DEPARTAMENT DE GENÉTICA

CARACTERIZACIÓN MOLECULAR DE LOS RECEPTORES DE GONADOTROFINAS DE LUBINA (*DICENTRARCHUS LABRAX*)

ANA MARÍA DOS SANTOS ROCHA

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Caracterización molecular de los receptores de gonadotrofinas de lubina (*Dicentrarchus labrax*)

Molecular characterization of sea bass (*Dicentrarchus labrax*) gonadotropin receptors

Memoria presentada por
Ana Maria dos Santos Rocha
para optar al grado de Doctor.

Fdo.
Ana Maria dos Santos Rocha

VNIVERSITAT DE VALÈNCIA
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El Dr. Manuel Carrillo Estévez, Profesor de Investigación del CSIC en el Departamento de Fisiología de Peces y Biotecnología del Instituto de Acuicultura Torre la Sal, la Dr. Ana María Gómez Peris, Científico Titular del CSIC en el Departamento de Fisiología de Peces y Biotecnología del Instituto de Acuicultura Torre la Sal y el Dr. João José Oliveira Dias Coimbra, Profesor Catedrático del Instituto de Ciencias Biomédicas de Abel Salazar de la Universidad de Oporto, hacen constar que:

Ana Maria dos Santos Rocha, licenciada en Ingeniería Zootécnica por la Universidade de Trás-os-Montes e Alto Douro, ha realizado en el Instituto de Acuicultura de Torre la Sal (CSIC) bajo nuestra dirección el trabajo de investigación recogido en esta memoria, que lleva por título: "Caracterización molecular de los receptores de gonadotrofinas de lubina (*Dicentrarchus labrax*)", para optar al grado de doctor por la Universitat de València.

Torre la Sal,

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Acronyms and Abbreviations

11-KT	11-ketotestosterone
3β-HDS	3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase
11β-HDS	11β-hydroxysteroid dehydrogenase
17,20βP	17α,20β-dihydroxy-4-pregnen-3-one
17β-HDS	17β-hydroxysteroid dehydrogenase
20βS	17α,20β,21-trihydroxy-4-pregnen-3-one
32P	phosphorous-32
35S	sulfur-35

A

Ala	alanine
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
Arg	arginine
Asn	asparagine
Asp	asparagine
ATP	adenosine triphosphate
AUAP	abridged universal amplification primer
α*L*	α-subunit loop

B

BLAST	basic local alignment search tool
bp	base pair
BPG	brain-pituitary-gonad
β*L*	β-subunit loop

C

cAMP	adenosine 3',5'-cyclic-monophosphate
cDNA	complementary deoxyribonucleic acid
CG	chorionic gonadotropin
CHO	chinese hamster ovary
CoA	coenzyme A
cpm	counts per minute
CRE	cAMP response element
Ct	threshold cycle

Cys	cysteine

D

dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
DMEM	dulbecco modified eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol

E

E. coli	*Escherichia coli*
E.U.	Ellman Units
E2	17β-estradiol
E-AChE	estradiol acetylcholinesterase conjugate
EDTA	ethylendiamin-tetra-acetate
Ef1-alpha	elongation factor 1-alpha
e.g.	*exemplī grātiā* (for example)
EIA	enzyme immuno assay exemplī grātiā
ELISA	enzyme-linked immunosorbent assay

F

FSH	follicle-stimulating hormone
FSHR	FSH receptor
FSHβ	FSHβ-subunit

G

g	centrifugal acceleration
G protein	heterotrimeric guanine nucleotide-binding protein
gDNA	genomic DNA
GDP	guanosine diphosphate
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor

GSI	gonadosomatic index
GTH I	gonadotropin I
GTH II	gonadotropin II
GTP	guanosine triphosphate
Gα	heterotrimeric G proteins α-subunit
Gβγ	heterotrimeric G proteins β and γ subunits

H

h	hour
HEK	human embryonic kidney
His	histidine

I

IgG	immunoglobulin G
Ile	isoleucine
i.e.	*id est* (that is)

K

kb	kilobase
kDa	kilodalton

L

Leu	leucine
LGR	leucine-rich-repeat containing GPCR
LH	luteinizing hormone
LHR	LH receptor
LHβ	LHβ-subunit
LRR	leucine-rich repeats
Lys	lysine

M

M	molar (mol/l)
MAP	mitogen-activated protein
Met	methionine
min	minute
MIS	maturation-inducing steroid
ml	mililiter

mM	milimol
mRNA	messenger RNA
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer

N

n	number of observations
ng	nanogram
nm	nanometre
no.	number

O

OD	optical density
ORF	open reading frame

P

P	probality value
P45011β	P450 11β-hydroxylase
P450arom	cytochrome P450 aromatase (CYP19)
P450c17	cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17)
P450scc	cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A)
PAF	paraformaldehyde
PB	phosphate buffered
PCR	polymerase chain reaction
Pfam	protein family
pg	picogram
PGC	primordial germ cell
pH	negative log of hydrogen ion concentration
Phe	phenylalanine
PKA	protein kinase A
Pro	proline

R

R	correlation coefficient
RACE	rapid amplification of cDNA ends

RI	ribonuclease inhibitor
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
rRNA	ribosomal RNA
RT	reverse transcription

S

sec	second
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Ser	serine
SSC	sodium-chloride sodium citrate
SSPE	sodium chloride-sodium hydrogen-phosphate-EDTA
StAR	steroidogenic acute regulatory protein

T

T	testosterone
Tris	tris-(hydroxymethyl)-aminomethan
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone receptor
Tyr	tyrosine

U

U	units
UTP	uridine triphosphate
UTR	untranslated region

V

Val	valine

The trivial names of the α-amino acids that are commonly found in proteins were written following the three-letter system of symbolism of the IUPAC--IUB Joint Commission on Biochemical Nomenclature. Other symbols and terms used also followed IUPAC recommendations.

Within each section and/or subsection, when referring to a species for the first time, the English common name appears followed by the scientific name in italics. Subsequently, only the former is used. Regarding fish species, both forms of nomenclature are based in the classification of an external information resource of the NCBI, the FishBase (http://www.fishbase.org/search.php).

In all sections, abbreviations are defined when used for the first time.

1. INTRODUCTION

Introduction

Sexual reproduction is one of the most fascinating biological processes occurring in Nature. It involves the fusion of two gametes allowing a unique recombination of the parental genes, increasing genetic diversity of the offspring. Successful sexual reproduction relies primarily on male and female gamete production in the gonads. Most fish of temperate climates have a defined breeding period that has developed throughout evolution to adapt spawning to occur at the optimal season for offspring survival.

European sea bass

European sea bass, *Dicentrarchus labrax*, belongs to the teleost order Perciformes, family Moronidae. It is essentially a Mediterranean species, although it as been recorded as far north as the southern coast of Norway. As the majority of fish, sea bass is a gonochoristic species, with male and female gonads residing in separate individuals. However, genders are difficult to distinguish due to the absence of external sexual characters. Growth is clearly related to sex, with females growing faster, reaching a larger size at first maturity. In the Mediterranean, first sexual maturity occurs generally during the second year of life in males and a year later in females. Sea bass is a batch spawner. One female can have up to 4 ovulations during the spawning period that may last 1-2 months. Spawning is totally dependent on water temperature and photoperiod meaning that, depending on the latitude, it takes place during the winter in the Mediterranean and in the spring near the British Isles. Eggs are pelagic, presenting 1-2 fat drops that fuse about 12 hours after laying. Embryo development lasts about three days at 13-14 °C and larval development takes about 40 days at 19 °C. Juvenile inhabit coastal waters and estuaries where they grow and progress towards puberty (Pickett and Pawson, 1994; Carrillo et al., 1995).

Endocrine control of reproduction

In vertebrates, gonadal function is controlled by a neuro-endocrine network consisting of the brain (mainly the hypothalamus), the pituitary gland and the gonads themselves, which is commonly termed as the brain-pituitary-gonad (BPG) axis (Fink, 2000) (**Fig. 1.1**). In order to maximize reproductive success and to avoid breeding during inappropriate conditions, external and internal factors are integrated by the brain, where different regions implicated in the control of reproduction respond by secreting the neuropeptide gonadotropin-releasing hormone (GnRH). The kisspeptins, and cognate receptor, the GPR54 (also designated KiSS1 receptor), act as a principal positive regulator of the reproductive axis by directly stimulating GnRH neuron activity (reviewed by Tena-Sempere, 2006; Dungan et al., 2006). In the anterior pituitary, GnRH, together with other factors, stimulate *de novo* synthesis and secretion of the gonadotropins, the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH), from the gonadotropes. Both gonadotropins are transported through the bloodstream to the gonads, where they bind to specific receptors, the FSH receptor (FSHR) and LH receptor (LHR), stimulating gametogenesis and the synthesis and secretion of gonadal hormones. Apart from being essential for gametogenesis, gonadal hormones together with peptide hormones can exert positive and negative feedback effects on gonadotropin synthesis and secretion, at all three levels

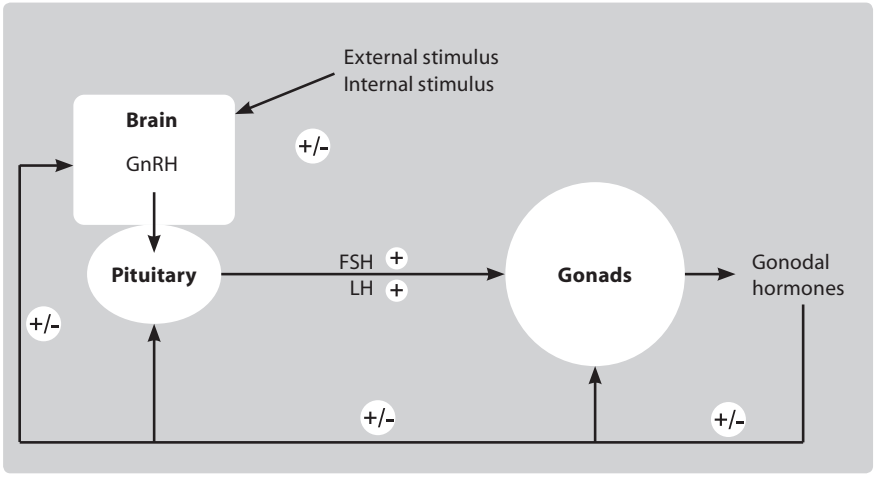

Figure 1.1 The brain–pituitary–gonad (BPG) axis in teleosts. The + symbol indicates stimulation whereas the – represents inhibition.

of the BPG axis, depending on the maturational and functional status of this axis (reviewed in Yaron and Levavi-Sivan, 2006).

Gonadotropins

Isolation, cloning and characterization of gonadotropins

In higher vertebrates, the trophic action of the pituitary on the gonads was shown for the first time by gonadal regression after hypophysectomy (reviewed in Bousfield et al., 1994). In 1931, Fevold and collaborators demonstrated that the gonadotrophic activity of pituitary extracts could be separated into two different fractions; one inducing luteinization and the other stimulating follicle maturation. The availability of purified preparations of these gonadotropins was followed by the discovery that the gonadotropins are dimers formed by two different subunits, and by the elucidation of their primary amino acid sequence (reviewed in Bousfield et al., 1994). Sequence analysis of gonadotropin subunit cDNAs in a variety of species confirmed the structural and functional conservation of these hormones.

The first biochemical studies of gonadotropins in fish suggested that a single LH like gonadotropin regulated gametogenesis (Burzawa-Gerard, 1982). Finally, in 1988 the presence and structure of two gonadotropins, GTH I and GTH II, was established in salmon (Suzuki et al., 1988a,b,c; Kawauchi et al., 1989; Itoh et al., 1990). From then until the present, the cDNAs encoding gonadotropin subunits of more than twenty three fish species have been reported in the GenBank™ data base, and structural analyses of their deduced amino acid sequences, together with functional data, clearly indicate that GTH I and GTH II are orthologues of the tetrapod FSH and LH, respectively (Prat et al., 1996; Li and Ford, 1998; Querat et al., 2000).

General structural features of gonadotropins

The pituitary gonadotropins, FSH and LH, and the placental chorionic gonadotropin (CG), together with the also pituitary-derived thyroid stimulating hormone (TSH), form an evolutionarily conserved family of glycoprotein hormones. They are glycosylated heterodimers, each composed of a common (within a given species) α-subunit noncovalently associated with a hormone

specific β-subunit. LH and CG are structurally similar, but CGβ-subunit is characterized by the presence of a unique carboxy-terminal peptide (CTP) (Pierce and Parsons, 1981; Bousfield et al., 1994). Gonadotropin subunits are encoded by paralogous genes (*i.e.* descending from a common ancestor), what explains the sequence identity shared by them. Heterodimerization,

