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Unique Expression of Gonadotropin-I and -II Subunit Genes in Male and Female Red Seabream (*Pagrus major*) During Sexual Maturation[1]

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ABSTRACT

Two distinct gonadotropins (GTHs) have been demonstrated in a number of teleost fishes. Although the physiological roles of GTHs have been extensively studied in salmonids, little is known about their biological functions in nonsalmonid fishes. In this study, to elucidate the role of GTH-I and GTH-II in reproduction, we cloned the α-glycoprotein subunit (αGSU) and gonadotropin β subunits (Iβ and IIβ) of red seabream using the 5′- and 3′-RACE methods and used these cDNA probes to reveal changes in mRNA levels of each subunit during sexual maturation of both male and female red seabream. The nucleotide sequences of αGSU, Iβ, and IIβ are 629, 531, and 557 base pairs long, encoding peptides of 117, 120, and 146 amino acids, respectively. The deduced amino acid sequence of each mature subunit showed high homology with those of other teleosts. Northern blot analysis showed that Iβ mRNA levels of males increase in association with gonadal development, whereas those of females remain low throughout sexual maturation, indicating sexual dimorphism in the expression pattern of Iβ. In contrast, IIβ mRNA levels of both sexes are maintained at high levels from the beginning of gametogenesis to spawning season. These results are different than those of salmonids and suggest that GTH-I may have important roles in male, but not female, gametogenesis. GTH-II may be involved in regulation of early and late gametogenesis in both male and female red seabream.

anterior pituitary, FSH, LH, seasonal reproduction

INTRODUCTION

Gonadotropins (GTHs), LH and FSH, are critical modulators of gametogenesis and gonadal steroidogenesis in almost all vertebrates, including teleosts. These glycoprotein hormones contain a common α-glycoprotein subunit (αGSU) that heterodimerizes with unique β subunits ([1, 2] for reviews). In many teleost species, biochemical analysis [3–5] and molecular cloning studies [6–8] have demonstrated that there are two distinct GTHs: namely, GTH-I, which is homologous to FSH; and GTH-II, which is homologous to LH ([9] for a review). In salmonids, plasma levels of GTH-I are elevated during vitellogenesis and spermatogenesis; they decline during final maturation and ovulation in females and during spermiation in males. GTH-II levels are low during the early stages of the reproductive cycle; they increase during final maturation and ovulation in females and during spermiation in males [10]. Both GTHs are equipotent in stimulating estradiol-17β (E_2) production, but GTH-II is more potent than GTH-I in stimulating maturation-inducing steroid, DHP (17α, 20β-dihydroxy-pregnen-3-one [11]). These findings tend to suggest that, in salmonids, GTH-I plays a significant role in vitellogenesis and spermatogenesis, whereas GTH-II is important for final maturation and ovulation or spermiation; however, very little is known at present about the involvement of GTHs, especially GTH-I, in reproduction of fish species other than salmonids [12, 13].

Red seabream, *Pagrus major* (order: Perciformes), provides an interesting and unique model to study the physiological roles of GTHs in fish reproduction. In contrast to salmonids, red seabream have an asynchronous-type ovary and spawn almost every day during the spawning season. Previous reports from our laboratory have shown that, in vitro, the biological activity of GTH-I is much lower than that of GTH-II in production of E_2 by vitellogenic follicles of red seabream [14], although GTH-I has a similar potency to that of GTH-II in stimulating in vitro 11-ketotestosterone (11-KT) production from testis slices of mature male fish [15]. In addition, more recent studies have demonstrated that GTH-II, not GTH-I, induces final maturation of oocytes of red seabream. These in vitro data raise the possibility that there may be functional differences between perciform and salmonid GTHs in regulation of reproduction, especially with the biological activity of GTH-I, and that sexual dimorphism may exist in the pattern of synthesis of GTHs between male and female red seabream.

In this study, to elucidate the roles of GTH-I and GTH-II in the reproduction of both male and female red seabream, the expression profile of GTH subunit genes was studied during sexual maturation using cDNA probes generated through a reverse transcription-polymerase chain reaction (RT-PCR) based cloning technique using degenerate oligonucleotide primers corresponding to the partial amino acid sequences of GTH subunits. We provide evidence showing a pattern of GTH subunit gene expression that is different from that of salmonids, and also a sexually dimorphic pattern of gene expression of Iβ during sexual maturation in male and female red seabream.

MATERIALS AND METHODS

Animals

Red seabream, reared under natural conditions in net pens in Gokasho Bay, Nansei, Mie, Japan, were collected in February, March, April, and June 1997, times that cor-

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responded to maturing (February–March), spawning (April–May), and regressed (June–July) phases. Fish were deeply anesthetized with 2-phenoxyehanol (Nacalai Tesque, Kyoto, Japan) before being killed by decapitation. The pituitary glands were removed and immediately frozen in liquid nitrogen and then stored individually at −80°C until extraction of mRNA. The body weight and length of each fish were recorded before dissection. The gonads were excised and weighed for calculation of the gonadosomatic index (GSI; [gonad weight/body weight] × 100%), then a small piece of the gonads was fixed in Bouins fixative for histological identification of maturation stage.

Amino Acid Sequences Analysis

Red seabream GTH-I and GTH-II were isolated from pituitary glands as previously described [5]. Separation of αGSU and β subunits was performed by reverse-phase (rp) HPLC [16]. Partial amino acid sequences of GTH subunits were determined by the method of Ito et al. [17]. Each of the purified subunits was treated with neuraminidase to remove sialic acid. Subsequently, the enzymatically modified subunits were reduced and carboxymethylated (RCM). Each RCM subunit was digested separately with several endopeptidases as follows: each RCM subunit (0.5 mg) was digested with lysyl endopeptidase (LE) at an enzyme-to-substrate ratio (E/S) of 1/60 (wt/wt) in 400 μl of 0.1 M ammonium bicarbonate buffer (pH 8.0) at 37°C for 4 h, with *Staphylococcus aureus* V8 protease (SP) at an E/S of 1/60 (wt/wt) in 200 μl of 0.05 M ammonium bicarbonate buffer (pH 8.0) at 37°C for 18 h, and with *Pseudomonas fragi mutant* metalloprotease (MP) at an E/S of 1/100 (wt/wt) in 300 μl of 0.05 M ammonium bicarbonate buffer (pH 8.0) at 37°C for 12 h. Fractionation of all fragments obtained from each RCM subunit was performed by rp HPLC on a TSK gel (Tosho, Tokyo, Japan) OSD-120T column (4.6 × 250 mm) with a linear gradient of isopropanol in 0.1% trifluoroacetic acid. The primary structures of peptides were determined by automated Edman degradation using an Applied Biosystem Model 471A protein sequencer with a Model 120A on-line phenylthiohydantoin analyzer. The sequence data were compared with known protein sequences in the EMBL and SwissProt protein database by the FASTA search program [18].

Oligonucleotides

Oligonucleotides (Custom Oligo Service, Tsukuba, Japan) used as PCR primers are listed here and shown in Figure 1. The degenerate PCR primers for GTH subunit cDNAs were designed from reverse translation of a partial amino acid sequence identified in this study. Primers for 18S rRNA were based on the nucleotide sequences of the other vertebrates [19].

Primer 1. Degenerate primer (DP) for αGSU: 5′-TAY CAR TGY ATG GGN TGY TG-3′ (IUB group codes were used: R=A+G, S=C+G, Y=C+T, W=A+T, D=A+G+T, H=A+C+T, N=A+C+G+T).

Primer 2. DP for αGSU: 5′-CAR TGR CAR TCN GTR TGR TT-3′.

Primer 3. αGSU gene specific primer (α-GSP) for 5′-RACE: 5′-TGG TTT CTC ACC CTT ATG CC-3′.

