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Molecular characterization of gonadotropin subunits and gonadotropin receptors in black porgy, *Acanthopagrus schlegeli*: Effects of estradiol-17β on mRNA expression profiles

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ABSTRACT

The cDNAs of three gonadotropin (GTH) subunits (GTHα, FSHβ, and LHβ) and two GTH receptors (FSHR and LHR) from pituitary and gonads of black porgy were cloned. The nucleotide sequences of the GTHα, FSHβ, and LHβ cDNA were 354, 363, and 414 base pairs (bps) in length with open reading frames (ORF) encoding peptides of 117, 120, and 137 amino acids, respectively. The FSHR and LHR cDNA was 2118 and 2076 bps in length with ORFs encoding peptides of 705 and 691 amino acids, respectively. To study the mechanism of the estradiol-17β (E_2) action, we examined the expression pattern of GTH subunit mRNAs in pituitary and GTH-receptor mRNAs in gonads, and the changes of plasma E_2 level when E_2 treatment was applied to immature black porgy. E_2 treatment increased mRNA expression levels of the genes and plasma E_2 levels, indicating that E_2 stimulated the increases in GTH subunit and GTH-receptor mRNAs. These data indicate that E_2 plays an important regulatory role in the brain–pituitary–gonad axis of immature black porgy. We provide the molecular characterization and expression of the GTH subunits and GTH receptors during sex change in the protandrous black porgy.

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1. Introduction

Gonadotropin-releasing hormone (GnRH) from the hypothalamus induces the synthesis and release of the hypophysial gonadotropins (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), which stimulate the secretion of the gonadal steroid hormones that regulate the reproductive function in teleost fish (Amano et al., 1997). FSH and LH are members of the glycoprotein hormone family, which also includes thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG). These hormones consist of a common α subunit and a hormone-specific β subunit. FSH and LH act by binding specific receptors (FSHR and LHR) in the gonads of vertebrates to induce steroidogenesis and gametogenesis (Nagahama et al., 1995).

The cDNA clones and sequences of the GTH subunits have been determined for a number of teleosts, such as European eel (*Anguilla anguilla*) (Querat et al., 1990), killifish (*Fundulus heteroclitus*) (Lin et al., 1992), striped bass (*Morone saxatilis*) (Hassin et al., 1995), channel catfish (*Ictalurus punctatus*) (Liu et al., 1997, 2001), red seabream (*Pagrus major*) (Gen et al., 2000), European sea bass (*Dicentrarchus labrax*) (Mateos et al., 2003), and greasy grouper (*Epinephelus coioides*) (Li et al., 2005), and many studies have shown a temporal pattern of pituitary FSH and LH expression and their circulating levels at different stages of the reproductive cycle (Hassin et al., 2000). In salmonids, plasma FSH levels are high during the early stages of gametogenesis and are believed to control vitellogenesis and spermatogenesis, whereas LH is believed to facilitate gamete maturation, spawning, and ovulation. In contrast, both FSHβ and LHβ mRNA levels of nonsalmonids increase during the spawning season (Kim et al., 2005).

FSHR and LHR cDNA has been isolated from the gonads of several teleosts (Jeng et al., 2007). FSHR and LHR are membrane-bound receptors belonging to the superfamily of G-protein coupled receptors (GPCRs), which contains seven transmembrane domains (7TMD). FSHR and LHR have a large extracellular domain (ECD) that is different from other GPCRs and constitute a subfamily of glycoprotein hormone receptors (GpHRs) that are members of the larger leucine-rich repeat (LRR) containing GPCR (LGR) family. The members of this family have been characterized as containing an ECD with multiple imperfect LRRs flanked by N- and C-terminal cysteine-rich subdomains, and a rhodopsin-like domain of 7TM helices with a C-terminal intracellular tail (Hsu et al., 2000).

The FSHR gene is only expressed in the granulosa cells of the ovary and in the Sertoli cells of the testis, whereas the LHR gene is expressed primarily in the theca and granulosa of preovulatory ovarian follicles

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and in the Leydig cells of the testis (Rocha et al., 2007). Moreover, the FSHR and LHR profiles during oogenesis are consistent with the FSH and LH profiles described in salmonids (Oba et al., 1999a,b).

Black porgy (*Acanthopagrus schlegeli*, Perciformes: Sparidae) is a widely distributed marine protandrous hermaphrodite and a candidate for commercial aquaculture in various parts of Asia, including Korea. Black porgy is functional male for the first two years of life, and then about 70% change sex to female during their third spawning season. To date, despite many studies on sex-change of black porgy (e.g. seasonal GnRH levels, exogenous hormone treatment, LH release and histological analysis) were reported (Chang et al., 1994; Lee et al., 2001, 2004; Du et al., 2005; An et al., 2008), a comprehensive study, investigating the cloning and expression of GTH subunits and their receptors during sex change is lacking for protandrous hermaphrodite fish.

Estradiol-17β (E_2) is involved in the control and release of GTHs in fish (Trudeau et al., 1991; Yen et al., 2002; Lee et al., 2004). Positive E_2 feedback on FSH expression has been reported in primary cultures of pre-pubertal European eel pituitary cells (Aroua et al., 2007) and also, in early and late recrudescent goldfish (*Carassius auratus*) pituitary (Huggard-Nelson et al., 2002). Positive feedback of E_2 on LH expression has been reported in fish, including chinook salmon (*Oncorhynchus tshawytscha*) (Xiong et al., 1994), African catfish (*Mocholkiella paynei*) (Schulz et al., 1995), goldfish (Huggard-Nelson et al., 2002) and black porgy (Lee et al., 2004). Despite the presence of positive and negative feedback of E_2 on GTH expression, its effects are dependent upon species, gonad maturity, hormone concentration, and the duration of exposure to the hormone (Banerjee and Khan, 2008).

The objectives of this study were to 1) first isolate the full-length cDNAs of the GTH subunits and their receptors in black porgy; 2) investigate the effects of E_2 on the brain–pituitary–gonad axis by measuring GTH subunits and GTH receptors mRNA expression following a single injection of E_2 in immature black porgy; and 3) describe the relationship among the GTH subunits, their receptors, and the brain–pituitary–gonad axis of black porgy.

2. Materials and methods

2.1. Experimental fish

The study was conducted with immature (51.0±2.3 g, 1-yr old), mature male (220±14.2 g, 2-yr old), sex-changing male (489.2±11.5 g, 3-yr old), and female (948.5±51.6 g, 4-yr old) black porgy. Sexual maturity was determined after excising the gonads defined by the presence of mature ova and sperm. All fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) and decapitated prior to blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 10,000 *g*, 5 min) and stored at −80 °C until analysis. Gonad and pituitary samples from black porgy at various stages of sex change and gonadal maturity (immature testis, mature testis, testicular portion of mostly testis, ovarian portion of mostly testis, testicular portion of mostly ovary, ovarian portion of mostly ovary, and mature ovary) were removed, immediately frozen in liquid nitrogen, and stored at −80 °C until the total RNA was extracted for analysis.

2.2. E_2 treatment

E_2 was dissolved in ethanol: 0.9% physiological saline (1:1) and then diluted in physiological saline to 1.5 μg/μL. Immature black porgy were anesthetized with MS-222 and injected at a dose of 1 μL/g body mass (BW; 1.5 μg/g). Pituitary, gonad, and blood were collected from three fish at 0, 3, 6, and 9 days following the injection. All of the fish survived the experimental period.

2.3. Total RNA extraction and reverse transcription (RT)

Total RNA from the pituitary and gonads of the E_2-treated black porgy at each stage of gonadal maturation and during sex change was extracted using the TRIzol method according to the manufacturer's instructions (Gibco/BRL, Grand Island, NY, USA). The concentration and purity of the RNA samples were determined by UV spectroscopy at 260 and 280 nm. Total RNA (2.5 μg) was reverse transcribed in a total volume of 20 μL using an oligo-d$(T)_{15}$ anchor primer and M-MLV reverse transcriptase (Bioneer, Korea) according to the manufacturer's instructions. The resulting cDNA was diluted and stored at −20 °C for use in a polymerase chain reaction (PCR) and quantitative PCR (QPCR).

