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Molecular cloning and characterization of gonadotropin subunits (GTHα, FSHβ and LHβ) and their regulation by hCG and GnRHa in Japanese sea bass (*Lateolabrax japonicas*) in vivo

Mei L. Chi · Meng Ni · Ji F. Li · Feng He · Kun Qian · Pei Zhang · Sen H. Chai · Hai S. Wen

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Abstract In this study, three cDNA sequences encoding common glycoprotein α subunit (GTHα), follicle-stimulating hormone β subunit (FSHβ) and luteinizing hormone β subunit (LHβ) were isolated from Japanese sea bass (*Lateolabrax japonicas*). Comparison of the deduced amino acid sequences with other gonadotropic hormones (GTHs) indicated that their cysteine residues and potential N-linked glycosylation sites were highly conserved, and high homology with those of other perciformes was showed in phylogenetic analysis. GTHs transcripts were present highly in the pituitary and brain and weakly in testis and other tissues. During testicular development, GTHs transcriptional levels in pituitary and brain (expect FSHβ subunit in brain) were significantly increased at spermiation period, stage V. Subsequently, the effects of hCG and GnRHa on the mRNA levels of GTHs subunits were examined. In brain, both hormones were detected to improve the expression of GTHα subunit mRNA. In pituitary, three GTHs subunits increased parallelly and abruptly in two hormone treatment groups. In testis, hCG was suggested to improve three GTHs subunits expression in Japanese sea bass for the first time. These results suggest that both gonadotropins are probably involved in the control of Japanese sea bass spermatogenesis and provide a framework for better understanding of the mechanisms of hormone-mediated reproduction control in Japanese sea bass and other teleosts.

Keywords Japanese sea bass · Gonadotropin subunits · Testis · hCG and GnRHa administration

Abbreviations

GTH	Gonadotropic hormone
FSH	Follicle-stimulating hormone
LH	Luteinizing hormone
hCG	Human chorionic gonadotropin
GnRHa	Gonadotropin-releasing hormone analogue
M-MLV	Moloney murine leukemia virus
PS	Physiological saline
PGC	Primordial germ cell
PMSG	Pregnant mare serum gonadotropin
CDS	Coding sequence
NCBI	National Center for Biotechnical Information

Introduction

The gonadotropins (follicle-stimulating hormone, FSH and luteinizing hormone, LH) are critical modulators of gametogenesis and gonadal steroidogenesis in

M. L. Chi · M. Ni · J. F. Li · F. He · K. Qian · P. Zhang · S. H. Chai · H. S. Wen (✉)
Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao 266003, China
e-mail: wenhaishen@ouc.edu.cn

almost all vertebrates. Both FSH and LH are heterodimers composed of a common alpha glycoprotein subunit (GTHα) and a unique β subunit (FSHβ or LHβ subunit) (Pierce and Parsons 1981). These three subunits are reported to carry O- and N-linked oligosaccharide chains, cysteine residues and highly conserved N-linked glycosylation sites, which are all important in hormone bioactivity (Guzmán et al. 2009a, b; Kamei et al. 2003).

Studies in salmonids have revealed that FSH and LH are differentially synthesized and released during the reproductive cycle (Schulz et al. 2001). FSH mediates vitellogenesis and spermatogenesis, which is higher at the beginning of the reproductive cycle and declines during final maturation (Prat et al. 1996), while LH regulates final gonadal maturation and spawning (Breton et al. 1998; Gomez et al. 1999). However, in some non-salmonids, including red seabream, *Pagrus major* (Gen et al. 2000); striped bass, *Morone saxatilis* (Hassin et al. 2000); and Japanese flounder, *Paralichthys olivaceus* (Kajimura et al. 2001), both FSHβ and LHβ mRNA levels fluctuate in parallel, and FSHβ is actively synthesized during gonadal maturation in these multiple spawners. This parallel fluctuation has been deduced that asynchronous gametogenesis takes place in the gonad at the same time (Levavi-Sivan et al. 2010).

Expression of GTHs subunits is regulated by various endocrine factors, including several neurohormones such as GnRH (Hassin et al. 1998), dopamine (Aizen et al. 2005) and kisspeptin (Kitahashi et al. 2009); gonadal steroids (Khan et al. 1999; Klenke and Zohar 2003); and some nonsteroidal factors (Yuen and Ge 2004). For a long time, the pituitary is considered as the only tissue where the gonadotropins reproduced and released, whereas increasing evidences have been gathered, indicating that other tissues can also produce FSHβ and LHβ subunits, such as brain of Nile tilapia (*Oreochromis niloticus*) (Parhar et al. 2003) and oocyte of gilthead seabream and hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) (Wong and Zohar 2004; Yaron et al. 2001), and brain, kidney and liver of zebrafish (*Danio rerio*) (So et al. 2005). In gonad, many works mainly focus on the fish ovary, except GTHα subunit in red drum testis (*Sciaenops ocellatus*) (Cohn et al. 2010), FSHβ and LHβ subunits in zebrafish testis (So et al. 2005) and three subunits in marbled eel (*Anguilla marmorata*) testis (Huang et al. 2009). Furthermore, the detections of some testis local regulatory factors, such as GnRH, kisspeptin and their receptors (Biran et al. 2008), also suggest that GTHs probably exist in fish testis and may play an important role in testicular development.

Japanese sea bass (*Lateolabrax japonicas*) is a highly priced fish for aquaculture in parts of East Asia countries including China. Puberty is attained at 2–3 years of age for male and at 3–4 years for female, and the fish presents an asynchronous type of gonadal development (Zhang and Li 2005). Considering of the sharp decline in population size of wild mature Japanese sea bass and the asynchronous of female and male gonadal development in cage culture condition, it is always difficult to get enough mature fish for their artificial breeding. Human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH) analogue-GnRHa have been employed to accelerate gamete maturation in many fish, such as coho salmon (*Oncorhynchus kisutch*) (Larsen and Swanson 1997), rainbow trout (*Oncorhynchus mykiss*) (Breton et al. 1998) and black porgy (*Acanthopagrus schlegeli*) (Choi et al. 2007). Furthermore, male fish has short development cycle and ready availability of animal. Therefore, hCG and GnRHa were chosen to administrate the testis development of Japanese sea bass. Hence, the objectives of present study were to: (1) isolate and characterize Japanese sea bass GTHα, FSHβ and LHβ subunits, (2) investigate the expression pattern of corresponding mRNA in the brain, pituitary and testis at different development stages, and (3) examine effects of exogenous hCG and GnRHa administration on GTHs mRNA in brain, pituitary and testis in maturing fish.