Primer 4. α-GSP for 5′-RACE: 5′-TCA TAG CTG TGC TTT GCG AC-3′.

Primer 5. α-GSP for 3′-RACE: 5′-TTC TCC AGA GCG TAC CCA AC-3′.

Primer 6. α-GSP for full length: 5′-GTG AAT GAA GAG GGT ACT TTC TC-3′.

Primer 7. α-GSP for full length: 5′-CAA TCT ACA GTT AAT CCT ATA CAT CTA TC-3′.

Primer 8. DP for Iβ: 5′-ATG CCN GTN GAR WSN TGY GG-3′.

Primer 9. DP for Iβ: 5′-GCN CAN GGR TAN GTD ATN GC-3′.

Primer 10. Iβ gene specific primer (Iβ-GSP) for 5′-RACE: 5′-TTC ACC TCA TAG GAC CAG TCC-3′.

Primer 11. Iβ-GSP for 5′-RACE: 5′-GTC CTT TGT TCA GCC CAG TC 3-′.

Primer 12. Iβ-GSP for 3′-RACE: 5′-AGC AAC GAG TTC ATC CAC AC-3′.

Primer 13. Iβ-GSP for full length: 5′-GCT GGT ACA GAT GTT CAG AGA G-3′.

Primer 14. Iβ-GSP for full length: 5′-TAA GTA CCA AGT TAC CAA AGA TG-3′.

Primer 15. DP for IIβ: 5′-CAR YTN ATH AAY CAR ACN GT-3′.

Primer 16. DP for IIβ: 5′-CRT TCA TRC ARA ART TNG G-3′.

Primer 17. IIβ gene specific primer (IIβ-GSP) for 5′-RACE: 5′-CTC AAA GGT GCA GTC AGA CG-3′.

Primer 18. IIβ-GSP for 5′-RACE: 5′-ATG CTG ATA AAC GTA CCG GG-3′.

Primer 19. IIβ-GSP for 3′-RACE: 5′-TGT CTC TTG AGA AGG AGG GC-3′.

Primer 20. IIβ-GSP for full length: 5′-CAC ACT GCC TGC AGA TAA CAG-3′.

Primer 21. IIβ-GSP for full length: 5′-TAC AAC AAT TGA CGC ACA GTT TC-3′.

Primer 22. Adapter primer: 5′-TGG AAG AAT TCG CGG CCG CAG-3′.

Primer 23. DP for 18S rRNA: 5′-AGA TAA CYT CGR GCC GAT CG-3′.

Primer 24. DP for 18S rRNA: 5′-CCA TCC AAT CGG TAG TAG CG-3′.

mRNA Isolation and Cloning of the Red Seabream GTH Subunit Genes

The pituitary gland was collected from a maturing female red seabream and total RNA was extracted from the gland (40 mg) according to the manufacturer's instructions (Pharmacia). Poly(A)$^+$ RNA was isolated using a Pharmacia mRNA extraction kit.

Complemetary DNA was synthesized from 100 ng poly(A)$^+$ RNA with *Not*I-d(T)$_{18}$ bifunctional primer using the first-strand cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden). An aliquot of the first-strand reaction was amplified with degenerate primers 1 and 2 for αGSU, 8 and 9 for Iβ, and 15 and 16 for IIβ, respectively. PCR was performed in 50 μl final volume containing 5 μl of 10× reaction buffer, 2 mM $MgCl_2$, 200 μM dNTP, 2 μM of each primer, and 2.5 U *Taq* DNA polymerase (Takara Biomedicals, Tokyo, Japan). After an initial 1.5-min denaturing step at 94°C, 30 cycles of amplification were performed using a cycle profile of 94°C for 30 sec, 50°C for 1 min, and 72°C for 1.5 min. After the last cycle, elongation was extended to 10 min at 72°C. PCR products were T-A cloned into pBluescript II KS- (Stratagene, La Jolla, CA) and sequenced.

5′-RACE was performed using a 5′-RACE kit (Gibco/BRL, Grand Island, NY) according to the manufacturer's instructions. Briefly, 100 ng of red seabream pituitary

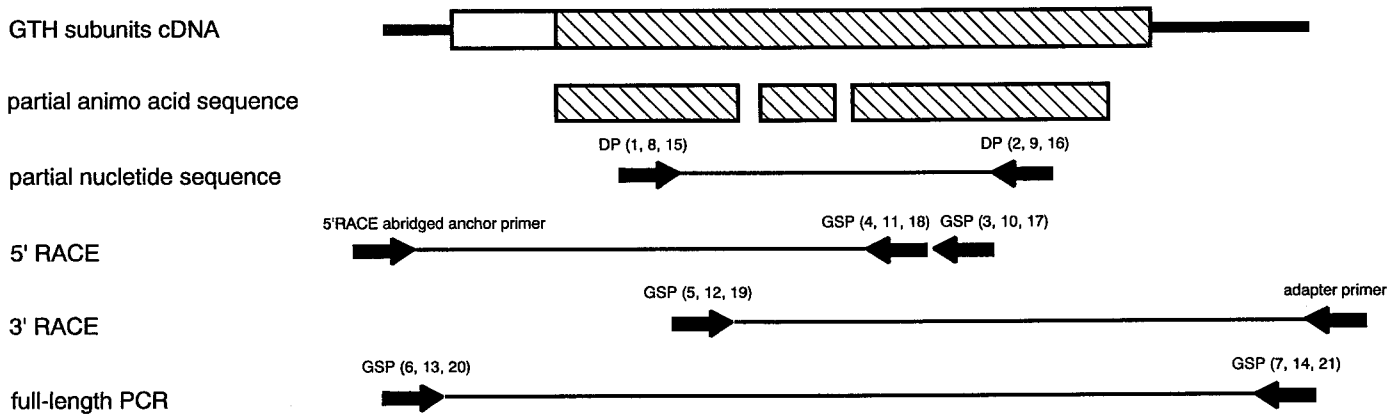


FIG. 1. Strategy for RT-PCR cloning of GTH subunit cDNAs from a red seabream pituitary gland. Location of DPs and GSPs are shown corresponding to oligonucleotides listed in *Materials and Methods*. Open box indicates signal peptide; hatched boxes indicate mature protein.

mRNA was reverse-transcribed using 20 units of Superscript II with each specific primer (αGSU: primer 3, Iβ: primer 10, IIβ: primer 17). The cDNA was C-tailed and then PCR-amplified using a second specific primer for each (αGSU: primer 4, Iβ: primer 11, IIβ: primer 18) and 5′-RACE abridged anchor primer. The PCR-amplified DNA fragments were fractionated in a 4% agarose gel, subcloned into pBluescript II KS- (Stratagene), and sequenced as just described.

For 3′-RACE, 100 ng of the pituitary poly(A)$^+$ RNA was reverse-transcribed using 50 pmol of *Not*I-d(T)$_{18}$ bifunctional primer by 200 units of MLVE (Pharmacia), followed by PCR between the primers (primers 5 and 22 for αGSU, primers 12 and 22 for Iβ, and primers 19 and 22 for IIβ). PCR was initiated at 94°C for 30 sec, followed by 30 cycles of amplification under conditions of 94°C for 30 sec, 50°C for 1 min, and 72°C for 1.5 min. After the last cycle, elongation was extended to 10 min at 72°C. The resultant DNA fragment was ligated and subcloned using pBluescript II KS- (Stratagene), and sequenced. The full-length cDNA was generated by PCR using ExTaq polymerase (Takara) with the same first-strand cDNA and primers 6 and 7 for αGSU, primers 13 and 14 for Iβ, and primers 20 and 21 for IIβ.