2.4. Identification of GTH-subunit cDNAs

The primers used for GTHα, FSHβ, and LHβ amplification were designed using highly conserved regions of other teleost fish: GTHα forward primer (5′-CTG GAC TGT CTC TTC TTC TG-3′), GTHα reverse primer (5′-TGT GGT TCC TCA CCC TTA TG-3′), FSHβ forward primer (5′-GGT TGT CAT GGT AGC AGT AG-3′), FSHβ reverse primer (5′-TAG AAG GGC AGA CAT TTG GG-3′), LHβ forward primer (5′-ATG TTG GGT TCC TTC CTG GGA-3′), and LHβ reverse primer (5′-TGC AGA AAT TGG GCT GCA GG-3′). Total RNA was extracted from the pituitary using a TRIzol kit (Gibco/BRL), and PCR amplification was performed using the 2× Taq Premix I (Solgent, Korea) according to the manufacturer's instructions. PCR was performed under the following conditions: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. Amplified PCR products were processed by electrophoresis using a 1% agarose gel containing ethidium bromide (Biosesang, Korea). The PCR product was purified and then cloned into a pGEM-T Easy Vector (Promega, USA). The transformed colony was cultivated in DH5α (RBC Life Sciences, Korea), and the plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoR (Fermentas, Glen Burnie, MD, USA). The GTH-subunit cDNA sequences were analyzed using an ABI DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Identification of GTH-receptor cDNAs

The primers used for FSHR and LHR amplification were designed using highly conserved regions of other teleostean fish: FSHR forward primer (5′-AAT CAA GGA GGT GGC AAG TG-3′), FSHR reverse primer (5′-ATC GCT GTC AAC GTG AAC A-3′), LHR forward primer (5′-CCA TCT CCA GAT TCC TCA TG-3′), and LHR reverse primer (5′-TGC ATA CGT AGC AGT AGC AG-3′). Total RNA was extracted from gonads using a TRIzol kit (Gibco/BRL). PCR amplification was conducted using the same method as the GTH subunits, and then the ligation and transformation was also performed using the same method as the GTH subunits.

2.6. Rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the PCR reaction, total RNA was extracted from the pituitary and gonads using a TRIzol kit (Gibco/BRL). Using 3 μg total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo-d$(T)_{18}$ anchor primer and a CapFishing™ adaptor (Seegene).

Gene-specific primers were selected from the PCR products obtained by RT-PCR. For the 3′ RACE, 50 μL PCR reaction mixture contained 5 μL of 3′ RACE cDNA, 1 μL of 10 μM 3′ target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL of 10 μM 3′ RACE GTHα-specific primer (5′-GTT TTC TCC AGG GAT CGT CCG ATT TAC CAG-3′), 1 μL of 10 μM 3′ RACE FSHβ-specific primer (5′-CAT GAC TGG GCT GAA CAA AGG ACC TGT AAT G-3′), 1 μL of 10 μM 3′ RACE LHβ-specific primer (5′-CAT CTG CAG TGG TCA

CTG CAT CAC CAA G-3′), 1 μL of 10 μM 3′ RACE FSHR-specific primer (5′-GTC ATA GCG ACT GTA GAC ATA GTC ACC C-3′), 1 μL of 10 μM 3′ RACE LHR-specific primer (5′-CAT GCT CGT CCT CAA TGT AGT CGC TTT CC-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, extension at 72 °C for 1 min, and 1 cycle of final extension at 72 °C for 5 min.

For the 5′ RACE, 50 μL PCR reaction mixture contained 5 μL of 5′ RACE cDNA, 1 μL of 10 μM 5′ target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL of 10 μM 5′ RACE GTHα-specific primer (5′-CTG GTA AAT CGG ACG ATC CCT GGA GAA AAC-3′), 1 μL of 10 μM 5′ RACE FSHβ-specific primer (5′-CAT TAC AGG TCC TTT GTT CAG CCC AGT CAT G-3′), 1 μL of 10 μM 5′ RACE LHβ-specific primer (5′-CTT GGT GAT GCA GTG ACC ACT GCA GAT G-3′), 1 μL of 10 μM 5′ RACE FSHR-specific primer (5′-CAT CTT TGT GCC GTT GAA GGC GTC ACT TG-3′), 1 μL of 10 μM 5′ RACE LHR-specific primer (5′-CAG GTT GCA CAT GAG GAA TCT GGA GAT GG-3′), and 25 μL of SeeAmp Taq Plus Master Mix. PCR was carried out for 40 cycles as follows: 1 cycle of denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min, followed by a final extension cycle at 72 °C for 5 min. After PCR, ligation and transformation were conducted using the methods described above.

2.7. Phylogenetic analysis

Phylogenetic analyses have been performed on the amino acid sequences of the full-length GTH subunit and the GTH receptor cDNAs from various fish. Sequences were aligned using the BioEdit Software (Hall, 1999). The sequences we used for comparison are as follows: GTHα: black porgy, red seabream, European sea bass, striped bass, swamp eel (*Monopterus albus*), longtooth grouper (*Epinephelus bruneus*), Hong Kong grouper (*Epinephelus akaara*), rockfish (*Sebastes schlegeli*), pejerrey (*Odontesthes bonariensis*), Mozambique tilapia (*Oreochromis mossambicus*), Nile tilapia (*Oreochromis niloticus*), bastard halibut (*Paralichthys olivaceus*), and Atlantic halibut (*Hippoglossus hippoglossus*). FSHβ: black porgy, red seabream, striped bass, European sea bass, blotched snakehead (*Channa maculate*), three spot gourami (*Trichogaster trichopterus*), convict grouper (*Epinephelus septemfasciatus*), orange spotted grouper (*E. coioides*), bastard halibut, and Atlantic halibut. LHβ: black porgy, yellowfin seabream

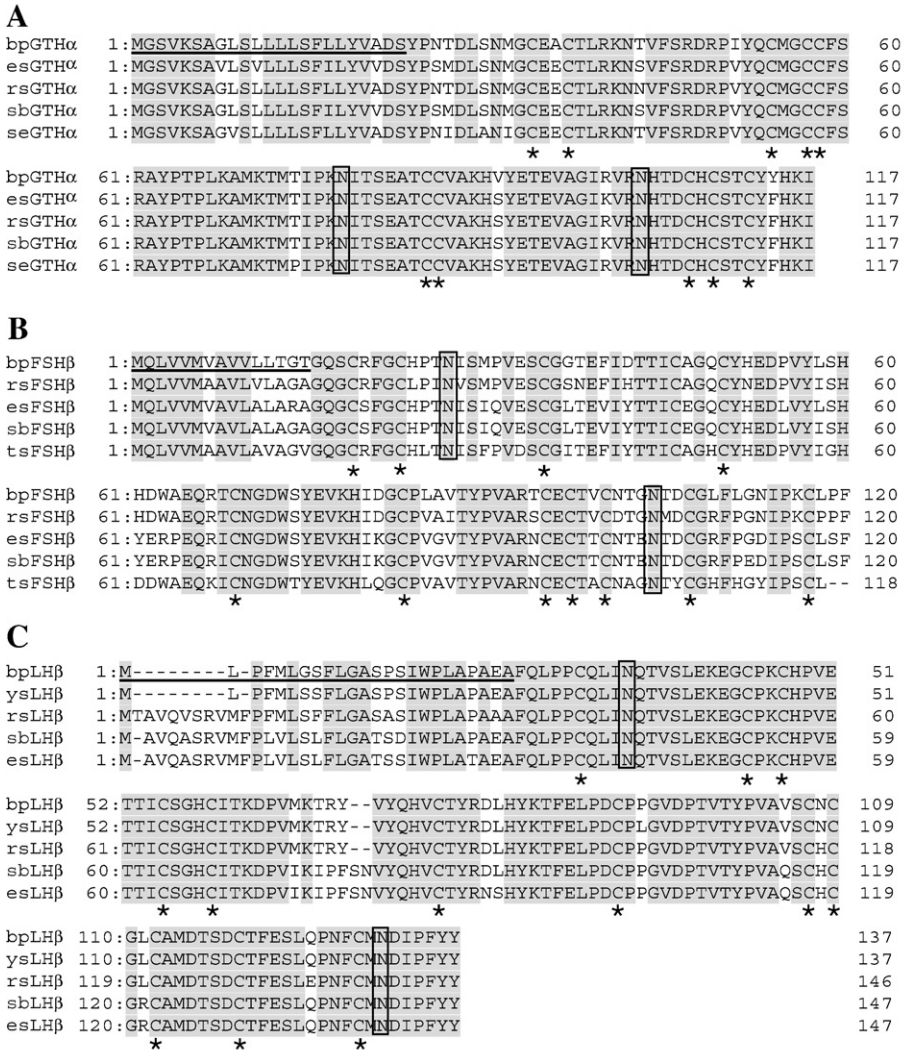

Fig. 1. Comparison of the amino acid sequence of GTHα (A), FSHβ (B), and LHβ (C). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. GenBank accession numbers of the sequences are: GTHα; black porgy (bpGTHα, EF605275), red seabream (rsGTHα, AB028211), swamp eel (seGPα, AF502395), striped bass (sbGTHα, L35071), European sea bass (esGTHα, AF269157), FSHβ; black porgy (bpFSHβ, AY921613), red seabream (rsFSHβ, AB028212), European sea bass (esFSHβ, AF543314), striped bass (sbFSHβ, L35070), three spot gourami (tsFSHβ, AF157630), LHβ; black porgy (bpLHβ, EF605276), yellowfin seabream (ysLHβ, L11722), red seabream (rsLHβ, AB028213), striped bass (sbLHβ, L35096), and European sea bass (esLHβ, AF543315). These sequences are optimally aligned to match identical residues, indicated by the shaded box. The two potential N-linked glycosylation sites are boxed, and cysteine residues of the N- and C-terminal cysteine-rich regions of the ECD are indicated by asterisk. Bold underline indicates signal peptide sequences.