Materials and methods

Animal treatment and sampling

Seventy-one individuals of male Japanese sea bass samples (body weight 671.21 ± 75.25 g and body length 37.78 ± 1.57 cm) were obtained from Shandong coastal area in November 2011 and acclimatized in 16 pools for 3 days prior to experiment. They were reared in natural sea water under controlled conditions (temperature 17.5 ± 0.7°; salinity 29.3 ± 0.8 ‰; dissolved oxygen >6 mg/l; 13 h light: 11 h dark cycle). Then, fish were randomly divided into three groups (23 fish in each group). Two treatment groups

were intraperitoneal injected with GnRHa and hCG at 3.5 μg/kg body weight and 1,000 units/kg body weight, respectively (Ningbo, China) (Dabrowski et al. 1994; Zhang et al. 2001), while the control group injected with physiological saline solution (PS, 0.7 % NaCl). Four male fish for each group were anesthetized in 100 mg/L tricaine methane sulfonate (MS-222, Sigma, St.Louis, MO) at 0, 6, 12, 24 and 48 h. After treatments, 13 tissues (including testis, liver, stomach, gills, heart, spleen, kidney, head kidney, intestine, caecus, brain, pituitary and muscle) were quickly removed under sterile condition; testes were sectioned into two parts, one was fixed in Bouin's solution for hematoxylin and eosin (HE) staining in order to identify the development stage and the other one was snap-frozen in liquid nitrogen along with other tissues.

For the research of testicular development cycle, six male Japanese sea bass were obtained every month during periods of spawning season (September–December, 2011), acclimatized in laboratory and anesthetized with MS-222. All 13 tissues were collected as described above.

Total RNA extraction and reverse transcription (RT)

Total RNA was extracted from tissue samples using RNAiso reagent (Takara, Japan) according to the manufacturer's protocol. Briefly, tissues were homogenized in RNAiso, precipitated isopropanol and washed in 75 % ethanol. After DNase treatment, the concentration and purity of each sample were quantified by the Nucleic acid analyzer, Biodropsis BD–1000 (OSTC, China), and a 1.5 % agarose gel was applied to detect RNA integrity. The reverse transcription of 2 μL total RNA was carried out using M-MLV Reverse Transcription Kit (Promega, USA), and the resulting first-strand cDNAs were used as templates.

Cloning and characterization of the coding sequences of Japanese sea bass GTHs genes

For the purpose of obtaining the coding sequences of GTHs genes from Japanese sea bass, three pairs of primers were designed (P1, P2 for GTHα; P3, P4 for FSHβ; and P5, P6 for LHβ, Table 1) according to previously reported GTHs sequences in teleost. PCR product was electrophoresed, purified using an Agarose Gel DNA Purification Kit (Tiangen, China), then cloned into pGEM-T vector followed by propagation in *Escherichia coli* DH5α, and subsequently sequenced the positive clones to get the nucleotide information. Blasting in National Center for Biotechnical Information (NCBI), it revealed that the cloned fragments shared high homologies with GTHs from other teleosts. Multi-sequences with deduced amino acid sequences of GTHs gene were gained from NCBI and aligned using Clustal W. In addition, MEGA 5.0 software package was applied to construct and analyze phylogenetic tree using the UPGMA method with 1,000 bootstrap trials. SignalP 4.1 was applied to predict signal peptide sequences and cleavage sites (Bendtsen et al. 2004) and NetNGlyc 1.0 (Julenius et al. 2005) program to analyze putative N-linked glycosylation sites, respectively.

Tissue distribution analysis

Total RNA was extracted from tissues (including testis, liver, stomach, gills, heart, spleen, kidney, head kidney, intestine, caecus, brain, pituitary and muscle) from three male fish collected in December. Their testes were all at stage V. Total RNA of these tissues was extracted and reverse transcribed as described above. Gene-specific primers (P7–P12) and the *18S* rRNA (internal control gene) primers (P13–P14) are listed in Table 1. They were optimized for concentration and annealing temperature to obtain apposite standard curve; the amplification efficiencies were between 95 and 105 %. Experiment was operated on Roche 480 light cycler System with SYBR green (TAKARA, Japan).

Quantitation of GTHs subunits mRNA during testicular development and hCG/GnRH administration in vivo

The expression patterns of four genes (GTHs and *18S*) were analyzed in the brain, pituitary and testis at four different development stages (stage II–V) and these three tissues from hCG/GnRH administration by qPCR. cDNAs were obtained following the protocol described above. The primers used are summarized in Table 1 (P7–P14). Each qRT-PCR was carried out in triplicate with the SYBR green on Roche 480 light cycler System. The thermal cycling parameters were

Table 1 Primers used for cDNA isolation and quantitative RT-PCR of GTHs subunits from Japanese sea bass

Primer name	Usage	Primer code	Sequence(5′ → 3′)
α-clone-for	Cloning CDS	P1	TCTCTCAACATGGTAACTGCTGCAAC
α-clone-rev	Cloning CDS	P2	TCATATCTTGTGGAAATAGCAGGTGC
fsh-clone-for	Cloning CDS	P3	GACGATGCAGCTGGTTGTCATGG
fsh-clone-rev	Cloning CDS	P4	CAGGTTTCTTTAAAAGGACAGACAGC
lh-clone-for	Cloning CDS	P5	CCAGAGAGRATGATGGCTGTRCAGGC
lh-clone-rev	Cloning CDS	P6	TTGAGACTAGTAGTARAAAGGTATGTC
α-expre-for	RT-PCR/q-PCR	P7	AAACATGGGCTGTGAGGAGT
α-expre-rev	RT-PCR/q-PCR	P8	CGGGATCGTCATTGTCTTCA
fsh-expre-for	RT-PCR/q-PCR	P9	CCAACCAACATCAGCATCCC
fsh-expre-rev	RT-PCR/q-PCR	P10	CCCACTGGACATCCTTGAATG
lh-expre-for	RT-PCR/q-PCR	P11	GAGTTTGTTTCTGGGAGCCTC
lh-expre-rev	RT-PCR/q-PCR	P12	TGGTTGTTTCCACTGGGTGA
18S-expre-for	RT-PCR/q-PCR	P13	GGGTCCGAAGCGTTTACT
18S-expre-rev	RT-PCR/q-PCR	P14	TCACCTCTAGCGGCACAA

as follows: an initial of activation at 95° for 2 min, followed by 40 cycles of 95° for 15 s; optimized temperatures for each gene for 15 s, 72° for 15 s; and a dissociation curve was produced starting from 55° (+1°/30 s) to 95°. After the PCR program, $2^{-\Delta\Delta CT}$ method was used to yield the average fold change of the target gene.

Statistical analysis

All data were expressed as mean ± standard error of the means (SEM). Differences between means were tested by one-way ANOVA followed by Duncan's multiple range tests using SPSS 13.0. Samples in testicular development cycle were relative to that of stage II, and samples from the hCG/GnRH-injected groups were expressed relative to those of initial point (0 h). In all cases, significance was accepted at $P < 0.05$.

Results

cDNA cloning of Japanese sea bass GTHs subunits

The complete coding sequences of gonadotropin subunits (GTHα, FSHβ and LHβ) were determined from the pituitary of Japanese sea bass. The GTHα subunit coding region of 372 nucleotides encoded a mature peptide of 124 amino acids, which was preceded by a signal peptide of 30 amino acids. The location of the 10 cysteine residues and two potential N-linked glycosylation sites were highly conserved (Fig. 1a). The predicted mature Japanese sea bass FSHβ peptide consisted of 120 amino acids. It was preceded by a signal peptide of 18 amino acids. The LHβ subunit coded as a mature peptide of 148 amino acids, which was preceded by a 33 amino acids signal peptide. Furthermore, 12 cysteine residues and one N-linked glycosylation site were conserved in Japanese sea bass FSHβ and LHβ subunits (Fig. 1b, c). Three GTHs subunits were submitted to NCBI with gene accession numbers: **AFN73131.1**, **AFN02626.1** and **AFN02625.1** for GTHα, FSHβ and LHβ subunits, respectively.