Cloning of Red Seabream 18S rRNA

Genomic DNA from red seabream was isolated by homogenizing the liver of a female red seabream and subsequently treating it with proteinase [20]. Using genomic DNA (100 ng) as a template, PCR was performed with degenerate primers 23 and 24. PCR conditions and subcloning into the plasmid vector pBluescript II KS- were as described earlier.

Nucleotide Sequencing Analysis

DNA was sequenced in both strands by the dideoxynucleotide chain-termination method [21] using a d-Rhodamine terminator cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Chiba, Japan) and an Applied Biosystems Model 377 DNA sequencer. At least four independent clones were sequenced by the dideoxy chain-termination method for each GTH subunit to eliminate misincorporation of nucleotides with *Taq* DNA polymerase. The nucleotide and amino acid sequences were analyzed using Geneworks software (release 2.45; IntelliGenetics, Inc., Mountain View, CA) and the BLAST network service of the National Center for Biotechnology Information [22]. The sequences discussed in this paper have been submitted to DDBJ/EMBL/GenBank (accession numbers AB028211, AB028212, AB028213, and AB028214).

Northern Blot Analysis

Three pituitary glands were isolated from fish at different maturational stages and pooled. Three replicate samples were made for each stage (i.e., 3 samples of 3 pooled pituitary glands). Total RNA from pooled sample was isolated by the guanidium isothiocyanate method [23]. Fifteen micrograms of total RNA per sample were denatured at 65°C for 15 min in 50% formaldehyde and subjected to electrophoresis on a 1% agarose gel in 0.2 M MOPS, pH 7.0, containing 2.2 M formamide, 50% formaldehyde, and then transferred onto a Hybond N$^+$ nylon membrane (Amersham Pharmacia Biotech). The membrane was air-dried and baked at 80°C for 2 h before hybridization with radiolabeled full-length red seabream GTH subunit cDNA probes. The membrane filter was washed at 65°C with several buffer changes of decreasing SSC concentrations from 2× to 0.1× (1× SCC = 0.1 M NaCl and 0.015 M sodium citrate). The radioactivity of each hybridization signals was counted using a BetaScope Model 603 Blot Analyzer (Aloka, Tokyo, Japan). Then, under the same conditions, the blot was rehybridized with 18S rRNA to control for loading variations. All values for mRNA levels were arbitrary radioactive units after standardizing with the levels of 18S rRNA. To check the specificity of hybridization signals, 0.1–100 pg of unlabeled plasmid DNA encoding red seabream GTH subunits were spotted on a nylon membrane and allowed to react with the same specific probes that were used for Northern blot analysis. The results indicated negligible cross-hybridization among the probes.

Statistical Analysis

All data were expressed as the mean ± SEM. Seasonal variation in the levels of the GTH subunits mRNAs in the pituitary of red seabream were examined by one-way ANOVA followed by Duncan's multiple-range test. GSI data were analyzed by the Kruskal-Wallis test.

RESULTS

Primary Structure of GTH Subunits

The amino acid sequence of αGSU is summarized in Figure 2. The solid line indicates the residues determined

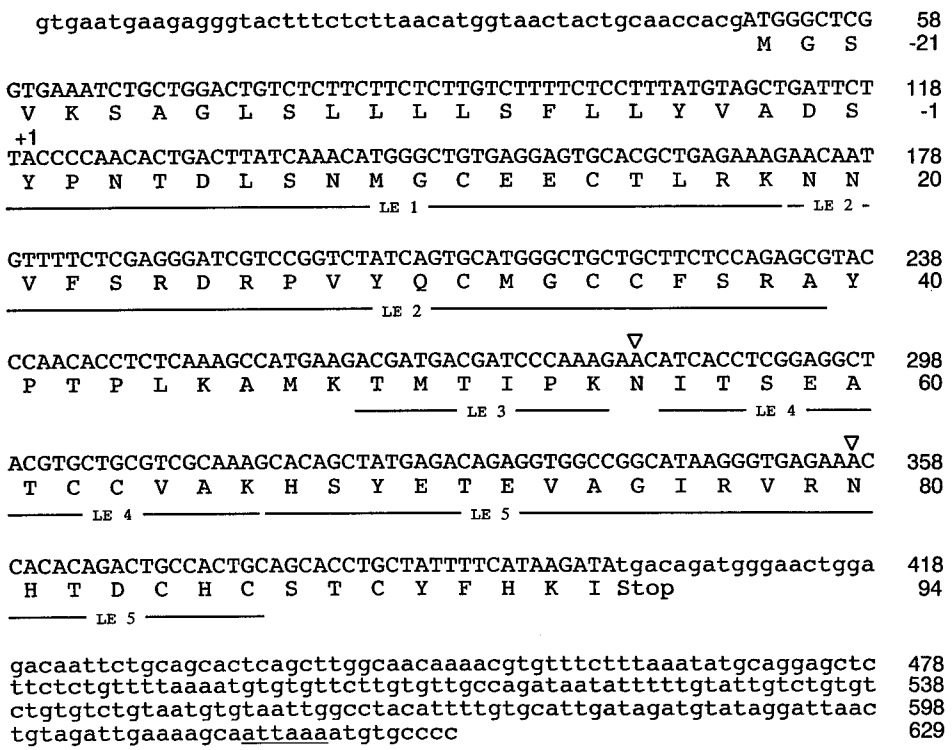


FIG. 2. Nucleotide and deduced amino acid sequence of cDNA encoding red seabream α-glycoprotein subunit. In the right-hand column, upper numbers refer to the nucleotide sequence and lower numbers refer to the amino acid sequence. The first amino acid of the mature peptide (Y) is numbered as +1. Negative numbers are used to indicate the amino acids that comprise the signal peptide. The putative N-linked glycosylation sites at positions 55 and 80 are indicated by arrowheads. The nucleotides corresponding to the polyadenylation signal are underlined. The solid lines represents the residues determined by amino acid sequence analysis. LE indicates fragment peptides prepared by digestion with lysyl endopeptidase.

by the automated Edman degradation. The αGSUs of GTH-I and GTH-II were digested separately by LE. Sequence analysis of the LE-digested RCM-αGSU of GTH-I resulted in determination of 76 residues, except for the 40th to 48th, 55th, and 87th to 94th. The amino acid sequence of GTH-II αGSU was determined by the same procedure that was used for GTH-I αGSU. Comparison of peptide maps of GTH-II αGSU with those of GTH-I αGSU indicated that GTH-II αGSU was identical to GTH-I αGSU in amino acid sequence (data not shown). The entire RCM-Iβ molecule was first evaluated to clarify the sequence from the N-terminal to the 42nd residue. Following sequence analysis of one LE, four SP, and six MP peptides, three residues (52nd, 53rd, and 102nd) remained undetermined (Fig. 3.). The N-terminal amino acid sequences of IIβ was determined up to the 40th residue. With amino acid sequences of LE, SP, and MP fragments, the complete amino acid sequence of IIβ can be proposed as summarized in Figure 4.

Isolation and Characterization of cDNA Encoding Red Seabream αGSU

A 629-base pair (bp) cDNA encoding αGSU was isolated by RT-PCR from a red seabream pituitary gland. During the first experiment, a fragment of ~200 bp amplified with a degenerate pair of primers 1 and 2 (see *Materials and Methods*) designed from the reverse translation of a partial peptide sequence. The deduced amino acid sequence of this cDNA fragment agreed with the partial amino acid sequence of αGSU. In the second step of cloning, gene-specific primers of identified sequences were used for 5′- and 3′-RACE, and the full cDNA sequence was determined. Subsequently, the full-length cDNA was amplified with a pair of primers targeted to the 5′ and 3′ ends, cloned, and sequenced.

