the chicken-II type gonadotropin-releasing hormone (GnRH) precursor in the rainbow trout. J Fish Biol 53:614–623.

Pham KX, Amano M, Amiya N, Kurita Y, Shimizu A, Yamamori K. 2007. Immunohistochemical localization of three GnRH systems in brain and pituitary of Japanese flounder. Fish Sci 73:1113–1122.

Powell JFF, Fischer WH, Park M, Craig AG, Rivier JE, White SA, Francis RC, Fernald RD, Licht P, Warby C, Sherwood NM. 1995. Primary structure of solitary forms of gonadotropin-releasing hormone (GnRH) in cichlid pituitary; three forms of GnRH in brain of cichlid and pumpkinseed fish. Regul Pept 57:43–53.

Rissman EF. 1996. Behavioral regulation of gonadotropin-releasing hormone. Biol Reprod 54:413–419.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108.

Senthilkumaran B, Okuzawa K, Gen K, Ookura T, Kagawa H. 1999. Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. J Neuroendocrinol 11:181–186.

Servili A, Le PY, Leprince J, Caraty A, Escobar S, Parhar IS, Seong JY, Vaudry H, Kah O. 2011. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. Endocrinology 152:1527–1540.

Servili A, Lethimonier C, Lareyre JJ, López-Olmeda JF, Sánchez-Vázquez FJ, Kah O, Muñoz-Cueto JA. 2010. The highly conserved gonadotropin-releasing hormone-2 form acts as a melatonin-releasing factor in the pineal of a teleost fish, the European sea bass *Dicentrarchus labrax*. Endocrinology 151:2265–2275.

Shahjahan M, Hamabata T, Eiji M, Doi H, Ando H. 2010. Differential expression of three types of gonadotropin-releasing hormone genes during the spawning season in grass puffer, *Takifugu niphobles*. Gen Comp Endocrinol 167:153–163.

Shi Y, Zhang Y, Li S, Liu Q, Lu D, Liu M, Meng Z, Cheng CHK, Liu X, Lin H. 2010. Molecular identification of the Kiss2/Kiss1ra system and its potential function during 17 alpha-methyltestosterone-induced sex reversal in the orange spotted grouper, *Epinephelus coioides*. Biol Reprod 83:63–74.

Soga T, Ogawa S, Millar RP, Sakuma Y, Parhar IS. 2005. Localization of the three GnRH types and GnRH receptors in the brain of a cichlid fish: insights into their neuroendocrine and neuromodulator functions. J Comp Neurol 487:28–41.

Soga T, Sakuma Y, Parhar IS. 1998. Testosterone differentially regulates expression of GnRH messenger RNAs in the terminal nerve, preoptic and midbrain of male tilapia. Brain Res Mol Brain Res 60:13–20.

Somoza GM, Lescheid D, Miranda LA, Lo Nostro FL, Magliulo-Cepriano L, Montaner AD, Schreibman MP, Rivier JE, Sherwood NM. 2002b. Expression of pejerrey gonadotropin-releasing hormone (pjGnRH) in three orders of fish. Biol Reprod 67:1864–1871.

Somoza GM, Miranda LA, Strobl-Mazzulla P, Guilgur LG. 2002a. Gonadotropin-releasing hormone (GnRH): from fish to mammalian brains. Cell Mol Neurobiol 22:589–609.

Suzuki M, Hyodo S, Kobayashi M, Aida K, Urano A. 1992. Characterization and localization of mRNA encoding the salmon-type gonadotrophin-releasing hormone precursor of the masu salmon. J Mol Endocrinol 9:73–82.

Takaaki K. 2005. Studies on techniques for reliable production of seed of barfin flounder *Verasper moseri*. Sci Rep Hokkaido Fish Exp Stn 69:1–116.

Uzbekova S, Lareyre JJ, Guiguen Y, Ferriere F, Bailhache T, Breton B. 2001. Expression of sGnRH mRNA in gonads during rainbow trout gametogenesis. Comp Biochem Physiol 129B:457–465.

Uzbekova S, Lareyre JJ, Madigou T, Davail B, Jalabert B, Breton B. 2002. Expression of prepro-GnRH and GnRH receptor messengers in rainbow trout ovary depends on the stage of ovarian follicular development. Mol Reprod Dev 62:47–56.

Von Schalburg K, Sherwood NM. 1999. Regulation and expression of gonadotropin-releasing hormone gene differs in brain and gonads in rainbow trout. Endocrinology 140:3012–3024.

Yahalom D, Chen A, Ben-Aroya N, Rahimipour S, Kaganovsky E, Okon E, Fridkin M, Koch Y. 1999. The gonadotropin-releasing hormone family of neuropeptides in the brain of human, bovine and rat: identification of a third isoform. FEBS Lett 463:289–294.

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. 2010. Neuroendocrinology of reproduction in teleost fish. Gen Comp Endocrinol 165:438–455.