Sequence analysis of Japanese sea bass GTHs subunits

In Table 2, the mature peptides of Japanese sea bass GTHs subunits showed high sequence identities (GTHα 80–92 %, FSHβ 64–84 % and LHβ 69–95 %) to the homologues of other perciformes (e.g., Taiwan snakehead, orange-spotted grouper, striped bass and sea bass) and pleuronectiform (Japanese flounder). In contrast, only moderate sequence identities were observed with salmoniformes (rainbow trout, Atlantic salmon), cypriniforms (Oriental weatherfish) and anguilliforms (European eel) (GTHα, 54–63 %; FSHβ, 37–42 %; and LHβ, 55–68 %). The identity between Japanese sea bass

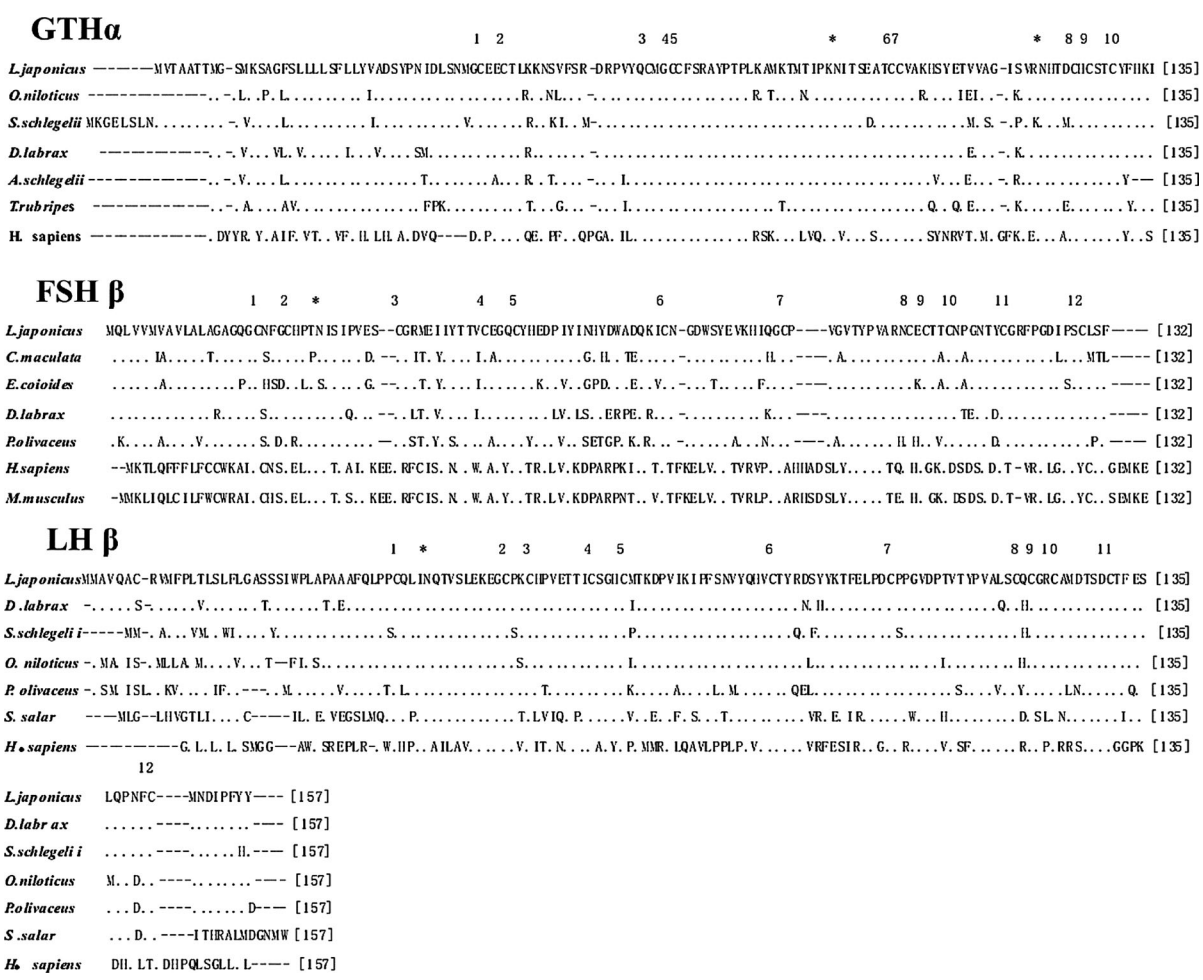

Fig. 1 Alignment of the amino acid sequence of Japanese sea bass GTH subunits to those of other vertebrates. For each subunit, the cysteine residues are numbered, and *asterisks* indicate putative N-linked glycosylation sites, respectively. Corresponding protein sequences and their Genbank accession numbers are listed in Table 2

FSHβ and LHβ mature peptides was 22 %, whereas GTHα subunit recorded similar identities to FSHβ and LHβ subunits (10 and 13 %, respectively). A phylogenetic analysis of the gonadotropin subunits observed in teleost and other species is presented in Fig. 2. These analyses revealed that Japanese sea bass GTHα, FSHβ and LHβ subunits belonged to the cluster of perciforme GTHα, FSHβ and LHβ, respectively.

mRNA expression of GTHs subunits in various tissues of male Japanese sea bass

The presence of GTHα, FSHβ and LHβ subunits mRNAs in different tissues of Japanese sea bass was investigated by qPCR (Fig. 3). Analysis revealed that Japanese sea bass GTHs transcripts expressed highly in the pituitary, brain and weakly in testis. In addition, slightly generate GTHα subunit mRNA in kidney and stomach and very weak signal of LHβ subunit in liver were also found in this study.

Changes of GTHs subunits mRNA levels in brain, pituitary and testis during the testicular development cycle

Twenty-four male Japanese sea bass were applied to test the expression profiles of GTHs subunits mRNA in brain, pituitary and testis at different stages ($N = 4$ for stage II, $N = 4$ for stage III, $N = 5$ for stage IV and $N = 11$ for stage V). In brain, the expressions of

Table 2 The identify of Japanese sea bass GTHs subunits to other vertebrate GTHs subunits