The nucleotide sequence of αGSU was derived from an open reading frame (ORF) of 351 bp, which encoded a protein of a calculated molecular weight of 13.1 kDa (Fig. 2.). The coding region was followed by a 3′-untranslated region (3′-UTR) of 229 bp that contained a polyadenylation signal, ATTAAA, 8 bp upstream from the poly(A)$^+$ tail. Northern blot analysis shows that one transcript of approximately 720 bp is present in red seabream pituitary (Fig. 5). Figure 6 shows the deduced amino acid sequence of the red seabream αGSU cDNA aligned with counterparts from other species. The red seabream αGSU shares 94% and 95% overall sequence identity with bonito and tuna, respectively; species that belong to the same order (Perciformes); 63%–73% αGSUs of other fishes; and 63% and 56% with those of rat and human, respectively. The positions of ten cysteines were conserved. In addition, two consensus N-linked glycosylation sites (N-X-S/T) were identified at Asn^{55} and Asn^{80}. On the basis of a comparison with N-terminal amino acid purified αGSU of red seabream and from studies by von Heijne [24, 25], the cleavage site of the signal peptide was determined to be between Ser (a.a.−1) and Tyr (a.a.+1).

Isolation and Characterization of cDNAs Encoding Red Seabream β Subunits

The cDNAs encoding mature β subunit peptides were amplified from the red seabream pituitary mRNA using RT-PCR. During the first experiment, partial β subunit cDNAs (Iβ: ~200 bp; IIβ: ~300 bp) were amplified with each degenerated pair of primers designed according to the par-

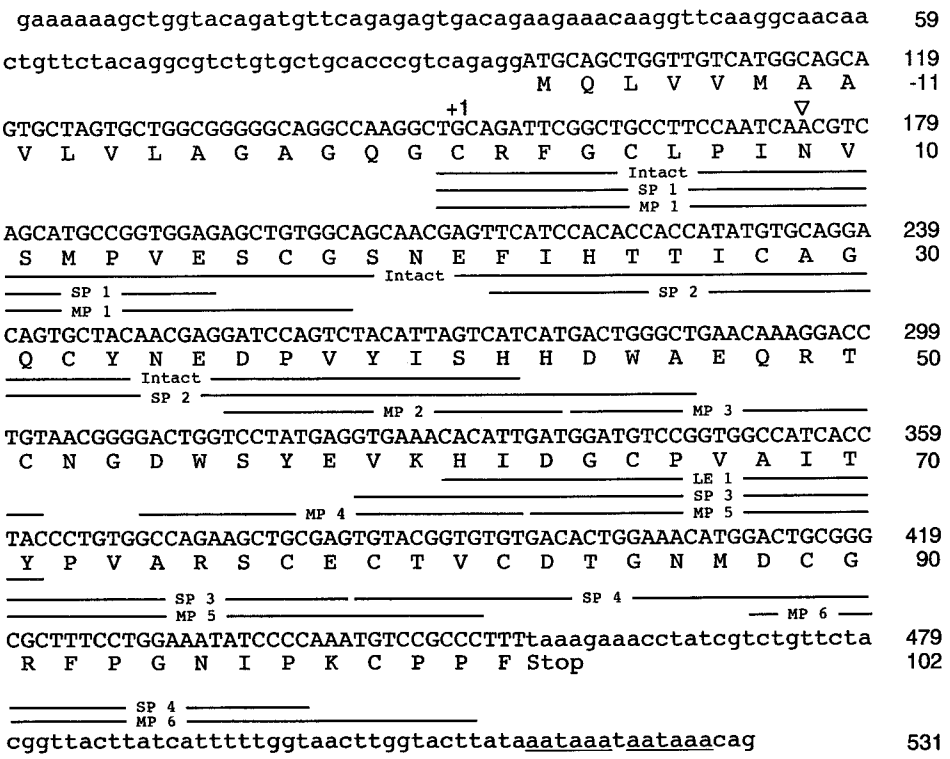


FIG. 3. Nucleotide and deduced amino acid sequence of cDNA encoding red seabream GTH-Iβ subunit. The first amino acid of the mature peptide (C) is numbered as +1. Negative numbers are used to indicate the amino acids that comprise the signal peptide. Arrowheads indicate the putative N-linked glycosylation site at position 9; the nucleotides corresponding to the polyadenylation signal are underlined; the residues determined by amino acid sequence analysis are indicated by solid lines; intact indicates N-terminal amino acid sequence of whole molecule; LE, SP, and MP indicate fragment peptides prepared by digestion with lysyl endopeptidase, *Staphylococcus aureus* V8 protease and *Pseudomonas fragi mutant* metalloprotease.

tial amino acid sequences of Iβ and IIβ subunits (see *Materials and Methods*). The sequences deduced from isolated cDNA fragments agreed with the partial amino acid sequences of β subunits. In the second step, by means of 5′- and 3′-RACE of newly identified sequences, the remaining sequence information was determined. Finally, the full-length cDNAs were amplified with a pair of primers targeted to the 5′- and 3′-ends, cloned, and sequenced. The nucleotide and deduced amino acid sequences of Iβ and IIβ subunits are shown in Figures 3 and 4, respectively. The Iβ cDNA consisted of 531 bp and included an ORF encoding a protein of 120 amino acids in length with a calculated molecular weight of 13.0 kDa. The 3′-UTR was 76 bp in length and contained two polyadenylation signals; 3 bp and 10 bp upstream from the poly(A)$^+$ tail. The 557-bp IIβ cDNA comprised an initiation codon (ATG: 28 bp), an ORF (465 bp), a stop codon (TAA), and the 3′-UTR, including a polyadenylation signal 12 bp upstream of the poly(A)$^+$ tail. The molecular weight of the protein encoded by this gene was 16.3 kDa. Northern blot analysis (Fig. 5) shows the mRNA transcripts of Iβ and IIβ subunits were approximately 700 bp and 720 bp, respectively. The alignment of the deduced amino acid sequence of the red seabream β subunits with other forms of β subunits is shown in Figure 6. The red seabream Iβ shared a high homology with bonito (68%) and tuna (67%), 37%–45% with other teleosts and 29% and 30% with rat and human, respectively. The positions of twelve cysteines and one N-linked glycosylation site (Asn^9) were completely conserved. For the IIβ sequence, the highest homology was shared with bonito (80%) and tuna (80%); 56%–67% with other teleosts; and 36% and 37% with rat and human, respectively. The positions of twelve cysteines and one N-linked glycosylation site (Asn^{20}) were completely conserved. Based on the N-terminal amino acid sequences and from studies by von Heijne [24, 25], cleavage sites appear to be between Ser (a.a.−1) and Typ (a.a.+1) for Iβ, and between Try (a.a.−1) and Cys (a.a.+1) for IIβ.

Seasonal Changes in mRNA Levels of GTH Subunits in the Red Seabream at Various Stages of Sexual Maturation

Changes in GSI of male and female red seabream sampled from February to June 1997 are shown in Figure 7. GSIs of both males and females gradually increased from February and reached a peak during the spawning phase, in April. Histological examination revealed that previtellogenic oocytes (oil droplet stage) were present in the ovaries of female red seabream collected in February, when GSI was 1.51 ± 0.31% (Fig. 8A). Vitellogenic oocytes were first observed in the ovaries of fish collected in March with a GSI of 2.88 ± 0.48% (Fig. 8B). In April, oocytes were observed at various developmental stages from perinucleolus to mature stage (Fig. 8C). At this time, the GSI was 7.08 ± 1.31%. In June, when GSI was 0.49 ± 0.06%, only perinucleolus-stage oocytes were found in the ovary (Fig. 8D). In male fish, the presence of all stages of germ cells in the testes collected from February (GSI: 1.39 ± 0.27%) to April (GSI: 7.24 ± 0.81%) (Fig. 8, E–G). In February and March with a GSI of 2.88 ± 0.48%, spermatocytes and spermatids predominantly occupied the testes of the fish (Fig. 8, E and F). As shown in Figure 8G, spermatozoa were predominantly occupied in April; however, spermatogonia were the only germ cells present in the testes in June with a GSI of 0.28 ± 0.03% (Fig. 8H).