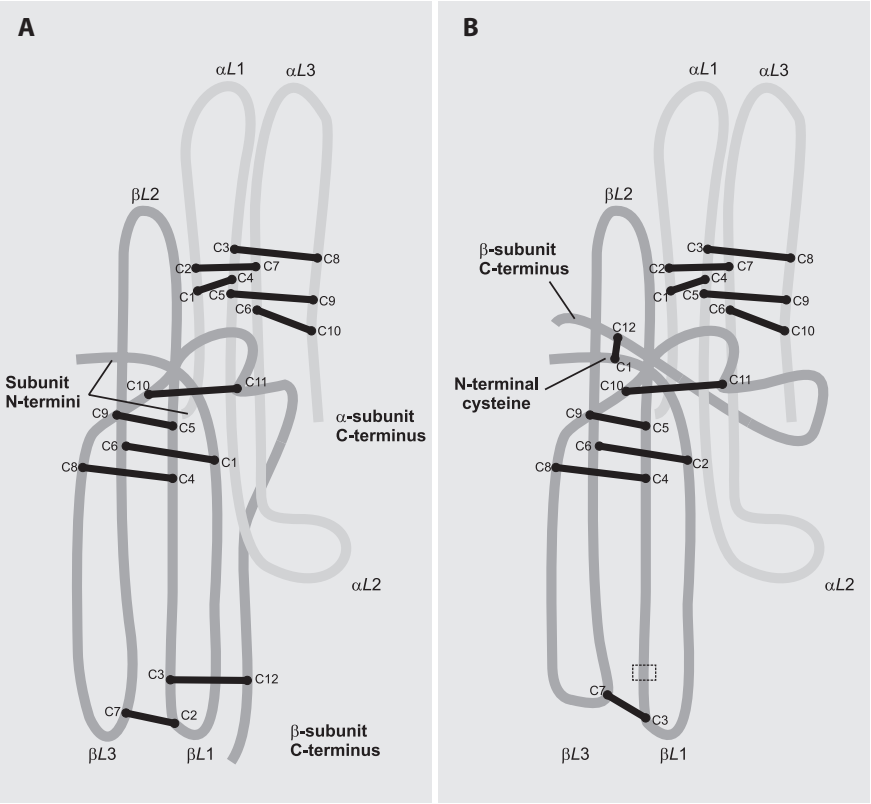

Figure 1.2 Schematic representations comparing the three-dimensional structures of two folding patterns found in vertebrate glycoprotein hormones. The heterodimeric glycoprotein hormones are composed of an α–subunit (light grey) and a β–subunit (dark grey) that are aligned head-to-tail. Each subunit contains a central cystine knot motif that divides the polypeptide in three loops (see text). The non-covalent heterodimeric association is stabilized by the "seat-belt" loop of the β–subunit that wraps around the αL2 and is tethered to βL1 (Lapthorn et al., 1994; Fox et al., 2001). (**A**) Folding pattern present in all tetrapod glycoproteins. (**B**) Folding pattern of some teleost fish FSHs. The β-subunits of some fish FSHs lack the third conserved cysteine in βL1 (open dashed rectangle), and their seat-belts are suggested to be latched to a cysteine in the N-terminus. This results in a marked difference in the spatial location of the hormone residues between the eleventh and the twelfth β–subunit cysteines. Adapted from Vischer (2003) and Moyle et al. (2005).

which occurs in the rough endoplasmic reticulum, is a prerequisite for glycoprotein hormones to achieve full biological activity. All known α-subunits contain 10 cysteines that are placed at identical sequence positions and form five disulfide bonds (Li and Ford, 1998). Likewise, nearly all gonadotropin and TSH β-subunits have 12 positionally conserved cysteines forming 6 disulfide bonds (**Fig. 1.2**). The crystal structures of deglycosylated human CG (Lapthorn et al., 1994) and human FSH (hFSH) (Fox et al., 2001) have been determined using X-ray diffraction. Despite considerable differences in their primary sequences, both α- and β-subunits share a similar overall structural organization. Each subunit contains a central cystine knot motif from which two β-hairpin loops extend at one end of the molecule (loops *L*1 and *L*3; **Fig. 1.2**), while one longer and more open loop extends at the opposite end (loop *L*2). The β-haipin loops are stabilized by disulfide bridges. In the heterodimer, the two subunits are tightly associated in a head-to-tail arrangement, forming an elongated, slightly curved structure with loop 2 of the β-subunit (β*L*2) and the loops 1 and 3 of the α-subunit (α*L*1 and α*L*3, respectively) situated on one end (**Fig. 1.2**), whereas loop 2 of the α-subunit (α*L*2) and loops 1 and 3 of the β-subunit (β*L*1 and β*L*3, respectively) form the opposite end. The β*L*1 and β*L*3 loops are linked at their distal ends by a conserved disulfide bond between cysteines 2 and 7, which is unique to β-subunits. The noncovalent heterodimer association is further stabilized by a loop at the C-terminus of the β-subunit that wraps around α*L*2 like a "seat-belt" and is tethered to β*L*1 by a disulfide bond between the conserved cysteines 3 and 12. In addition to its role in heterodimer stability, the "seat belt" contains receptor-binding specificity determinants, a fact that has implications in the manner in which these ligands dock with their receptors (Moyle et al., 1994).

Similar to the situation in higher vertebrates, fish FSH and LH are heterodimeric glycoproteins each consisting of a common α- and hormone specific β-subunits that are noncovalently linked (Boime and Ben-Menahem, 1999). Analyses of the evolutionary history of the gonadotropin subunits have demonstrated that the FSHβ-subunit has evolved at a higher rate than the LHβ-subunit in the lineage leading to the teleosts (Querat et al., 2004). Thus, the amino acid sequences of fish LHβ-subunits are highly conserved, particularly in regions thought to be important for receptor interaction (Moyle et al., 1994) such as the "seat-belt" and a portion of β*L*2 and the tip of β*L*3. The positions of the 12 cysteine residues in LHβ-subunit are strictly conserved throughout vertebrates (**Fig. 1.2, A**). In contrast, the primary

structures of fish FSHβ-subunits are more variable, even in regions thought to be important for ligand specificity. The majority of teleost FSHs differ to some degree from the tetrapod paradigm of 12 cysteine residues. The FSHβ-subunits from perciform, salmonid and pleuronectiform fish lack the third conserved cysteine in β*L*1, and have an additional cysteine near the N-terminus (**Fig. 1.2, B**) (reviewed in Swanson et al., 2003; Yaron et al., 2003; Weltzien et al., 2004). Given the importance of the "seat belt" region for receptor interactions and heterodimer formation, the variation in structure among fish FSHβ-subunits in this region may result in considerable species differences in the nature of receptor interactions, and possibly in the stability of the heterodimer (Moyle et al., 2005).

Gonadotropin receptors

In fish, the first investigations on gonadotropin receptors were elusive regarding their duality (see Bieniarz and Kime, 1986; Breton et al., 1986; Kanamori et al., 1987, Kanamori and Nagahama, 1988). The studies performed by Yan et al. (1992) in coho salmon (*Oncorhynchus kisutch*) demonstrated for the first time that in the ovaries of teleost fish two types of gonadotropin receptors exist, type I and type II. Type I receptor (FSHR) interacted with both gonadotropins, with merely a slight preference for FSH, and type II receptor bound exclusively LH (Yan et al., 1992). Subsequently, Miwa et al. (1994) localized both types of receptors in the ovaries and testis of coho salmon in several stages of gonadal recrudescence. At the same time, Quesnel and Breton (1993) identified a receptor for LH in the ovary of common trout (*Salmo trutta trutta*).

Since the establishment of the duality of fish gonadotropin receptors, efforts devoted to their characterization have been scarce and far less pronounced than the ones related to their ligands, what has hampered our knowledge concerning the biological actions of gonadotropins in teleost fish.

Cloning of gonadotropin receptors

Cloning of the rat (*Rattus norvegicus*) *LHR* in 1989 by McFarland et al. (1989) and simultaneously the porcine (*Sus scrofa*) *LHR* by Loosfelt et al.

(1989) paved the way for the cloning of similar cDNAs encoding the *LHR*, *FSHR* and TSH receptor (*TSHR*) from numerous mammalian species, including human (*Homo sapiens*) (Sprengel et al., 1990; Nagayama et al., 1989; Gudermann et al., 1992). In 1994, the first non-mammalian *LHR* was partially cloned from the testis of Japanese quail (*Coturnix japonica*) (Akazome et al., 1994), followed by the cloning of *FSHR* fragments from Japanese quail (Akazome et al., 1996b), tortoise (*Geoclemys reevesii*), gecko (*Gekko japonicus*) and lizard (*Eumeces latiscutatus*) (Akazome et al., 1996a). In fish, Dittman et al. (1995) reported for the first time, the cloning of a glycoprotein hormone receptor cDNA from coho salmon gonads that exhibited LHR-like function but TSHR-like structure. In a similar communication, Bogerd et al. (1999) reported the isolation of an African catfish (*Clarias gariepinus*) gonadotropin hormone receptor whose identity was uncertain. Later, and for the first time in a teleost fish, Oba et al., (1999a,b) cloned and functionally characterized the complete cDNAs coding for both *LHR* and *FSHR* in the amago salmon (*Oncorhynchus rhodurus*). The duality of gonadotropin receptors, at least in salmonids, was proven.

Currently, the existence of both gonadotropin receptors, by cloning their respective complete cDNAs, has been reported in Perciformes: Nile tilapia (*Oreochromis niloticus*) (Oba et al., 2001), gilthead seabream (*Sparus aurata*) (Wong et al., 2004) and European sea bass (*Dicentrarchus labrax*) (described in this thesis, Sections 3.1 and 3.2); Siluriformes: channel catfish (*Ictalurus punctatus*) (Kumar et al., 2001a,b) and African catfish (Bogerd et al., 2001; Vischer and Bogerd, 2003a); Cipriniformes: zebrafish (*Danio rerio*) (Laan et al., 2002; Kwok et al., 2005) and in other species of Salmoniformes: rainbow trout (*Oncorhynchus mykiss*) (Bobe et al., 2003) and Atlantic salmon (*Salmo salar*) (Maugars and Schmitz, 2006).

General structural features of gonadotropin receptors

The FSHR, LHR and TSHR constitute the subfamily of glycoprotein hormone receptors, which belongs to the large family of rhodopsin-like G protein-coupled receptors (GPCRs) that act as signal transducers through the cellular membrane (Vassart et al., 2004). Within the GPCR family, the glycoprotein hormone receptors, together with a number of structurally related invertebrate and vertebrate orphan receptors recently identified, represent the subfamily of leucine-rich-repeat containing GPCRs (LGR) (Hsu

et al., 2000). LGRs have the same basic structure with seven transmembrane domains and a C-terminal intracellular segment as other GPCRs, but diverge from them in having a large extracellular N-terminal domain which is involved in selective and high-affinity ligand binding (Jiang et al., 1995; Kajava, 1998).

Structures of the extracellular domains of the glycoprotein hormone receptors were not available until very recently (see below), but inspection of their sequences revealed the presence of a central portion containing nine leucine-rich repeats (LRR), flanked by N- and C-terminal cysteine rich regions (**Fig. 1.3**) that are thought to protect the hydrophobic core of the LRR from the solvent (Jiang et al., 1995; Kajava et al., 1995; Bhowmick et al., 1996; Kobe and Kajava, 2001). The C-terminal cysteine rich region is sometimes referred as a hinge region or as a signalling-specificity domain, and has been shown to play a significant role in hormone-receptor specific recognition and signal transduction (Moyle et al., 2004; Vassart et al., 2004; Bonomi et al., 2006). LRR motifs have been found in a large number of distinct proteins (Kobe and Kajava, 2001). The crystal structure of the ribonuclease inhibitor (RI) showed that its LRRs are arranged in a horseshoe-like conformation, in which each LRR (approx 20-24 amino acid residues) is organized into a short β-strand connected to a parallel α-helical segment (Kobe and Deisenhofer, 1993). The consecutive β-strands organize themselves as a parallel β-sheet, forming a concave surface to which the ligand (in this case the RNase) binds using multiple contact points, whereas the helical segments are aligned to form the outer convex side of the RI structure. Characteristically, the short β–strands of all LRRs correspond to the invariant consensus sequence X-L-X-L-X. In this sequence motif, X can be any amino acid and L refers to leucine, isoleucine or other hydrophobic residues such as valine, methionine, phenylalaline or alanine. Based on the RI structure, the LRR portions of the extracellular domains of the TSH and LH receptors have been modelled (Kajava et al., 1995; Jiang et al., 1995; Bhowmick et al., 1996). Binding of LH and TSH to their cognate receptors was predicted to involve multiple contact points with the concave β-sheet of their curved extracellular domains. The report of the crystal structure of hFSH in complex with the extracellular domain of the human FSHR (hFSHR) (amino acids 1-268) has corrected and further refined the existing models (Fan and Hendrikson, 2005). The main features are that the curvature of the hormone-biding concave β-sheet is less pronounced. The β-sheet contains nine parallel strands

and a tenth that is contributed by the N-terminal cysteine-rich domain (Fan and Hendrikson, 2005).

Like all other GPCRs, glycoprotein hormone receptors have a common transmembrane core domain composed of seven α-helical transmembrane spanning domains connected by three intracellular and three extracellular loops (**Fig. 1.3**). The α-helices made up of 22-28 hydrophobic amino acids traverse the lipid bilayer in a counter-clockwise manner. Many of the cognate signatures of the serpentine region of the GPCRs that may be involved in receptor activation or conformation are also present in the primary structures of glycoprotein hormone receptors (Vassart et al., 2004).

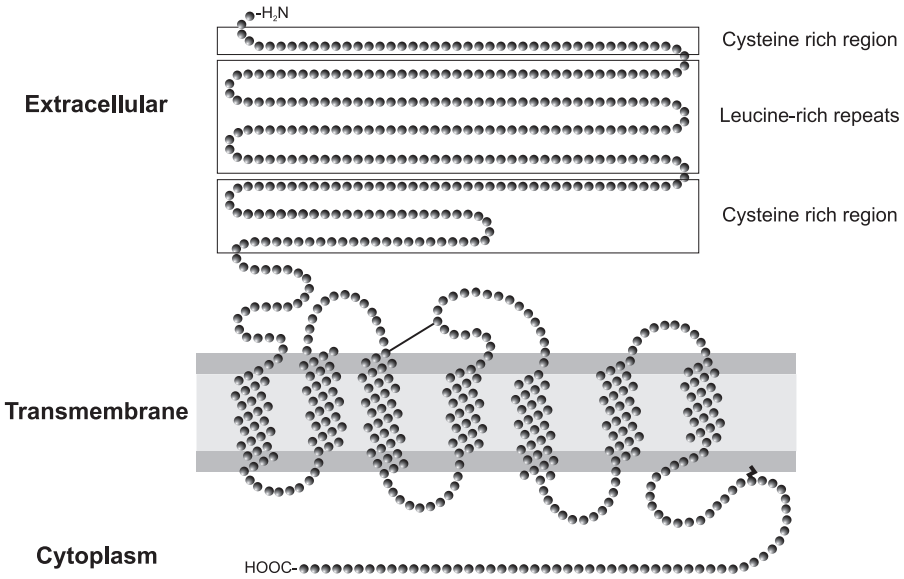

Figure 1.3 Schematic representation of the generalized structure of glycoprotein hormone receptors, illustrating the N-terminal extracellular domain, the seven transmembrane helices and the C-terminal intracellular tail. The extracellualr domain comprises leucine-rich repeats (LRR) flanked by N-terminal and C-terminal cysteine-rich regions. A disulfide bond between two highly conserved cysteine residues in the extracellular loop one and two of the transmembrane domain is represented by a black line. The lipid bilayer of the cell membrane is represented by two grey bars. A conserved cysteine residue in the C-terminal end of the eighth transmembrane α-helix is suggested to be palmitoylated anchoring that part of the tail into the plasma membrane (indicated by a black zigzagging bar). Adapted from Vassart et al. (2004).