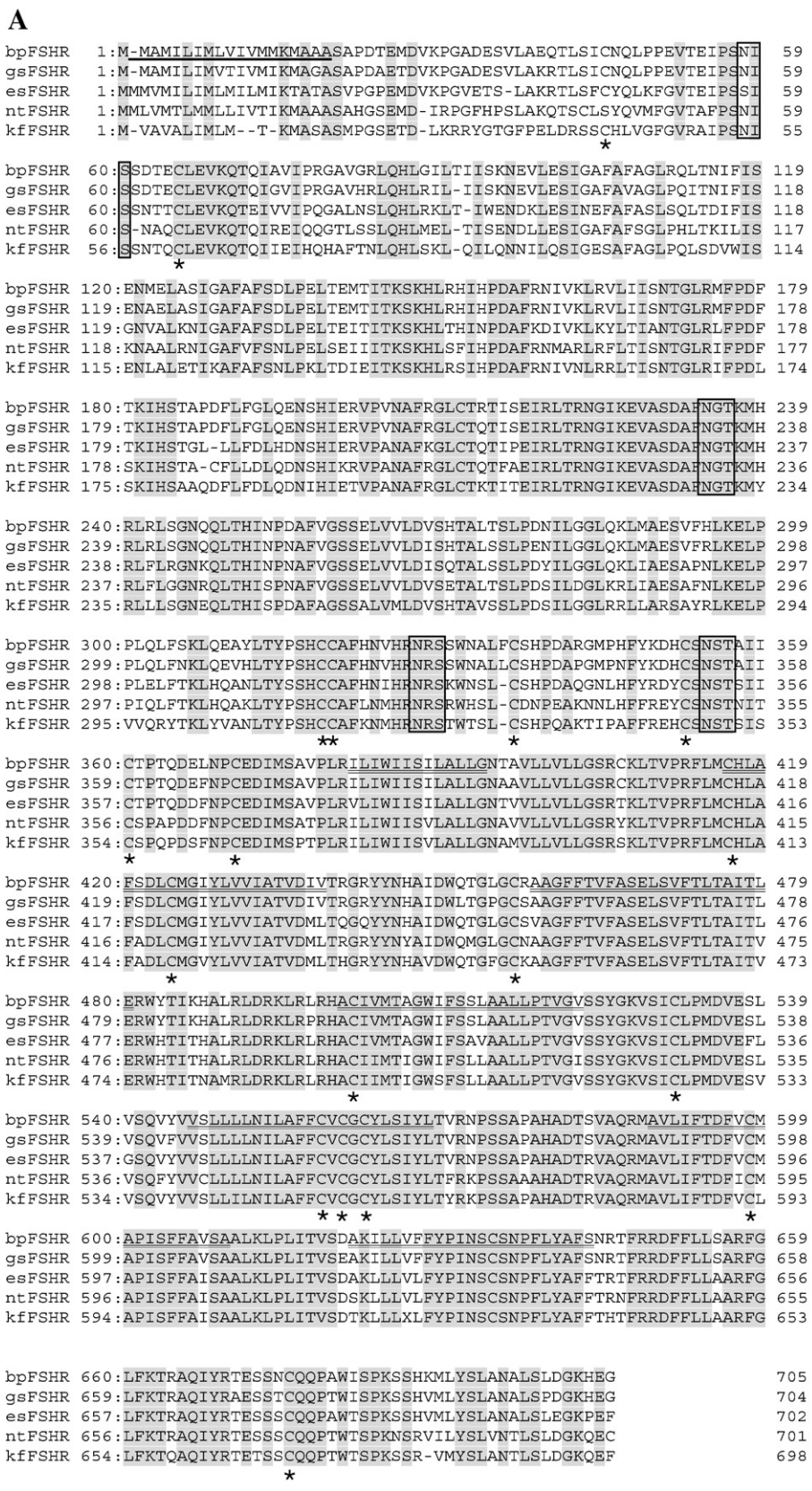

Fig. 2. Comparison of the amino acid sequence of FSHR (A) and LHR (B). The sequences were taken from the GenBank/EMBL/DDBJ sequence databases. GenBank accession numbers of the sequences are: FSHR; black porgy (EU095497), gilthead seabream (gsFSHR, AY587262), European sea bass (esFSHR, AY642113), Nile tilapia (ntFSHR, AB041762), and killifish (kfFSHR, AB295490), LHR: black porgy (EU339125), Nile tilapia (ntLHR, AB041763), channel catfish (ccLHR, AF285181), gilthead seabream (gsLHR, AY587261), and Atlantic salmon (asLHR, AJ579790). These sequences are optimally aligned to match identical residues, indicated by the shaded box. The four potential N-linked glycosylation sites are boxed, and cysteine residues of the N- and C-terminal cysteine-rich regions of the ECD are indicated by asterisk. Bold underline indicates signal peptide sequences and double underline indicates TMD.