Expression of GTH subunit genes during sexual matu-

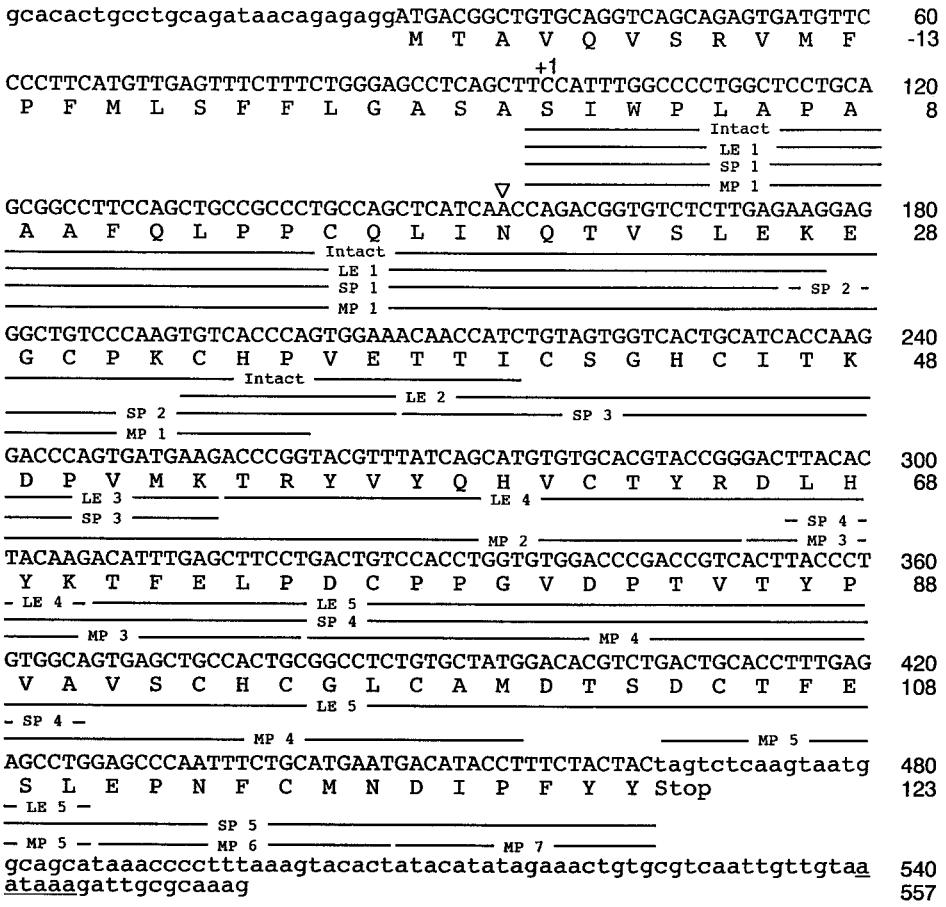


FIG. 4. Nucleotide and deduced amino acid sequence of cDNA encoding red seabream GTH-IIβ subunit. The first amino acid of the mature peptide (S) is numbered as +1. Negative numbers are used to indicate the amino acids that comprise the signal peptide. The putative N-linked glycosylation site at position 20 is indicated by an arrowhead; the nucleotides corresponding to the polyadenylation signal are underlined; the solid lines represent the residues determined by amino acid sequence analysis. Intact indicates N-terminal amino acid sequence of whole molecule. LE, SP, and MP indicate fragment peptides prepared by digestion with lysyl endopeptidase, *Staphylococcus aureus* V8 protease and *Pseudomonas fragi mutant* metalloprotease.

ration was assessed by Northern blot analysis and levels of 18S rRNA were used for internal standardization. There were no significant changes observed in 18S rRNA levels during sexual maturation, indicating that 18S rRNA is an appropriate internal standard in these experiments. Figure 9A shows the changes in mRNA levels of αGSU in males and females. In males, the levels of αGSU mRNA gradually increased from February and reached a maximum in April. Similarly, the levels of αGSU mRNA of females were high from February to April. Subsequently, αGSU

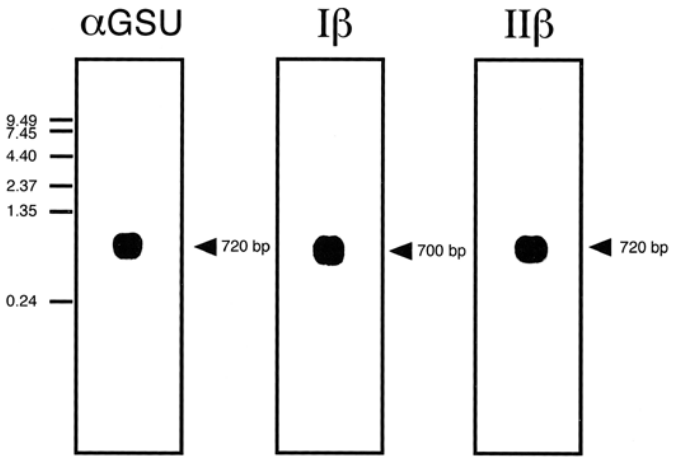
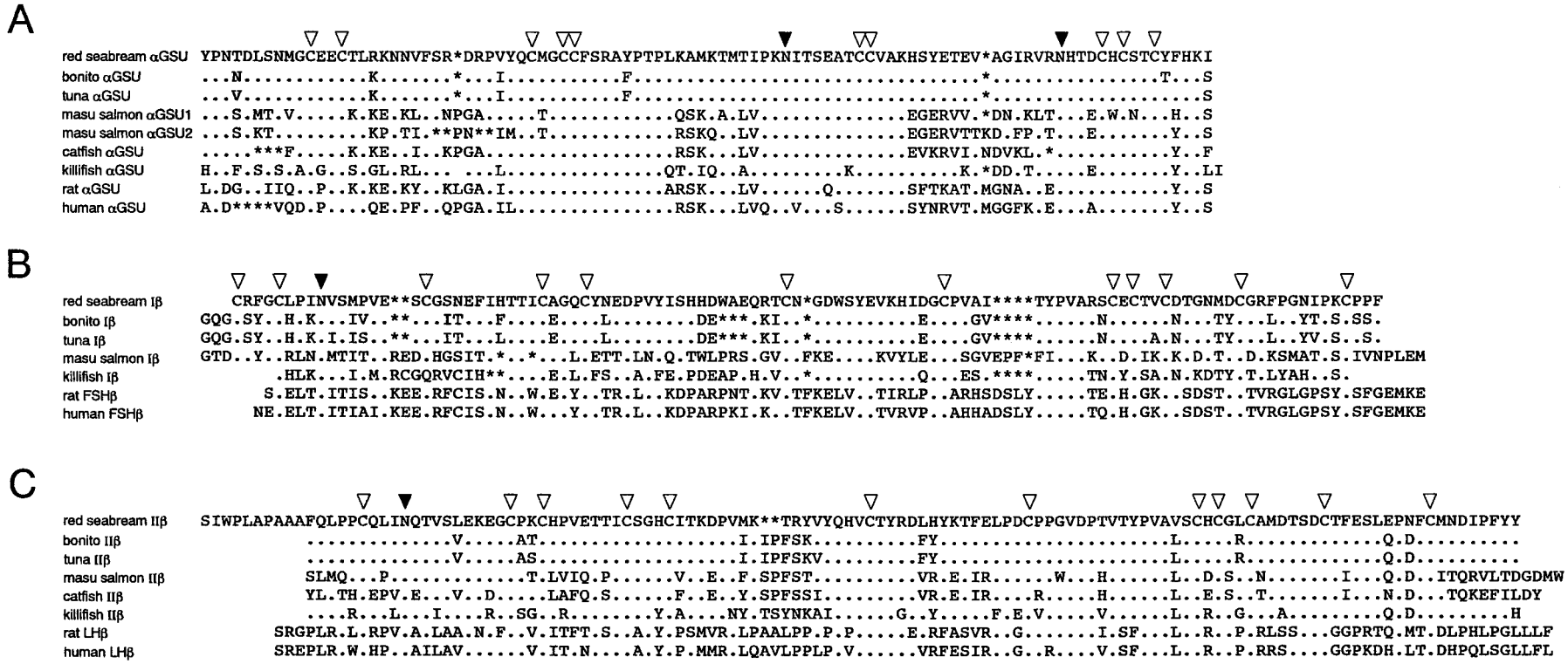