The short intracellular domain of the glycoprotein hormone receptors interacts with cytoplasmic proteins. This is a highly divergent region, rich in serine, threonine and tyrosine residues, which are potential phosphorylation sites. As in all the members of the rhodopsin/β2-adrenergic receptor-like subfamily (*i.e.* subfamily A) of the GPCRs, the intracellular domain of the glycoprotein hormone receptors contains a highly conserved cysteine that is palmitoylated, fixing this domain in the cell membrane, and creating a fourth intracellular loop (Palczwski et al., 2000; Gether, 2000) (**Fig. 1.3**). While the transmembrane domains of individual glycoprotein hormone receptors are functionally interchangeable and display high sequence identity, the extracellular domains are less similar. This reflects the observation that ligand recognition specificity is completely encoded within the extracellular domain (Braun et al., 1991).

Structure and organization of gonadotropin receptor genes

Shortly after the cloning of the first gonadotropin receptor cDNAs in mammals, their corresponding genes were isolated in rat (Tsai-Morris et al., 1991; Koo et al., 1991; Heckert et al., 1992) and human (Atger et al., 1995; Gromoll et al., 1996). The glycoprotein hormone receptor genes are single-copy, have

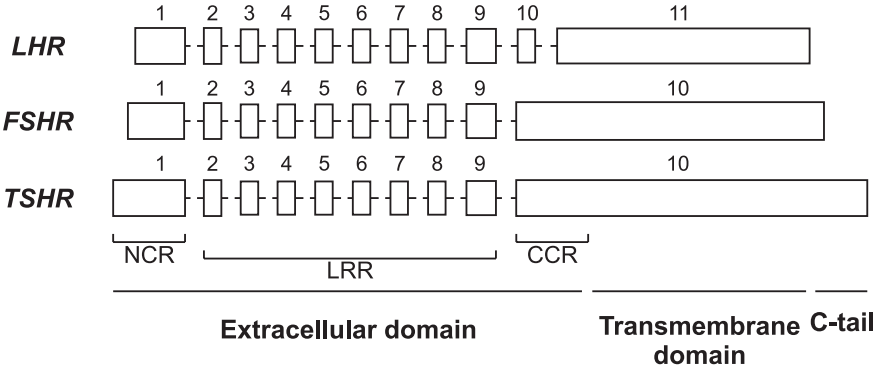

Figure 1.4 General structural organization of most glycoprotein hormone receptor genes. *LHR* is encoded by 11 exons while *FSHR* and *TSHR* are partitioned into 10 exons. The last exon codes for the transmembrane domain, whereas the first 9 or 10 exons code for the N-terminal extracellular domain of the FSHR and TSHR, and LHR, respectively. The N-terminal (NCR) and C-terminal (CCR) cysteine-rich regions and the leucine-rich repeats (LRR) region of the extracellular domains are indicated.

equal chromosomal localization and share a high structural similarity (**Fig. 1.4**). Mammalian *FSHR* and *TSHR* genes comprise ten exons and nine introns and have a length ranging from 192 to 235 kilobases (kb). Mammalian *LHR* genes cover a DNA region that can range from 38 to 69 kb and have eleven exons and ten introns. Compared to *FSHR* and *TSHR* genes, the additional exon present in *LHRs* appears between exons 9 and 10. This exon is specific for the *LHR* gene considering that all the mammalian and bird genes analysed to date have this exon, while *FSHRs* do not have an exon in a homologous position. This difference seems to have emerged early in vertebrate evolution since the *LHR*-specific exon is also present in fish (Oba et al., 1999a; Kumar et al., 2001a; Vischer and Bogerd, 2003a; Kwok et al., 2005; Maugars and Schmitz, 2006). Thus, the presence of this exon may serve as a valid criterion for the identification of *LHRs*. In all gonadotropin receptors, exons 2-8 code for most of the LRRs and have a similar size ranging from 69 to 75 base pairs (bp). They also share a high degree of sequence similarity. Each of these exons is interrupted by an intron in a homologous position of each LRR motif. Exon 9 is twice as long as any of the exons 2-8, and codes for two consecutive LRRs. The last exon of each of the mammalian glycoprotein hormone receptor genes (*i.e.* exon 10 of the *FSHR* and *TSHR* genes, and exon 11 of the *LHR* genes) encodes a small part of the extracellular domain and the totality of the transmembrane and the C-terminal intracellular domains. The partial structural organization of these genes has been described in some fish species and, contrary to their mammalian counterparts, their transmembrane domains are not free of introns (Oba et al., 1999a,b; Oba et al., 2001).

Expression of gonadotropin receptors

Tissue expression

Gonadotropin receptor genes are highly expressed in gonadal tissues. This expression is established in fish and retained in all higher vertebrates. However, cumulative data from different sources has confirmed that these receptors are not exclusively expressed in the gonads. The presence of *FSHR* has been demonstrated in the reproductive tract and in osteoclasts of human and rodents (Mariani et al., 2006; Dahia et al., 2006; Sun et al., 2006), while *LHR* transcripts have been found in an extremely wide variety of extragonadal reproductive and non-reproductive tissues (Rao, 2001). The occurrence of these

receptors in extragonadal tissues, particularly in non-endocrine organs, is an emerging but controversial area of investigation. In many cases, the available information is based only on mRNA detection, but not in the presence of protein. The biological roles FSH and LH receptors in these tissues are still under investigation. The expression pattern of the majority of fish gonadotropin receptors cloned so far is similar to the one described for mammals. Exceptions include gonadotropin receptors from amago salmon whose transcripts were exclusively detected in the gonads (Oba et al., 1999a,b). Low expression levels of the *FSHR* were also found in the spleen of the channel catfish (Kumar et al., 2001b), liver of the zebrafish (Kwok et al., 2005) and gills of the Atlantic salmon (Maugars and Schmitz, 2006).

Cellular localization

Functional gonadotropin receptors have been localized on the membrane surface of somatic cells in both the ovary and testis of coho salmon, by use of *in vitro* ligand autoradiography (Miwa et al., 1994). In the testis, the FSHR was observed on Sertoli cells and the LHR on Leydig cells. In the ovary, the FSHR was expressed on the thecal cells, the granulosa cells and in interstitial connective tissue, while the LHR was expressed on granulosa cells (Yan et al., 1992; Miwa et al., 1994). The above cellular distribution resembles the one described for mammalian gonadotropin receptors (Heckert and Griswold, 1991; Saez, 1994; Camp et al., 1991). In coho salmon males, FSHR was found at all examined stages of spermatogenesis while the LHR was found only in the Leydig testis cells of spermiating fish (Miwa et al., 1994). In vitellogenic coho salmon females, FSHR was localized intensely on granulosa cells and also on the theca cell layer while the presence of the LHR was undetectable. In preovulatory follicles of coho salmon females, the FSHR was found in the theca cell layer and in interstitial connective tissue, but not on the granulosa layer. Among all examined stages of coho salmon oogenesis, only granulosa cells of the preovulatory follicle exhibited LHR (Miwa et al., 1994). *In situ* hybridizations performed on amago salmon and Nile tilapia gonadal tissues (Oba et al., 2001) localized *FSHR* transcripts in granulosa cells of early vitellogenic oocytes and in Sertoli cells. On the other hand, *LHR* expression was found in granulosa cells of mature oocytes and in Leydig cells, partially confirming the previous studies (Miwa et al., 1994).

Transcript variants

In mammals, transcription of the gonadotropin receptor genes gives rise to multiple mRNA variants that differ in their length (reviewed in O'Shaughnessy et al., 1996; Simoni et al., 2002). There seems to be no clear species specificity in mRNA variants, and no systematic differences have been found between the ovary and testis. The longer forms result from using different polyadenylation sites, giving rise to longer 3'-untranslated regions, and the shorter transcripts result from alternative splicing (Simoni et al., 1997; Dufau et al., 1998). Many of the analysed transcript variants result from exon skipping. Since all introns are in the same phase (Koo et al., 1991; Heckert et al., 1992), the open reading frame is preserved. Potentially, alternative splicing in the LRR encoding region may modulate the hormone binding properties or create receptors with differential activity state conformations. Splicing of the last exon, on the other hand, affects the transmembrane domain, possibly creating soluble products. It has been speculated that isoforms resulting from alternative splicing, if expressed at the cell surface, could modulate ligand binding action. However, when tested in connection with the full-length receptor in transfected cell lines, these variants were generally unable to modify the function of the full-length protein (Xie et al., 1990; Tsai-Morris et al., 1990; VuHai-LuuThi et al., 1992; Kraaij et al., 1998; Tena-Sempere et al., 1999; Peterson et al., 2000). In fish, no alternative mRNA forms of gonadotropin receptor genes have been described so far.

Ligand binding specificity of gonadotropin receptors

From earlier experiments, in which the extracellular domains of the LH and FSH receptors were exchanged (Braun et al., 1991), it is widely accepted that the LRRs are the structures implicated in ligand binding specificity. In addition, it has been demonstrated that purified extracellular domains of these receptors do bind their cognate hormones with high affinity (Cornelis et al., 2001; Remy et al., 2001; Schmidt et al., 2001). Until recently, the mechanism of hormone binding and consequently the mode of receptor activation remained puzzling. By solving the crystal structure of hFSH in complex with the extracellular domain of the hFSHR (Fan and Hendrickson, 2005), it has become clear that the C-terminal segments of both human FSHα- and β-subunits as well as the α*L2* and β*L2* loops, contact the concave surface of the LRR domain. The hormone orientation is such that causes

the long axis of the hFSH to be roughly "perpendicular" to the long axis of the ligand-binding domain of the hFSHR. These contacts are thought to stabilize the hormone in a position that enables $\alpha L1$ and/or $\alpha L3$ to contact the outer loops of the transmembrane domain (**Fig. 1.5**). The finding that the hormone–receptor complex crystallized as a dimer in which two LRR domains contact each other (Fan and Hendrickson, 2005) was considered as support for the notion that signal transduction occurs by ligand-induced receptor dimerization. The described hormone-receptor interaction in the crystal structure is consistent with previous extensive mutagenesis studies (Smits et al., 2003; Vischer et al., 2003a; Vischer et al., 2006).

The electrostatic potential surfaces of the hormone and receptor in the interface are predominantly positive and negative, respectively. This reflects direct interactions between residues with complementary charges. Al-

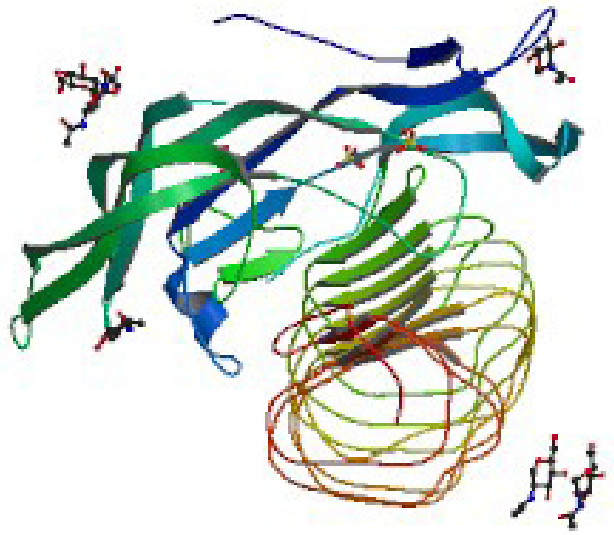

Figure 1.5 Ribbon diagram showing the crystal structure of human FSH in complex with the extracellular domain of the human FSHR. The long axis of the FSH (above) is perpendicular to the concave face of the curved extracellular domain of the FSHR (below) and aligned with the ten parallel receptor LRR β-strands (arrows). Both FSHα (blue) and FSHβ (green) interact with the receptor. The interface between hormone and receptor is bare of carbohydrate groups. Image from the PDB Protein Data Bank (PDB ID: 1XWD; Fan and Hendrickson, 2005).

though collective interfaces must contribute to specificity, detailed analysis of the structure revealed that a small number of residues in the hFSHR may function as important hormone-selective determinants precisely at the interface where the hormone "seat-belt" contacts the hormone-binding site of the receptor (Fan and Hendrickson, 2005).

In mammals, the specificity barriers between each gonadotropin-receptor couple are such that no cross-signaling occurs under physiological conditions in which hormone concentrations are low (Braun et al., 1991; Moyle et al., 1994). In contrast, there are evidences indicating that the specificity of the piscine gonadotropin receptors is less obvious (Bogerd et al., 2005). Studies with recombinant receptors of two species of catfish and zebrafish suggests that the FSHRs preferentially bind FSH but they also bind LH although with less affinity, whereas the LHRs are highly selective for the LH (Bogerd et al., 2001; Vischer and Bogerd, 2003a; Kumar et al., 2001a,b; Kwok et al., 2005; So et al., 2005). Similar results were previously obtained in the coho salmon as the FSHR was unable to distinguish between FSH and LH while the LHR only responded to LH (Yan et al., 1992; Miwa et al., 1994). Nevertheless these results oppose to data obtained in other salmonid species. The amago salmon recombinant FSHR responded in a preferential manner to chum salmon FSH while the recombinant LHR responded with a high sensibility to chum salmon LH and, with a lower magnitude also to the FSH (Oba et al., 1999a,b). Results of studies on the functional characterization of the gonadotropin receptors in fish are summarized in **Table 1.1**.

