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Molecular characterization of two sea bass gonadotropin receptors: cDNA cloning, expression analysis, and functional activity.

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Abstract

The follicle-stimulating hormone (FSH) and the luteinizing hormone (LH) play central roles in vertebrate reproduction. They act through their cognate receptors to stimulate testicular and ovarian functions. The present study reports the cloning and characterization of two sea bass (*Dicentrarchus labrax*) cDNAs encoding a FSH receptor (sbsFSHR) and a LH receptor (sbsLHR). The mature proteins display typical features of the glycoprotein hormone receptor family members, but the sbsFSHR also contains some remarkable differences when compared with other fish or mammalian FSHRs. Among them, a distinct extracellular N-terminal cysteine domain as regards to its length and cysteine number, and the presence of an extra leucine-rich repeat. Expression analysis revealed that the *sbsFSHR* is exclusively expressed in gonadal tissues, specifically in the follicular wall of previtelogenic and early-vitelogenic follicles. On the contrary, *sbsLHR* mRNA was found to be widely distributed in sea bass somatic tissues. When stably expressed in mammalian cell lines, sbsFSHR was specifically stimulated by bovine FSH, while sbsLHR was activated by both bovine LH and FSH. Nevertheless, specific stimulation of the sbsLHR was observed when recombinant sea bass gonadotropins were used. The isolation of a FSHR and a LHR in sea bass opens new ways to study gonadotropin action in this species.

Introduction

It is well established that the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), secreted by the pituitary gland, play central roles in vertebrate reproduction (Chappel and Howles, 1991). In ovarian follicles, FSH regulates granulosa cell proliferation, the synthesis of cell cycle-regulatory proteins and induces the expression of differentiation-specific genes (Richards, 1994). On the other hand LH promotes follicular maturation, ovulation and the synthesis of ovarian steroid hormones. In the testes, FSH determines Sertoli cell proliferation and attends germ cell maturation (Allan and Handelsman, 2005), while LH supports Leydig cell functions and stimulates the synthesis of androgens, regulating the final stages of spermatogenesis. FSH and LH are members of the glycoprotein hormone family, which also includes the thyroid stimulating hormone (TSH) and the chorionic gonadotropin (CG) (Pierce and Parsons, 1981).

The glycoprotein hormones exert their biological actions by interacting with specific receptors present on target cell surfaces. Accordingly, the FSH receptor (FSHR) gene is expressed only in granulosa cells in the ovary and in Sertoli cells in the testis. The LH receptor (LHR) is expressed primarily in theca and granulosa cells of ovarian preovulatory follicles and in the Leydig cells in the testes (reviewed in refs. Themmen and Huhtaniemi, 2000; Ascoli et al., 2002; Vassart et al., 2004). In primates and equines, it also binds the CG, a placental hormone essential for the maintenance of pregnancy, structurally similar to LH (Bousfield et al., 1994). These receptors are encoded by paralogous genes belonging to the large family of G protein-coupled receptors (GPCRs) (Simoni et al., 1997; Ascoli et al., 2002; Dias et al., 2002; Szkudlinski et al., 2002), which includes in addition the TSH receptor that binds TSH. They constitute the subfamily of glycoprotein hormone receptors (GpHRs), themselves members of the wider leucine-rich repeat containing GPCR (LGR) family. The members of this family are characterized by a large extracellular (EC) domain with multiple

imperfect leucine-rich repeats (LRRs), flanked by N- and C-terminal cysteine-rich subdomains. This is followed by a rhodopsin-like domain of seven transmembrane (7TM) helices and a C-terminal intracellular tail (Hsu et al., 2000).

Evidence for the presence of gonadotropin receptors in fish was first demonstrated more than ten years ago, by binding studies of coho salmon FSH and LH to membranes of isolated granulosa cells and theca-interstitial layers of coho salmon ovary (Yan et al., 1992; Miwa et al., 1994). The isolation of the cDNAs from these receptors remained elusive for years. Finally, the cloning of two types of gonadotropin receptors from the ovaries of amago salmon was reported (Oba et al., 1999a; Oba et al., 1999b), thereby providing new experimental tools to understand fish GpHRs biology.

Ligand binding and receptor activation appear to be distinct activities of the GpHRs. Ligand binding specificity and affinity are determined by residues present in the β -strands of the LRRs (Braun et al., 1991; Smits et al., 2003), whereas the TM domain is responsible for receptor activation and signal transduction through G proteins. Hormone binding to the EC domain of a GpHR causes changes in the 7TM domain promoting cAMP accumulation via activation of heterotrimeric Gs, thereby initiating a signalling cascade that leads to steroid synthesis (Ascoli et al., 2002; Dias et al., 2002; Szkudlinski et al., 2002).

Ligand specificity of the mammalian GpHRs is well defined, with no cross-stimulation occurring under physiological conditions. Conversely, promiscuous activation of fish GpHRs has been described. *In vitro* experiments with African catfish, channel catfish and zebrafish recombinant FSHRs suggest that these are not selective for FSH, as LH can also activate them, whereas, LHRs are highly selective for homologous LH (Bogerd et al., 2001; Kumar et al., 2001a; Kumar et al., 2001b; Vischer and Bogerd, 2003; Kwok et al., 2005). These data are consistent with results obtained in coho salmon, whereby type I receptor (i.e., the putative FSHR), localized in both the theca and granulosa cells, did not discriminate between FSH and

LH, while a type II receptor i.e., the putative LHR, located only in granulosa cells, was highly selective for LH (Yan et al., 1992; Miwa et al., 1994). However, the ligand selectivity of the amago salmon receptors appeared to be different from this (Oba et al., 1999a; Oba et al., 1999b). Heterologous cells transiently transfected with the amago salmon *FSHR* were specifically activated by chum salmon FSH but not by LH, whereas *LHR* transfected cells were highly responsive to LH and in a lesser extent to FSH.

This different ligand selectivity could imply that GpHR actions in teleosts are not totally overlapping those of their mammalian counterparts, and reinforces the need of learning more from fish, the most diverse group of vertebrates with dissimilar modes of gonadal development and reproductive strategies. Although the cloning of a *FSHR* and a *LHR* has been reported in two species of Perciforms, Nile tilapia and seabream (Oba et al., 2001; Wong et al., 2004), there is no information regarding the functional characterization of these receptors in this lineage of fish.

To bring more knowledge on fish gonadotropin receptors, in the present study we report the isolation and functional characterization of the cDNAs coding for a sea bass *FSHR* (sbsFSHR) and a *LHR* (sbsLHR).

Materials and Methods

Isolation of Nucleic Acids

Sexually mature female and male sea bass (*Dicentrarchus labrax*) were obtained from the stock raised in the Instituto de Acuicultura de Torre la Sal facilities. Animals were treated in accordance with the European Union Animal Care Regulations.

Total RNA was isolated from sea bass tissues (n = 2 animals/ tissue) following the TRI Reagent protocol (Molecular Research Center, Inc. Cincinnati, OH). Poly(A)⁺ RNA was isolated using PolyATtract mRNA Isolation Systems (Promega Corp., Southampton, UK).

Genomic DNA (gDNA) was isolated from sea bass whole blood, using a protein salting-out method (Martínez et al., 1998).

Reverse transcription-Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction

To perform PCR amplification on sea bass gDNA 150 ng were used as template.

Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) was used for cDNA synthesis by priming 4 µg of RQ1-DNase (Promega Corp.) treated total RNA with 100 ng of random hexamers in a total volume of 20 µl. Briefly, RNA was mixed with 100 ng of random hexamers, 1 µl of dNTPs (10 mM each dNTP) and denatured at 65°C for 5 min. After a quick chilling on ice, 40 units of RNAsin (Promega Corp.), and 200 units of SuperScript II reverse transcriptase were added. Incubation took place at 42°C for 50 min. The reaction was stopped by heating to 70°C for 15 min.

One to 2.5 µl of the cDNA were used for PCR reactions. The sequences of the primers used in this study are shown in Table 1. Touchdown PCR programs comprising a 10 to 15°C span of annealing temperatures were used following these conditions: an initial denaturation at 94°C for 2 min followed by 30 (15°C span) or 20 (10°C span) cycles of 94°C for 30 s, the highest annealing temperature for 30 s, and 72°C for 45 s. The annealing temperature was decreased 0.5°C per cycle.

Primer pairs fshr25-fshr26 and lhr35-lhr23 (Table 1) were designed to amplify an intron-containing region in order to exclude false positive bands arising from potential contaminating gDNA. Also, they share limited sequence identity. As internal control a 495 bp fragment of the sea bass 18S ribosomal RNA (rRNA) gene was amplified, using primers 5'18S and 3'18S. Cycling conditions consisted of 25 cycles at 94°C for 30 s, 65°C for 45 s, and 72°C for 45 s.

The resulting PCR products were cloned into the pGEM-T Easy Vector (Promega Corp.) and sequenced.

Screening of a sea bass cDNA testicular library

A 1175 bp cDNA fragment from the *sbsFSHR* and a 678 bp cDNA fragment of the *sbsLHR* were labelled with [α - 32 P]-dCTP by random octamer priming (Rad Prime DNA Labelling System Kit, Invitrogen Corp.) and used as probes to screen, following standard methods (Sambrook & Russel 2001), a sea bass testicular directional cDNA library constructed in the UNI-ZAP XR vector.