FIG. 5. Northern blot analysis of red seabream GTH subunits mRNA. Total RNA (15 μg) from pituitary glands as fractionated on a denaturing 1% agarose gel and transferred to Hybond-N$^+$ membrane. The blot was hybridized with ^{32}P-labeled red seabream αGSU, Iβ and IIβ cDNA, respectively. The bands were detected by autoradiography for 18 h. The molecular markers appear on the left in kb. The hybridization signal for each subunit is marked with an arrowhead.

FIG. 6. Alignments of red seabream GTH subunits to counterparts of other vertebrates. The amino acid sequences of (**A**) αGSU, (**B**) Iβ, and (**C**) IIβ of the red seabream are shown. Identical amino acids in other species are marked by dots (.), gaps are marked by asterisks (*), the conserved cysteine residues are indicated by open arrowheads, and the putative N-linked glycosylation sites are indicated by dark arrowheads. The sequences were extracted from GenEMBL and SwissProt databases and from published papers. For αGSU: bonito (Koide et al. [30]), tuna (P37204), masu salmon α1 and α2 (Gen et al. [7]), catfish (P53542), killifish (P47744), rat (P11962), and human (P01215). For Iβ: bonito (Koide et al. [30]), tuna (P37205), masu salmon (P48252), killifish (P30971), rat (P18472), and human (P01225). For IIβ: bonito (Koide et al. [30]), tuna (P37206), masu salmon (P48253), catfish (P53543), killifish (P30972), rat (P01230), and human (P01229).



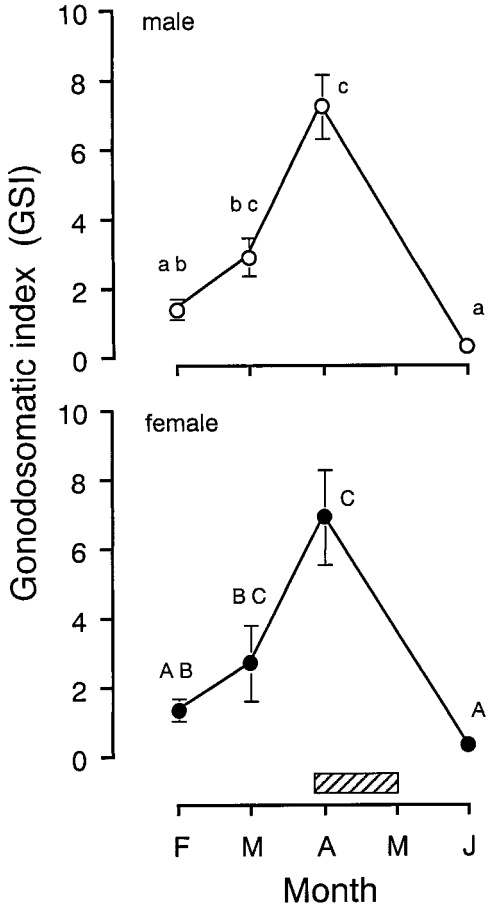


FIG. 7. Changes in the GSI of male and female red seabream during sexual maturation. Each point represents the mean ± SEM of 9 observations. Hatched box indicates spawning season. GSIs were subjected to ANOVA followed by the Kruskal-Wallis test. Significant ($P < 0.05$) differences between samples are denoted by different letters.

mRNA levels in both males and females significantly decreased in June. A one-way ANOVA followed by a Duncan's multiple range test revealed that significant differences ($P < 0.01$) between sexes in February, April, and June. Iβ mRNA levels in males gradually increased from February to March, reached a peak in April, and then declined in June (Fig. 9B). In contrast, Iβ mRNA levels in females were found to be considerably lower than those of males over the entire sampling period. Statistical analysis revealed that mRNA levels of Iβ in both sexes in April (the spawning phase) were significantly different ($P < 0.05$) from those in any other months studied (Fig. 9B). Conversely, mRNA levels of IIβ in both males and females were high from February to April, decreased in June, and no significant differences were observed between levels in males and females during the experiment period (Fig. 9C).

DISCUSSION

In this study, the cDNAs encoding red seabream αGSU and GTH β subunits were cloned and their nucleotide sequences determined. We were then able to describe the expression of GTH subunit genes in males and females during sexual maturation.

The deduced amino acid sequence of mature αGSU shares a high homology with those of other teleosts. The positions of all ten cysteines and two putative N-glycosylation sites are well conserved. From a comparison with amino acid sequences of other vertebrates, it appears that one region is highly conserved; this region is located between amino acids 33 and 66, and consists of two paired adjacent cysteines and the first putative N-linked glycosylation site. This region of αGSU is believed to be partially involved, totally involved, or both in subunit assembly, receptor binding, or both as suggested in human αGSU [26]. In this study, only one type of cDNA for αGSU was cloned from red seabream pituitary. There is only one type of αGSU in pituitaries of higher vertebrates [27], whereas two types of αGSU exist in several tetraploid teleosts [7, 28]. Southern blot analysis showed that the red seabream genome contained only one copy of the αGSU gene (data not shown); thus, red seabream has only one type of αGSU in the pituitary. This is further supported by the peptide map showing that GTH-I αGSU and GTH-II αGSU are identical.

High sequence homology was observed between the deduced amino acid sequence of mature Iβ and IIβ subunits of red seabream and their counterparts from other teleosts, especially perciform species. Furthermore, the position of all cysteines, which are related to the folding tendency of the protein and the rigid conformation of the GTH family, are well conserved. Putative N-linked glycosylation sites are also conserved among teleosts. (Fig. 6). Because the glycosylation of human FSH is important for the coupling of the receptor to adenylate cyclase [29], the sugar chain in teleost Iβ may also be involved in receptor binding or signal transduction mechanisms. Multiple protein sequence alignments of LHβ/IIβ also showed that red seabream IIβ had an extended N-terminus (Fig. 6). Although deduced amino acid sequences have been described for many teleosts, to our knowledge, there is little information about amino acid sequences of IIβ obtained by biochemical analyses [13, 14, 30]. Efforts to analyze the N-terminus of IIβ in other teleosts will help to resolve knowledge of the molecular evolution of red seabream IIβ.

Northern blot analysis of red seabream pituitary total RNA revealed a single mRNA transcript of about 720 bp for αGSU, 700 bp for Iβ, and 720 bp for IIβ (Fig. 5). The size of each mRNA transcript of GTH subunits was longer than the respective cloned cDNA sequences. This discrepancy can be attributed to the length of the poly(A)$^+$ tail. Alternatively, it is possible that the 5′-UTRs of the subunits were are not completely cloned. Further work is required to confirm the 5′-UTR results using a different approach, such as a primer extension, S1 nuclease protection analysis, or both.