Receptor activation and signal transduction pathways

GPCRs transduce the information provided by extracellular signals into specific intracellular messages, thereby modulating diverse cellular processes. In principle, a certain GPCR can exist in an active or inactive state. Each state involves a different receptor conformation. In the absence of an agonist most receptors are in the inactive state conformation, and the binding of an agonist shifts the balance of the receptor conformation from inactive to active, which allows G protein activation (reviewed by Gether et al., 2000). Hormone binding to most members of the subfamily A of the GPCRs takes place on or close to their transmembrane domains, and can directly influence the receptor transmembrane conformation. Glycoprotein

hormones, on the other hand, bind solely to the extracellular domain of their equivalent receptors and the mechanism by which these receptors are activated is still unclear. Several mechanisms of receptor activation have been proposed (for review see Ascoli, 2002; Simoni et al., 1997; Nurwakagari et al., 2007). According to one model, once glycoprotein hormone is bound by the extracellular domain of the receptor, residues in the hormone, most likely in the common α-subunit, interact with conserved amino acids present in the transmembrane domain of the receptor (Yoo et al., 1993; Ji et al., 1993; Zeng et al., 1995; Grossmann et al., 1995; Ryu et al., 1996a). In a second model, particularly proposed for the TSHR, the extracellular domain serves as an inverse agonist stabilizing the transmembrane helices in an inactive conformation. This model implies that hormone binding and activating mutations disengage the inhibitory interaction between the extracellular and transmembrane domains. This is supported by findings where removal of the complete extracelluar domain leads to high constitutive receptor activity (Zhang et al. 2000; Nakabayashi et al., 2000). In a third scenario, binding of the hormone to the extracellular domain would lead to a conformational change of the receptor so that the extracellular domain would be able to act as an endogenous agonist on the transmembrane domain (Ryu et al., 1996b; Alvarez et al., 1999). A combination of the second and third model gives rise to an additional mechanism implying that the extracellular domain initially acts as an inverse agonist, but changes its conformation to become an agonist by hormone binding or activating mutations (Vlaeminck-Guillem et al., 2002).

In the classical model of GPCR signaling, the activation of the GPCR leads to the activation of heterotrimeric G (guanine nucleotide binding) proteins located at the cytosolic side of the plasma membrane, which dissociate into α- and βγ-subunits. These subunits activate effector molecules, which include second messenger generating systems.

The best characterized, and probably the main signalling pathway stimulated by gonadotropin hormone receptors is the cAMP-mediated activation of protein kinase A (PKA). Binding of gonadotropin hormones to their cognate receptors catalyzes the exchange of GDP for GTP that leads to the dissociation of Gα and Gβγ. The GTP bound form of Gα protein stimulates adenylate cyclase that catalyzes the conversion of ATP to cAMP, the major second messenger of glycoprotein hormone action (Zhang et al., 1991).

Increased intracellular cAMP concentrations release the catalytic subunit of PKA from the repressor subunits allowing phosphorylation of numerous cellular proteins. One target of the cAMP–PKA pathway is a class of transcription factors that bind to specific sequences known as cAMP response elements (CREs). Specifically, the CRE binding transcription factor is rapidly activated by PKA phosphorylation in response to glycoprotein hormone stimulation (Walker et al., 1995). Signaling pathways other than the cAMP-

Table 1.1 Activation of fish gonadotropin receptors by fish and mammalian gonadotropins.

			LIGANDS						
			Fish gonadotropins		Mammalian gonadotropins				
			FSH	LH	human FSH	human CG	bovine FSH	bovine LH	References
FISH GONADOTROPIN RECEPTORS	FSHR	sea bass	+	–			+	–	Section 3.1 of this thesis; Gómez et al., unpublished
		amago salmon	+	–			–	–	Oba et al., 1999a
		coho salmon	+	+					Yan et al., 1992; Miwa et al., 1994
		zebrafish	+	+			+	–	So et al., 2005; Kwok et al., 2005
		channel catfish			+	+			Kumar et al., 2001a
		African catfish	+	+	+				Bogerd et al., 2001; Vischer et al., 2003b
	LHR	sea bass	–	+			+	+	Section 3.2 of this thesis
		amago salmon	+	+			+	+	Oba et al., 1999b
		coho salmon	–	+					Yan et al., 1992; Miwa et al., 1994
		zebrafish	–	+			+	+	So et al., 2005; Kwok et al., 2005
		channel catfish			–	+			Kumar et al., 2001b
		African catfish	–	+	+	+			Vischer et al., 2003b; Vischer and Bogerd, 2003a

PKA pathway have also been identified as beeing stimulated by gonadotropin hormone receptors but their physiological significance remains under investigation. They include the MAP kinase, calcium, phosphatidylinositol 3-kinase/protein kinase B and phospholipase A_2 pathways.

Gonadal functions

Gametogenesis in males: the teleost testis

Morphology

The teleost testis is usually an elongated paired organ attached to the dorsal part of the body cavity by the extension of the mesentery (Grier et al., 1981; Nagahama, 1983). They can be divided into two compartments: the germinal compartment composed of germ cells and the associated somatic Sertoli cells, and the interstitial compartments, generally consisting of connective tissue, blood vessels, and Leydig cells (**Fig. 1.6, A**). In teleosts, two testicular types have been described, depending on the morphology of the germinal compartment and the distribution of germ cells within it (Grier, 1993). In lower fish the germinal compartment is organized in "anastomosing tubules", while those of higher teleosts are organized in "branching lobules" that terminate at the testis periphery and open into the central efferent duct. Both testis types can further be divided based on the distribution of spermatogonia in the germinal compartment. In the "unrestricted spermatogonial testis-type", spermatogonia can be found anywhere along the length of the tubule or lobule. In the "restricted spermatogonial testis-type", so far only observed in atherinomorph teleosts, the spermatogonia are restricted to the testis periphery (Selman and Wallace, 1986; Grier, 1993). Following the nomenclature of Grier (1993), sea bass has an unrestricted spermatogonial testis-type with a branching network of lobules.

In teleost fish, including sea bass, the germ cells and their associated Sertoli cells are organized in spermatocysts. Each spermatocyst contains a isogenic clone of germ cells all at the same stage of development and surrounded by a specific number of Sertoli cells attach to one another by specialized junctional complexes that result in a blood-testis barrier (Billard et al., 1982; Pudney, 1995). Individual germinal tubules or lobules are separated by connective tissue containing fibroblast cells, blood vessels and Leydig cells.

Spermatogenesis

Three major phases compose spermatogenesis: (1) asynchronous mitotic proliferation of spermatogonia, (2) meiosis of spermatocytes, and (3) spermiogenesis that consists in restructuring of spermatids into flagellated spermatozoa (Pudney, 1995). The process is fuelled by undifferentiated cells provided by the self-renewing stem cell population of the testis, the A_0 spermatogonia, also referred to as A_s, primordial, or primary spermatogonia (de Rooij and Grootegoed, 1998). Spermatogonia undergo a species-specific number of mitotic cell divisions, increasing exponentially their number (Ando et al., 2000). The last generation (B spermatogonia) emerges from the final mitosis as primary spermatocytes, which enter into meiosis. Short-lived secondary spermatocytes are produced during the first meiotic division. These cells quickly start the second meiotic division without a DNA synthesis phase, giving rise to genetically unique, small round haploid spermatids. During spermiogenesis, spermatids develop into spermatozoa. This transformation is marked by their elongation, associated with the formation of the sperm head with a condensed nucleus, a mid-piece in which mitochondria are concentrated and a flagellum. A hallmark event of spermiogenesis is the loss of 80–90% of the cellular and nuclear volume (Sprando et al., 1988). This is achieved by maximal chromatin condensation, shedding of the nucleoplasm into the cytoplasm, and by extrusion of cellular material into the lumen of the spermatocyst, which is then phagocytosed by the Sertoli cells that form the wall of the spermatocyst. After completion of spermiogenesis the cyst wall opens to release the spermatozoa (spermiation) that attain full fertilizing capacity after a process known as capacitation (for a review see Schulz and Miura, 2002; Miura and Miura, 2003).

The process of spermatogenesis depends on the production by the Sertoli cells of factors that are required for the development of germ cells (reviewed in Griswold, 2005). In teleosts, as in mammals, the number of Sertoli cells determines testicular size, germ cell number and spermatozoa output (Schulz et al., 2005). Furthermore, Sertoli cells provide a specialized, protected environment for germ cell development. Adjacent Sertoli cells form tight junctions with each other restricting what crosses from the outside to the inside of the germinal compartment. This characteristic of Sertoli cells creates what is known as the blood-testis barrier that separates the haploid

germ cells in a compartment shielded from the host tissues (Abraham et al., 1980; Loir et al., 1995). In mammals, Sertoli cell proliferation in the adult testis has not been observed under natural conditions. On the contrary, fish Sertoli cells proliferate during spermatogenesis, allowing the increase in space required for the development of spermatogenic cysts (Schulz et al., 2005). In catfish and Nile tilapia, this proliferation occurs mainly when spermatogonia undergo mitosis and cyst volume increases dramatically. Sertoli cell proliferation is strongly reduced when germ cells enter into meiosis, and stops in postmeiotic cysts (Schulz et al., 2005).

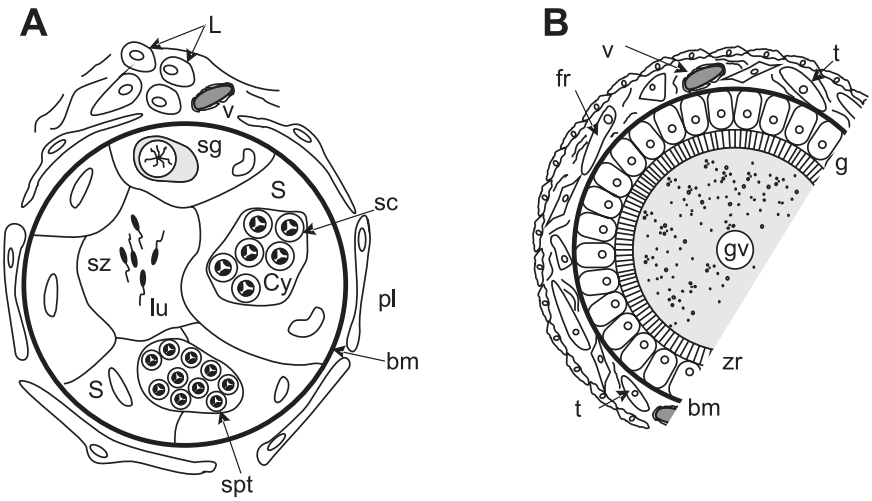

Figure 1.6 Schematic representation of the anatomical arrangement of a testicular lobule and an ovarian follicle in teleost fish. (**A**) Cross section of a testicular lobule during spermatogenesis. Leydig cells (L) and blood vessels (v) reside in the interstitial area between adjacent lobules; Sertoli cells (S) are inside the lobule and nurture the developing germ cells in different phases of spermatogenesis. Spermatogonia (sg), spermatocytes (sc), spermatids (spt), spermatozoa (sz), cyst (cy), lobular lumen (lu), basement membrane (bm), perilobular cells (pl). (**B**) Follicle layer surrounding an early vitellogenic oocyte. The granulosa layer (g) is separated from the thecal layer by a basement membrane (bm). The theca layer is composed of fibroblasts (fr), blood vessels (v), and large special thecal cells (t). Zona radiate (zr), germinal vesicle (gv). Adapted from Billard et al. (1982) and Nagahama (1983).

Gametogenesis in females: the teleost ovary

Morphology

The ovaries of teleosts, unlike those of mammals, are highly variable reflecting the diversity of fish reproductive strategies. Generally, they are paired structures located along the abdominal cavity (Nagahama, 1983). Typically, the mature ovary comprises a sac with walls consisting of ovigerous lamellae containing oocytes enclosed in follicles and an ovarian matrix. Each oocyte is delimited by an acellular envelope, the chorion (also termed vitelline membrane or zona radiata), which is surrounded by a single layer of granulosa cells and a thin heterogeneous theca layer consisting of fibroblasts, collagen fibers, capillaries and large cells designated special theca cells (**Fig. 1.6, B**).

Oogenesis

In its widest sense, oogenesis is the process by which primordial germ cells (PGCs) become ova that are ready to be fertilized. Oogenesis can be described in three major steps: (1) differentiation of oogonia into primary oocytes with the onset of meiosis which is arrested at prophase I, (2) oocyte growth, and (3) oocyte maturation when resumption of meiosis takes place (Wourms, 1976; Wourms and Sheldon, 1976).

In adult females of most teleost fish a fraction of the oogonia present in the ovaries undergo a series of mitotic divisions at the beginning of each reproductive cycle. Oogonia differentiate into primary oocytes when they begin meiosis (Tokarz, 1978). The first of two meiotic arrests during oogenesis takes place at the diplotene stage of chromosomal development (prophase I). The mechanisms controlling oogonial selection, proliferation and meiotic commitment are largely unknown. Following the onset of meiosis and after the formation of the ovarian follicle a significant growth of the oocyte begins. Follicular growth can be generally classified into previtellogenic and vitellogenic stages. The diameter of the ovarian follicle can increase by more than an order of magnitude during the previtellogenic period. Large amounts of ribosomal and heterogeneous RNA are produced by the nucleoli of the oocyte. Much of the mRNAs present in full-grown oocytes seems to be produced during previtellogenic growth (Wallace and Selman, 1990). Huge amounts of glycoproteins (polysialoglycoproteins) are also synthesized by the oocyte during mid- to late previtellogenic growth (Wallace and Selman 1990). This material is incorporated into alveoli newly formed at the oocyte's periphery known as cortical alveoli. Moreover, the oocyte lipid

deposition generally also begins during previtellogenic growth and structural changes as the formation of microvilli occur in the wall of the ovarian follicle (reviewed in Patiño and Sullivan, 2002). Vitellogenic growth is characterized by the accumulation within the oocyte of vitellogenin, a large glycophospholipoprotein precursor of yolk proteins, synthesized by the liver, and transported via the blood stream to the ovary. When vitellogenic growth of the ovarian follicle is completed a sequence of events occurs. They include acquisition of oocyte maturational competence or in other words, the ability of the follicle-enclosed oocyte to resume meiosis when stimulated with maturation-inducing hormone (MIS), and cytoplasmic maturation. Upon completion of the first meiotic division and expulsion of the first polar body, meiosis in the matured oocyte (egg) is arrested again, this time at metaphase II. The completion of the second meiotic division and expulsion of the second polar body is induced by fertilization. Cytoplasmic maturation includes the MIS-dependent yolk protein hydrolysis and the associated oocyte hydration that in some marine or brackish-water fishes is remarkable (for a review see Nagahama et al., 1995; Patiño and Sullivan, 2002).