Prehybridizations and hybridizations were done at high stringency conditions (50% formamide, 42°C). Final washes were carried out at 55°C with 1xSSPE and 0.5% SDS. Several positive clones were obtained after first round screening of approximately 1×10^6 phages. Secondary and tertiary screenings were performed and the resulting positive phages were in vivo excised from the Lambda ZAP XR vector as pBluescript SK (-) phagemids, following the protocol described by the manufacturer (Stratagene Corp.).

DNA Sequence and Phylogenetic analysis

DNA sequences were determined for both strands on an automated ABI PRISM 3730 DNA Analyser (Applied Biosystems) using the Rhodamine Terminator Cycle Sequencing Kit (Perkin-Elmer Inc., Wellesley, MS). The generated sequences were assembled using Sequencher version 4.0.5 software (Gene Codes Corpor., Ann Arbor, MI). BLASTN and BLASTP (version 2.2.9, National Center for Biotechnology Information) were used for database searching. The presence and location of the putative signal peptide cleavage site, the 7TM helices, Ser, Thr, and Tyr phosphorylation sites and potential N-glycosylation sites in the amino acid sequences, were predicted using the prediction servers of the Center for Biological

Sequence Analysis (<http://www.cbs.dtu.dk/services/>). The Protein Families (Pfam) Database of the Sanger Institute (UK) was used to search for common protein domains. The UniProt name for the sbsFSHR and the sbsLHR are Q4L192_DICLA and Q4L191_DICLA respectively. Multiple sequence alignments were carried out using ClustalX version 1.81 (Thompson et al., 1997). Phylogenetic analyses, of full length amino acid sequences, were conducted using MEGA version 2.0 (Kumar et al., 2001). A rooted phylogenetic tree was constructed by means of the Neighbor-joining algorithm, using the LGR sequence of *Caenorhabditis elegans* as an outgroup. Gaps or missing data were pairwise deleted. One thousand bootstrapping pseudo replicates were used to assess the robustness of the inferred nodes of the tree.

Northern blot analysis

Poly (A)⁺ RNA (10 µg) from sea bass ovaries and total RNA (30 µg) from testes and ovaries were denatured and electrophoresed in the presence of formaldehyde using standard conditions (Sambrook and Russel, 2001). RNAs were blotted on a Hybond-NX nylon membrane (Amersham Biosciences UK Ltd.) and stained with methylene blue to control for equal loading. Prehybridization and hybridization were carried out overnight at 42°C with 50% formamide. The full length *sbsFSHR* and *sbsLHR* cDNAs were labelled with [α -³²P]-dCTP and used as probes. Final washes were performed in 2xSSC /0.5% SDS at 50 °C. The membranes were exposed to HyperfilmTM MP autoradiography films (Amersham Biosciences UK Ltd.).

In situ hybridization

In situ hybridization experiments were carried out as described previously (Cerdá-Reverter et al., 2003). Briefly, sea bass gonads were fixed with 4% paraformaldehyde (PAF)

overnight at 4°C, dehydrated, and embedded in Paraplast (Sherwood, St. Louis, MO). Before hybridization, 6µm sections were deparaffinized, rehydrated, and postfixed. Slides were later washed in phosphate buffer (PB) and treated with Proteinase-K. Next, they were washed in PB and post fixed again in PAF, subsequently rinsed in sterile water, and acetylated in a triethanolamine (0.1 M, pH 8)/acetic anhydride solution. Sections were then dehydrated and dried at room temperature.

A pBluescript SK (-) phagemid containing an 800 bp fragment from the *sbsFSHR* 3' UTR was used to prepare riboprobes. Antisense and sense RNA probes were synthesized in vitro by linearizing the phagemid with *Xba*I or *Xho*I, and in vitro transcription was carried out with T7 or T3 RNA polymerase, respectively. The probes were labelled with ³⁵S-UTP using the Riboprobe In Vitro Transcription System Kit (Promega Corp.). After in vitro RNA synthesis, samples were treated with 1U of RQ1-DNase RNase-free (Promega Corp.) and purified on Sephadex G50 columns. The two fractions containing the highest radioactivity were pooled, precipitated, stored at -20 °C and used within 1 week.

The ³⁵S-UTP riboprobes were pelleted and dissolved in an appropriate volume of 100 mM DTT to obtain 2x10⁵ cpm/µl. After 5 minutes incubation at 80°C, ³⁵S-UTP riboprobes were diluted 1/10 (final concentration of probes, 10 mM DTT and 2x10⁴ cpm/µl) in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8), 10% Dextan sulphate, 1X Denhardt's solution and 0.5 µg/ml of yeast RNA type III. Subsequently, 60 µl of hybridization solution were added to each pre-treated slides (see above) that were cover-slipped and incubated in a humidified chamber overnight at 55°C.

After washing and ribonuclease treatment, slides were finally dehydrated and dried at room temperature. Finished the hybridization process, slides were dipped in Hypercoat Photographic Emulsion RPN40 (Amersham Biosciences UK Ltd) and exposed under dry

conditions at 4°C for 15–20 days, developed in Kodak D-19 Film Developer and counterstained with Cleveland & Wolfe staining (Herlaut 1960). Anatomical locations were confirmed by reference to Mayer et al. (1990) and Alvariño et al. (1992).

Expression plasmids, cell culture and transfections

The pcDNA-sbsFSHR plasmid was generated by inserting a 2.8 kb fragment containing the complete open reading frame (ORF) of the *sbsFSHR* into the pcDNA3 expression vector (Invitrogen Corp.). The expression vector pcDNA-sbsLHR, containing the complete ORF of the *sbsLHR* was generated in several steps. First, two fragments corresponding to the EC domain of *sbsLHR* were PCR amplified with primer pairs lhr30-lhr24 and lhr35-lhr21 respectively (Table 1). Equal quantities of each fragment were used as template in an overlapping PCR reaction with primers lhr30-lhr21 (Table 1). The resulting PCR product was inserted into the pPCR-Script Amp SK (+) cloning vector (PCR-Script® Amp Cloning Kit, Stratagene, La Jolla, CA). A *Hind*III-*Vsp*I fragment from this plasmid containing most of the EC domain (nucleotides 1 to 1048) was in-frame ligated to a *Vsp*I-*Eco*RV fragment (nucleotides 1048 to 3172) obtained from a cDNA library phagemid, which corresponds to the rest of the *sbsLHR* cDNA. Finally, they were introduced into the *Hind*III-*Eco*RV-digested pcDNA3 vector (Invitrogen Corp.). All the above PCR reactions were performed with the proofreading *PfuTurbo*® DNA polymerase (Stratagene, La Jolla, CA) and further checked by sequencing. pCRE-luc (BD Clontech, Palo Alto, CA) contains the firefly luciferase gene under the control of a promoter with cAMP Responsive Elements (CRE) binding sites. The tgCMV/HyTK plasmid harbours a hygromycin resistance gene (Wellbrock et al., 1998).

Human embryonic kidney (HEK) 293 cells were grown in DMEM (Invitrogen Corp) containing 10% foetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml)

in a humidified atmosphere of 5% CO₂ at 37°C. Cells were transfected using a modified calcium phosphate transfection method (Chen and Okayama, 1987) or Lipofectamine 2000 reagent according to manufacturer's guidelines. pcDNA-sbsFSHR and tgCMV/HyTK were co-transfected (50:1) in cells growing in 24-well plates. pcDNA-sbsLHR, pCRE-luc and tgCMV/HyTK were co-transfected (50:50:1) in cells growing in 6 well culture plates. To generate stable clones, 48 hours after transfection cells were replated in 96-well plates and selected in medium containing 400 µg/ml of hygromycin B (Invitrogen). Hygromycin resistant colonies were isolated 2 weeks after transfection and subsequently expanded. In the sbsFSHR stable clones, the expression level of this gene was analyzed by Northern blot. Transient transfections of the pCRE-luc plasmid were performed in 9 cm dishes. Forty eight hours after transfection, cells were spread in 24-well plates and stimulated by adding the specified amounts of hormones in the growth medium for the indicated times.

The sbsLHR single clones were screened for luciferase activity by treating the cells with 100 µM forskolin (Sigma-Aldrich Co.) for five hours. Untreated cells were used as controls. The clone presenting the highest difference in luciferase activity between treated and untreated cells (LHR-LUC10) was further expanded. For functional characterization of the sbsLHR, LHR-LUC10 cells were spread in 48 well plates and stimulated by adding the specified amounts of hormones on the growth medium for five hours.

Bovine FSH (bFSH, AFP-5332B, biopotency 68 x NIH-FSH-S1) and bovine LH (bLH, AFP-11743B, biopotency 2,3 x NIH-LH-S1) were obtained from the National Hormone & Peptide Program, Harbor-UCLA Medical Center (California, USA). Cells were also challenged with conditioned medium of cultured Chinese hamster ovary (CHO-K1) stable clones producing recombinant sea bass LH and FSH (Gómez et al., unpublished).