	Amino acid			NCBI Accession No.		
	GTHα	FSHβ	LHβ	GTHα	FSHβ	LHβ
Morone saxatilis	92	84	93	Q91119.2	Q91120.2	AAC38019.1
Channa maculate	91	80.8	83.1	AAP87114.1	AAS01610.1	AAS01609.1
Epinephelus coioides	90.6	80.0	95.3	ABQ57398.1	AAO31971.1	BAJ05297.1
Dicentrarchus labrax	90.6	83.3	93.2	AF269157.1	AAN40506.1	AAN40507.1
Acanthopagrus schlegelii	90.4	73.1	87.6	AAX21763.1	ADX31689.1	ABQ96864.1
Sebastes schlegelii	88.7	66.0	88.8	AAU14140.1	AAU14141.1	AAU14142.1
Oreochromis niloticus	87.2	63.7	86.2	AAP49577.1	XP_003450841.	XP_003438397
Takifugu rubripes	86.3	73.3	68.9	DAA06175.1	DAA06176.1	DAA06177.1
Paralichthys olivaceus	80.3	76.7	78.6	AAK58600.1	AAK58601.1	BAB47388.1
Oryzias latipes	66.1	65.3	67.8	BAK61760.1	ABQ08583.1	NP_001131125
Oncorhynchus mykiss	63.4	37.2	68.1	NP_001117676	NP_001118058	NP_001117677
Salmo salar	61.0	38.2	64.4	NP_001139928	AAD34594.1	NP_001167142
Mus musculus	55.0	29.2	33.3	NP_034019.1	NP_032071.1	EDL22853.1
Misgurnus anguillicaudatus	54.4	42.2	59.6	BAK39637.1	BAK39638.1	BAK39639.1
Homo sapiens	54.3	28.3	37.5	AAD13690.1	ABQ08583.1	AAL69719.1
Anguilla Anguilla	53.6	38.2	54.6	CAA43373.1	AAN64352.1	AAL37629.1
Coturnix coturnix	50.8	–	35.8	AAB30866.1	–	AAB30867.1
Alligator mississippiensis	50.4	32.9	49.3	BAJ14505.1	BAJ14506.1	BAJ14507.1
Lateolabrax japonicus GTHα	–	10.1	12.6	AFN73131.1	–	AFN02625.1
Lateolabrax japonicus FSHβ	–	–	22.3		AFN02626.1	–

GTHα and LHβ subunits mRNA were increased during stage II to V, peaking at stage V with levels of 3.5- and 2.7-fold higher than those of stage II (Fig. 4a). In pituitary, all three subunit transcripts increased parallelly and significantly from early spermatogenesis to spermiation (stage II–V), at stage V, and they were 3.5-, 2.9- and 3.3-fold higher than those of stage II ($P < 0.05$, Fig. 4b). Meanwhile, in testis, the maximum expression of these three subunits was found at stage IV, stage III and stage IV (for GTHα, FSHβ and LHβ subunit, respectively), and all subunits mRNA levels declined slightly at stage V when compared to stage IV. The significant can only be detectable at stage III for FSHβ subunit (Fig. 4c, $P < 0.05$).

Regulation of expressions of GTHs subunits in brain, pituitary and testis of Japanese sea bass by hCG/GnRHa administration

In hormone administration experiment, typical early period of stage V testes was detected in fish without any treatment, and after injecting exogenous hormone, especially hCG, large quantities of mature spermatozoa were detected in testis, and the development level had been improved greatly (data not shown). In an effort to create a comprehensive frame work of gene changed during this process, we investigated associated changes of GTHs mRNA expression in brain, pituitary and testis by qPCR.

In brain, it showed that levels of GTHα subunit in hCG and GnRHa treatments at 6 h were 6.8- and 4.2-fold over PS-injected control ($P < 0.01$ and $P < 0.05$, respectively). In the case of FSHβ subunit, its relative transcript level was significantly increased in hCG group when compared to PS- and GnRHa-treatment groups at 24 h ($P < 0.05$-, 1.7- and 1.6-fold, respectively). The mRNA level of LHβ subunit increased abruptly in GnRHa group at 6, 12 and 48 h when compared to those of PS group ($P < 0.05$), while in hCG group, the significance can only be detected at 48 h (Fig. 5).

In pituitary, all three gonadotropin subunits transcripts changed in parallel during hormone

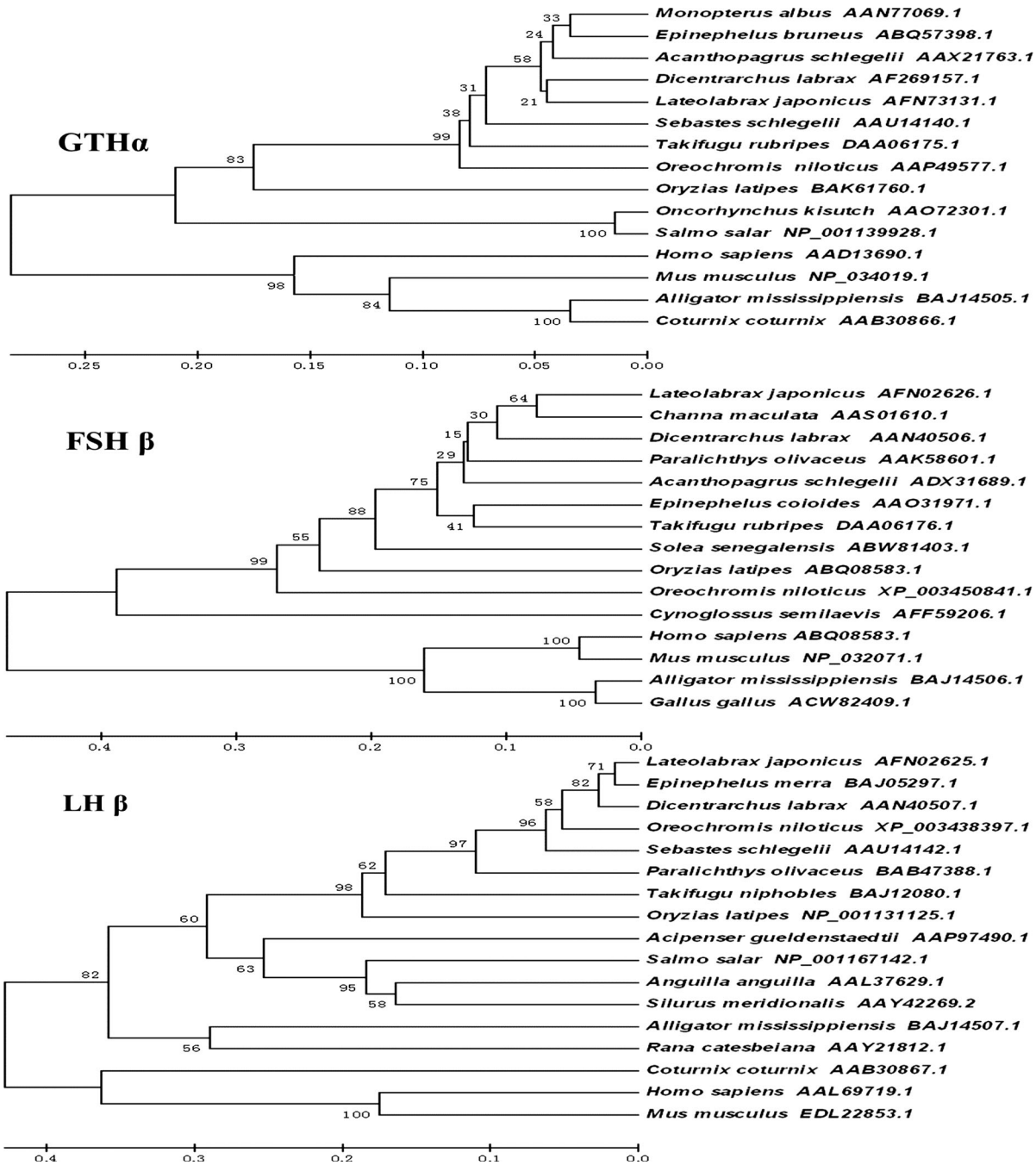

Fig. 2 Phylogenetic analyses of GTHs subunits. They are conducted in MEGA 5.0 using the UPGMA method with bootstrap values at 1,000 resampling replicates. Protein sequences used for comparison and their Genbank accession numbers are listed at the *right* of the branches

administration, extremely higher in two hormone groups than those in PS groups at 6 h ($P < 0.01$ for GTHα, FSHβ and $P < 0.05$ for LHβ). The mRNA levels of three subunits were significantly increased at 12 h except GTHα subunit in hCG group and FSHβ subunit in GnRHa group. During 24–48 h, the mRNA