Ovarian development in most teleosts has been classified into three groups according to the growth pattern of the oocytes present in the ovary at a given time (reviewed by Nagahama, 1983; Wallace and Selman, 1990; Nagahama et al., 1995) : a) synchronous, when egg production is an annual single event, as in the brown trout (*Salmo trutta*), or a single occurrence in the life time of a fish, as in the coho salmon; b) group synchronous, when the ovary contains two or more clutches of oocytes in different stages of development that are successively ovulated. Spawning is a seasonal event occurring during a restricted period of time. Sea bass has this type of ovarian development and females can have up to four ovulations during the spawning period that may last 1-2 months; c) asynchronous, when the ovary contains oocytes in different developmental stages and no clear clutches are observed. Eggs are recruited from this heterogeneous population of developing oocytes and are subsequently ovulated in several batches during each spawning season, as it happens in the zebrafish.

Gonadal steroid hormone biosynthesis

Sex steroids regulate diverse functions, including gametogenesis, the development of secondary sexual characters and reproductive behaviour. Testosterone (T) and specially 11-ketotestosterone (11-KT) are the major produced androgens in male fish (Borg, 1994), and collectively with the progestagens 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) are known to be essential for spermatogenesis and spermiation (Fostier et al., 1983). In the sea bass, plasma levels of 11-KT and T are highest during prespawning season and decline at spawning, whereas progestagen levels sharply rise prior to final spawning (Prat et al., 1990; Asturiano et al., 2002). These studies suggest that shifts in the testis steroidogenic pathway, may be responsible for the regulation of spermiation in this species. A similar mechanism occurs in the testis of salmonids (Nagahama et al., 1994; Planas and Swanson, 1995).

In female fish, 17β-estradiol (E2) and the progestagens 17,20βP and 20βS, regulate the reproductive breeding cycle. The production of T is also considerable in some teleost females. In female sea bass, plasma T and E2 levels increase in parallel with vitellogenic oocyte growth and decrease during the spawning season (Prat et al., 1990). The highest plasma level of 17,20βP is observed during postvitellogenesis while the highest level of 20βS coincides with final maturation (Asturiano et al., 2000). In addition, a second peak of plasma T has been observed at the end of the post-spawning period, beginning of the pre-gametogeneis period, in both female and male sea bass. This elevation is not correlated with 11-KT plasma levels in males or E2 in females.

It is known that 11-KT, the most abundant androgen in the plasma of male fish (Schulz and Goos, 1999), has an important role in the stimulation of fish spermatogenesis (reviewed in Cavaco et al., 2001). Several studies have demonstrated that 11-KT can induce all stages of spermatogenesis (spermatogonial proliferation, meiotic division and spermiogenesis) (reviwed by Schulz and Miura, 2002). In the sea bass, there is a positive correlation between testicular growth and circulating levels of 11-KT (Rodríguez et al., 2004) and there are data suggesting that 11-KT is more effective than T in inducing spermatogenesis (Rodríguez et al., 2005). The mode of action of androgens in spermatogenesis is still a major enigma in male reproduction.

In the absence of androgens, mammalian spermatogenesis becomes a very inefficient process due to increased apoptosis (Sinha Hikim et al., 1997), and germ cell development eventually is blocked well before the completion of spermiogenesis (El Shennawy et al., 1998).

Several reports have demonstrated that the entire process of vitellogenesis is regulated by E2 (for review see Nagahama, 1994). Vitellogenin is synthesized by the liver under the stimulating action of E2 and transported via the blood stream to the ovary. The hepatic synthesis of vitellogenin under E2 stimulus has also been proved in the sea bass (Mañanós et al. 1994). As mentioned above, oocyte maturational competence is stimulated by the production of MIS. In several teleosts, 17,20βP is considered to be the MIS, while in a sciaenid fish, 20βS is thought to be the MIS. In the sea bass, both 17,20βP and 20βS are believed to be this species MISs (Asturiano et al., 2000).

Leydig cells were early described as the main source of testis steroids in teleost fish (van der Hurk et al., 1978). These cells contain vesicles filled with cholesterol esters needed for steroid synthesis. Although little information is available regarding Leydig cell proliferation, differentiation and regression during spermatogenesis in fish, studies performed in trout (Loir, 1990) and sea bream (Chaves-Pozo et al., 2005) point to the proliferation of Leydig cells or a cellular type that may represent proliferative Leydig cell precursors during spermatogenesis. In mammals, LH is the physiological stimulator of Leydig cell steroidogenesis and plays a critical role in the regulation of maturation and differentiation of these cells (reviewed in Chamindrani Mendis-Handagama and Siril Ariyaratne, 2001).

In the ovary, the cellular contribution to steroidogenesis is very different from that in the testis, and both granulosa and theca cells are involved in steroid production. These joint actions form the basis of the two-cell type model that has been proposed to describe the biosynthesis of different steroid hormones in fish ovarian follicles. The theca cells are responsible for androgen synthesis, and the granulosa cells are responsible for conversion of androgens to estrogens, as well as progestagens synthesis. In the proposed model, during the phase of oocyte growth, the thecal cells secrete the androgen substrate (probably T), which diffuses into the avascular granulosa cell layer where it is converted to E2. During oocyte maturation, there is an increase in the

availability of a different androgen substrate (17α-hydroxyprogesterone) in the theca cells which serves as substrate for the production of progestagens in the granulosa cells (Nagahama et al., 1994). The steroidogenic shift from estrogens to progestagens production in granulosa cells, preceding oocyte maturation, is well documented (Senthilkumaran et al., 2004). In the sea bass, successive elevations of plasma T and E2 levels are observed prior to peaks of progestagens, which results from a shift in gonadal steroidogenesis and coincides with the maturation-ovulation of the different clutches of oocytes, or with increases in the sperm production. Following each progestagen wave, a new shift in gonadal steroidogenesis occurs, resulting in a new elevation in plasma T and E2. This hormonal pattern is repeated several times depending on the number of ovulations per female and spermatogenic waves in males (Asturiano et al., 2002).

Steroid hormones are synthesized in the gonads, but also in the adrenal tissues (equivalent to the head kidney tissue in fish) and in the brain. Steroidogenesis occurs in the mitochondria and the smooth endoplasmic reticulum, and involves a complex cascade of oxidative enzymes that convert cholesterol into different functional steroids. Based primarily on the receptor to which they bind, steroid hormones are classified into five groups: androgens, estrogens, progestagens, glucocorticoids and mineralcorticoids. Synthesis of steroids depends on the availability of cholesterol inside the mitochondria. The transport of this substrate across the mitochondrial membrane is mediated by a protein known as the steroidogenic acute regulatory protein (StAR) (Stocco and Clark, 1996) and constitutes a restrictive step of the whole process. Inside the mitochondria, cholesterol is converted to pregnenolone by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc, CYP11A1), which resides on the matrix side of the mitochondrial inner membrane. Pregnenolone itself is not a hormone, but is the immediate precursor for the synthesis of all the steroid hormones. A summary of the steroid synthesis pathway in teleosts gonads is shown in (**Fig. 1.7**). Two other cytochrome P450-associated enzymes, 17α-hydroxylase/17,20-lyase (P450c17, CYP17) in the cytoplasm and 11β-hydroxylase (P45011β, CYP11B) in the mitochondria, catalyze key steps in the further metabolism of pregnenolone to T and the 11-oxygenated androgen 11-KT. Finally, the cytochrome P450 aromatase (P450arom, CYP19) transforms the androgens T and androstenedione in E2 and estrone (E1), respectively.

Unlike cells that produce polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store very little steroid. Thus, a rapid steroidogenic response requires rapid synthesis of new steroids. Steroid hormone biosynthesis has an acute and a chronic hormonal regulation, which is known to be mediated by cAMP sig-

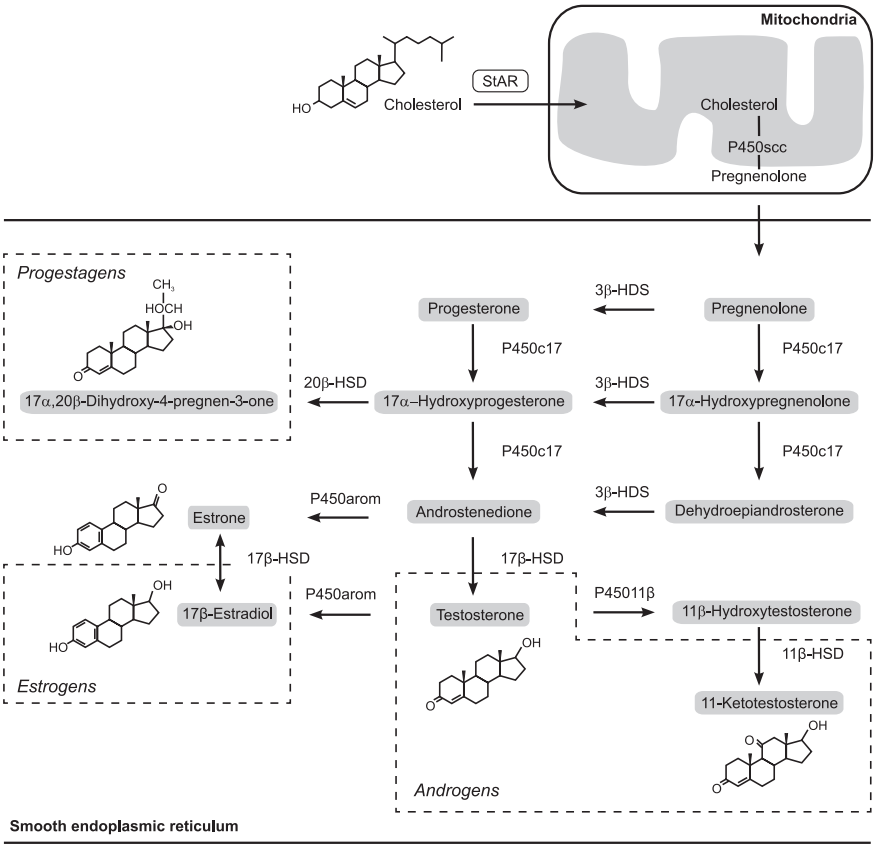

Figure 1.7 Gonadal steroid biosynthesis pathways in teleost fish. Steroidogenic acute regulatory protein (StAR); P450 cholesterol side-chain cleavage enzyme (P450scc); P450 17α-hydroxylase/17,20-lyase (P450c17); 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HDS); 11β-hydroxysteroid dehydrogenase (11β-HSD); 17β-hydroxysteroid dehydrogenase (17β-HSD); 20β-hydroxysteroid dehydrogenase (20β-HSD); P450 aromatase (P450arom); P450 11β-hydroxylase (P45011β); 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP); 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS). Open dashed rectangles indicate the predominating androgens, estrogens and progestagens in plasma of teleost.

naling. Whereas chronic, long-term regulation of steroidogenic capacity involves increased transcription/ translation of the genes encoding steroidogenic enzymes, the acute regulation of steroidogenesis occurs at the level of substrate access to P450scc, and depends on cholesterol transport into the mitochondria (Miller, 1988; Stocco and Clark, 1996). Orme-Johnson first showed that this acute steroidogenic response was accompanied by the rapid synthesis of a 37 kDa phosphoprotein, which was later cloned and named as steroidogenic acute regulatory protein, StAR (Pon and Orme-Johnson, 1986; Clark et al., 1994). This nuclear-encoded mitochondrial protein is synthesized in the cytosol and carries an amino terminal targeting sequence that directs its import to the mitochondrial matrix, where it is proteolytically processed to yield a mature 30 kDa form that is rapidly degraded (Clark et al., 1994; Miller, 2007). The cDNA that codes for the StAR protein has been cloned from many species, and is highly conserved among mammals, birds, amphibians and fish (Bauer et al., 2000). In the testis of adult mammalian males, *StAR* mRNA levels are highest in Leydig cells, although low levels are also detected in Sertoli cells. In female ovaries, *StAR* mRNA is found in the interstitum, atretic follicles, granulosa and theca cells of ovulatory follicles and corpora lutea. The acute, steroidogenic effect of LH in mammalian Leydig cells is well documented and it is known to be achieved via induction of *StAR*. It has also been proven the stimulatory effect of FSH on *StAR* expression in granulosa cells and luteinized granulosa cells (for a review see Manna and Stocco, 2005).

Gonadotropins regulate gonadal functions

The known roles of FSH in the mammalian ovary are to stimulate follicular maturation, including follicular estrogen production through aromatization of androgens. LH stimulates androgen production in theca cells, thus providing substrate for granulosa cell estrogen production (Richards, 1994). LH also triggers ovulation, and thereafter maintains the progesterone production by the corpus luteum (reviewed in Dufau, 1998). The specific roles of FSH in mammalian testicular function are still unclear, but functions such as stimulation of Sertoli cell proliferation in the immature testis and maintenance of sperm number and quality, by indirect effects mediated

through Sertoli cells, have been proposed (Allan and Handelsman, 2005). Moreover, FSH stimulates the aromatase activity and production of, *e.g.*, inhibin, lactate, transferrin and androgen receptor by Sertoli cells (Bicsak et al., 1987; Mita et al., 1982; Skinner et al, 1989; Verhoeven and Cailleau, 1988). The role of LH is to stimulate Leydig cell androgen production and thereby to maintain the endocrine (systemic) and paracrine (spermatogenic) effects of androgens.

Similar to the situation in higher vertebrates, in teleost fish FSH is considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH is responsible for the final maturation processes, such as oocyte maturation, ovulation, spermiation, and milt production (reviewed in Yaron et al., 2003). However, the functional duality between FSH and LH has yet to be clarified in fish. During female vitellogenesis in common carp (*Cyprinus carpio carpio*) (Van Der Kraak et al., 1992), tuna (*Thunnus obesus*) (Okada et al., 1994), and salmon (Suzuki et al., 1988c; Planas et al., 2000), both FSH and LH stimulate the *in vitro* production of ovarian estrogens. During male spermatogenesis in salmon (Planas and Swanson 1995) and red seabream (*Pagrus major*) (Kagawa et al., 1998), both LH and FSH stimulate testicular androgen production *in vitro*. Furthermore, several studies have suggested cross-ligand binding of fish gonadotropin receptors (reviewed in Bogerd et al., 2005). Nevertheless, functional duality in the activities of fish gonadotropins may arise from their different temporal expression pattern. In salmon, for example, FSH is present during the beginning and early stages of gametogenesis whereas LH is mainly detected during final maturation and ovulation or spermiation (reviewed in Swanson et al., 2003).