Luciferase assay

To measure luciferase activity, cells were lysed in Reporter Lysis Buffer (Promega Corp.) as indicated by the manufacturer. Cell debris were separated by centrifugation for 30 s at 15000xg, and 20 μ l of the supernatant were mixed with 200 μ l of luciferin reagent (20 mM TricineKOH, pH 7.8, 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 270 μ M CoA, 530 μ M ATP, 400 μ M luciferin). The light emitted was measured in a luminometer (Junior, EG&G, Berthold) and expressed as relative light units (RLU).

Results

Cloning and sequence analysis of a *sbsFSHR* and a *sbsLHR* cDNAs

Two pairs of degenerate oligonucleotides (fshr6-fshr2 and fshr1-fshr2) were designed based on published fish and mammalian *FSHR* and *LHR* sequences respectively, and corresponding to a highly conserved and intronless region of the TM domain. They were used as primers in a PCR reaction using sea bass gDNA as template. A 330 bp and a 680 bp products were amplified, and subsequently subcloned and sequenced. The obtained sequences displayed the highest identity to other *FSHRs* and *LHRs* respectively. A *sbsFSHR* specific primer (fshr9), based on the sequence of the amplified product, was used in a PCR reaction on testis cDNA in combination with a degenerated primer designed on the EC domain (fshr8). The amplified fragment was about 1.1 Kb. Screenings of a cDNA testicular library using the *sbsFSHR* 1.1 Kb and the *sbsLHR* 680 bp fragments as probes rendered a high number of positive clones for the *sbsFSHR* and a single specific clone for the *sbsLHR*.

The sbsFSHR sequence. PCR and sequencing analysis of the 5' region of the *sbsFSHR* clones showed that eight of them were full length and contained the ATG initiation codon. One of them was chosen for in vivo excision and sequencing. This phagemid contained a 3134 bp fragment (Fig. 1), consisting of an ORF of 2109 nucleotides that was flanked by

leader and trailer sequences of 206 and 819 nucleotides respectively. This ORF encodes a protein of 702 amino acids of which the first 20 amino acids were predicted to constitute the putative signal peptide (Fig. 1). The mature protein displayed typical features of members of the GpHR family. The predicted EC domain contains 377 amino acids, including the signal peptide, followed by a putative 7TM domain and an intracellular C-terminal domain.

The sbsFSHR protein showed the highest identity with FSHRs of other fish species (81%-53%), followed by mammalian FSHRs (46%-45%), chicken FSHR (45%), mammalian LHRs (44%-43%), chicken LHR (41%), fish LHRs (41%-38%), reptile FSHRs (47%-46%) and fish and mammalian TSHRs (39%-35%). Amino acid sequence alignment of the sbsFSHR with other glycoprotein receptors, made clear that the most conserved regions of the receptor are the TM domain and the intracellular loop between TM I and TM II, whereas the least conserved areas are the EC domain (Fig. 2) and the intracellular loop between TM V and TM VI. The ClustalX alignment also revealed the presence of specific signature sequences (e.g. ³¹⁶CCAF, ³⁶⁴FNPCED, ⁴⁷⁷ERW, ⁵⁹⁰FTD, ⁶³⁴NPFLY), highly conserved in GpHRs (Vassart et al., 2004) (Fig. 2).

When aligning the amino acid sequence of the sbsFSHR with other fish and human FSHR sequences, two insertions and one deletion were identified in the EC domain of the sbsFSHR (Fig. 2). The first insertion, of 9 amino acids, is placed just after the signal peptide. The second one, considerable larger (25 amino acids), begins in amino acid position 80. Finally, a 30 amino acid deletion, with respect to the human FSHR (hFSHR), is easily identified in the C-terminal region of the EC domain, in the middle of the cysteine-rich cluster. This deletion is present in other fish FSHRs.

Predictions for N-glycosylation sites identified five motifs in the sbsFSHR at positions ⁶²NTT, ²³²NGT, ³⁰⁹NLT, ³²⁵NRS and ³⁵¹NST (Fig. 1). In other fish species, these potential

sites are also present, but only the first two are conserved in the hFSHR (Fig. 2) (Davis et al., 1995).

Pfam database searching and further comparison with the hFSHR allowed the identification of 10 imperfect LRRs in the sbsFSHR, with lengths ranging from 22 to 25 residues. One of these repeats is within the 25-amino acid insertion mentioned above, and thus would correspond to an additional LRR (Fig. 1 and 2). In the areas flanking the LRR region, two (Cys⁴⁵ and Cys⁶⁵) and six cysteines (Cys at positions 316, 317, 333, 349, 357 and 367) exist in the sbsFSHR, which could represent the N- and C-terminal cysteine-rich clusters respectively.

The TM domain of the sbsFSHR consists of 263 amino acids, and includes seven stretches of 21-23 predominantly hydrophobic residues predicted to form α -helices and connected by three intracellular and three EC loops (Fig. 1). The first and second EC loops of this domain contain two highly conserved cysteines (Cys⁴⁵³ and Cys⁵²⁸), predicted to form an intramolecular disulfide bond, which constrains the protein (Gudermann et al., 1995). The conserved ERW motif can be found at the bottom of the predicted TM III, and contains Arg⁴⁷⁸ and Glu⁴⁷⁷, that would form an ionic lock with Asp⁵⁷⁸ at the cytoplasmic end of the TM VI. Activation of the receptor involves the disruption of this ionic lock, which causes a crucial movement of these two α -helices. The NPxxY motif is also present, and includes Asn⁶³³ one of the most conserved residues in rodopsin-like GPCRs. It has been suggested that this residue would be implicated in the activation mechanism by switching its interaction between two aspartic residues, both existing in the sbsFSHR (Asp⁵⁹² and Asp⁴¹⁹) (Vassart et al., 2004). Phosphorylation site predictions identified two potential phosphorylation sites, Thr⁵⁶⁶ and Ser⁵⁷¹, in the third intracellular loop and 5 potential phosphorylation sites, Thr⁶⁴³, Ser⁶⁶⁹, Ser⁶⁷¹, Ser⁶⁷⁹ and Ser⁶⁸³, in the intracellular C-terminal domain. From them, Thr⁵⁶⁶ and Thr⁶⁴³

are potential phosphorylation sites for protein kinase C, while Ser⁵⁷¹ and Ser⁶⁸³ are potential sites for protein kinase A phosphorylation.

The *sbsLHR* sequence. The single isolated clone for the *sbsLHR* contained a cDNA insert of 2228 bp. Sequence analysis revealed that this clone did not contain the full length *sbsLHR* cDNA, as it was lacking coding sequence at its 5' end. Based on information obtained from the genomic sequence of the *sbsLHR* (unpublished data), a specific primer (lhr30) annealing to the 5' UTR of *sbsLHR* gene was designed. It was used for PCR amplification of testis cDNA in order to obtain the remaining *sbsLHR* cDNA sequence. The combination of this PCR product plus the cDNA sequence obtained from the library yield a *sbsLHR* cDNA sequence of 3172 bp. It consists of an ORF of 2166 bp that codes for a 721 amino acid polypeptide, flanked by 5' UTR and 3' UTR of 158 bp and 848 bp, respectively. The first 21 amino acids were predicted to constitute the putative signal peptide. The complete nucleotide and deduced amino acid sequence are shown in Fig. 3. *sbsLHR* contains all the features of a GpHR. The large EC amino-terminal domain consists of 388 amino acids, including the signal peptide. Searches in the Pfam database and alignments with other GpHRs allowed us to identify, within this domain, nine imperfect LRRs, with sizes ranging from 21 to 25 amino acids each. These LRRs are flanked by ten conserved cysteines, four of them in an N-terminal cluster (Cys at positions 27, 31, 33 and 40) and six in a C-terminal group (Cys at positions 283, 284, 308, 360, 368 and 378). The predicted TM domain, of 261 amino acids, includes 7 stretches of hydrophobic residues. The intracellular C-terminal domain consists of 72 amino acids and has two highly conserved contiguous cysteines (Cys⁶⁶⁸ and Cys⁶⁶⁹). Three N-glycosylation motifs were identified in the *sbsLHR* EC domain at positions ⁴²NVT, ¹⁰³NLS, and ¹⁹⁹NGT (Fig. 3). Only two of them, the second and third, are conserved between mammalian and sea bass LHRs. (Vu-Hai et al., 2000). NetPhosK 1.0 prediction server found one site, Thr⁵⁹⁰, in the third intracellular loop of the TM, and six sites, Thr⁶⁵², Ser⁶⁷¹, Ser⁶⁷⁴,

Ser⁶⁸³, Ser⁶⁹³ and Ser⁷¹¹ in the intracellular C-terminal domain of the *sbsLHR*, predicted to be phosphorylated by protein kinase C.