According to Northern blot analysis, Iβ mRNA levels of males increase in association with an increase in GSI levels and gonadal development during sexual maturation. This is consistent with findings reported for rainbow trout, in which Iβ mRNA levels predominate in early stages of gonadal development [31]. Furthermore, GTH-I stimulates in vitro production of 11-KT, a main androgen in teleosts [32] by testes of male red seabream [15]. Taken together, these results lend support to the hypothesis that in male red seabream, GTH-I has an important role in spermatogenesis, similar to that of salmonids. In contrast to salmonids, the Iβ mRNA levels of red seabream were still high in the spawning season. Because spermatogenesis occurs in the spawning season simultaneously with spermiation, high levels of Iβ in this season are not unusual. IIβ mRNA levels are high from early spermatogenesis to the spawning season in red seabream. This is completely different from the data obtained in salmonids, in which IIβ mRNA levels are very

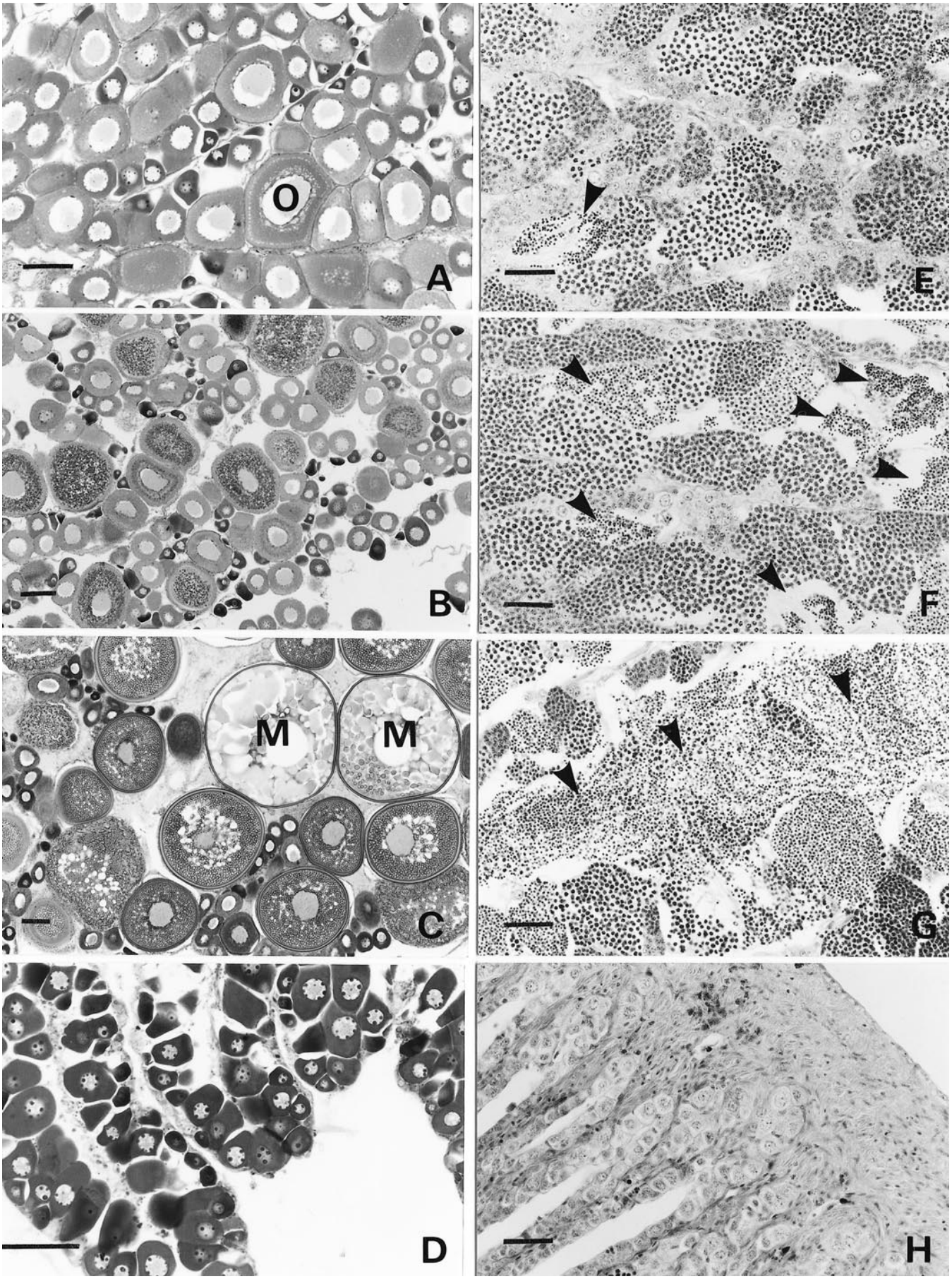


FIG. 8. Representative light micrographs of ovaries (**A–D**) and testes (**E–H**) of red seabream in February (**A**, **E**), March (**B**, **F**), April (**C**, **G**) and June (**D**, **H**). O, Oocyte at the oil stage; M, oocyte at the mature stage. Arrow heads indicate lobules filled with spermatozoa. Bars on left and right lanes indicate 100 μm and 30 μm, respectively. Photographs were taken under ×90 magnification (**A**), ×60 (**B**), ×50 (**C**), ×150 (**D**), and ×300 (**E–H**).