B

```
bpLHR   1:M----ALREAWLLVALSGVLHVRSCCAFTCPTICRCSADSFHCSGATQLASR-AGPRSVT  55
ntLHR   1:M----ALREVWLLFALSGVLNARSCCAYTCPAICRCTADSFQCSKETQLASR-TGPTSVL  55
ccLHR   1:M----VSRCSVAALLLAVVMRLSRGGAFTCPPICSCTADTLSCTAQTERRTRVPSSTTST  56
gsLHR   1:MRTAPPLLLFL-IV-L-ISCGWKSTSGFVCPRICRCFANTIRCNNVT-QGSALTMEHRDK  56
asLHR   1:MMSISLLFLFYPSVLLFFGFGCRYASSFVCPGICRCSSNTIRCNNIT-E-KSVPMSERGP  58
                                    *   * *     *

bpLHR  56:RLRLTHLPMKEVPTHAFRELINITTIEISQSDRVTRIRRHAFLSLHSLAQISLQSINSLR 115
ntLHR  56:RLRLTHLPLKRVPSHAFKELINITTIEISQSDCITHIQTHAFLSLYSLAQISVQNINSLR 115
ccLHR  57:NLRLAHLPLKEVQSGTFRGLINVSRIEISQSDSIRKIKGEAFLSIHNVIEISILNIIHLS 116
gsLHR  57:RLFLYHLPLHTISSHSFEGLKGVQRIDIAQSVTLETIETLAFNNLLNLSEISIQNTRSLM 116
asLHR  59:RLVLKHLTMSTIASHTFDGLRRVQHIEIGQSVALETIETLAFNNLLDLNEIFIKNTRSLV 118

bpLHR 116:VIEKGAFTDLPKLEYLSISNTGLIHFPDFTTIASL---VPNIILEMADNMRIDIIPANCF 172
ntLHR 116:FIEKGAFADLPKLEYLSISNTGIAHFPDFTTISSL---SPNIILEMADNMEIDIIPANSF 172
ccLHR 117:AMEKGAFTDLPRLRYLSICNTGIRHFPDFSRISSL---GLEFFLDMGDNIQLNNIPANAF 173
gsLHR 117:RIGRGTFNNLPKLRYLSISNTGITVFPDITSIYSLE--PE-FILDIYDNLYLLEIPPNAF 173
asLHR 119:HIARRTFNNLPKLRYLSISNTGITVFPDMTSIHSLEPWNQNFVLDICDNLYLLSIPVNAF 178

bpLHR 173:QGITEEYVDMNLVRNSFTEITAHAFNGTKLNTLNLRDNTYLSDIQEDAFEGATGPSFLDV 232
ntLHR 173:QGITEEYVDMNLVRNGFKEIKSHAFNGTKLNTLVLRDNWYLRNIQEDAFEGATGPTLLDV 232
ccLHR 174:QGISKADMYMNLVRNGFTQIESHAFNGTRLEKLIMKDNWHLIKIHDDAFEGAEGPTLLDV 233
gsLHR 174:IGLTKEYVTMNLYNNGIREIHDHAFNGTKIDKLVLKNNRNLRGIHRDAFKGATGPEVLDV 233
asLHR 179:VGMTTEYTAMNLFNNGIREIQDYAFNGTKINKLVLKNNRNLRVIHREAFKGAVGPRILDV 238

bpLHR 233:SSTALRSLPPKGLRQVRFLKASAAFALKTLPPLESLAELMEAELTYPSHCCAFHTW--RR 290
ntLHR 233:SSTALRSLPPNGLRHVKFLKASHAYALKSLPLLESLAELLEAELTYPSHCCAFHTW--RR 290
ccLHR 234:SSTALKSLPTRGLRGVKVLIARSTPSLKTLPPLDNLAELQEADLTYPSHCCAFHTW--KR 291
gsLHR 234:SATALKKLPAEGLESVLVLFAQSAYALKSLPPLQGLWSLREAHLTYNSHCCALLSWSTHR 293
asLHR 239:SSTAIETLPSHGLNSVVELVARTAYGLKRLPPFRGLGNLQKAHLTYNSHCCALLTWDTHR 298
                                                  **

bpLHR 291:-KQRESA-------LK-------------NSTKFCDLSRTEIEPTA-D-GMNDD----I 322
ntLHR 291:-KQRESA-------LK-------------NLTKFCDLMNTEIDPTADDTSLIND----I 324
ccLHR 292:-NNRETAVFD--R-FK-------------NLTMLCNM-DDQANNMDPSGDDLND----I 328
gsLHR 294:DFTFNPAWNNGS--TSCDESDSTARVQHVIGGSAEGTLPKDLNIFSDADLFVDDESFGDV 351
asLHR 299:DSPINAAQHNGSRPTYCDD-SQSEKFPAGMVDSSDTSLLVEIHG-TNKDV--EDESYGGV 354
                                        *

bpLHR 323:NFQYPDLELDCLNNPFVKCLPKPDASNPCEDLLGFPFLRCLTWIITVFAVAGNLAVLIIL 382
ntLHR 325:NFQYPDLEFDCFSNPFVKCSPKPDAFNPCEDLLGFSFLRCLTWIIMVFAVAGNLAVLVIL 384
ccLHR 329:NFHYPDLEL-CASSSSFKCTPEPDAFNPCEDLLGHTFLRAITWIVTVFALVGNLAVLLVL 387
gsLHR 352:NFHYPELDF-CQTRPTLLCTPEADAFNPCEDIAGFSFLRVAIWFINILAITGNLTVLLVS 410
asLHR 355:DFQYPELGLYCQTRPTLQCTPEADAFNPCEDIAGFSFLRVAIWFINILAIIGNLTVLLIF 414
                  *       *       *

bpLHR 383:LISHSKFTISRFLMCNLAVADLCMGLYLMLIAFMDYHSHHEYYNHATDWQTGPGCGIAGF 442
ntLHR 385:LIGHHKLTVSRFLMCNLAFADLCMGLYLILIAFMDYHSHHEYYNHATDWQTGPGCGIAGF 444
ccLHR 388:LLSHQKLSVSRFLMCNLAFADLCMGFYLLLIAVADYRSRQEYYNHATDWQTGMGCGVAGF 447
gsLHR 411:FTSRNKLTVPRFFMCNLAFADLCIGIYLLMIATVDLRTHGHYSQHAIEWQTGPGCSAAGF 470
asLHR 415:FTSRCKLTVPRFLMCHLAFADFCIGVYLLMIAAVDLHTRGHYSEHAIDWQTGAGCSAAGF 474
                                                  *

bpLHR 443:LTVFASELSVYTLTVISLERWHTITNAMHVNKRLRMHHVAAIMGAGWGFSLLVALLPLVG 502
ntLHR 445:LTVFSSELSVYTLTVISLERWHTITNAMHVNKRLRMHHVTAMMVGGWAFSLLVALLPLVG 504
ccLHR 448:LTVFASELSIYTLTMITLERWHTITHAMQMSRRLRLRHMVTMMAIGWGFSFVIALLPVVG 507
gsLHR 471:LSVFGGELSVYTLSTITLERWHTITNALQVERHLLLTQAASIMAAGWLISLGMGMLPLVG 530
asLHR 475:LSVFGGELSVYTLSTITLERWHTITHALQLEKRLGLAQAAGIMAGGWLICLGMAMLPLVG 534

bpLHR 503:VSSYSRVSICLPMDIDTLGSQVYVVAMLVLNVVAFLVVCYCYVCIYLSVRNPEHSTRHGD 562
ntLHR 505:VSSYSKVSICLPMDIDTLGAQVYVVAVLILNVVAFLVVCYCYICIYLSVHNPEHSTRRGD 564
ccLHR 508:VSSYSKVSICLPMDIETPVSQGYVVAVLVINVAAFLMVCSSYAGIYLSVRNPNVPTRHGH 567
gsLHR 531:VSSYSKVSMCLPMDIETPLAQTFIIIILLFNVGAFIVVCVCYVLIYLAVKNPEVPRRSAD 590
asLHR 535:VSSYSRVSMCLPMDVKTPLAQAFILLLLFFNVGAFLVVCVCYVLIYLAVRNPQFPSRSAD 594

bpLHR 563:TKIAKRMAVLIFTDFVCMAPISFSAISAALRMPLITVSHSKILLILFYPIDSLC-NPFLY 621
ntLHR 565:TKIAKRMAVLIFTDFLCMAPISFFAISAALRMPLITVSHSKILLILFYPINSLC-NPFLY 623
ccLHR 568:TRMAKRMAVLIFTDFLCMAPISFFAISAALHMPLISVSQSKILLILFYPINSLC-NPFLY 626
gsLHR 591:TKMAKRMAVLIFTDFLCMAPISFFAISAAFKVPLITVTNSKILLVLFFPINS-CANPFLY 649
asLHR 595:AKIAKRMAVLIFTDLLCMAPISFFAISAAFKVPLITVTNSKILLVLFFPINS-CANPFLY 653

bpLHR 622:TLFTRAFRRDVCLLLGRCGCCHASADFYRSQTLASHLNSTQKTSTKKTHSLGFYAYHIKM 681
ntLHR 624:TIFTRAFRKDVCLLLSRCGCCNSHADFYRSQTLGSHLTCTQKMSKREPHSLGFYAYHIKM 683
ccLHR 627:TIFTRAFRRDMCLLLSRCGCCHAQAEFYRSQGLLGFTLAPRKKRVRKPHSKNFRAYHVKL 686
gsLHR 650:AIFTKAFRKDAYQLMSALGCCKSKASVHRMNAHCGEKAI--NFGSSYKGSGTAVRLAVME 707
asLHR 654:AIFTKAFRKDVYLLLSNMGCCENKANMYRMKAYCSENLVKSSSGNKGTLICTTQMMDPLP 713
                              **

bpLHR 682:QGCFLNKGST----- 691
ntLHR 684:KGCFLNKGTT----- 693
ccLHR 687:QGCIFNKAAT----- 696
gsLHR 708:GQSHHPKEEGELT-- 720
asLHR 714:LQSQQLKDDGDLGTI 728
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Fig. 2. (*continued*).

(*Acanthopagrus latus*), red seabream, Atlantic croaker (*Micropogonias undulatus*), striped bass, European sea bass, bamboo leaf wrasse (*Pseudolabrus sieboldi*), orange spotted grouper, longtooth grouper, Nile tilapia, Mozambique tilapia, three spot gourami, and blotched snakehead. FSHR: black porgy, gilthead seabream (*Sparus aurata*), European sea bass, Nile tilapia, killifish, Atlantic salmon (*Salmo salar*), amago (*Oncorhynchus rhodurus*), rainbow trout (*Oncorhynchus mykiss*), Japanese eel (*Anguilla japonica*), and zebrafish (*Danio rerio*). LHR: black porgy, gilthead seabream, European sea bass, Atlantic salmon, rainbow trout, African catfish (*Clarias gariepinus*), zebrafish, grass carp (*Ctenopharyngodon idella*), Japanese eel, channel catfish, and Nile tilapia. The phylogenetic tree was constructed using the neighbor-joining method and the Mega 3.1 software package (Center for Evolutionary Functional Genomics, Tempe, AZ, USA).