The *sbsLHR* protein has the highest identity to LHRs of other fish species (86%-45.5%), followed by chicken LHR (51%), mammalian LHRs (49%-48%), mammalian FSHRs (46%-45%), reptile FSHRs (44.5%-38%), fish FSHRs (43.5%-37%) and mammalian and fish TSHRs (41%-37%). Sea bass FSHR and LHR EC domains are 28% identical, while their TM and intracellular domains are 64% identical. The overall amino acid identity between these two sea bass proteins is 41%. Alignment of the *sbsLHR* amino acid sequence with other LHRs, showed that the most divergent areas are the C-terminus of the EC domain and the intracellular domain (data not shown). Similarly to the *sbsFSHR*, highly conserved GpHR signature sequences (e.g. ³⁷⁵FNPCEIDMSA, ⁴⁸⁸ERW, ⁶⁰¹FTD, ⁶⁴⁴NPFLY) (Vassart et al., 2004) were also found in the *sbsLHR*.

Phylogenetic analysis

The evolutionary relationship of the *sbsFSHR* and *sbsLHR* to other members of the GpHR family was inferred by performing a phylogenetic analysis by the Neighbour-joining method. The topology of the resulting rooted tree (Fig. 4) shows three main groups: *LHR*, *FSHR* and *TSHR* lineages. The *LHRs* are separated from the other GpHRs in 73% of the replicates while the *FSHR* and *TSHR* clades presented a bootstrap value of 78. As predicted by fish evolutionary relationships (Nelson, 1994), *sbsFSHR* and *sbsLHR* sequences exhibit the closest relation with the seabream gonadotropin receptors.

Expression analysis

The tissue expression pattern of the *sbsFSHR* and *sbsLHR* was analyzed by RT-PCR (Fig. 5A, B). *sbsFSHR* mRNA was only detected in gonads, and higher levels were seen in

testis compared to the ones observed in ovary. No transcripts were amplified in the somatic tissues analyzed (Fig. 5A). On the other hand, the *sbsLHR* showed the highest expression in gonadal tissues, but lower expression levels were detected in different somatic tissues, from which the head and ventral kidneys as well as the spleen presented the strongest amplification signal (Fig. 5B). Sequencing results from two randomly selected RT-PCR products (liver and gills) confirmed the authenticity of these extragonadal amplicons (Fig. 5B).

Northern blot analysis of poly (A)⁺ RNA and total RNA from sea bass gonads, using the full length *sbsFSHR* cDNA as a probe, revealed two faint hybridization signals corresponding to two different transcripts. One of approximately 3 kb, which corresponds to the full length mRNA and another of 1.8 kb that would be consistent with the existence of an alternatively spliced *sbsFSHR* transcript (Fig. 5C). When the *sbsLHR* cDNA was used as a probe, a faint and diffuse hybridization signal, of approximately 3 kb, was detected; this could contain *sbsLHR* transcripts slightly different in size (Fig. 5C). No signal could be detected in Northern blot when total RNA was used (data not shown). The level of expression obtain for both receptors in the Northern blot analysis, indicates that the *sbsLHR* is expressed to a much lower level than the *sbsFSHR*.

To establish the cellular localisation and maturation stage dependent expression of the *sbsFSHR*, we carried out an in situ hybridization on ovary sections of mature sea bass female. When an antisense probe was used, a strong expression was observed in previtelogenetic oocytes (Fig. 6A). Positive signals were also detected in the follicular cells of oocytes in early and late stages of the vitelogenesis process (Fig. 6B, C, D). Little or no expression was found in mature oocytes. No specific signal was generated with a *sbsFSHR*-cRNA sense probe (data not shown).

Activation of the sbsFSHR and sbsLHR by gonadotropins

To test the functionality of the isolated sbsFSHR and sbsLHR we expressed their cDNAs in HEK293 cells. It has been described (Tao et al., 2000) that the expression of a high number of gonadotropin receptors per cell can result in an increase in the basal level of activation of the receptor. With the aim of obtaining low amounts of receptor expressed per cell we developed stable HEK293 clones containing the *sbsFSHR* or the *sbsLHR* cDNAs. In the case of the *sbsFSHR* we analyzed those clones by Northern blot (data not shown) and selected the one that rendered the lowest expression of the receptor. On this clone we performed transient transfections with the pCRE-luc plasmid. To assess the functionality of the sbsLHR, we developed stable transfected HEK293 cells expressing the *sbsLHR* and the luciferase based reporter construct pCRE-luc (LHR-LUC10 cells). The activation of gonadotropin receptors results in the activation of the cAMP pathway (reviewed in Means et al., 1980) what finally leads to the expression of genes containing CRE binding sites in their promoters. Thus, the increase in luciferase activity as a result of its expression from the pCRE-luc plasmid constitutes an indirect measurement of the increase in intracellular cAMP.

The sbsFSHR/pCRE-luc double transfectants were stimulated with different amounts (0-5 $\mu\text{g/ml}$) of bFSH and bLH. When stimulated with bFSH we observed that the luciferase activity increased following a dose-response curve, while those cells treated with bLH did not show an increase in luciferase activity different from the untreated cells, even at high concentration (5 $\mu\text{g/ml}$) (Fig. 7B). In a time course experiment we show that maximum luciferase activity is obtained 6 hours after bFSH stimulation and no increase in luciferase activity is obtained in those cells treated with bLH (Fig. 7A). All together these data show that the sbsFSHR is specifically stimulated by FSH but not by LH.

LHR-LUC10 cells were stimulated with different amounts (0-5 $\mu\text{g/ml}$) of bLH and bFSH. Treatment with bLH increased luciferase activity in a clear dose-dependent manner.

Interestingly, bFSH was also effective in stimulating the sbsLHR in a similar way as the one observed for the bLH, with luciferase activity being only slightly lower (Fig. 8A). On the contrary, when LHR-LUC10 cells were stimulated with conditioned medium from CHO-K1 stable clones producing recombinant sbsFSH, no increase in intracellular cAMP was observed compared to the cells treated with CHO-K1 wild type medium. (Fig. 8B). On the other hand, conditioned medium of CHO-K1 stable clones producing recombinant sbsLH was effective in stimulating the sbsLHR (Fig. 8B).

Discussion

This study describes the cloning of two sea bass cDNAs that code for a FSHR and a LHR. They contain all the general structural features of a GpHR, but important differences were observed in the sbsFSHR. Unlike hFSHR, sbsLHR and many other LRR-containing proteins that have four cysteines in their N-terminal cysteine-rich region, the sbsFSHR has only two cysteines (Cys⁴⁵ and Cys⁶⁵). This allows the formation of a single disulphide bridge in the sbsFSHR with a different location than the two bridges found in the hFSHR (Fan and Hendrickson, 2005), what suggests a different folding for each receptor in this region. In other fish FSHRs the number of cysteines in this region is variable (Fig 2). Besides, the sbsFSHR, as well as Nile tilapia and seabream FSHRs, have an insertion of nine amino acids in this domain, precisely where one of the two cysteines is found (Fig. 2).

The N-terminal region is followed by the LRR domain. LRRs are usually involved in protein-protein interactions, and are constituted of β -strand/ α -helix units connected by a turn and arranged in a horseshoe-like configuration (Kobe & Kajava 2001). We identified nine LRR motifs in the sbsLHR, which match with the nine LRRs described for the hLHR. In the sbsFSHR ten LRRs were identified, nine of them correspond with repeats of the hFSHR, and the additional one is encoded by the 25-amino acid insertion described previously (Fig. 2).

The presence of this extra LRR indicates that the binding domain of the sbsFSHR could have a distinct curvature and size, influencing hormone recognition.

The crystal structure of the human FSH (hFSH) in complex with a fragment of the hFSHR EC domain (hFSHR₁₋₂₅₀) has revealed how the hFSH- α and - β subunits bind the receptor making contacts with all the β -strands of the LRRs (Fan and Hendrickson, 2005). From the residues buried at the receptor/ligand interface either by one subunit alone or by both hFSH- α and - β , few are conserved in the sbsFSHR. A higher preservation is found in sbsFSHR LRR5 (amino acids 163 to 171) that is predicted to interact mostly with FSH- α residues. A key feature in this area is an aromatic ring interaction between Tyr⁸⁸ of hFSH- α and Tyr¹²⁴ of hFSHR, which correspond to Tyr⁹⁰ and Tyr¹⁶⁴ in their sea bass counterparts. In addition, Asn¹²⁹ and Gly¹³¹ make important contacts with different residues in the α -subunit, and both are conserved in sbsFSHR (Asn¹⁶⁹ and Gly¹⁷¹).