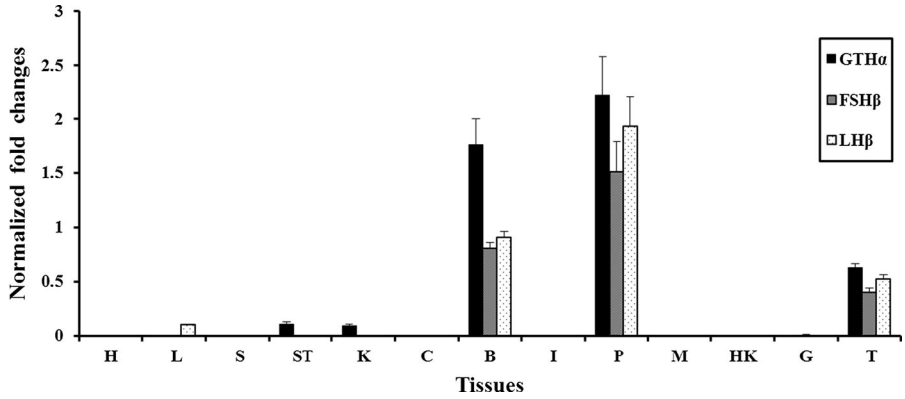

Fig. 3 Tissue expression patterns of GTHα, FSHβ and LHβ subunits in male Japanese sea bass. Gene expression profiles were analyzed by qPCR using specific primers, and *18S* ribosomal RNA was used as internal control for relative quantity. Heart (H), liver (L), spleen (S), stomach (ST), kidney (K), caecus (C), brain (B), intestine (I), pituitary (P), muscle (M), head kidney (HK), gills (G) and testis (T)

levels of three GTHs subunits in hormone groups resume to the levels of those in PS groups in addition to GTHα mRNA, which was still strongly stimulated by GnRHa, showing approximately 5.3- and 4.2-fold over PS group at 24 and 48 h, respectively ($P < 0.05$, Fig. 6).

In testis, similar trend of three GTHs subunits mRNA expression in response to hCG administration is illustrated in Fig. 7. They increased significantly at 12 h after injection, showing 3.1-, 2.0- and 2.6-fold over PS group for GTHα, FSHβ and LHβ, respectively ($P < 0.05$). Meanwhile, in GnRHa administration, GTHα subunit showed no statistical change during experiment phrase, while FSHβ subunit increased significantly at 24 and 48 h. However, the trend of LHβ subunit mRNA in GnRHa administration was similar with that of hCG group. In addition, all three subunits transcript levels remained relatively constant throughout the administration in PS-treatment groups.

Discussion

In present study, GTHα, FSHβ and LHβ cDNAs with complete ORFs were cloned from the pituitary of Japanese sea bass for the first time. Multi-alignment of these deduced amino acids and phylogenetic studies of vertebrate GTHs revealed that Japanese sea bass GTHs subunits had high homology with other vertebrates. Japanese sea bass GTHα subunit included two putative N-linked glycosylation sites and ten cysteine residues, which have been proved to involve in the processes of receptor binding and subunit assembly in vertebrates (Gen et al. 2000; Zenkevics et al. 2000). The alignment results also showed that fish GTHα subunit was more conservative than their FSHβ and most of the LHβ subunits (Table 2). Because α subunit is shared by all pituitary glycoprotein hormones, it might under higher selective pressure during evolution (Huang et al. 2009). Analysis of the primary structure of Japanese sea bass FSHβ and LHβ subunits revealed that they both contained 12 cysteine residues and one putative N-linked glycosylation site. It is reported that there were different numbers of glycosylation site in fish FSHβ subunit, like one for gilthead seabream and Senegalese sole (*Solea senegalensis*) (Wong and Zohar 2004; Mechaly et al. 2012), two for channel catfish ((*Ictaluris punctatus*) and Japanese eel (*Anguilla japonica*) (Ulloa-Aguirre et al. 1999; Swanson et al. 2003). Moreover, there was none glycosylation site for Atlantic halibut (*Hippoglossus hippoglossus*) and red-spotted grouper (*Epinephelus coioides*) (Weltzien et al. 2003; Li et al. 2005). Considering of the importance of N-linked glycosylation site on biological potency of the hormone (Swanson et al. 2003), it has been suggested that lack or absence of FSHβ glycosylation site might be supplied by GTHα subunit (Zenkevics et al. 2000). Furthermore, the primary structure of Japanese sea bass LHβ subunit appeared to be more conserving than that of FSHβ subunit (Table 2). This phenomenon has also been previously found in other fish such as sea bass and zebrafish