2.8. QPCR

QPCR was conducted to determine the relative expression of GTH-subunits and GTH-receptors mRNA using the total RNA extracted from pituitary and gonads of black porgy. The primers used for the QPCR were GTHα forward primer (5′-AAG ACG ATG ACG ATC CCG AAG-3′), GTHα reverse primer (5′-GTG TGG TTC CTC ACC CTT ATG C-3′), FSHβ forward primer (5′-TGC CAT CCA ACC AAC ATC AGC-3′), FSHβ reverse primer (5′-ATC CTC GTG GTA GCA CTG TCC-3′), LHβ forward primer (5′-ACC AAG GAC CCA GTG ATG AAG AC-3′), LHβ reverse primer (5′-GGG CGG ACA GTC AGG AAG C-3′), FSHR forward primer (5′-CCG ACC CAA GAC GAA CTC AAC-3′), FSHR reverse primer (5′-CCA GCA GGA CCA GAA GCA C-3′), LHR forward primer (5′-GAC GGT GTT CGC CAG TGA G-3′), LHR reverse primer (5′-GCG TTG GTG ATG GTG TGC-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′), and β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and the iQ™ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. The QPCR conditions were 1 cycle of denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, and annealing at 55 °C for 20 s. Each experimental group was run in triplicate. As an internal control, experiments were duplicated with β-actin, and all data were normalized to the β-actin calculated threshold-cycle (Ct) level. The Ct was defined as the PCR cycle at which the fluorescence signal crossed a threshold line that was placed in the exponential phase of the amplification curve. After the PCR, QPCR data from three replicate samples were analyzed with the software of the cycler system to estimate the transcript copy numbers for each sample. The efficiencies of the reactions were determined by performing the QPCR. The efficiencies were as follows: β-actin=94.3%, GTHα=92.8%, FSHβ=96.0%, LHβ=96.5%, FSHR=95.1%, and LHR=92.4%. We performed a melt curve at one temperature to ensure that the primers amplified a specific product.

2.9. Plasma parameter analysis

Plasma E_2 levels were analyzed by radioimmunoassay (RIA) using an E_2 RIA kit (Adaltis, Bologna, Italy).

2.10. Statistical analysis

A one-way analysis of variance followed by a *post hoc* Newman Keuls' test was used to compare differences at a significance level of $P<0.05$. The data were analyzed with the Statistical Package for the Social Sciences version 10.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Identification of GTHα, FSHβ, and LHβ cDNAs

A single PCR product of the expected size (GTHα, 294 base pairs [bp]; FSHβ, 354 bp; and LHβ, 397 bp) was obtained for each subunit. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone the full-length cDNA encoding each of the three GTH subunits.

The full-length GTHα cDNA contained 354 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 117 amino acids. The full-length FSHβ cDNA consisted of 363 nucleotides, including an ORF that was predicted to encode a protein of 120 amino acids. The full-length LHβ cDNA contained 414 nucleotides, including an ORF that was predicted to encode a protein of 137 amino acids. The three GTH subunits contained signal peptides (GTHα, residues 1–23; FSHβ, residues 1–15; and LHβ, residues 1–24), cysteine residues, and a highly conserved N-linked glycosylation site (Fig. 1).

Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that the three GTH-subunit sequences displayed a high similarity with those of other teleosts, and these were compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid similarities were as follows: GTHα, 95% with red seabream, 92% with swamp eel, 91% with striped bass, and 89% with European sea bass; FSHβ, 81% with red seabream, 75% with European sea bass, 74% with striped bass, and 74% with the three spot gourami; LHβ, 98% with yellowfin seabream, 94% with red seabream, 87% with striped bass, and 86% with European sea bass.

3.2. Identification of FSHR and LHR cDNAs

A single PCR product of the expected size (FSHR, 759 bp; LHR, 471 bp) was obtained for the GTH receptors. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone the full-length cDNA encoding each of the two GTH receptors.

The full-length FSHR cDNA contained 2118 nucleotides, including an ORF that was predicted to encode a protein of 705 amino acids. The full-length LHR cDNA consisted of 2076 nucleotides, including an ORF that was predicted to encode a protein of 691 amino acids.

The FSHR ECD was predicted to contain 380 amino acids, including the signal peptide (positions 1–19), followed by a putative 7TMD (263 amino acids) and an intracellular C-terminal domain (62 amino acids). ClustalX alignment revealed the presence of specific signature sequences (e.g. ^{318}CCAF, ^{480}ERW, ^{593}FTD, and ^{635}NPFLY), which are highly conserved in GpHRs (Vassart et al., 2004). In the areas flanking the LRR region, two cysteines (^{45}C and ^{65}C) and six cysteines (^{318}C, ^{319}C, ^{336}C, ^{353}C, ^{360}C, and ^{370}C) were found that could represent the N- and C-terminal cysteine-rich clusters, respectively. The putative N-linked glycosylation sites identified four motifs at positions ^{58}NIS, ^{234}NGT, ^{326}NRS, and ^{354}NST in the bps of the FSHR. The TMD of the FSHR consisted of 263 amino acids, including seven stretches of 13–23 predominantly hydrophobic residues predicted to form α-helices connected by three intracellular and three extracellular (EC) loops. The conserved ^{480}ERW motif was in the bottom third of the TMD, and the NPXXY motif was also present as a form of ^{633}NPELY, one of the most conserved residues in rhodopsin-like GPCRs. This residue is implicated in receptor activation by switching its interaction between ^{422}D and ^{595}D aspartic residues (Vassart et al., 2004). There were two phosphorylation sites (^{569}T and ^{574}S) in the third intracellular loop and four potential phosphorylation sites (^{646}T, ^{672}S, ^{682}S and ^{686}S) in the intracellular C-terminal domain. Among them, ^{569}T and ^{646}T are potential phosphorylation sites for protein kinase C, and ^{574}S and ^{686}S are potential sites for protein kinase A phosphorylation (Fig. 2A).