Although collective interfaces may contribute to the specificity of binding, three potential determinants of specificity have been identified in the hFSHR (Fan and Hendrickson, 2005), namely residues Leu⁵⁵, Lys¹⁷⁹ and the combination of Glu⁷⁶ and Arg¹⁰¹. In the hFSHR, Leu⁵⁵ makes hydrophobic contacts with Arg⁴² of hFSH- α , and Leu⁹⁹ and Tyr¹⁰³ of hFSH- β . In the sbsFSHR, Gln⁷⁰ is equivalent to hFSHR Leu⁵⁵, which still allows hydrophobic interactions with Lys⁴⁵ of sbsFSH- α and Gly⁹⁴ and Ser⁹⁸ of sbsFSH- β (Mateos et al., 2003). In the second specificity pocket of the hFSHR the side chain of Lys¹⁷⁹ hydrogen bonds with Ser⁸⁹ and Asp⁹⁰ of hFSH- β . In sbsFSH- β , Thr⁸⁴ corresponds to human Ser⁸⁹; both hydroxyl side chain amino acids, and human Asp⁹⁰ is substituted by Glu⁸⁵, both negatively charged. Thus, these sbsFSH residues could host the side chain of sbsFSHR Arg²²⁰, a polar hydrophilic positively charged residue equivalent to hFSHR Lys¹⁷⁹. The sides of this channel are formed by the basic residues Lys⁵⁴ and Lys⁹³ of sbsFSH- α (Lys⁵¹ and Lys⁹¹ in hFSH- α), which would make salt bridges with two conserved acidic residues in the sbsFSHR, Asp¹⁹⁰

and Asp¹⁹³. The third specificity determinant is based on polar interactions of the contiguous residues Arg⁹⁷ and Val⁹⁶ of hFSH- β with the hFSHR residues Glu⁷⁶ and Arg¹⁰¹ respectively. These two residues are spatially adjacent in the human receptor, however, in the sbsFSHR the additional LRR would originate an extra turn there, spatially separating the equivalent sea bass residues, and therefore preventing the formation of equivalent bonds. Consistently, those human residues are not conserved in sea bass.

For the LHR there is no crystal structure available, but six ionizable amino acids of the rat LHR (rLHR) EC domain involved in ligand binding (Bhowmick et al., 1999) are conserved in the sbsLHR, or substituted by residues with similar size, shape and chemical composition. These are rLHR Glu¹³², Asp¹³⁵, Lys¹⁵⁸, Lys¹⁸³, Glu¹⁸⁴ and Asp²⁰⁶, which correspond to Asp¹⁵⁸, Asp¹⁶¹, Asn¹⁸⁴, Lys²⁰⁹, Asn²¹⁰ and Asp²³² in the sbsLHR. Besides, two residues located in the LRR3 of the hLHR, namely Asn¹⁰⁴ that is an LH-selective determinant (Vischer et al., 2003a), and Ile¹¹⁴ that is essential for ligand binding (Leung et al., 2006), are conserved in sbsLHR (Asn¹¹¹, Ile¹¹⁸). On the other hand, the rat and human LHRs have in their LRRs two cysteines important for hormone binding (Cys¹⁰⁹ and Cys¹³⁴ in the rLHR), which are probably bonded (Zhang et al., 1996). But, sbsLHR, and all known fish LHRs, contain a serine (sbsLHR Ser¹³³) instead of rLHR Cys¹⁰⁹, what prevents disulfide bonding. Even so, the sbsLHR, like other fish LHRs, can be activated by mammalian LH, indicating that its presence is not essential for fish LHR functionality.

Following the LRRs is the C-terminal cysteine-rich domain, which has six conserved cysteines in all known FSHRs and LHRs, including the ones of sea bass. A crucial ligand binding site has been found in hFSHR between the third and fourth cysteine of this domain (Kene et al., 2005), but, interestingly, this site does not exist in fish FSHRs, due to a 30 amino acid deletion (Fig. 2). Unfortunately, direct evidence of specific contacts is not available, as the crystal structure of the hFSH-FSHR₁₋₂₅₀ complex does not contain this domain.

Despite the distinct features found in the EC regions of fish and mammalian FSHRs, fish receptors respond to mammalian FSH, suggesting that those differences can be important but not fundamental for ligand binding. Besides, this is consistent with the existence of multiple FSH selective determinants in the hFSHR (Vischer et al., 2006).

sbsFSHR expression was exclusively detected in gonads, as found in other fish species (Oba et al., 1999b; Bogerd et al., 2001). On the contrary, the *sbsLHR* is mainly expressed in gonads, but also in other sea bass tissues, which is in line with reports in mammals (Frazier et al., 1990; Meduri et al., 1997; Rao, 2001) and different fish species (Oba et al., 1999a; Kumar et al., 2001b; Vischer and Bogerd, 2003; Kwok et al., 2005). In the sea bass, extragonadal expression of *LHR* was remarkable in head kidney. Similarly, *LHR* expression in African catfish was found to be higher in head kidney than in gonads (Vischer and Bogerd, 2003). Moreover, in coho salmon two gonadotropin preparations effectively stimulated cortisol and androstenedione secretion by the interrenal tissue, which it is located in the head kidney (Schreck et al., 1989). Despite many reports on the presence of *LHR* in diverse extragonadal tissues, their significance remains poorly understood due to the absence of *in vivo* data on their functionality (Pakarainen et al., 2005).

In situ hybridization showed a strong expression of the *sbsFSHR* in previtelogenic and early vitelogenic oocytes, as observed in Nile tilapia (Oba et al., 2001) and zebrafish ovaries (Kwok et al., 2005). This follicle-stage dependent expression strongly suggests a role for the *sbsFSHR* in oocyte growth. However, in adult sea bass (Mateos et al., 2003) and other fish species (Yoshiura et al., 1997; Kajimura et al., 2001; So et al., 2005), maximum levels of *FSH-β* expression are detected at the final stages of gamete maturation. This mismatch between the expression of *FSH-β* and *sbsFSHR* could be explained by the spawning strategy of sea bass. The sea bass has a group-synchronous ovarian development and spawns several times per reproductive season. Steady high levels of FSH would ensure follicle growth of

different clutches of oocytes during a rather long period, but only those ones producing FSHR would be responsive.

In mammals, glycoprotein hormone/receptor interactions are very specific. Conversely, functional studies in salmonids, catfish and cyprinids question the binding specificity between teleost gonadotropins and their respective receptors (Yan et al., 1992, Miwa et al., 1994; Oba et al., 1999a,b; Bogerd et al., 2001; Vischer et al., 2003b; Kumar et al., 2001a,b; Basu and Bhattacharya, 2002; Kwok et al., 2005). In this study, sbsFSHR displayed ligand selectivity as it was only activated by bFSH, but not by bLH. Besides, the same results were obtained with conditioned medium of CHO-K1 stable clones producing recombinant sea bass FSH and LH; only recombinant sbsFSH could stimulate cAMP production (unpublished data). On the contrary, the sbsLHR was activated by both bLH and bFSH. The same behaviour was observed in zebrafish and amago salmon, bLH and bFSH could activate their LHRs, while the FSHRs were only responsive to bFSH (Kwok et al., 2005, Oba et al., 1999a,b). Nevertheless, other mammalian hormones can act in a different way in other fish receptors (Kumar et al., 2001a,b; Bogerd et al., 2001; Vischer and Bogerd, 2003). This complex cross-activation warns about the use of mammalian hormones in aquaculture, where they have been applied pharmacologically to overcome reproductive dysfunctions caused by confinement.

Despite the promiscuous activation of the sbsLHR by bovine gonadotropins, this receptor was only activated by recombinant sbsLH, but not sbsFSH. This divergent response of the sbsLHR to bFSH and sbsFSH is probably due to sequence differences in their β -subunits. In the mammalian LH β -subunits, the presence of a net positive charge in the stretch between conserved Cys¹⁰⁻¹¹ (small seat-belt loop) is important for LHR binding. However, bFSH, sbsFSH and sbsLH, like other mammalian FSHs, have a clear negative net charge in this area; thus, charge differences are not related to the promiscuous activation of

the sbsLHR. Indeed, fish β -subunits also contain a specificity determinant in this loop, but it is not related to charge differences (Vischer et al., 2004). Considering the whole sequence of the bovine and sea bass β -subunits, bFSH is more similar to sbsLH than to sbsFSH, particularly between Cys¹⁻² and Cys⁶⁻⁷. Furthermore, sbsFSH has five amino acids less than the other three gonadotropins between Cys⁶⁻⁸, resulting in a shorter β 3 loop. This fact could influence hormone binding, as it has been suggested that the tips of β 1 and β 3 loops in the hCG contact the receptor (Moyle et al., 2004). Another different feature of the sbsFSH is the spatial position of the C-terminal seat-belt loop, between Cys¹¹⁻¹². In the bovine subunits and sbsLH- β these residues are near β 1 and β 3 loops, due to a disulfide bond between Cys¹² and Cys³. Nevertheless, in sbsFSH, as in other perciform and salmonid species, Cys³ is missing (Mateos et al., 2003; Swanson et al., 2003). Instead, a cysteine is found in the very N-terminus, which bonds with Cys¹², placing the tail of the seat-belt far from β 1 and β 3 loops. This difference could denote a different binding mode for the gonadotropin receptors of sea bass and other fish species.

In summary, we have characterized, for the first time, both gonadotropin receptors in a Perciform species. sbsLHR sequence is more conserved than sbsFSHR when compared to other fish and mammalian receptors; particularly in the EC domain, where the sbsFSHR contains an additional LRR. While *sbsFSHR* is exclusively expressed in gonads, *sbsLHR* shows a lower and more ubiquitous expression. Finally, the sbsLHR was activated by both bovine gonadotropins, but our results suggest that the interactions between the sea bass gonadotropins and their receptors are specific. However, additional experiments with different hormone doses are needed to further assess this specificity.