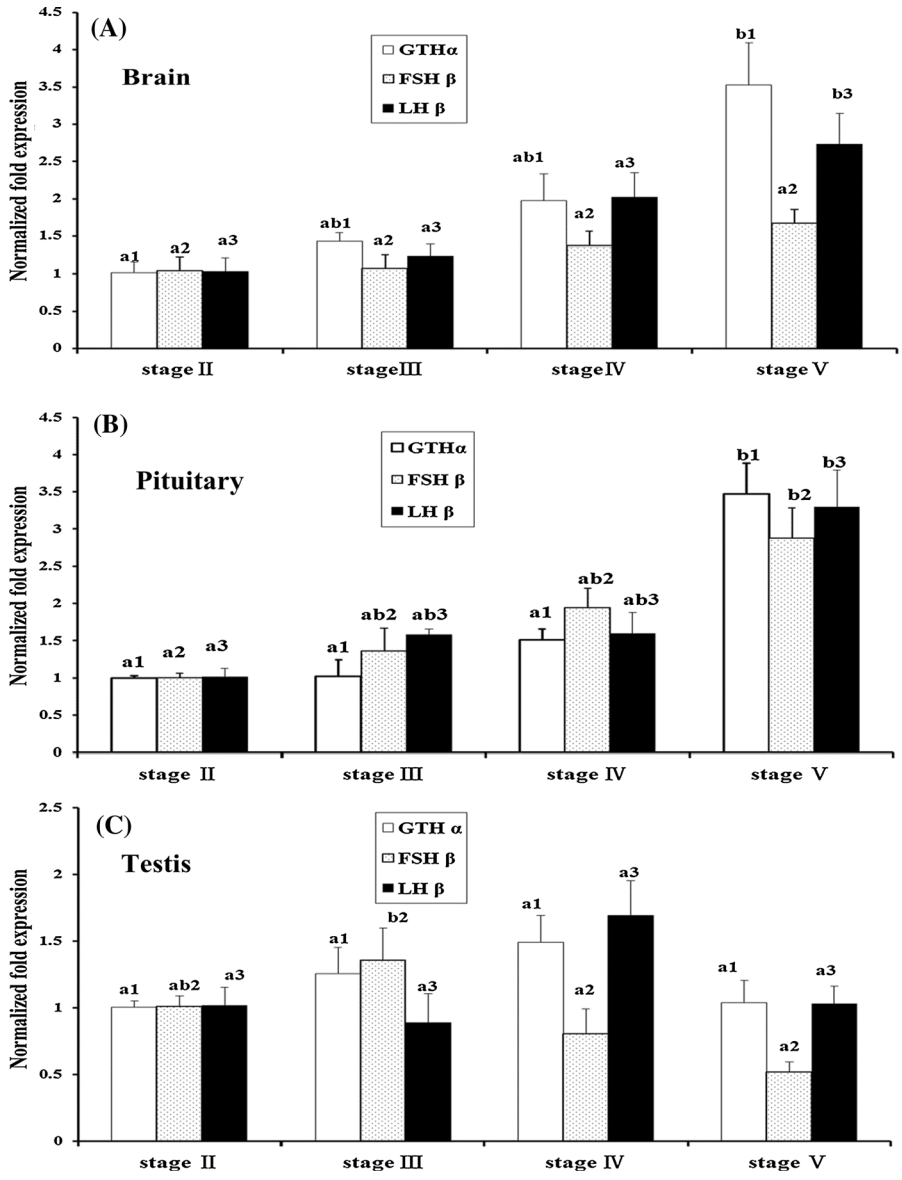

Fig. 4 mRNA expressions of GTHs subunits in brain (**a**), pituitary (**b**) and testis (**c**) of male Japanese sea bass during testicular development cycle by qPCR. *18S* ribosomal RNA is used as internal control gene. Data are expressed relative to stageII ($N = 4$ for stage II, $N = 4$ for stage III, $N = 5$ for stage IV and $N = 11$ for stage V). Values are expressed as mean ± standard error of mean. Values with different letters indicate statistical significance by one-way ANOVA, Duncan's multiple tests

(Mateos et al. 2003). It is supposed that fish FSHβ subunit diverges more rapidly than LHβ subunit during teleost evolution (Quérat et al. 2000).

Except for the pituitary, extra-pituitary expression of gonadotropin may be a common phenomenon (So et al. 2005). In marbled eel, three gonadotropin subunits mRNAs were detected in ovary, testis, brain, kidney besides the pituitary (Huang et al. 2009). Furthermore, localizations of different GTH subunits in the pejerrey testis were analyzed by in situ hybridization (Elisio et al. 2012). Many results of other fish including Japanese sea bass further support this hypothesis (Parhar et al. 2003; Pandolfi et al. 2009). However, the physiological significance of GTHs subunits in brain, testis and other tissues remains unknown and still need further investigation.

The GTHα, FSHβ and LHβ subunits cDNA were first detected in Japanese sea bass brain. Similar findings have been reported in other teleosts (Wong and Zohar 2004; Wu et al. 2009). Distribution of GTH subunits in the preoptic and hypothalamic areas of rat (Hostetter et al. 1987; Emanuele et al. 1984), South

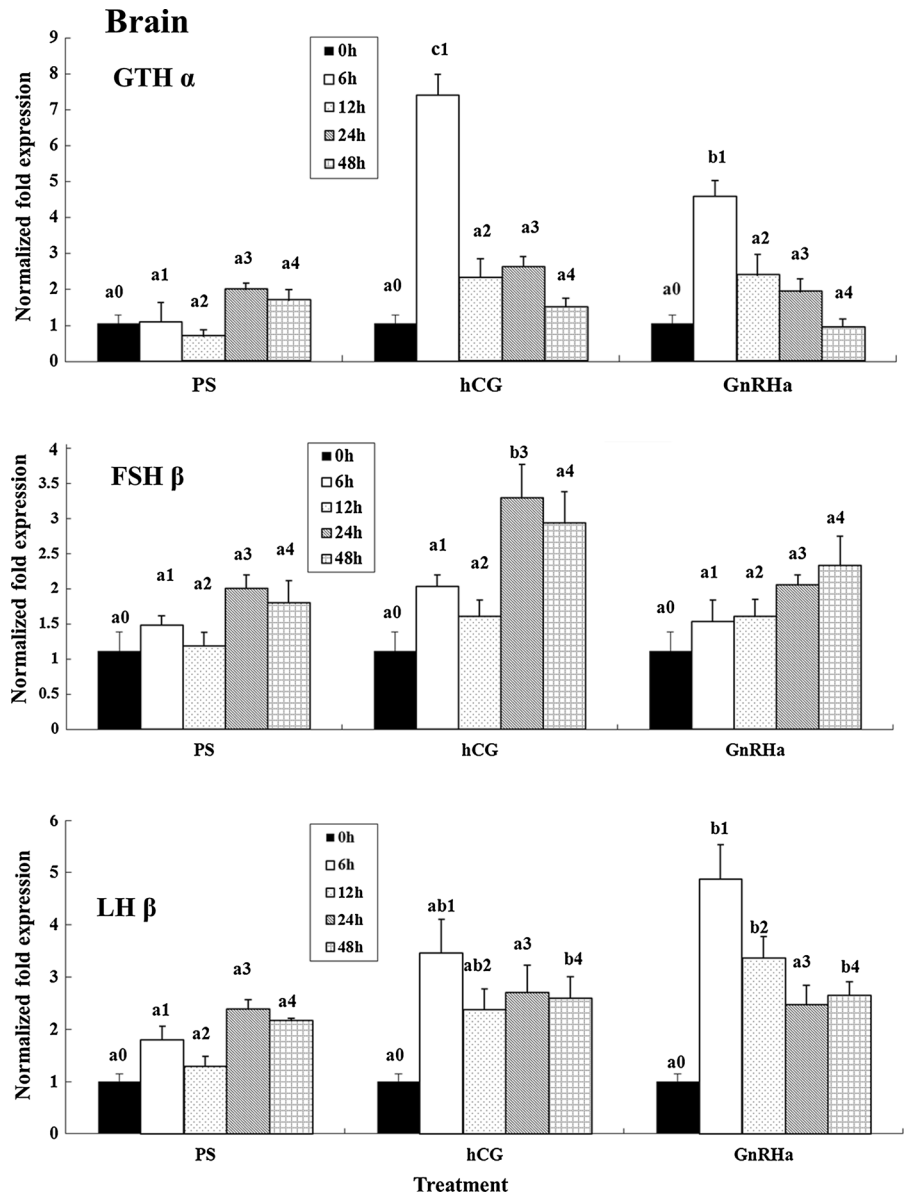

Fig. 5 Regulation of GTHα, FSHβ and LHβ subunits expression in brain of Japanese sea bass by hCG and GnRHa treatment. The expressions of these genes were analyzed by qPCR by real-time qPCR. Data are expressed relative to control, PS-injected fish (Mean ± SEM). *Different letters* are indicated significant differences between each group on same time after PS/hormones injection ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