Understanding the scope of this doctoral thesis

Research in the Group of Reproductive Physiology of Fish at the Instituto de Acuicultura de Torre la Sal (Spanish National Research Council) focuses on the molecular, cellular, neuroendocrine and endocrine mechanisms that control reproduction in the European sea bass (*Dicentrarchus labrax*). This perciform is among the most studied marine fish species with a substantial history of research carried out in physiology, nutrition, pathology, genetics and ecology. It was also the first marine non-salmonid species to be commercially cultured in Europe and at present is one of the most important commercial fish widely cultured in Mediterranean areas. Despite major progress in our understanding of the reproductive physiology of sea bass, significant gaps still exist in our knowledge as regards the physiological regulation of important reproductive processes including sex differentiation, the timing of the first sexual maturation (puberty), gamete maturation and egg quality. In all vertebrates, the gonadotropins (FSH and LH) are the main regulators of gametogenesis and gonadal hormone production. Regarding sea bass gonadotropins, except from some structural data, expression profiles and LH plasma levels, little is known related to their mode of action and specific roles. To exert their biological actions, gonadotropins must bind to and activate specific receptors. This implies that understanding the molecular mechanisms that allow gonadotropins to exert their distinct effects requires a profound knowledge of the function of their cognate receptors.

Aims of this doctoral thesis

The overall aim of this thesis was to study the role of gonadotropins in the sea bass reproductive function by studying their cognate receptors. The specific aims were:

- To investigate the molecular structural characteristics of the sea bass FSHR and LHR
- To determine the conservation of their genomic primary structures
- To identify where these genes are expressed
- To evaluate the ligand binding specificity of the sea bass gonadotropin receptors
- To study how the expression of gonadotropin receptor genes changes during the sea bass reproductive cycle
- To investigate how changes in the expression of gonadotropin receptor genes are related to gonadal development and growth, and to the temporal profile of plasma sexual hormones and *StAR*
- To investigate if the TSH, acting through its gonadal receptor, could participate in the regulation of the sea bass reproductive function

2. MATERIALS AND METHODS

Materials and Methods

Animals and sample collection

European sea bass (*Dicentrarchus labrax*) were bred and grown at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain, 40ºN), under natural conditions of photoperiod and temperature. Fish were reared in fiberglass tanks provided with well-aerated flow-through sea water and were fed a commercial diet at a ratio adjusted to fish size and water temperature (ranging from 0.5% to 1.5% of body weight).

To obtain the starting material needed for experimentation, various tissue samples of adult fish were collected, frozen in liquid nitrogen and stored at -70ºC until RNA extraction. Over a period of two years, young male and female sea bass, entering their first year of reproductive activity (pubescent) were sampled monthly. At each sampling point, five fish of each sex were anesthetized by submersion in a water bath containing 0.025% of 2-phenoxyethanol (Sigma-Aldrich, Inc.), weighed, sized and sacrificed by decapitation in accordance with the European Union Animal Care Regulations. Their blood was collected via the caudal vein using heparinized syringes, centrifuged at 2500 x *g* for 25 min at 4ºC and the obtained plasma was stored at -20ºC until analysis. Gonads were dissected, weighed and one portion was flash frozen in liquid nitrogen and stored at -70ºC. The other portion was processed for histological analysis. Gonadosomatic index (GSI) was determined by the following formula: gonad weight/ body weight x 100.

Molecular biology techniques

The most common solutions used in these techniques were prepared following the protocols described in Sambrook and Russell (2001). Solutions and media were prepared using distilled water and filtered or autoclaved. Sterile disposable material and autoclaved glass/ plastic material were routinely used.

Isolation of nucleic acids

Sea bass total RNA was isolated from different tissues (n = 2 animals/ tissue), using the TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) which is an improved version of the single-step total RNA isolation reagent developed by Chomczynski et al. (1987). Poly $(A)^+$ RNA was isolated using the PolyATtract mRNA Isolation Systems (Promega Corp.). The method is based in the hybridization, in the presence of high-salt concentrations, of the poly$(A)^+$ tail to a biotinylated oligo(dT) primer. The hybrids are bound to streptavidin (a solid-phase matrix) coupled to paramagnetic particles, which are then captured using a magnetic separation stand and washed at high stringency. The mRNA is eluted from the solid phase by the addition of ribonuclease-free, deionized water. During the RNA work a series of precautions were taken to avoid RNA degradation. Glassware was baked at 200°C for at least 4 hours and double-distillated water was treated with 0.1% diethyl pyrocarbonate (DEPC), and later autoclaved. Genomic DNA (gDNA) was isolated from sea bass whole blood, using a protein salting-out method (Martínez et al., 1998). The integrity of the nucleic acids was checked by running a sample aliquot in an agarose gel. Ethidium bromide staining was used for nucleic acid visualization. Comparison of the fluorescence intensity of the samples to DNA molecular weight markers provided an estimation of the amount of nucleic acid in the samples. Quantification of the nucleic acids was also done by spectrophotometry at 260 nm (GeneQuant, Pharmacia Biotech, Cambridge, UK). The optical density (OD) absorbance ratio, OD 260 nm / OD 280 nm, gave an estimate of the purity of the solution. When needed, total RNA was subjected to RNase-free DNase digestion with RQ1-DNase (Promega Corp.) to remove gDNA contamination.

Reverse transcription-polymerase chain reaction (RT-PCR) and polymerase chain reaction

Single-stranded complementary DNA (cDNA) was synthesized by using 4 μg of total RNA which was denatured at 65°C for 5 min, in the presence of 100 ng of random hexamers and 1 μl of dNTPs (10 mM each dNTP), and then chilled on ice. RT was performed at 42°C for 50 min using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Protection of mRNA from ribonucleases during the cDNA synthesis was assured by usage of 40 units of RNasin (Promega Corp.). The reaction was stopped by heating at 70°C for 15 min.

PCR was used to amplify DNA segments using either 150 ng of gDNA or

0.5 to 2.5 μl of cDNA as template. **Table 2.1** summarizes the features of the primers used in this work. For general purposes, the highly thermostable DNA *Taq* polymerase (Bioron GmbH, Germany) was used. For an efficient, accurate and convenient amplification of long DNA fragments, a combination of a DNA *Taq* polymerase and a minor amount of the proofreading *PfuTurbo*® DNA polymerase (Stratagene, La Jolla, CA) was used instead. Improved specificity was achieved by using touchdown PCR thermal cycling programmes (Don et al., 1991), comprising a 10 to 15°C span of annealing temperatures. In general the following conditions were used: an initial denaturation step at 94°C for 2 min followed by 30 (15°C span) or 20 (10°C span) cycles of 94°C for 30 sec, the highest annealing temperature for 30 sec, and an extension temperature of 72°C for a period of time corresponding to 1 min per kb of target. The annealing temperature was then decreased 0.5°C per cycle. Final extension was a single cycle of 72°C for 5 min.

Primer pairs fshr25-fshr26, lhr35-lhr23 and tshr6-tshr9 (**Table 2.1**) were designed to amplify intron-containing regions in order to exclude false positive bands arising from potential contaminating gDNA. Also, they share limited sequence identity between them. As internal control a 495 bp fragment of the sea bass 18S ribosomal RNA (*sbs18S rRNA*) gene was also amplified, using primers 5'18S and 3'18S (**Table 2.1**). Cycling conditions consisted of 25 cycles at 94°C for 30 sec, 65°C for 45 sec, and 72°C for 45 sec.

In order to obtain a fragment of sea bass *StAR* (*sbsStAR*) cDNA, a PCR was performed using 2 μl of cDNA and the degenerate primers star1 and star2 designed to conserved regions of *StAR* from the largemouth bass (*Micropterus salmoides*; GenBank™ accession no. DQ166820). The PCR reaction using primer pair tshr17-tshr8 was performed with the FideliTaq™ DNA polymerase (USB Corp, Cleveland, OH).

Genome walking PCR was carried out according to the instructions of the Universal GenomeWalker™ Kit (Clontech Laboratories, Inc. CA). In detail, four GenomeWalker "libraries" were prepared by blunt-end digestion of sea bass gDNA with *Dra*I, *EcoR*V, *Pvu*II and *Stu*I. Each batch of digested gDNA was ligated to the GenomeWalker adaptor provided in the kit. Primary PCR was performed using 1 μl (containing approximately 0.1 ng of DNA) of each library with the gene-specific primer lhr 22 and the AP-1 primer provided in the kit (**Table 2.1**). Seven initial cycles were carried out, with 20 sec denaturation at 94°C, and 3 min annealing and DNA extension at 72°C, followed by 32 cycles in which the annealing and extension tempera-

Table 2.1 Primers used in the PCR experiments[a]

Primer	Nucleotide position	Sequence (5'→3')[b]	Touchdown[c] Annealing Temp	
	sbsFSHR			
fshr 2	2113	AGGAARGGRTTGGCRCAIGARTTKAT	70ºC max → 55ºC min	Reverse
fshr 6	1779	RYSAGYATCTGYCTICCCATGGATGT	65ºC max → 55ºC min	Forward
fshr 8	699	CTGACTATYTCMAACACNGGNCT	65ºC max → 50ºC min	Forward
fshr 9	1865	GAAAGCCAGGATGTTGAGGAG	65ºC max → 50ºC min	Reverse
fshr 19	1598	TGAGCTGTCAGTGTTCACCTTAAC	65ºC max → 55ºC min	Forward
fshr 23	1189	AGTGGAACTCCCTGTGCTCC	65ºC max → 55ºC min	Forward
fshr 24	1392	GAAGTACCACTGTGTTCCCCA	65ºC max → 55ºC min	Reverse
fshr 25	1339	TCCTCATCTGGATCATCTCCAT	65ºC max → 55ºC min	Forward
fshr 26	1621	GTTAATGTGAACACTGACAGCTCA	65ºC max → 55ºC min	Reverse
fshr 24B	373	CCTCCAGCATCTCCAGCAACA	65ºC max → 55ºC min	Forward
fshr 26B	448	TCAACAGCCTGCAGCACCTCA	65ºC max → 55ºC min	Forward
fshr 28	1274	TCCAACTCCACCTCCATCATC	65ºC max → 55ºC min	Reverse
fshr 12	823	CTCAGGGGCGGTTACGAAAGTT	65ºC max → 55ºC min	Reverse
	sbsLHR			
fshr 1	1419	TTCCTKATGTGCMACCTIKCMTTTGC	70ºC max → 55ºC min	Forward
fshr 2	2098	AGGAARGGRTTGGCRCAIGARTTKAT	70ºC max → 55ºC min	Reverse
lhr 1	1588	TGTCAGTCTACACGCTTTCCACC	70ºC max → 60ºC min	Forward
lhr 3	1655	AGAGCGCCATCTGGTACTCACA	68ºC max → 60ºC min	Forward
lhr 6	1484	TGTGGCTATCATTAGGAGGTAGA	65ºC max → 50ºC min	Reverse
lhr 12	942	AGTCTGCCTCCTCTGCAG	65ºC max → 50ºC min	Forward
lhr 19	744	YAYGCMTTYAAYGGRACHARRMTV	65ºC max → 55ºC min	Forward
lhr 21	1090	CTCTCATCACAATAAGTAGAGTCA	60ºC max → 55ºC min	Reverse
lhr 22	847	ACTCTAGGTCCTGTGGCTCCTTCGAAA	Genome Walking	Reverse
lhr 23	811	TGGATCACTCTGAGGTTTCGATTATTC	70ºC max → 55ºC min	Reverse
lhr 24	535	AGGTTATTGAATGTCCTTCTGCCAATGTG	60ºC max → 55ºC min	Reverse
lhr 25	502	AGACTCCTCGTGTTCTGGACTGAG*CTG*	Genome Walking	Reverse
lhr 27	Intron 2	TTGATACCTACATCCTCTGGACTCCTT	Genome Walking	Reverse
lhr 28	Intron 2	GGTTACACTCTGAGCAATCTCACTGTG	Genome Walking	Reverse
lhr 30	1	CACTTGATGAGAAGTTGAGTAACA	60ºC max → 55ºC min	Forward
lhr 35	484	TCCAGAACACGAGGAGTCTGAT	70ºC max → 55ºC min	Forward
	sbsStAR			
star1		CCHCCTGCTTCYTGGCKGGR	70ºC max → 60ºC min	Forward
star2		GCATCTTGTGTCAGCAGGCRTG	70ºC max → 60ºC min	Reverse
	sbsTSHR			
fshr 1	1665	TTCCTKATGTGCMACCTIKCMTTTGC	70ºC max → 55ºC min	Forward
fshr 2	2284	AGGAARGGRTTGGCRCAIGARTTKAT	70ºC max → 55ºC min	Reverse
tshr 1	1774	TATCTGTCTACACCTTGACGGTG	70ºC max → 60ºC min	Forward
tshr 3	1841	GGATCGTAAGCTGCATCTGCAC	68ºC max → 60ºC min	Forward
tshr 6	231	CAGGTGATAACATGYGCGYTGTT	62ºC max → 52ºC min	Forward
tshr 7	618	TACCTGGKGATTTTCAAYACYGG	65ºC max → 50ºC min	Forward
tshr 8	1671	CAGAGGCAATAAGCAGCAGATAA	65ºC max → 50ºC min	Reverse
tshr 9	661	AAGTCAGGGAAAAAAGTAAGGCC	62ºC max → 52ºC min	Reverse
tshr 17	2	GCATCCCCACCTTCACAA	65ºC max → 50ºC min	Forward
	sbs18S rRNA			
5'18S		TCAAGAACGAAAGTCGGAGG	65ºC	Forward
3'18S		GGACATCTAAGGGCATCACA	65ºC	Reverse
	Universal			
SKL		GCCGCTCTAGAACTAGTGGATCC	62ºC max → 52ºC min	Forward
AP1		GTAATACGACTCACTATAGGGC	Genome Walking	Forward
AP2		ACTATAGGGCACGCGTGGT	Genome Walking	Forward
AUAP		GGCCACGCGTCGACTAGTAC	70ºC max → 60ºC min	Reverse

a - Primers were obtained from Invitrogen Corp. (Carlsbad, CA); **b** - Y = C or T, R = G or A, K = T or G, S = C or G, M = A or C, H = A or T or C, V = A or C or G, N = A or C or T or G and I = deoxyinosine; **c** - Maximum and minimum ºC achieved during annealing step of touchdown-PCR are indicated for each primer. Italic underlined letters correspond to intronic sequence.

tures were both 67ºC, and a final extension step of 7 min at 67ºC. Nested PCR was performed on a 1/50 dilution of the primary PCR product with the gene-specific primer lhr 23 and the AP-2 primer from the kit (**Table 2.1**). The PCR reaction mixture was subjected to five cycles at 94ºC for 20 sec, and 72ºC for 3 min, followed by 20 cycles where the annealing/extension temperature was 67ºC during 3 min, and a final extension step at 67ºC for 7 min. The primers used in the second (primers pair lhr 24 and lhr 25; **Table 2.1**) and third (primers pair lhr 27 and lhr 28; **Table 2.1**) rounds of genome walking PCR were subsequently synthesized based on the sequences of PCR products from previous rounds.