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Figure Legends

Figure 1 Nucleotide and deduced amino acid sequence of the *sbsFSHR* cDNA. Numbers on the left refer to position of the amino acid (top) and the nucleotide residues (bottom). Amino acid numbering begins with the proposed initial methionine. The predicted signal peptide is indicated in bold italics. Cysteine residues of the N- and C-terminal cysteine-rich regions of the extracellular domain are indicated by grey boxes. The ten β -strand motifs (X-L-X-L-X) of the LRRs, identified by Pfam Blast and sequence alignment with the hFSHR, are shown as arrows. Two potential N-linked glycosylation sites, conserved in the hFSHR, are indicated by open boxes. The position of the seven predicted transmembrane helices is shown as black boxes. The nucleotide sequence has been submitted to the GenBank and is available under the accession number [AY642113](#).

Figure 2 Amino acid sequence alignment of the extracellular domain and the region that links with the transmembrane domain of the FSHRs. Accession numbers: human (Hum) AY429104, sea bass (Sbs) [AY642113](#), Nile tilapia (Til) AB041762, seabream (Sb) AY587262, amago salmon (As) AB030012, Atlantic salmon (Ats) AJ567667, rainbow trout (Rtr) AF439405, zebrafish (Zbf) AY278107 African catfish (Ac) AJ012647, channel catfish (Cc) AF285182. The numbers on the right refer to amino acid position. *sbsFSHR* (Sbs) sequence is highlighted in bold letters. Amino acid residues conserved in all sequences are boxed in grey. Asterisks indicate the cysteines conserved in the *sbsFSHR*. Black bars over the sequence indicate the β -strand motif (X-L-X-L-X) of the LRRs.

Figure 3 Nucleotide and deduced amino acid sequence of the *sbsLHR* cDNA. Numbers on the left refer to position of the amino acid (top) and the nucleotide residues (bottom). Amino acid numbering begins with the proposed initial methionine. The predicted signal peptide is

indicated in bold italics. Cysteines of the N- and C-terminal cysteine-rich regions of the extracellular domain are indicated by grey boxes. Two conserved adjacent cysteines, present in the intracellular domain and predicted to be palmitoylated are also indicated by grey boxes. The nine β -strand motifs (consensus sequence: X-L-X-L-X) of the LRRs, found by Pfam Blast and sequence alignments, are shown as arrows. Two potential N-linked glycosylation sites, conserved in the hLHR, are indicated by open boxes. The position of the seven predicted transmembrane domains is shown as black boxes. The nucleotide sequence has been submitted to the GenBank and is available under the accession number **AY642114**.

Figure 4 Phylogenetic tree of fish GpHR amino acid sequences inferred from the Neighbor-Joining method. Accession numbers: sbsLHR (**AY642114**), amago salmon LHR (AB030005), channel catfish LHR (AF285181), Nile tilapia LHR (AB041763), gilthead seabream LHR (AY587261), African catfish LHR (AF324540), rainbow trout LHR (AF439404), Atlantic salmon LHR (AJ579790), zebrafish LHR (AY424302), amago salmon TSHRa (AB030954), amago salmon TSHRb (AB030955), striped sea bass TSHR (AF239761), Nile tilapia TSHR (AB047390), Atlantic salmon TSHR (AF406603), African catfish TSHR (AY129556), sbsFSHR (AY642113), African catfish FSHR (AJ012647), channel catfish FSHR (AF285182), amago salmon FSHR (AB030012), Atlantic salmon FSHR (AJ567667), rainbow trout FSHR (AF439405), Nile tilapia FSHR (AB041762), gilthead seabream FSHR (AY587262), zebrafish FSHR (AY278107). The LGR sequence from the *Caenorhabditis elegans* (AF224743) was used as the outgroup. Bootstrap values (in %) from 1000 replicates are indicated for each tree node.

Figure 5 RT-PCR expression analysis of *sbsFSHR* (A) and *sbsLHR* (B) in adult fish tissues. Specific primers encoding a segment of the transmembrane or extracellular domain were used

for cDNA amplification of *sbsFSHR* and *sbsLHR* respectively. The presence of introns in these areas of the genes guarantees that amplification comes exclusively from the mRNA. The integrity of the RNAs was verified by uniform amplification of the sea bass *18S rRNA* transcript. **(C)** Northern blot analysis. Poly (A)⁺ RNA from ovary (lane 1) and total RNA from testis (lanes 2 and 3) and ovary (lane 4) from 2 adult animals were probed with the full length *sbsFSHR* cDNA (left panel). Poly (A)⁺ RNA from ovary was probed with the complete *sbsLHR* cDNA (right panel), the RNA in this membrane is identical to lane 1 in the left panel. This blot was much longer exposed than the *sbsFSHR* blot. The numbers on the left correspond to the localization of size marker RNAs (in Kb). Arrows indicate the position of two *sbsFSHR* mRNAs of about 1.8 and 3 Kb, and one *sbsLHR* transcript of about 3 Kb.

Figure 6 In situ hybridization of *sbsFSHR* antisense riboprobe in ovary sections of sexually mature females. Expression of *sbsFSHR* (dark grains) was observed on the follicular wall of oocytes in previtelogenesis **(A)**, in cortical alveoli stage **(B)** and in later stages of vitelogenesis **(C, D)**. Higher magnification in photomicrograph **D** clearly shows expression in the follicular wall (**fw**) while the surrounding areas are free of signal (**zp**, zona pellucida; **cyv**, cortical alveoli; **yv**, yolk vesicle; **oo**, ooplasm). Scale bar: 50 μ m.

Figure 7 Functional analysis of *sbsFSHR* expressed in HEK293 cells. A stable clone containing the *sbsFSHR* was transiently transfected with the reporter plasmid pCRE-Luc. **(A)** Temporal luciferase activity in response to 1 μ g/ml of bFSH, bLH or untreated (Control). **(B)** Luciferase activity after 6-h treatment with different dosages of bFSH and bLH.

Figure 8 Functional analysis of *sbsLHR* expressed in HEK293 cells. A double stable clone expressing the *sbsLHR* and the reporter construct pCRE-Luc was developed. **(A)** Luciferase

activity after 5-h treatment with different doses of bFSH and bLH. **(B)** Luciferase activity after 5-h treatment with a single concentration of conditioned medium of cultured CHO-K1 wild type cells (Control) or stable clones producing recombinant sea bass LH or FSH.

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Table 1. Primers used in the PCR experiments^a.

Primer	Nucleotide position	Sequence (5'→3') ^b	Touchdown ^c Annealing Temp	
fshr 1	1419	TTCCTKATGTGCMACCTIKCMTTGC	70°C max → 55°C min	Forward
fshr 2	2113	AGGAARGGRTTGGCRCAIGARTTKAT	70°C max → 55°C min	Reverse
fshr 6	1779	RYSAGYATCTGYCTICCCATGGATGT	65°C max → 55°C min	Forward
fshr 8	699	CTGACTATYTCMAACACNNGNCT	65°C max → 50°C min	Forward
fshr 9	1865	GAAAGCCAGGATGTTGAGGAG	65°C max → 50°C min	Reverse
fshr 25	1339	TCCTCATCTGGATCATCTCCAT	65°C max → 55°C min	Forward
fshr 26	1621	GTTAATGTGAACTGACAGCTCA	65°C max → 55°C min	Reverse
lhr 21	1090	CTCTCATACAATAAGTAGAGTCA	60°C max → 55°C min	Reverse
lhr 23	811	TGGATCACTCTGAGGTTTCGATTATTC	70°C max → 55°C min	Reverse
lhr 24	535	AGGTTATTGAATGTCCTTCTGCCAATGTG	60°C max → 55°C min	Reverse
lhr 30	1	CACTTGATGAGAAGTTGAGTAACA	60°C max → 55°C min	Forward
lhr 35	484	TCCAGAACACGAGGAGTCTGAT	70°C max → 55°C min	Forward
5'18S		TCAAGAACGAAAGTCGGAGG	65°C	Forward
3'18S		GGACATCTAAGGGCATCACA	65°C	Reverse

^a Primers were obtained from Invitrogen Corp.(Carlsbad, CA).

^b Y = C or T, R = G or A, K = T or G, M = A or C, and I = deoxyinosine.

^c Touchdown-PCR was used for amplification. Maximum and minimum temperatures achieved during annealing step, are indicated for each primer.