American cichlid (*Cichlasoma dimerus*) (Pandolfi et al. 2009) and Nile tilapia (Parhar et al. 2003) has also been demonstrated by immunocytochemistry. These results suggested that FSH- and LH-producing cells may play important roles in brain as well as in pituitary. Moreover, brain-derived gonadotropins may function as hypophysiotropic hormones to regulate pituitary cells (Apaja et al. 2004). We also found the expression of GTHα and LHβ subunits dramatically increased during reproduction cycle and hormone administration, while the FSHβ subunit remained constant during these periods. This phenomenon has not been described in other fish before. It may suppose that in Japanese sea bass brain-derived FSH may not as much associate with gonadal development as brain-derived LH during maturing period. Though lacking information about the determinate effects of hCG and GnRHa on GTHs expression in fish brain, the LH and FSH in rat were proved to act on the hypothalamic level to alter secretion of gonadotropins (McCann et al. 1998). It is worth noting that the highest expression of GTHα and LHβ subunits in brain was

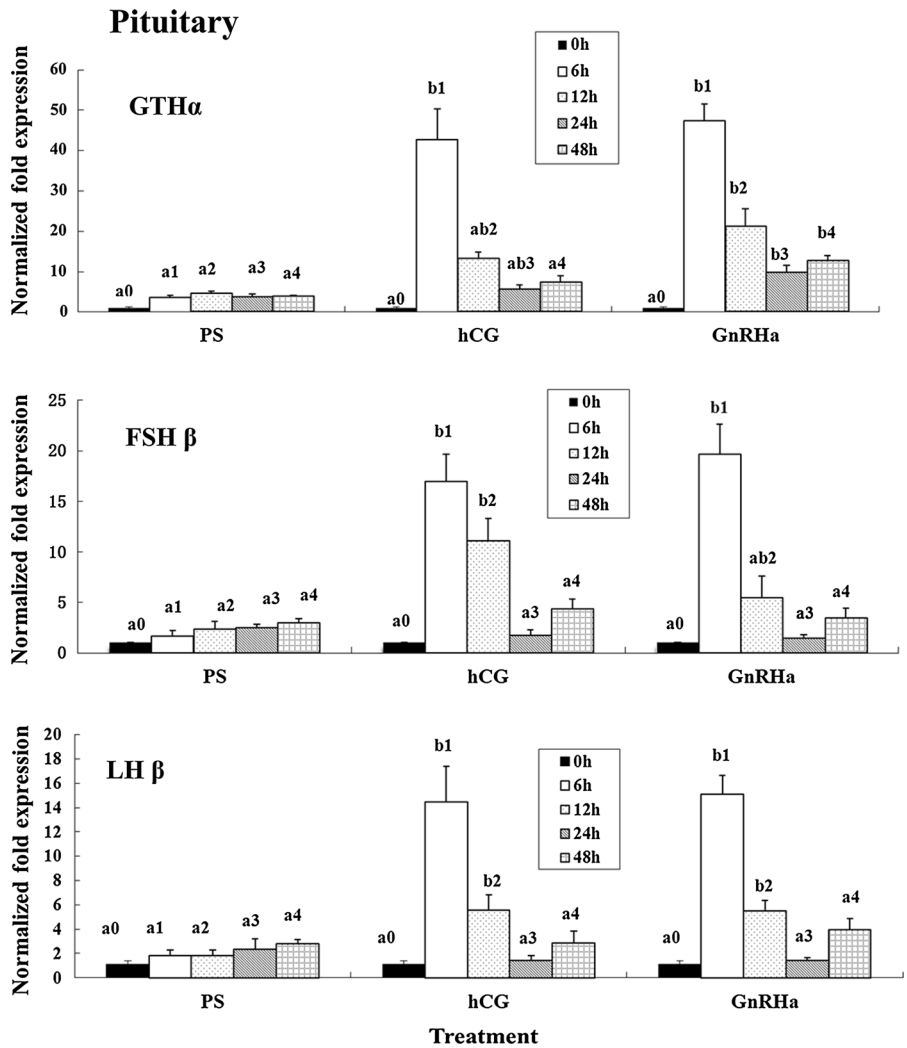

Fig. 6 Regulation of GTHα, FSHβ and LHβ subunits expressions in pituitary of Japanese sea bass by hCG and GnRHa treatment. Relative mRNA levels of Japanese sea bass GTHα, FSHβ and LHβ subunits are normalized with those of *18S* rRNA level. Data are expressed relative to control, PS-injected fish (mean ± SEM).*Different letters* are indicated significant differences between each group on same time after PS/hormones injection ($P < 0.05$, one-way ANOVA, followed by Duncan's test)

6 h earlier than that in testis, suggesting that these two hormones may directly/indirectly act on the brain and exert a stimulatory effect on brain-derived gonadotropins of Japanese sea bass.

In pituitary, all three GTHs subunits transcripts increased in parallel during testicular reproduction and hormone administration. During testicular reproduction, the mRNA levels of GTHs subunits increase in correlation with the increments of the GSI and testosterone level in serum, attaining maximum at late-spermatogenesis and spermiation period, stage V (data not shown). This profile was similar to other teleost fish, such as Japanese flounder (Kajimura et al. 2001) and red seabream (Gen et al. 2000), suggesting that these three GTHs genes may regulate steroidogenesis of Japanese sea bass. Though the GTHα subunit is expressed in both gonadotrops and thyrotrops of the pituitary, the increased GTHα mRNA expression in Japanese sea bass pituitary would use to reflect the increasing activity from gonadotrops instead of thyrotrops (Nagae et al. 1996; Huang et al. 2009; Cohn et al. 2010). Additionally, during stage II–V, FSHβ and LHβ mRNA were present equally in pituitary, which suggested that FSH, like LH, may also contribute to the regulation of final maturation and spermiation in Japanese sea bass (Gen et al. 2000; Mateos et al. 2003). High transcription of FSHβ during the spermiation period might due to the asynchronous spawner feature of Japanese sea bass; spermatogenesis occurs in the spawning season