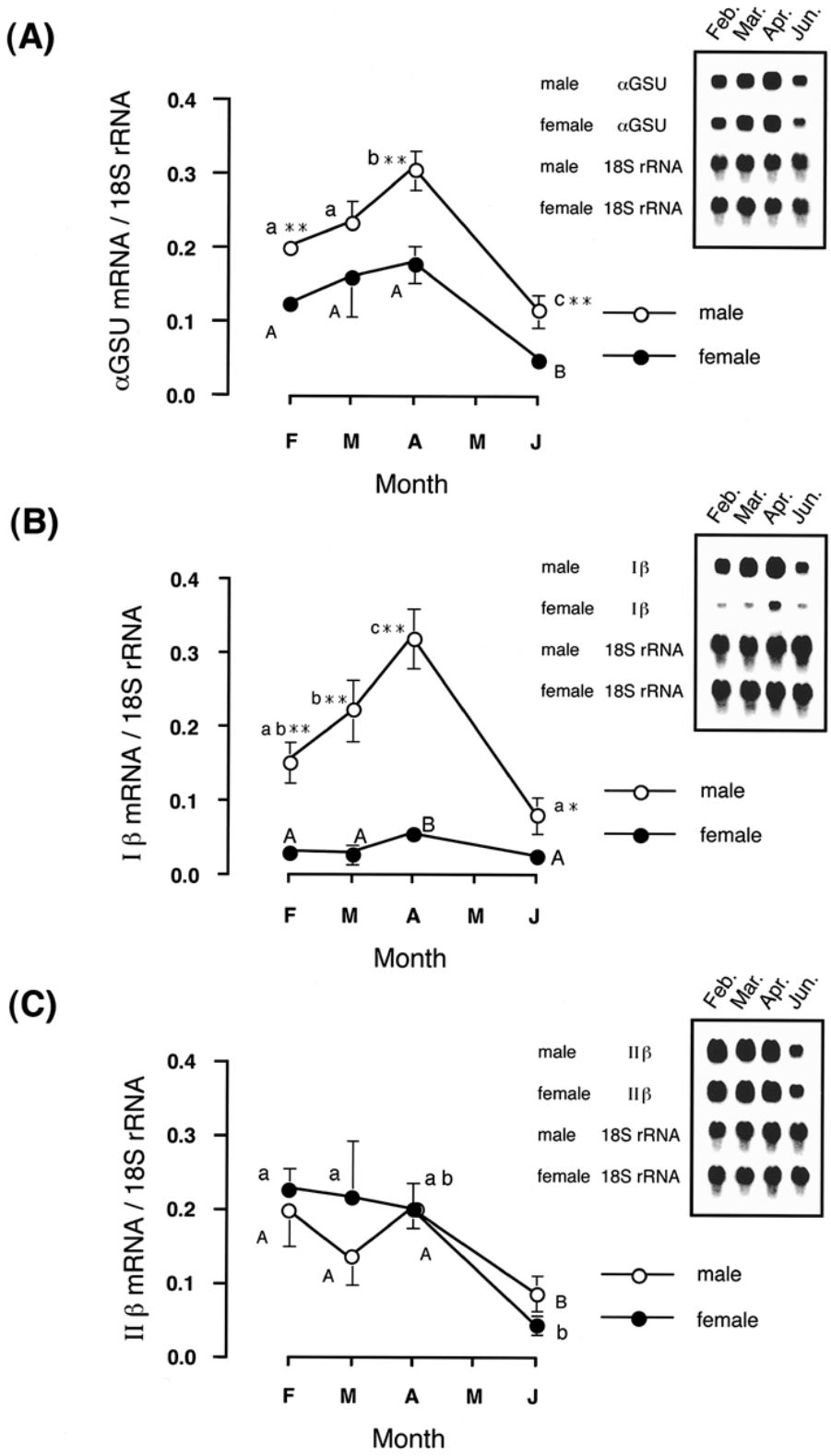


FIG. 9. Changes in mRNA levels of αGSU (**A**), Iβ (**B**) and IIβ (**C**) in male and female red seabream during sexual maturation. Fifteen micrograms of total RNA per sample obtained from 3 pituitary glands was electrophoresed, blotted onto nylon membranes, and sequentially hybridized with each subunit (αGSU, Iβ, and IIβ) and 18S rRNA probes. Radioactivity of the hybridization signal was counted using a BetaScope Model 603 Blot Analyzer and data for each subunit mRNA levels were normalized to the18S rRNA signals. Values are the means ± SEM of 3 samples. Data were 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. Asterisks (* $P < 0.05$, ** $P < 0.01$) indicate that mRNA levels in male pituitaries are significantly higher than the corresponding female levels. Representative autoradiograms for GTH subunits and 18S rRNA are shown in the inset.

low during early gametogenesis and increase rapidly during late gametogenesis [31]. Similar to GTH-I, in vitro, GTH-II stimulated 11-KT production in testis slices of male red seabream [15]. These data, together with the present data, suggest that GTH-II is also involved in spermatogenesis in this fish. Unlike salmonids, in this study, all developmental stages of gametes in red seabream (from spermatogonia to spermatozoa) were present in the testes used; thus, we could not conclude that there are differences between two GTHs in their physiological roles in spermatogenesis and spermiation in male red seabream.

In contrast to males, the Iβ mRNA levels of females were maintained at low levels during sexual maturation, although levels increased slightly in the spawning season. The expression pattern of Iβ mRNA in female red seabream is different from that reported in salmonids [31], in which Iβ mRNA levels and the plasma levels of GTH-I predominate during the initial phase of vitellogenesis [31, 33]. Moreover, the biological activity of GTH-I is much lower than that of GTH-II in in vitro E_2 production of vitellogenic follicles [14] and GTH-I is ineffective in the induction of germinal vesicle break down (GVBD; [15]); therefore, unlike salmonids, GTH-I may not have any significant roles in steroidogenesis and gonadal development of female red seabream. GTH-I has been shown to increase the rate of vitellogenin uptake by the oocyte of rainbow trout in vitro and in vivo [34]; hence, it may be interesting to examine whether GTH-I has additional roles, such as uptake of vitellogenin into oocytes of female red seabream.

Currently, it remains unclear whether the pattern of Iβ mRNA levels is correlated with that of plasma GTH-I levels, because there is no specific radioimmunoassay available to measure plasma GTH-I levels in red seabream. Immunocytochemical analysis, however, shows that the number of GTH-I immunoreactive cells increases during sexual maturation in male red seabream [16], suggesting that Iβ mRNA levels in the pituitary may correlate with those of the protein. This is consistent with observations that Iβ mRNA levels and plasma GTH-I levels rise in parallel during gametogenesis in salmonids [35]; therefore, plasma GTH-I levels may positively correlate with the expression of Iβ subunit genes in the pituitary of red seabream during sexual maturation.

This study also demonstrates that IIβ mRNA levels are high from the beginning of early vitellogenesis to the spawning season in female red seabream. A previous study showed that plasma levels of GTH-II increased with gonadal development and reached a peak during spawning season in female red seabream [16]. These results are different from the pattern of changes observed in plasma levels of GTH-II in the rainbow trout [31] and striped bass [36]. In these species, IIβ levels are low or nondetectable during vitellogenesis and increase at final oocyte maturation. In female red seabream, GTH-II is more potent than GTH-I in stimulating E_2 production by vitellogenic oocytes [14]. Moreover, GTH-II, but not GTH-I, induced final maturation of red seabream oocytes [15]. Plasma GTH-II increased in association with the final maturation and ovulation induced by injection of a gonadotropin-releasing hormone analogue [5] and a daily rhythm of increased GTH-II levels linked to oocyte maturation was observed in females that spawned naturally during the spawning season [16]. Thus, these data suggest that GTH-II is also involved in final oocyte maturation in red seabream. In female red seabream, GTH-II may have important roles both in final maturation of oocytes and, unlike salmonids, in vitellogenesis. African catfish (*Clarias gariepinus*) GTH-I-like hormone could not be isolated and only one type of gonadotroph is present in the pituitary [37, 38]; thus, it is assumed that GTH-II performs a major role in female reproduction [39] similar to that proposed for red seabream.

In both sexes, the αGSU mRNA levels were high during vitellogenesis and spermatogenesis and at the spawning phase, then sharply declined in the regressed phase, although, overall, mRNA levels in males were higher than those in females. In situ hybridization shows that an increase in mRNA levels of αGSU is correlated with active transcription of the αGSU gene in the gonadotrophs of the rainbow trout [35]. Although it has not yet been analyzed, it is likely that the increase in αGSU mRNA levels detected in our study is the result of enhanced gene transcription in gonadotrophs. However, because αGSU mRNA is potentially expressed in both gonadotrophs and thyrotrophs, we can not rule out the possibility that thyrotrophs contribute to the observed increases in αGSU mRNA levels.

This study clearly shows sexual dimorphism in Iβ gene expression during sexual maturation in the red seabream. These data are in agreement with previous data showing that, in gilthead seabream, Iβ mRNA levels were 2.5-fold higher in males compared with females during the spawning phase [40]. The mechanisms underlying gender-specific patterns of Iβ gene expression in the pituitary of male and female red seabream are currently unclear. The expression of GTH subunit genes are regulated by various endocrine factors, including hypothalamic releasing hormones [36, 41] and gonadal steroids [42–44]. In the male rat, androgens positively regulate higher steady-state levels of FSHβ expression, but not αGSU or LHβ, than in females [45, 46]. More recently, Borg et al. [47] showed that 11-ketoandrostenedione increases the pituitary and plasma GTH-I levels in male Atlantic salmon; thus, it is possible that sex steroids may be involved in sexual dimorphism in gene expression of Iβ in red seabream. Therefore, additional studies, including castration and steroid hormone replacement, and primary pituitary cultures obtained from male and female red seabream, would be helpful to draw more definite conclusions.

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