Similar to FSHR, the LHR ECD was predicted to contain 361 amino acids, including a signal peptide (positions 1–23), followed by a putative 7TMD (261 amino acids), and an intracellular C-terminal domain (69 amino acids). The LRRs were flanked by 10 conserved cysteines, four of them in an N-terminal cluster (^{26}C, ^{30}C, ^{32}C, and ^{39}C) and six in a C-terminal group (^{282}C, ^{283}C, ^{305}C, ^{333}C, ^{341}C, and ^{351}C). The putative N-linked glycosylation sites identified four motifs at positions ^{71}NIT, ^{198}NGT, ^{299}NST, and ^{375}NLA in the bps of the FSHR. The predicted TMD included seven stretches of hydrophobic residues. The

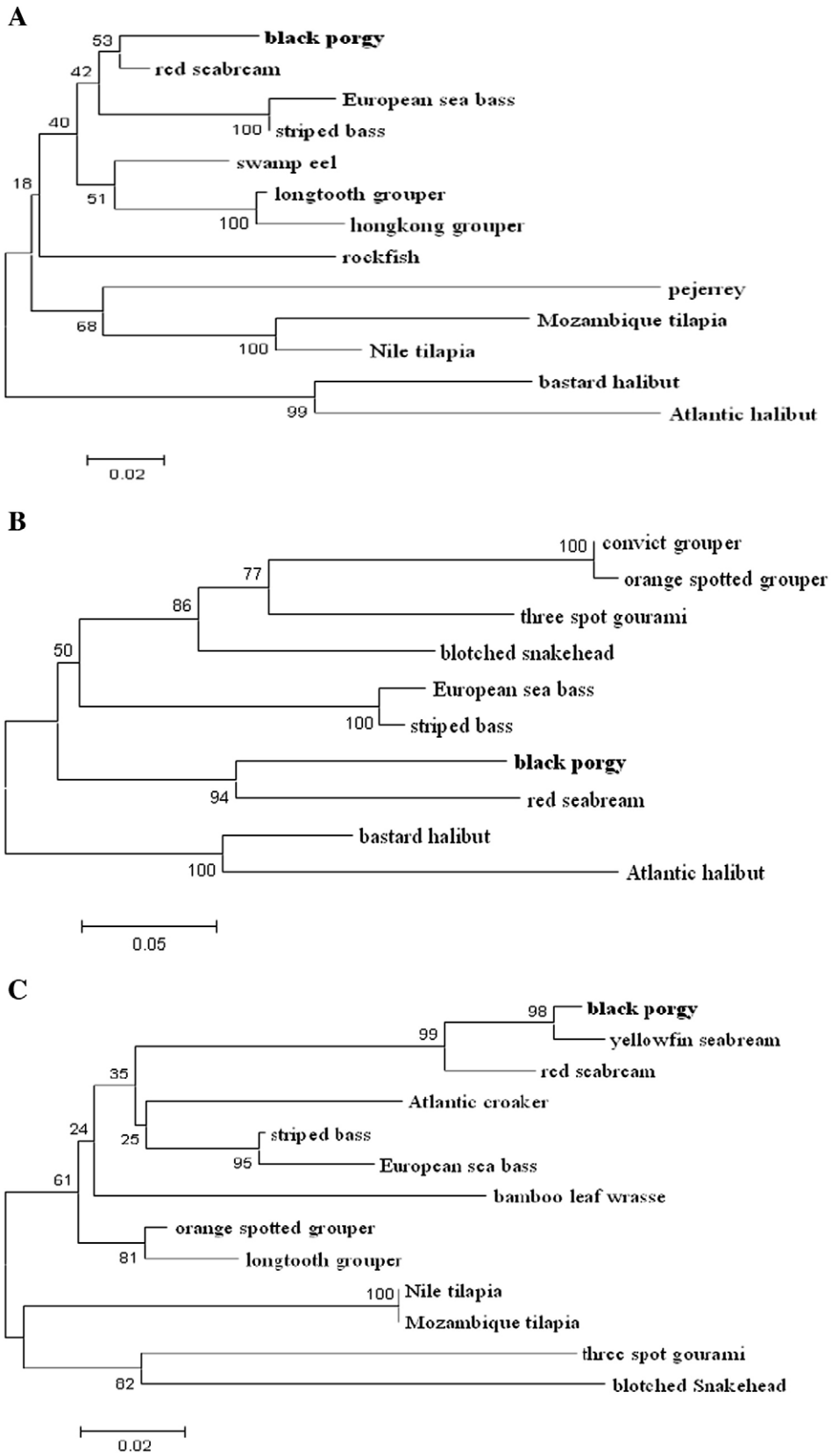

Fig. 3. Phylogenetic tree based on an amino acid alignment for GTH subunits in teleost fish. Bootstrap values (%) are indicated 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: GTHα; black porgy, red seabream, European sea bass, striped bass, swamp eel, longtooth grouper (EF583918), Hong Kong grouper (AY207430), rockfish (AY609078), pejerrey (DQ382280), Mozambique tilapia (AF303087), Nile tilapia (AY294017), bastard halibut (AF268692), and Atlantic halibut (AJ417770), FSHβ; black porgy, red seabream, striped bass, European sea bass, blotched snakehead (AY447038), three spot gourami, convict grouper (AB111457), orange spotted grouper (AY186242), bastard halibut (AB042422) and Atlantic halibut (AJ417768), LHβ; black porgy, yellowfin seabream, red seabream, Atlantic croaker (EF433429), striped bass, European sea bass, bamboo leaf wrasse (AB300391), orange spotted grouper (AF507939), longtooth grouper (EF583920), Nile tilapia (AY294016), Mozambique tilapia (AY541609), three spot gourami (AF157631), and blotched snakehead (AY447037).

intracellular C-terminal domain consisted of two highly conserved contiguous cysteines (^{641}C and ^{642}C). Similar to the FSHR, the LHR contained highly conserved GpHR signature sequences (e.g. ^{461}ERW, ^{574}FTD, and ^{617}NPFLY). Moreover, the NPXXY motif was also present as a form of ^{617}NPELY, one of the most conserved residues in rhodopsin-like GPCRs (Vassart et al., 2004; Fig. 2B).

Using Blastp, we found that the FSHR and LHR sequences displayed a high similarity to those of other teleosts. The amino acid sequences of the three receptors were compared to those deduced from the cDNA of other teleost species (Fig. 2). The amino acid similarities were as follows: FSHR, 94% with gilthead seabream, 82% with European sea bass, 76% with Nile tilapia, and 74% with killifish; LHR, 84% with Nile tilapia, 62% with channel catfish, 51% with gilthead seabream, and 50% with Atlantic salmon.

3.3. Phylogenetic analysis

The phylogenetic trees of these genes were very similar and generally agreed with the known taxonomic relationships among these species. The phylogenetic analysis indicated the expected relationship among the GTH subunits and GTH receptors. Therefore, we named these genes based on our proposed nomenclature and phylogenetic analysis. The black porgy GTH subunits were most closely related to red seabream (GTHα and FSHβ) and yellowfin seabream (LHβ), which include the Perciformes (Fig. 3). The porgy FSHR was most closely related to gilthead seabream, and the LHR was most closely related to Nile tilapia (Fig. 4).

3.4. Effects of E_2 on GTH-subunit mRNA expression in pituitary

The mRNA of each of the GTH subunits increased significantly after E_2 injection (Fig. 5). The GTHα mRNA increased significantly after 3 days (approximately 5.4-fold that of the control) and then decreased 9 days after the injection (Fig. 5A). The FSHβ mRNA was at its highest after 6 days (approximately 5.9-fold that of the control) and then decreased after 9 days (Fig. 5B). The LHβ mRNA was at its highest after 3 to 6 days (approximately 5.1-fold that of the control) and then decreased after 9 days (Fig. 5C).

3.5. Effects of E_2 on GTH-receptor mRNA expression in gonad

The mRNA of both GTH receptors increased significantly after E_2 injection. The FSHR mRNA was at its highest after 6 days (approximately 5.7-fold that of the control) and then decreased after 9 days (Fig. 6A). The LHR mRNA was at its highest after 6 days (approximately 6.8-fold that of the control) and then decreased after 9 days (Fig. 6B).

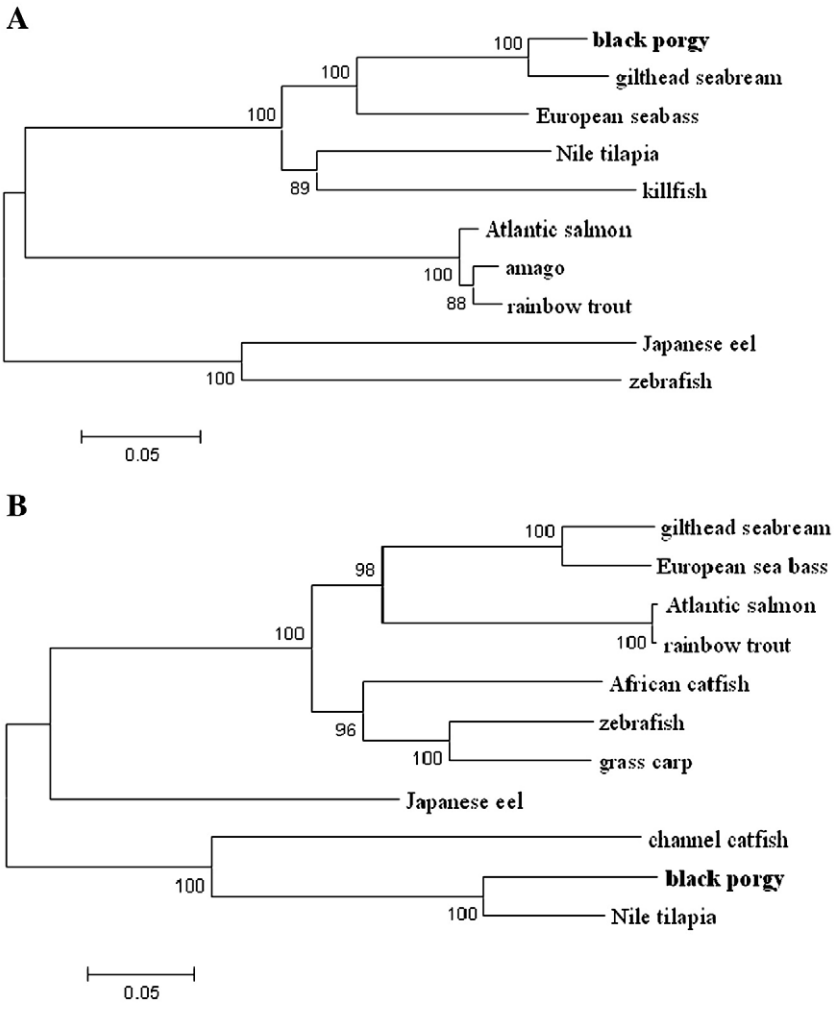

Fig. 4. Phylogenetic tree based on an amino acid alignment for GTH receptors in teleost fish. Bootstrap values (%) are indicated 1000 replicates. The number associated with each internal branch is the local bootstrap probability. GenBank accession numbers of the sequences are: FSHR; black porgy, gilthead seabream, European sea bass, Nile tilapia, killifish, Atlantic salmon (AJ567667), amago (AB030012), rainbow trout (AF439405), Japanese eel (AB360713), and zebrafish (XM_001337064), LHR; black porgy, gilthead seabream, European sea bass (EU282005), Atlantic salmon, rainbow trout (AF439404), African catfish (AF324540), zebrafish (AY714133), grass carp (EF194761), Japanese eel (AY742795), channel catfish, and Nile tilapia.

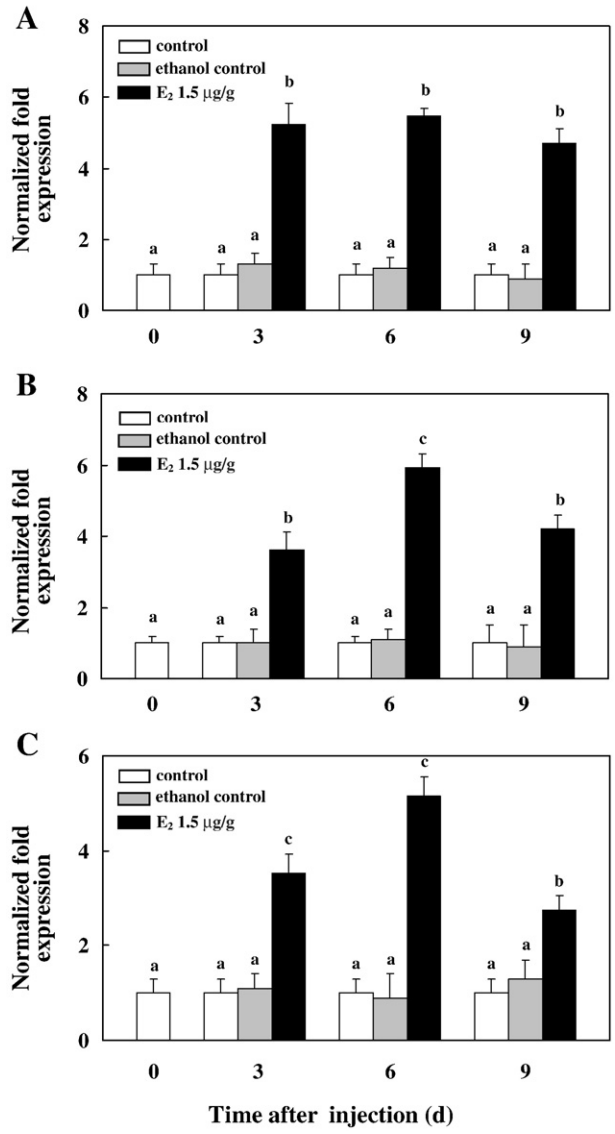

Fig. 5. Expression of GTHα (A), FSHβ (B) and LHβ (C) mRNA in pituitary of black porgy using quantitative PCR by E_2 (1.5 μg/g) injection. 2.5 μg of total RNA prepared from pituitary was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