Rapid amplification of sea bass *LHR* (*sbsLHR*) and sea bass *TSHR* (*sbsTSHR*) mRNA 3´ ends (3' RACE) was performed by using the 3' RACE System (Invitrogen Corp., Carlsbad, CA), following the manufacturer indications. This system takes advantage of the natural poly(A)$^+$ tail found in mRNA as a generic priming site for PCR. In this procedure, mRNAs are converted into cDNA using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) and an oligo(dT) adapter primer. A specific cDNA is then amplified by PCR using a gene-specific primer that anneals to a region of known exon sequence and an adapter primer that targets the poly(A)$^+$ tail region. The initial PCR amplifications were carried out using the sense gene-specific primers lhr1 or tshr1 followed by a nested amplification with primers lhr3 or tshr3, both in combination with the adapter primer AUAP (**Table 2.1**). Thermal cycling was performed using touchdown PCR programs as described above.

cDNA library screening

cDNA fragments of 1175 bp, 678 bp and 1054 bp from the sea bass *FSHR* (*sbsFSHR*), *sbsLHR* and *sbsTSHR* respectively, were labelled with [α-^{32}P]-dCTP by random octamer priming (Rad Prime DNA Labelling System Kit, Invitrogen Corp., Carlsbad, CA) and used as probes to screen, following standard methods (Sambrook and Russel, 2001), a sea bass testicular directional cDNA library constructed in the UNI-ZAP XR self-replicating lambda vector.

To obtain the highest and specific signal, prehybridizations and hybridizations were done at high stringency conditions (50% formamide, 42ºC). Final washes were carried out at 55ºC with 1xSSPE and 0.5% SDS to achieve a low background signal. Several positive clones were obtained after first round

screening of approximately $1x10^6$ 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, La Jolla, CA). The phagemids were then analysed by restriction enzymatic digestion and PCR screening.

Plasmid generation, propagation and purification.
PCR products generated with *Taq* polymerase, which contain a 3'-A overhang at both ends, were ligated to the pGEM-T Easy Vector (Promega Corp.) and those amplified with a proofreading thermostable polymerase, which contain blunt ends, were ligated to pPCR-Script (Stratagene, La Jolla, CA) following the protocols of each manufacturer. Other sub-clonings of different fragments were done in the pUC18 vector (Fermentas International Inc., Ontario, Canada).

Plasmids for expression in eukaryotic cells were generated by inserting the complete open reading frame (ORF) of the gene of interest into the pcDNA3 (Invitrogen Corp., Carlsbad, CA), which is a eukaryotic expression vector that contains the cytomegalovirus (CMV) enhancer-promoter for high-level gene expression. The pcDNA-sbsFSHR plasmid was generated by inserting a 2.8 kb fragment containing the complete ORF of the *sbsFSHR* into this expression vector. The expression vector pcDNA-sbsLHR, containing the complete ORF of the *sbsLHR* was generated in several steps. First, two fragments corresponding to the extracellular domain of *sbsLHR* were PCR amplified with primer pairs lhr30-lhr24 and lhr35-lhr21 respectively (**Table 2.1**). Equal quantities of each fragment were used as template in an overlapping PCR reaction with primers lhr30-lhr21 (**Table 2.1**). 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 extracellular domain (nucleotides 1 to 1048) was in-frame ligated to a *Vsp*I-*EcoR*V 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-*EcoR*V-digested pcDNA3 vector (Invitrogen Corp., Carlsbad, CA). 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).

All these ligations were done with T4 DNA Ligase (Invitrogen) which catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA with blunt or cohesive-end termini. This enzyme requires ATP as cofactor and has no activity on single-stranded nucleic acids. Insert:vector molar ratios from 3:1 to 1:3 were generally used in the ligation reactions. Incubations were performed overnight at 16°C.

Plasmid propagation was done by transformation of the *Escherichia coli* strains DH5α or XL1Blue that had been pre-treated to make cells temporary permeable to small DNA molecules (*i.e.*, competent). The new phenotype conferred by the plasmid (*i.e.*, resistance to an antibiotic) allowed selection of bacteria that had been successfully transformed. Isolated bacterial colonies were checked either by PCR using primers located in the vector and insert or by small scale plasmid DNA preparations (minipreps) and subsequent restriction enzyme analysis. Plasmid DNA was purified from *E. coli* liquid cultures grown overnight at 37°C with the appropriate antibiotic (usually ampicillin) to ensure plasmid maintenance. Purification was done following the alkaline lysis method, using home-made solutions. For large scale preparations (maxipreps) plasmid DNA was purified using a commercial kit (Qiagen GmbH, Germany) that is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low salt and pH conditions.

Before ligation, DNA fragments were run in an agarose gel, extracted and purified. All the fragments generated by PCR and those isolated from the cDNA library were sequenced to verify that they contained the desired inserts and to control for undesired mutations.

Northern blot analysis

Poly $(A)^+$ RNA and total RNA from sea bass gonads were denatured and electrophoresed in the presence of formaldehyde. RNAs were blotted on a nylon membrane and stained with methylene blue to control for equal load-

ing, integrity and transfer efficiency. The full length *sbsFSHR* and *sbsLHR* cDNAs were labelled with [α-^{32}P]-dCTP and used as probes. Membranes were prehybridized for at least 4 h in hybridization solution and hybridization were carried out overnight at 42ºC with 50% formamide. Final washes were performed in 2xSSC /0.5% SDS at 50 ºC. The membranes were exposed to Hyperfilm ™ MP autoradiography films (Amersham Biosciences UK Ltd.) and later developed.

In situ hybridization

The principle of *in situ* hybridization is very similar to *Northern* hybridization and is based on the annealing of a labelled nucleic acid probe (RNA or DNA) to a complementary sequence of mRNA. The two techniques differ in the starting material that in a *Northern* consists of isolated RNA, whereas for *in situ* hybridization is a histological tissue section.

In situ hybridization experiments were carried out as described previously (Cerdá-Reverter et al., 2003). Sea bass gonads were fixed with 4% paraformaldehyde (PAF) overnight at 4ºC, dehydrated, and embedded in Paraplast (Sherwood, St. Louis, MO). Before hybridization, 6 µm 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.

Riboprobes were prepared from a pBluescript SK (-) phagemid containing an 800 bp fragment from the *sbsFSHR* 3' UTR, and linearized with *Xba*I or *Xho*I. Antisense and sense RNA probes were *in vitro* transcribed using T7 or T3 RNA polymerase, respectively. The probes were labelled with ^{35}S-UTP (the most sensitive type of label) using the Riboprobe *In Vitro* Transcription System Kit (Promega Corp.). After *in vitro* RNA synthesis, samples were treated with 1unit 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 ºC and used within one week.. The ^{35}S-UTP riboprobes were pelleted and dissolved in an appropriate volume of 100 mM DTT to obtain 2x10^5 cpm/µl. After 5 min incubation at 80ºC, ^{35}S-UTP riboprobes were diluted 1/10 (final concentration of probes, 10 mM DTT and 2x10^4 cpm/µl) in hybridization buffer containing 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8),

10% Dextran 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 55ºC. After washing and ribonuclease treatment, slides were finally dehydrated and dried at room temperature. Once finished the hybridization process, slides were dipped in Hypercoat Photographic Emulsion RPN40 (Amersham Biosciences UK Ltd) and exposed under dry conditions at 4ºC for 15–20 days, developed in Kodak D-19 Film Developer and counterstained with Cleveland & Wolfe staining (Herlaut, 1960) for histological analysis. Anatomical locations were confirmed by reference to Mayer et al. (1988) and Alvariño et al. (1992).

RNA isolation and reverse transcription for real-time quantitative RT-PCR assays

Quantitative expression profiling of the genes of interest was done by real-time RT-PCR using the iCycler iQ™ (Bio-Rad Laboratories, Inc.) platform and the TaqMan® sequence detection chemistry.

Low molecular weight RNAs are massively produced and accumulated in the sea bass ovary during previtellogenesis and declined in amount thereafter (**Fig. 2.1**). To avoid an inaccurate quantification of RNA samples and potential interferences of these RNAs with the RT reaction, poly $(A)^+$ enriched RNA, instead of total RNA, was used in the female seasonal expression study. For males, total RNA, obtained as described above, was used. To prepare mRNA, total RNA was isolated from ~ 100 mg of frozen tissue using the FastRNA® Pro Green Kit (Qbiogene, Inc., CA) and the FastPrep® Instrument (Qbiogene, Inc., CA). RNA purity was verified by an OD absorption ratio, OD 260 nm/ OD 280 nm >1.9, and quantified by spectrophotometry at 260 nm (GeneQuant, Pharmacia Biotech, Cambridge, UK). The Oligotex® mRNA Kit (Qiagen GmbH, Germany) was used to isolate poly $(A)^+$ mRNA from ~ 240 μg of total RNA preparations. The ULTRA Evolution 384™ (Tecan Group Ltd., Männedorf, Switzerland) fluorescence-based microplate reader along with the RediPlate™ 96 RiboGreen® RNA Quantitation Kit (Molecular Probes, Invitrogen Carlsbad, USA) were used for poly $(A)^+$ mRNA concentration determination.

RT was performed as described above using 1 μg of DNase I RNase-free (Ambion, Inc., Austin, TX) treated total RNA or 150 ng of poly $(A)^+$ mRNA.

The volume of poly $(A)^+$ mRNA RTs was then increased to 300 µl.

The TaqMan® chemistry (also known as "fluorogenic 5′ nuclease chemistry") uses a forward primer, a reverse primer and a fluorogenic probe that enables the detection of a specific PCR product as it accumulates during PCR cycles. The oligonucleotide probe is constructed containing a reporter fluorescent dye on the 5′ end and a quencher dye on the 3′ end. While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5′ nuclease activity of *Taq* DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal, and removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. With each cycle, additional

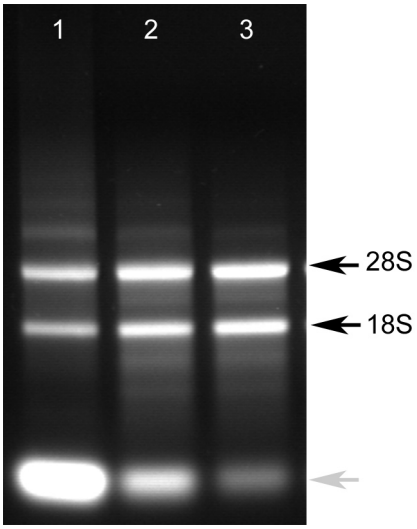

Fig. 2.1 Total RNA from sea bass ovary ran in an agarose gel and stained with ethidium bromide. RNA from females in early vitellogenesis (lane 1), advanced vitellogenesis (lane 2) and post-vitellogenesis (lane 3) is shown. 28S and 18S rRNAs, and small RNAs (grey arrow) are indicated.

reporter dye molecules are cleaved from their respective probes resulting in an increase in fluorescence intensity which is proportional to the amount of amplicon produced. The increase in fluorescence emission is read by the sequence detector in real time, during the course of the reaction. The computer software program calculates the magnitude of the fluorescence signal by subtracting from the fluorescence emission of the product at each time point, the fluorescence emission of the baseline. Using the fluorescence emission data collected during PCR amplification, the computer software constructs amplification plots. The fluorescence signal (PCR baseline subtracted curve fit relative fluorescence units [CF RFU]) is plotted versus the cycle number. Based on the variability of the baseline (normally set from cycles 3 to 15), an arbitrary threshold is chosen. Threshold cycle (C_t) values are then calculated by determining the point at which the fluorescence exceeds this chosen threshold limit. C_t is reported as the cycle number at this point. Therefore, C_t values decrease linearly with increasing input target quantity. This can be used as a quantitative measurement of the input target.

Probes and primers for real-time quantitative RT-PCR assays were designed using Primer Express software (Applied Biosystems, Inc., Foster City, CA). All assays were run in triplicate, using 96 well optical plates (Thermo-Fast® 96 Semi-Skirted PCR Plate; Advanced Biotechnologies Ltd, UK) and the iCycler iQ™ default settings. For each 25 µl PCR reaction, 1 µl of RT reaction was mixed with the corresponding amount of primers and probe (**Table 2.2**) in 1 x ABgene's Absolute™ QPCR Mix (Advanced Biotechnologies Ltd, UK). To correct for variability in amplification efficiency between different samples, standard curves were prepared from plasmids containing sea bass target genes (*sbsFSHR*, *sbsLHR*, *sbsTSHR* and *sbsStAR*) or sea bass endogenous control genes (*sbs18S rRNA* and elongation factor 1-alpha (*sbsEf1-alpha*; GenBank™ accession number: AJ866727)). Ten-fold serial dilutions of known concentrations of the plasmids containing each of the genes were used. By using the same stock DNA to prepare standard curves for multiple plates, the relative quantities determined could be compared across the plates. Data were capture and analyzed by the iCycler iQ™ software (version 3.0.6070). Correlation coefficients of the standard curves ranged from 0.99 to 1.00. PCR efficiencies are shown in **Table 2.2**. For each experimental sample, the amount of target and endogenous reference was determined from the appropriate standard curve.