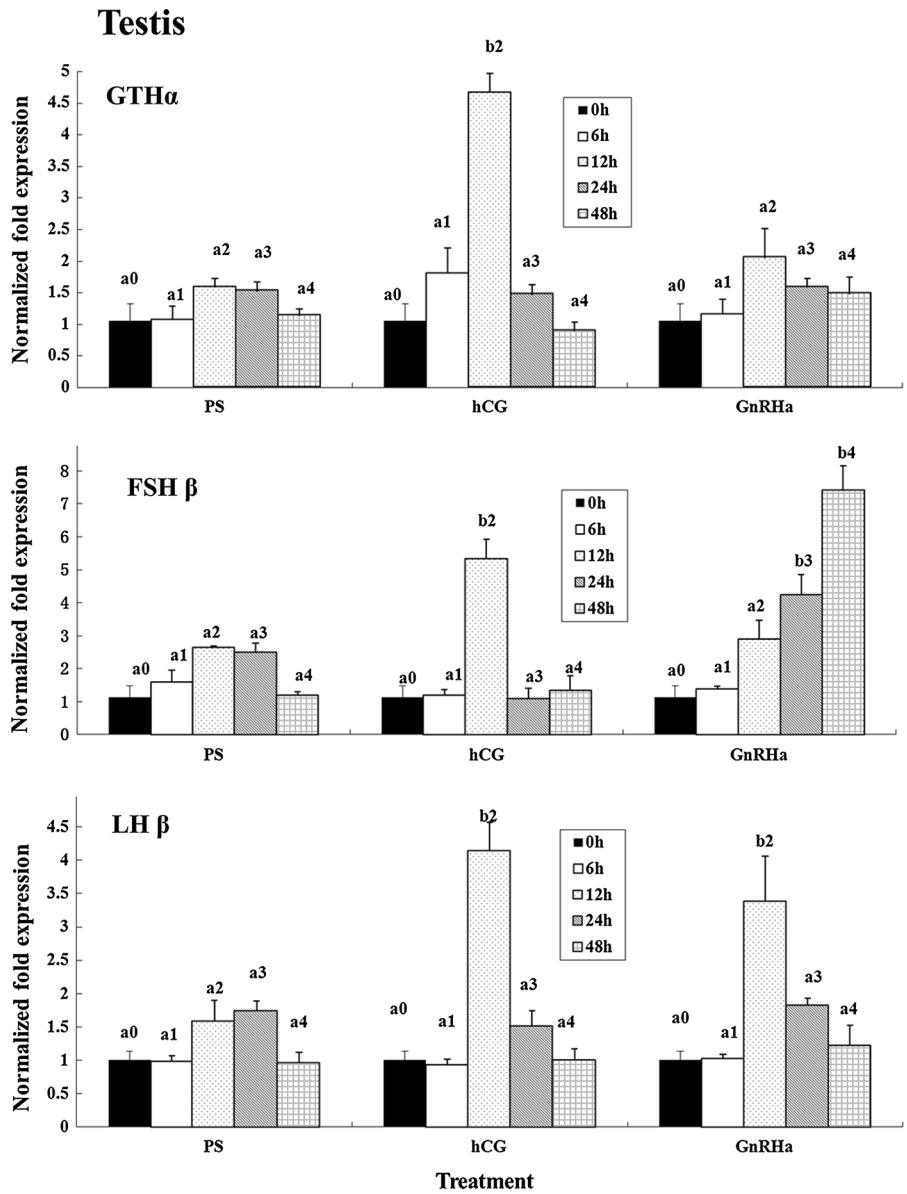

Fig. 7 Regulation of GTHα, FSHβ and LHβ subunits expressions in testis of Japanese sea bass by hCG and GnRHa treatment. Data are expressed relative to PS-injected fish. Values are Mean ± SEM. Data are subjected to an ANOVA followed by a Duncan's multiple range test. Significant differences ($P < 0.05$) in each series are denoted by *different letters*

simultaneously with spermiation (Levavi-Sivan et al. 2010). GnRH has been shown to significantly alter the expression of gonadotropin subunits mRNA in many teleost pituitaries (Yaron et al. 2003), and there are many different effects in the control of two gonadotropins. The increments of GTHα and LHβ mRNA expression were found after GnRHa treatment in sockeye salmon (Ando and Urano 2005). In coho salmon, sGnRH resulted in increasing of GTHα and FSHβ subunits mRNA profile, but not LHβ (Dickey and Swanson 2000). While in goldfish (Klausen et al. 2002), the result was similar with our study three subunits all increased. The discrepancy between these studies may attribute to different species, reproductive phase (immature or mature) or experimental procedures. Different forms of GnRH and multiple treatment methods will also be needed to detect the determinate effects to Japanese sea bass reproduction. Though hCG, LH analogue, has been employed in spawning induction trials of many male species in aquaculture (Mayes et al. 1993; Watanabe et al. 1998), the report about its effect on pituitary GTHs subunits

expression is scant. Significantly increase of GTHs subunits found in this study may suggest that hCG can activate the brain–pituitary–gonad axis pathway in Japanese sea bass. Recently, more and more researches focused on the recombinant LH and FSH of different fish (Ko et al. 2007; Kobayashi et al. 2006; Vischer et al. 2003). These observations will also provide information about up-regulatory mechanisms underlying GTH-mediated control of reproduction in fish pituitary.

The present study demonstrated that gonadotropic hormones were synthesized in the Japanese sea bass testis for the first time. During testicular development, three GTH subunits mRNA levels declined at stage V to a certain extent. A similar phenomenon was also found in male pejerrey (*Odontesthes bonariensis*) in which three subunits expressed a less extent in spermatocytes than in spermatogonia (Elisio et al. 2012). In addition, the expression of LH mRNA decreased in the morning of estrus when compared to the LH surge on proestrus in rat ovary (Schirman-Hildesheim et al. 2008). These may due to increasing circular hormone levels, like testosterone or gonadotropins, which down-regulated endogenous testicular gonadotropins. Further, the expression of FSHR and LHR, through which FSH and LH exert their effects on gonadal functions (So et al. 2005; Mechaly et al. 2012), will be urgently needed to support this hypothesis. For GnRHa, distinct tendencies were found in this study when it manipulated testicular FSHβ and LHβ expression. Although significant increase of FSHβ mRNA expression was still needed to be confirmed at multiple molecular levels, it is suggested that GnRHa may regulate the expression of FSHβ and LHβ subunits expression through different mechanisms. It is interesting to note that the high expression of FSHβ and LHβ subunits at GnRH groups was not accompanied by the increase of GTHα subunit, suggesting that in testis FSHβ and LHβ may be expressed as monomers, which has been reported in zebrafish (So et al. 2005). In addition, in rat ovary, the free LHβ subunit has been demonstrated to bind to LHR without increasing cAMP level (Muralidhar and Moudgal 1976). The physiological significance of free GTHs subunits still need to be explored. In this study, injecting hCG significantly improved three subunits mRNA expression at 12 h. Although significant reduction in ovarian LH mRNA abundance after pregnant mare serum gonadotropin (PMSG) administration was described in rat (Schirman-Hildesheim et al. 2008), this may also indirectly indicate that the gonadal LH was homologously regulated. It is worth noting that the highest GTHs mRNA in testis was 6 h later when compared with those in brain and pituitary. It may due to the feedback mechanism between the brain and/or pituitary and the local testicular hormones. These observations provided novel information on regulatory mechanisms underlying hCG- and GnRH-mediated control of reproduction in Japanese sea bass testis. It is possible that testis may possess a greater degree of autonomy than we have conceived (Schirman-Hildesheim et al. 2008).

In summary, the complete CDS of GTHα, FSHβ and LHβ subunits were cloned and characterized from Japanese sea bass pituitary. In addition to the pituitary, they also appeared to be expressed in many extra-pituitary tissues especially brain and testis. Determination of the significant changes of GTHs subunits expressions in the brain, pituitary and testis during the reproductive cycle suggested that they may play an important role in testicular development and maturation. Subsequently, this study found positive effects of hCG and GnRHa on GTHs subunits expression in Japanese sea bass brain, pituitary and testis. These findings enhanced our understanding of hormonal regulation of reproduction in Japanese sea bass. Additional studies will be necessary to determine the precise roles of the non-pituitary GTHs in gonadal development. In addition, the role of other factors in the regulation of GTHs biosynthesis would be instrumental in further elucidating.

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