3.6. Effects of E_2 on E_2 levels in plasma

The plasma E_2 level was 9.4±0.7 pg/mL at the start of the experiment, increased to a maximum of 260.1±23.54 pg/mL after 3 days, and then decreased to 96.0±13.2 pg/mL after 9 days (Fig. 7).

3.7. Expression of pituitary GTH subunit mRNA during sex change

GTHα mRNA was at its lowest when the fish were immature (Fig. 8A), and FSHβ mRNA was higher in mature males and females but then decreased when the mature gonad became primarily ovary (Fig. 8B). LHβ mRNA was higher in mature males and females (Fig. 8C) than during the other maturation stages.

3.8. Expression of gonadal GTH receptor mRNA during sex change

The FSHR and LHR mRNA was highest in mature males and females than the other maturation stages (Fig. 9).

4. Discussion

In the present study, we first isolated the full-length GTH subunit (GTHα, FSHβ, and LHβ) cDNAs in the pituitary and the GTH receptor (FSHR and LHR) cDNAs in the ovaries of mature females. We studied the effects of E_2 on GTH subunit and GTH receptor mRNA levels in the pituitary and gonads of immature black porgy after the fish were injected with E_2, and investigated interaction between GTH-subunits and GTH receptors during sex change of black porgy.

Using Blastp, we found that the amino acid sequences for the three GTH subunits were highly similar to those of other fish species (GTHα, 89–95%; FSHβ, 74–81%; and LHβ, 86–98%). The cysteine and N-linked glycosylation sites, which are reportedly sites for receptor binding in mammals and fish, were conserved in all three of the GTH subunits of black porgy (Xia et al., 1994; Gen et al., 2000). Similar to the GTHs, we found that the amino acid sequences for the GTH receptors were highly similar to those of other fish species (FSHR, 74–94%; LHR, 50–84%). N-linked glycosylation sites were identified in black porgy GTH receptors and have been linked to binding and expression of specific hormones (Maugars and Schmitz, 2006). The black porgy FSHR and LHR contained the general structural features of a GpHR (Fig. 2). Unlike FSHR, LHR had four cysteines in their N-terminal cysteine-rich region, whereas the FSHR had only two (^{45}C and ^{65}C). This structure allows for the formation of a single disulfide bridge that differs from the location of the two bridges found in the human FSHR (Fan and Hendrickson, 2005), suggesting a difference in the folding of the receptors in this region. There are variable numbers of cysteines in this region of other fish FSHRs (Rocha et al., 2007).

The phylogenetic analysis indicated that the GTH subunits and receptors were closely related to other Perciforms (red seabream GTHα and FSHβ, yellowfin seabream LHβ, gilthead seabream FSHR,

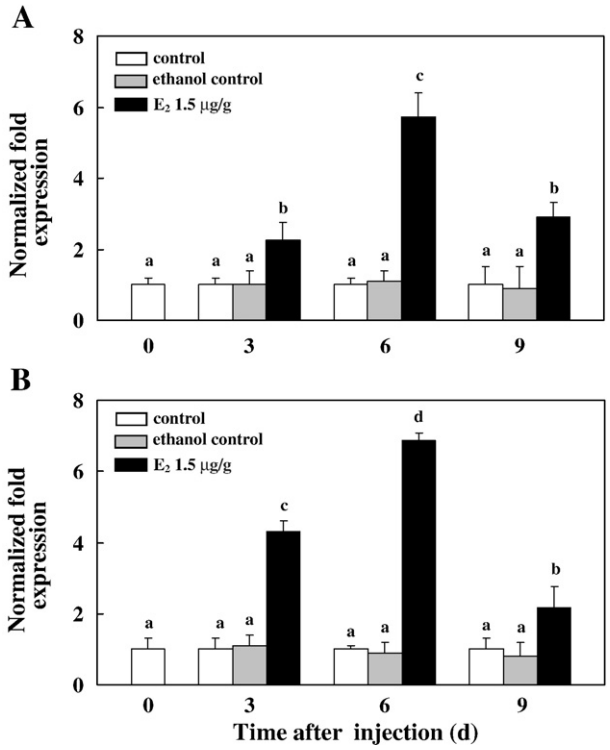

Fig. 6. Expression of FSHR (A), and LHR (B) mRNA in gonads of black porgy using quantitative PCR by E_2 (1.5 μg/g) injection. 2.5 μg of total RNA prepared from gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample, and the mean value of the control was set to 1. Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

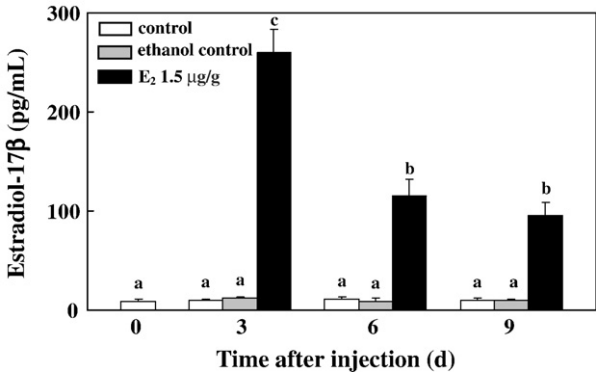

Fig. 7. The plasma estradiol-17β (E_2) levels by estradiol-17β injection in black porgy. Values with dissimilar letters are significantly different ($P<0.05$) from each other. Values are means±SD ($n=3$).

and Nile tilapia LHR). We also found that each of the GTH subunits and receptors was well conserved among other fish species (Figs. 3 and 4).

E_2 was a regulator of the GTH subunits and their receptors as shown by their expression patterns during sex change. The increase of pituitary GTHα mRNA following E_2 injection agreed with the result of a previous study of goldfish (Huggard-Nelson et al., 2002). In agreement with the results of a study of mature grouper (Li et al., 2005), the mRNA expression of the GTH subunits was detected in the pituitary of black porgy at all stages of gonadal maturity, and this expression was relatively higher in mature males and females (Fig. 8). In particular, the GTHα expression increased at mature testis and remained elevated thorough sex change, suggesting that it may be involved with sex change in black porgy; however, it is the common subunit in the pituitary among TSH, FSH, and LH (Salmon et al., 1993).

FSHβ mRNA expression increased following the administration of E_2. This result agreed with a previous study of goldfish that reported increased expression of GTHα, FSHβ, and LHβ (Huggard-Nelson et al., 2002). In the European eel, FSHβ mRNA was increased specifically by E_2, not by testosterone (T) or dihydrotestosterone (DHT) (Aroua et al., 2007). Although FSHβ expression was higher than LHβ in black porgy, we found that the increase was more closely associated with sexual maturation. This result agreed with previous studies demonstrating that FSHβ expression is high during early maturational stages in fish (Gen et al., 2000; Hassin et al., 2000). In mammals, FSH regulation depends not only on steroid hormones but also on gonadal peptides such as activin, inhibin, and follistatin. Activin has been detected in several fish (Pang and Ge, 1999; Yam et al., 1999) and has been shown to regulate FSH in the goldfish pituitary (Yam et al., 1999) and in the zebrafish ovary (Pang and Ge, 1999). Although activin has been detected in the black porgy, FSH expression is dependent on the reproductive stage of the fish (Yaron et al., 2003), so the mechanism of FSH regulation in black porgy remains unclear.