1 CACACTTCTTCCCTCTTCAACCTGGCGGTGAAACCCAGAGTTGTGAGACGAAAAAACAACCCATTATCATGATATCAAAATGTGGAGTGTGGGCTCTGT
 101 GAGAAGGCTTGAACACACGTGAACACAGAGGGAACAACAGTGTGAGAGAGTGTGAGGAGAAGCAGAAAGAAGAAGAGAAACAACCTGGAGCTGTGGACAG
 1 M M M V M I L I M L M I L M I K T A T A S V P G P E M D V K P
 201 ACTGCGATGATGATGGTGTGATTCTCATAATGTGTGATGATTCTGATGATAAAGACGGCAACAGCCTCGGTGCCGTCAGAGATGGACGTTAAACAG
 32 G V E T S L A K R T L S F C Y Q L K F G V T E I P S S I S S N T T C
 301 GAGTGGAGACCAAGCTTGGCCAAACGAAACCTTCAGCTTCTGATACCAGTTGAAGTTTGGGGTACACAGAGATCCCTCCAGCATCTCCAGCAACACCCACATG
 66 L E V K Q T E I V V I P Q G A L N S L Q H L R K L T I W E N D K L
 401 CCTGGAAGTTAAGCAGACGGAGATCGTAGTGATCCCCAGGGCGCCCTCAACAGCCTGCAGCACCTCAGGAAACTCACCATATGGGGAACGACACAGCTG
 99 E S I N E F A F A F A S L S Q L T D I F I S G N V A L K N I G A F A F
 501 GAGAGTATCAATGAGTTTGCCTTCGCCAGCCTCTCAGCTCACCAGTATCTTCATCTCTGGGAATGTTGCGTTAAAAAATATAGGAGCTTTTGCCTTTCT
 132 S D L P E L T E I T I T K S K H L T H I N P D A F K D I V K L K Y L
 601 CTGATCTGCCTGAACCTCAGTGAATAACAAAGTCAAAACACCTGACACACATCAATCCAGATGATTCAGGACATGTTGAAACTGAAATGATCT
 166 T I A N T G L R L F P D F T K I H S T G L L L F D L H D N S H I E
 701 GACCATCGCAACACTGGACTGAGGCTTTTCCAGACTTCACCAAGATCCACTCCACAGGCTGCTTCTGTTGACCTGCAGCAACAGCCACATAGAG
 199 R V P A N A F K G L C T Q T I P E I R L T R N G I K E V A S D A F
 801 AGAGTCCCGCCAAATGCTTTCAAGGCCTCTGCACTCAAACATCCCTGAGATCAGGCTCACCAGAAATGGCATCAAGGAGTGGCAAGTACGCTTCA
 232 N G T K M H R L F L R G N K Q L T H I N P N A F V G S S E L V L D
 901 ACGGCACAAAGTGCACAGATTGTTCTAAGAGGCAACAAACAGCTTACTCACAATCAATCCCAACGCCTTTGTGGTTCAGTGAGTTGGTGGTACTGGA
 266 I S Q T A L S S L P D Y I L G G L Q K L I A E S A P N L K E L P P
 1001 CATTTCAGCAGACGCTCAGCTCCTGCGGACTACATCCTCGTGAGCTCCAGAAGCTGATCGCAGAGTCCGCCCAATCTGAAGGAGCTTCTCCT
 299 L E L F T K L H Q A N L T Y S S H C C A F H N I H R N R S K W N S
 1101 CTGGAGCTTCCACAAACTGCACAGGCCAACCTGACGTACTCGTCACACTGCTGCGCCTTCCATAACATACACAGGAACAGATCAAAGTGAACCTCC
 332 L C S H P D A Q G N L H F Y R D Y C S N S T S I I C T P T Q D D F N
 1201 TGTGCTCCACCCCGATGCTCAGGGCAACCTTCACTTCTACAGGACTACTGCTCCAACCTCCACCTCCATCATCTGCACCCCAACCCAAAGATGACTTTAA
 366 P C E D I M S A V P L R V L I W I I S I L A L L G N T V V L L V L
 1301 CCCCTGTGAGGATATCATGTCCGCCGTCGCCCTCGGGTCTCATCTGGATCATCTCCATCCTCGCACTGCTGGGGAACACAGTGGTACTTTTGTGCTTAA
 399 L G S R T K L T V P R F L M C H L A F S D L C M G I Y L V V I A T
 1401 TTAGGAGCGCCACCAACTGACTGTTCCCGGTTTCTCATGTGCCACTTGGCTTTTCTGACCTTGCATGGGCATCTACCTGGTAGTCATAGCAACTG
 432 V D M L T Q G Q Y Y N H A I D W Q T G L G C S V A G F F T V F A S E
 1501 TAGACATGCTCACTCAAGGCCAGTACTACAACACGCCATAGACTGGCAGACGGGCTCGGCTGCAGTGTTCGGGGCTTCTTACGGGTTTGGCCAGTGA
 466 L S V F T L T A I T L E R W H T I T H A L R L D R K L R L R H A C
 1601 GCTGTCAAGTGTACATTAACAGCGATCACCCTGGAGCGTGGCACACCATCACACATGCTCTGCGGCTCGACCGTAAACTTCGCTGAGACATGCTGCT
 499 I V M T A G W I F S A V A A L L P T V G V S S Y G K V S I C L P M
 1701 ATTGTCAAGACAGCGGGTGGATCTTCTCCGCGTAGTCTGCTTTGCTTCCACAGTCCGGAGTCAAGCAGTACGGCAAGTGGTATCTGTCTGCCCATGG
 532 D V E F L G S Q V Y V V S L L L L N I L A F F C V C G C Y L S I Y L
 1801 ACGTGGAGTTCGTTGGGCTCAGGCTATGTGGTGTCACTTCTCCTCCTCAACATCCTGGCTTTCTTCTGCGGTGCGGCTGTTACCTCAGCATCTACCT
 566 T V R N P S S A P A H A D T R V A Q R M A V L I F T D F V C M A P
 1901 GACCGTCCGCAACCCCTCGTACGCGCGGCCACCGCCACACAGTGTGGCCCAACGATGGCAGTCCCTCATCTTACCAGCTTTGTCTGCATGGCTCC
 599 I S F F A I S A A L K L P L I T V S D A K L L L V L F Y P I N S C
 2001 ATCTCCTTCTTGGCATCTCAGCAGCCCTCAAGTCCCTCTCATCACAGTGTGAGCAGCAAACTCCTGCTGGTCTCTTACCAGATCAACTCCTGCT
 632 S N P F L Y A F F T R T F R R D F F L L A A R F G L F K T R A Q I Y
 2101 CCAACCCCTTCTGTACGCTTCTTCAACAGCAGCTTCAAGCGGGATTTCTTCTCCTCGCAGCTGCTTCCGCGCTGTTAAGACCAGGACACAGATTTA
 666 R T E S S S C Q Q P A W T S P K S S H V M L Y S L A N A L S L E G
 2201 CCGGACAGAGAGTTCCTCCTGTGAGCAGCGGCGTGACCTTCCAAAGAGCAGCCAGTGTGATGCTGACTCCTGGCCAAATGCATTAAGTCTAGAAGG
 699 K P E F *
 2301 AAACCAGAGTTCAGCTGTTATCAAAAAGCAGATAGAAAATTTTGCCTAATTTTGTGGTTTTCATGTATCTCTGATATGTTGCCAGTATTTTGGCCA
 2401 GCACATCAGCTGCTGATCTTTCAGACCTGACCTCAGACCTGCAACAAACACAAGTATCAGTGTCTTCTTAAACCATGTGTGACAACCTACTGTATGTA
 2501 CAGTGTATAGAAAACCAAGTAATAGCTACAGAACAACCTGAGATACTTTGACCAAACTAGGTCAAATGGCTGTCCAGATAATTCCTTGTGTTTGT
 2601 TTAATTTGATTGGACCGCCAGATGGGTGGAGTTACTGCATAATAACAATGAGTTCACAAGTAAATGTAGGAGGAGTATCAGAGAGGTCACACTGACTT
 2701 TTACTTTTCATGCATGCTTATATAACATCCATCTGGGTGCCAAGATAAATAAACAAGCTTAAATACCTTTGAATCTGTTAATAAAGGTTTGGACACTCG
 2801 GGATATCAGCATATGAACGTCAAGAAATCAACATTCATTTAAACATCAACGTCATGATTGAATGATATTCATTAAGAAGCACCCGAGGGACTTACTGT
 2901 TTAATAATCTCCTATACACCGCTGCTGTAGCTTACATCCCTCCACATCCCTCTTACCCTCAGTGTCCATAATTTTAAATGAAGGACGTGTAATTA
 3001 TCTTTGTAATGTAATAGTTGTGTAATGAAAGTACCAGTGTTTGTATATGTGTGTTTGTACAGAATATATCTTGGAGATGTAATTTTCATGTCAAT
 3101 ATAAATTTGTTTATTCAAAAAATAAAAAAAAAA

Hum -----MALLVSLLAFLSLGSGCHHRIHCNSNRVFLCQESK-----VTEIPSDLPRNAIELRFVLTCLRVIQKGAFGSGFDLEKI 75
Sbs **MMVMILIMLIMLMIKTATASVPGPEMDVKPGVETS-LAKRTLSTFCYQLKFGVTEIPSSISSNTTCLLEVKTQTEIVVIPQAGALNSLQHLRKL 90**
Sb -MMAMILIMVTIVMIKMAASAPDAETDVKPGADESVLAKRTLSTFCYQLKFGVTEIPSSISSNTTCLLEVKTQTEIVVIPQAGALNSLQHLRKL 90
Til MMLVMTLMMLLIVTIKMAAASAHGSEMDIRPGFHPS-LAKQTSCLSYQVMFGVTAFAFBNIS-NAQCLEVKQTQIREIQQGTLSLQHLRKL 89
As -MMKMKKIMKMLLVCVLCVMSQAEVAMVNSGTTFTTYLCMGNT-----ITHMPTHIPKNTTDLFEKQTHIRVFPQEAFTNLQQLTAI 81
Ats -MMKMKKIMKMLLVCVLCVMSQAEVAMVNSGTTFTTYLCMGNT-----ITHMPTHIPKNTTDLFEKQTHIRVFPQEAFTNLQQLTAI 81
Rtr -MMKMKKIMKMLLVCVLCVMSQAEVAMVNSGTTFTTYLCMGNT-----ITHMPTHIPKNTTDLFEKQTHIRVFPQEAFTNLQQLTAI 81
Zbf MVLSMMLCFILGCSIANATEDTLAASQCAFNSTRSFICLGNK-----VHEIPRRIPNTTTFVEIKLTQISVFRRAALSELHELKRI 82
Ac -----MLRYILSWLVMHTGNMFLGSYACLASGTTTRSFCLGSK-----VHQMPPHIPINTTYVEIKLTQIIFPYRAMSSSLHDLKRI 77
Cc -----MMCFILSWLMMHAGNMLCGSYACLANGTTRSFCLGSK-----VHQMPPHIPINTTYVEIKLTQIIFPYRAMSSSLHDLKRI 77