E_2 also increases LHβ mRNA expression in fish (Chang et al., 1994; Xiong et al., 1994; Yen et al., 2002). Also, treatment with exogenous E_2 increases LH levels in goldfish during the spawning season (Huggard-Nelson et al., 2002) and induces sex change in black porgy (Chang et al., 1994, 1995; Lee et al., 2001). It is well established that E_2 is involved with the activation of estrogen response elements (EREs). The presence of EREs on the upstream portion of the LHβ gene has been demonstrated in mammals and fish (Xiong et al., 1994). In chinook salmon, a proximal ERE (pERE) increases LHβ gene transcription by de-repressing the proximal silencer (Xiong et al., 1994), indicating that the stimulatory effects of E_2 may occur, in part, at the level of the LHβ gene in chinook salmon. However, the presence of EREs has not been demonstrated in black porgy. E_2 treatment stimulates seabream GnRH mRNA expression in the preoptic anterior hypothalamic area (POAH), a brain region that projects GnRH neurons to the pituitary (Mohamed et al., 2005). Thus, the positive feedback of E_2 on LH appears to involve GnRH activation, which may stimulate LH synthesis and release.

In mammals, E_2 indirectly stimulates GTH production by upregulating the number of pituitary GnRH receptors (Adams et al., 1981), altering the GnRH receptor mRNA level (Quinones-Jenab et al., 1996), or by increasing GnRH release (Nett et al., 1984). Further studies have demonstrated that the two forms of GnRH present in the European silver eel may be differentially regulated by gonadal steroids (Montero et al., 1995), supporting the view that indirect regulation of GTH production by E_2 may involve GnRH regulation. Treatment with E_2 increases pituitary LH and brain GnRH content (Dufour et al., 1983, 1985). Taken together, these results suggest that E_2 may indirectly regulate GTH production. Further studies are needed to determine the interactions among E_2, GTHs, and GnRHs.

FSHR and LHR expression increased in the gonad following E_2 injection (Fig. 6), which was consistent with pituitary GTH mRNA expression (Fig. 5). The stimulatory effect of E_2 increases GnRH levels in the brain resulting in an increase in the pituitary GTHs (Adams et al.,

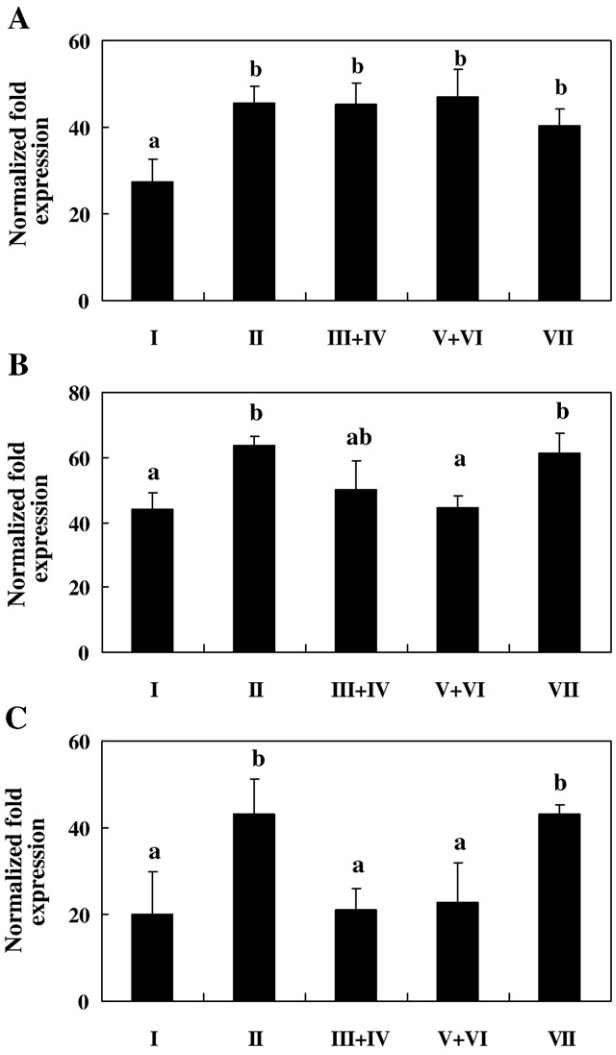

Fig. 8. Expression of GTHα (A), FSHβ (B) and LHβ (C) mRNA in pituitary of black porgy by quantitative real-time PCR. 2.5 μg of total RNA prepared from pituitary was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex change process from male to female (I: immature testis, II: mature testis, III+IV: mostly testicular gonad, V+VI: mostly ovarian gonad, VII: mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means±SD ($n=3$).

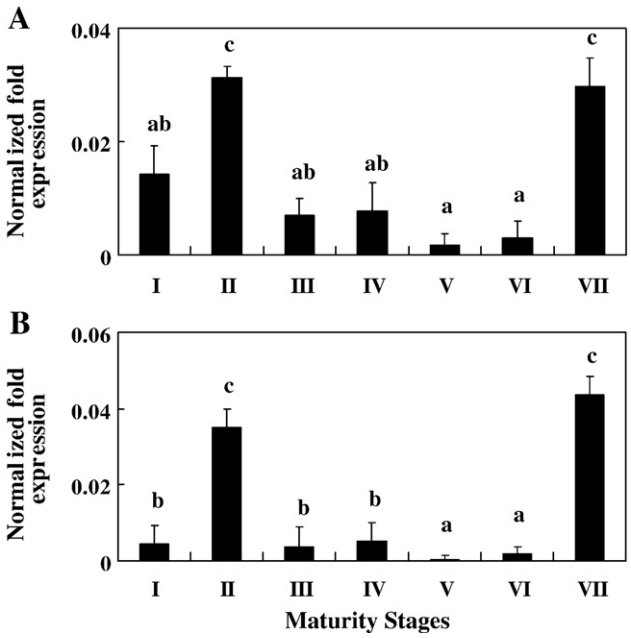

Fig. 9. Expression of FSHR (A) and LHR (B) mRNA in gonads of black porgy by quantitative real-time PCR. 2.5 μg of total RNA prepared from gonads was reverse transcribed and amplified using gene-specific primers. Results are expressed as normalized fold expression with respect to β-actin levels for the same sample. Maturity stages were divided into seven stages during the sex change process from male to female (I: immature testis, II: mature testis, III: testicular portion of mostly testis, IV: ovarian portion of mostly testis, V: testicular portion of mostly ovary, VI: ovarian portion of mostly ovary, VII: mature ovary). Values with dissimilar letters are significantly different ($P<0.05$). Values are means ± SD ($n=3$).

1981), indicating that GTHs are involved in gonadal development by binding to FSHR and LHR (Nagahama et al., 1995).

Although E_2 did not affect FSHR mRNA in Japanese eel (Jeng et al., 2007), E_2 increased pituitary GTHs mRNA as well as FSHR and LHR mRNA expression in gonads of black porgy, demonstrating the involvement of E_2 in gonadal development. However, further studies are necessary to fully establish this association.

In particular, E_2 was found to induce sex change in 1-yr old black porgy by E_2 fed for 7 months, however, the ovary remained at the primary oocyte state (Chang et al., 1994, 1995), and vitellogenic oocytes were observed in 2-yr old black porgy after treatment with E_2 (4–6 mg/kg feed) for at least 5 months (Lee et al., 2000). According to these studies, it is suggested that E_2 induces sex change of black porgy. We found that the maximum plasma E_2 level (3 days; 260.1 ± 23.54 pg/mL) following E_2 injection was lower than that of mature female black porgy (705.6 ± 70 pg/mL) during sex change determined in our previous study (An et al., 2008). These data suggest that the plasma E_2 concentration attained following the exogenous administration of E_2 did not affect sex change but only temporarily increased plasma levels in immature black porgy. Therefore, these results combined with these previous reports suggest that E_2 is involved in ovarian maturation as well as the male-to-female sex change in black porgy.

The mRNA of the GTH subunits and GTH receptors increased in mature male and female black porgy. The increased FSHR and LHR mRNA implies that pituitary-stimulated FSH and LH release is involved in gonadal development.

In conclusion, GTH-subunit and GTH-receptor cDNAs were isolated from mature female black porgy, and the mRNA expression of the genes was investigated following E_2 injection of immature fish. We also compared the mRNA expression at each stage of the sex change process. We found that E_2 stimulated GTH-subunits mRNA in the pituitary and GTH-receptors mRNA in the gonads. Our results showed a high level of expression in the immature gonads, indicating that E_2 affects the brain–pituitary–gonad axis and leads to gonadal development. Additional studies, including those with different doses and treatments during gonadal development, will be necessary to fully identify the roles of E_2 in maturation. The results of this study can be used to elucidate the endocrinological mechanism and relationship between E_2 and the GTH subunits in black porgy.

Acknowledgements

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