Hum ETSQNDVLEVI-----EADVFSNLPKLEIRIEKANNLLYINPEAFQNLNPIQYLLISNTGIKHLDPDVHKI 141
Sbs **TIWENDKLESINEFAPASLSQLTDIFISGNVALKNIGAFASDLPPELTEITITKSKHLTHINPDAFKDIVKLYLTIANTGLRFLPDDFTKI 181**
Sb IISKNEVLESI GAFAVAGLPQITNIFISENAELASIGAFASDLPPELTEMITKSKHLRHIHPDAFRNIVKLRVLIISNTGLRMPDFTKI 181
Til TISENDLESIGAFAFSLPHLTKLISKNAALRNIGAFVSNLPELSEIITKSKHLRHIHPDAFRNMARLRFITISNTGLRIFPDSKI 180
As VLTENGMELESI-----GAFAFANLPRLEITITKSKHLVIHQQAFIGLPKLSHLTICNTGLRVLPNFSRI 147
Ats VLTENGMELESI-----GAFAFANLPRLEITITKSKHLVVIHQAFMGLPKLSHLTICNTGLRVLPNFSRI 147
Rtr VLTENGMELESI-----GAFAFANLPRLEITITKSKHLVIHQQAFIGLPKLSHLTICNTGLRVLPNFSRI 147
Zbf VVSENGALERI-----EALAFANLLEETITKSKNLV-MHKDAFWRLPKLRYLTIISNTGLKILPDFSQI 147
Ac MVSENGALQRI-----EAYAFANLLEETITKSKNLVMDRDTFWGLPKLRYLTIISNTGLTVLPDFSQI 143
Cc LVSENGVLRQRI-----EAYAFANLTKLEEITITKSKNLVCMRDTFWGLPKLRYLTIISNTGLTVLPDFSQI 143

Hum HSLQ-KVLLDIQDNINIHTIERNFVGLSFESV-ILWLNKNGIQEIHNCANFGTQDDELNLSDNNNLEELPNDVFHGASGPVILDISRTRI 230
Sbs **HSTG-LLLFDLHDNSHIERPANAFKGLCTQTIPEIRLTRNGIKEVASDAFNGTKMHRFLRGNKQLTHINPNAFVGSSELVVDISQTAL 271**
Sb HSTADFLFLGQENSHIERVPANAFKGLCTQTIPEIRLTRNGIKEVASDAFNGTKMHRFLRGNKQLTHINPNAFVGSSELVVDISHTAL 272
Til HSTA-CFLLDLQDNSHIKRVPANAFKGLCTQTFAEIRLTRNGIKEVASDAFNGTKMHRFLRGNKQLTHINPNAFVGSSELVVDISSETAL 270
As HSAAMTFLLDLQDNVHIVIPSNAPLGLTNTIDELRLTKNGISEVESHAFFNGTKIHKLYLMGNLQLSHMHNNSFKGAEGPGFLDISRTAL 238
Ats HSTALTFLLDLQDNVHIVIPSNAPLGLTNTIDELRLTKNGISEVESHAFFNGTKIHKLYLMGNLQLSHMHNNSFKGAEGPGFLDISRTAL 238
Rtr HSAALTFLLDLQDNVHIVIPSNAPLGLTNTIDELRLTKNGISEVESHAFFNGTKIHKLYLMGNLQLSHMHNNSFKGAEGPGFLDISRTAL 238
Zbf NSAALEFLFDLQDNMHIERIPSNAPLGLTNTIDELRLTKNGIREIDSHAFNGTKIHKLYLMGNLQLSHMHNNSFKGAEGPGFLDISRTAV 238
Ac QSAAEFFLFDLEDNMHIERIPSNAPLGLTNTIDELRLTKNGIREIDSHAFNGTKIHKLYLMGNLQLSHMHNNSFKGAEGPGFLDISRTAI 234
Cc QSAAEFFLFDLEDNMHIERIPSNAPLGLTNTIDELRLTKNGIREIDSHAFNGTKIHKLYLMGNLQLSHMHNNSFKGAEGPGFLDISRTAI 234

Hum HSLPSYGLENLKLRARS*TYNKKLPLEKLVALMEASLTYP SHCCAFANWRRQISELHP-ICNKSILRQEVDMYMTQTRGQRSSSLAEDNES 320
Sbs **SSLPDYILGGLQKLIASAPNLKELPPELFTKLHQANLTYSSHCCAFHNIHRNRSKWNS-LCSDPDAQNLHFFY----- 345**
Sb SSLPENILGGLQKLIASAPNLKELPPELFTKLHQANLTYSSHCCAFHNIHRNRSSWNALLCSHPDAPGMPNFFY----- 347
Til TSLPDSILDGLKRLIAESAFNLKELPPIQLFTKLHQAKLTYSSHCCAFHNIHRNRSRWNS-LCDNPEAKNNLHFF----- 344
As SSLPEVSVLGEVEHL SAVSVFSLRLLPPLSFTKLRQANLTYSSHCCAFHNIHRNRTRFRMTSACFKPGAQNNLHFF----- 313
Ats SSLPEVSVLGEVEHL SAVSVFSLRLLPPLSFTKLRQANLTYSSHCCAFHNIHRNRTRFRMTSACFKPGAQNNLHFF----- 313
Rtr SSLPEVSVLGEVEHL SAVSVFSLRLLPPLSFTKLRQANLTYSSHCCAFHNIHRNRTRFRMTSACFKPGAQNNLHFF----- 313
Zbf HTLPESMLKTLKLLMAVSVYSLRKLPSLELFTLQANLTYSSHCCAFKFNKFKKSVKNQ-MCNVTGAHEEPDFNFF----- 315
Ac SSLPENMLRRLKLLIATS SVYSLRWLPLEIFALFTQANLTYSSHCCAFKFNKFKKLEKNH-LCNVSTIRNQEPYF--F----- 309
Cc SSLPENMLRRLKLLIATS SVYSLRWLPLEIFALFTQANLTYSSHCCAFKFNKFKKSEKNR-LCNDSTIRNQEPYF--F----- 309

Hum SYSRGFDMTYTEFDYDL*CNVVDVIT*GSPKPAFNPCEDIMGYNIRVLIWFI*SLAITGNIIVLVILITTSOYKLTVPFRFLMCHLAFADLCI 411
Sbs **-----RDYCSNSTSIICPTQDDFNPCEDIMSAVPLRVLIIWII*SVLALLGNAVLLVLLGSRCKLTVPRFLMCHLAFADLCM 422**
Sb -----KDHCSNSTAIICPTQDEFNPCEDIMSAVPLRVLIIWII*SVLALLGNAVLLVLLGSRCKLTVPRFLMCHLAFADLCM 424
Til -----REYCSNSTAIICPTQDEFNPCEDIMSAVPLRVLIIWII*SVLALLGNAVLLVLLGSRCKLTVPRFLMCHLAFADLCM 421
As -----MDFCLNWT*SVACSPAPDAFNPCEDIMSAVPLRVLIIWII*SVLALLGNTIVLLVLLGSRKMTVPRFLMCHLAFADLCM 390
Ats -----MDFCLNWT*SVACSPAPDAFNPCEDIMSAVPLRVLIIWII*SVLALLGNTIVLLVLLGSRKMTVPRFLMCHLAFADLCM 390
Rtr -----MDFCLNWT*SVACSPAPDAFNPCEDIMSAVPLRVLIIWII*SVLALLGNTIVLLVLLGSRKMTVPRFLMCHLAFADLCM 390
Zbf -----NDHKDVI*EVT*CYPTPDAFNPCEDIMGFTFLRVLIIWII*SVLAVGNTVLLVLLGSRKMTVPRFLMCHLAFADLCM 392
Ac -----EEHKDVI*EVS*CYPEPDAFNPCEDIMGFTFLRVLIIWII*SVLAVLGNFTVLLVLLGSRKMTVPRFLMCHLAFADLCM 386
Cc -----EEHKDVI*EVR*CYPEPDAFNPCEDIMGFTFLRVLIIWII*SVLAVLGNFTVLLVLLGSRKMTVPRFLMCHLAFADLCM 386