Normalization of gene expression

All real-time RT-PCR assay results are subject to variability caused by technical as well as biological variation. It is essential that technical variability is kept to a minimum to optimize the chances of identifying biologically relevant changes in mRNA levels. Consequently, data normalization is an essential part of a meaningful real-time RT-PCR assay. Unfortunately, normalization is a rather problematic area and, at present, there is not a universally accepted method for data normalization that accounts for all variables encountered during the course of a real-time RT-PCR experiment. Several strategies have been proposed for normalizing real-time RT-PCR data. The most popular one is the use of an endogenous reference gene with constitutive expression. However, recent studies have shown that, often, the expression of the most commonly used reference genes change significantly under many experimental conditions (Dheda et al., 2004). In this study the *sbs18S rRNA* and *sbsEf1-alpha* endogenous genes were tested for their ability to be

Table 2.2 Primers and TaqMan™ fluorogenic probes[a]

Target	Primer or Probe	Sequence (5'→3')	nM[c]	Amplicon size; PCR efficiency
sbsFSHR	fshr 1074 fw	CCGCCCCCAATCTGAAG	50	63 bp; 0.89
	fshr 1136 rv	GGTTGGCCTGGTGCAGTTT	900	
	fshr 1092 pr	[6~FAM]AGCTTCCTCCTCTGGAGCTCTTC[TAMRA]	75	
sbsLHR	lhr 1231 fw	ACTTCTGTCAGACCCGACCAA	900	67 bp; 0.92
	lhr 1297 rv	TCCTCACAGGGATTGAAAGCA	900	
	lhr 1253 pr	[6~FAM]TTTGGTTTGCACACCTGAAGCA[TAMRA]	125	
sbsStAR	star 142 fw	GGCTGGATCCCGAAGACAA	900	72 bp; 0.98
	star 213 rv	CCTGAGGTGGTTGGCAAAGT	900	
	star 162 pr	[6~FAM]CATAAACAAAGTGCTCTCTCAGACGCAGGTG[TAMRA]	75	
sbsTSHR	tshr 1834 fw	TGCGGCTGGATCGTAAGC	900	68 bp; 0.94
	tshr 1901 rv	GCAGAAGAGCCAGCCACCTA	900	
	tshr 1854 pr	[6~FAM]CATCTGCACCATGCAGCGGC[TAMRA]	125	
sbs18S	18S fw	GCATGCCGGAGTCTCGTT	900	71 bp; 0.91
	18S rv	TGCATGGCCGTTCTTAGTTG	900	
	18S pr	[6~FAM]TTATCGGAATTAACCAGAC[TAMRA]	200	
sbsEf1-α[b]	Ef1-α 156 fw	GGAGTGAAGCAGCTCATCGTT	50	69 bp; 0.99
	Ef1-α 224 rv	GCGGGCCTGGCTGTAAG	300	
	Ef1-α 179 pr	[6~FAM]AGTCAACAAGATGGACTCCACTGAGCCC[TAMRA]	200	

a - Forward (fw) and reverse (rv) primers were obtained from Invitrogen Corp. (Carlsbad, CA). Fluorogenic probes were purchased from Operon Biotechnologies GmbH (Cologne, Germany)
b - GenBank™ accession number for sea bass elongation factor one-alpha (sbsEf1-α): AJ866727.
c - Amount of primer or probe in the PCR reaction.
d - Values represent the average numbers of two, three or five assays.

used as control genes.

Data of *sbsTSHR* expression level were normalized. For male data normalization, the target amount was divided by the *sbs18S rRNA* amount, while female target values were normalized using the *sbsEf1-alpha* amount. Data are presented as relative mRNA levels. In males, the mean of samples in April was set as 1, while in females the mean of samples from August was the chosen value to be set as 1.

Regarding data normalization of the genes whose expression was compare (*sbsFSHR*, *sbsLHR* and *sbsStAR*), male data normalization was done by dividing the target amount by the *sbs18S rRNA* amount. Concerning females, the expression of the genes of interest was analysed using two separate methods: (1) Raw arbitrary input amount (nonnormalized) and (2) Input amount normalized against adjusted *sbsEf1-alpha* values. The first method has been supported by studies that demonstrate that normalized results often oppose the trend seen in raw data (Neuvians et al., 2005), as well as by contradictory results obtained when using different reference genes in an experiment (Zhang et al., 2003). The second method involves the standardization of expression of the reference gene in each sample of each month to a randomly chosen "control" group and it has been used in the characterization of the expression levels of several genes at different stages of ovarian follicular development in zebrafish (*Danio rerio*) (Ings and Van Der Kraak, 2006). This is done by using the following formula according to Billiau et al. (2001) and Essex-Fraser et al. (2005): individual value within a group/(mean value within a group/mean value of control group), where August was chosen as the control group. This allows the use of an internal control that takes into account loading errors and RT efficiency, and corrects for changes between months. Nevertheless, this method has the disadvantage of using artificial values for normalization. In our studies, the expression patterns of the genes of interest normalized to adjusted *Ef1-alpha* values were similar to those of nonnormalized expression, implying that both methods are feasible. Data are presented as relative mRNA levels. In males, the mean of samples in April was set as 1, while in females the mean of samples from May was the chosen value to be set as 1.

To account for changes in gene expression related to tissue growth and cellular composition, Kusakabe et al. (2006) and Maugars and Schmitz (2008) have proposed in their studies performed on rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) that, in addition to the

endogenous reference gene normalization, transcript levels should also be normalized to the amount of total RNA per entire organ corrected by body weight, assuming that the changes in gonad growth correspond mostly to an increase in germ cells. The total amount of RNA per organ is estimated by measuring the concentration of the total RNA extracted per weight of a tissue fragment. Thus gene expression in the gonads would be calculated according to the following formula: Target mRNA levels = (PCR target gene quantity/PCR reference gene) x (amount of extracted RNA/tissue weight for RNA extraction) x (gonad weight/body weight). During the sea bass reproductive cycle, values of gonad weight per body weight dramatically change, being very low during the immature/ previtellogenic and early recrudescence/ early vitellogenesis stages and extremely high during the maturation-spermiation/ovulation. Thus, when using the above method to normalize the transcription levels of sea bass genes, we got values that vary greatly in magnitude. Data had to be split into two different graphs (or axes breaks introduced) to be clearly and effectively shown. This is because the intergroup difference being measured, in this case the expression level of the gene of interest in each month, is much lower than the GSI variation. So the level of variability of the GSI levels masks the differences of the mRNA quantification data. Nevertheless, analysis of the observed results shows profiles similar to the ones obtained with the former normalization methods (data not shown). An exception is found in testis *sbsFSHR* expression in November where a less pronounced decrease is observed which could be associated to a dilution effect correction. This subject will be further developed in the discussion section.

Cell culture techniques

Human embryonic kidney (HEK) 293 cells were used to generate stable cell lines, constitutively expressing the sea bass genes. Cells were grown routinely in Dulbecco's modified Eagle medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% foetal bovine serum and the antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml) to avoid microbial contamination, in a humidified atmosphere of 5% CO_2 at 37 ºC. Cells were transfected using a modified calcium phosphate transfection method (Chen and Okayama, 1987) or Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA) 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 Corp., Carlsbad, CA). 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 to 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, Inc.) 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 to 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 (CA, 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).

Histological analysis

Freshly extracted gonads were fixed by immersion in 4% formaldehyde : 1% gluteraldehyde (McDowell and Trump, 1976), embedded in 2-hydroxyethyl methacrylate polymer resin (Technovit 7100, Heraeus Kultzer, Germany), sectioned (3 μm) and stained according to Bennet et al. (1976). The stages of testicular development were classified by light microscopy, following the previously established criteria (Begtashi et al., 2004): stage I, immature; stage II, early recrudescence; stage III, mid recrudescence; stage IV, late recrudescence; stage V, full spermiating testes; stage VI, post-spawning. The ovarian stages were as follows: previtellogenesis, vitellogenesis, post-vitellogenesis,

maturation-ovulation and atresia (Asturiano et al., 2000).

Biochemical analysis

Hormone measurements

Plasma E2 was measured by a conventional competitive enzyme-linked immunosorbent assay (ELISA), developed and validated for the sea bass in our laboratory (Crespo et al., unpublished). The assay uses purchased E2 (Sigma-Aldrich, Inc.) to built a standard curve, a specific rabbit antisera against E2 as primary antibody and estradiol acetylcholinesterase conjugate (E-AChE, Cayman Chemical MI, USA) as tracer. The separation between free and bound fractions of tracer is achieved by coating the plates with mouse anti-rabbit IgG monoclonal antibody (Clone RG-16, Sigma-Aldrich, Inc.). Plasma was extracted with methanol (Panreac Química S.A., Spain). The organic solvent was evaporated and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA) by vortexing. The assay was performed in a final volume of 150 µl in mouse anti-rabbit IgG coated wells (200 µl of a 1:1800 dilution/well; 96-well microtiter plates). Each component, E-AChE tracer (diluted to 1:10 Ellman Units (E.U.)/ml), anti-E2 rabbit antiserum (diluted to1:845,000), E2 standards (ranging from 80 ng/ml to 0.078 ng/ml), or samples, were added in a volume of 50 µl. Plates were incubated overnight at 37°C. After incubation, plates were rinsed and 200 µl of Ellman's reagent were added to each well. Colour development was performed at 20°C in the dark under constant gentle agitation for 2 h. OD was read at 405 nm using a microplate reader (Bio-Rad microplate reader model 3550). The sensitivity of the assay was around 0.156 ng/ml (Bi/B0 = 90%) and half-displacement (Bi/B0 = 50%) occurred around 2.35 ng/ml.

The plasma levels of 11-ketotestosterone (11-KT) were determined by enzyme immuno assay (EIA), using an assay developed for the Siberian sturgeon and modified for its use in sea bass (Rodríguez et al., 2005). The protocol was similar to that described by Cuisset et al. (1994) except that primary antibodies were used at a final dilution of 1:200,000 and the tracer (Cayman Chemicals, MI, USA) was diluted at 1:10 E.U. /ml. The assay sensitivity of 11-KT was 1.75 pg/well.

Plasma LH levels were measured by a homologous competitive ELISA according to Mateos et al. (2006). Ninety six-well microtiter plates were coated with sea bass LHβ-subunit (1 ng/0.1 ml/well) and incubated over-

night at 4°C. Samples were preincubated (overnight at 4°C) with polyclonal antibodies against sea bass LHβ-subunit (final dilution 1/80,000), and then dispensed in duplicate (100 µl/well) into the coated wells and incubated for 90 min at 37°C. The antibodies bound to the coated antigen were detected by incubation (100 µl/well, 30 min at 37°C) with HRP-labelled goat anti-rabbit IgG (GAR-HRP, affinity purified EIA grade, Bio-Rad Laboratories, Inc.). Colour development was performed by addition of 100 µl/ well of TMB peroxidase substrate solution (Bio-Rad Laboratories, Inc.). OD was read at 405 nm using a microplate reader. The intra- and inter-assay coefficients of variation were 11.7% and 11%, respectively, and the sensitivity of the assay was 0.65 ng/ml.

Luciferase assay

Luciferase was measured from cell lysates. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase, catalyzes luciferin oxidation using ATP-$Mg2^{+}$ as a co-substrate. A flash of light is generated and measured using a luminometer. To improve reaction kinetics, coenzyme A is incorporated, allowing greater enzymatic turnover resulting in increased light intensity that is nearly constant for at least one minute.

Cells were lysed in Reporter Lysis Buffer (Promega Biotech Ibéria SL, Madrid, Spain) as indicated by the manufacturer. Cell debris were separated by centrifugation for 30 sec at 15000 x *g*, and 20 µl of the supernatant were mixed with 200 µl of luciferin reagent (20 mM TricineKOH, pH 7.8, 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 270 µM CoA, 530 µM ATP, 400 µM luciferin). The emitted light was measured in a luminometer (*Junior*, EG&G, Berthold) and expressed as relative light units (RLU).

Computer assisted analysis

cDNA sequence and phylogenetic gene analyses

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, USA). BLASTN and BLASTP (version 2.2.9, National Center for Biotechnology Information) were used for

database searching. Predictions of translation start in nucleotide sequences, the presence and location of the putative signal peptide cleavage site, potential *N*-glycolylation sites and Ser, Thr, and Tyr phosphorylation sites in the amino acid sequence, were predicted using the prediction servers of the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). The seven transmembrane helixes were predicted using the combined results of the HMMTOP server (http://www.enzim.hu/hmmtop/index.html) and the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). The Protein Families (Pfam) Database of the Sanger Institute (UK) was used in order to search for common protein domains. The UniProt name for the sbsFSHR is Q4L192_DICLA, for the sbsLHR is Q4L191_DICLA and for the sbsTSHR is A3QP57_DICLA. Multiple sequence alignments were carried out using ClustalX version 1.81 (Thompson et al., 1997). Phylogenetic analysis, incorporating full-length amino acid sequences, were conducted using MEGA version 2.0 (Kumar et al., 2001). Rooted phylogenetic trees were constructed by means of the Neighbour-Joining algorithm, using the LGR sequence of *Caenorhabditis elegans* as outgroup. Gaps or missing data were pairwise deleted. The reliability of the inferred branching pattern was assessed by 1000 bootstrapping pseudo replicates.

Exon/intron boundaries were determined by loss of identity between the gDNA and cDNA sequences, and also by the presence of consensus donor and acceptor signals at the point of divergence, helped by the predictions of splice sites given by the NetGene2 Server (http://www.cbs.dtu.dk/services/).

Statistical analysis

SigmaStat version 3 software (SYSTAT Software Inc., Richmond, CA) was used for the statistical analysis of the seasonal changes in gene expression levels and plasma hormone levels. Data were analyzed by one-way ANOVA followed by the Holm-Sidak multiple comparison test. Before the analysis, and whenever necessary, mathematical transforms were performed to ensure normality and equal variances (homoscedasticity) across samples. The Kolmogorov-Smirnov test (with Lilliefors' correction) was used to test data for normality while the Bartlett's test was used to verify the homoscedasticity. When data did not meet the criteria for parametric statistics, the Kruskal-Wallis nonparametric test was used, followed by a multiple comparison test established by Conover (1980) according to the formula:

$$|R_i/n_i - R_j/n_j| > t_{1-(\alpha/2)}[S^2(N-1-T)/(N-k)]^{½}(1/n_i+1/n_j)^{½}$$

where R_i is the sum of ranks of a level (group), n_i is the number of observations in that level, N is the total number of observations, k is the number of levels, α is the signification level and t is the Student's t value for the significance level 1-(α/2) with N-k degrees of freedom. T is the Kruskal-Wallis test value and S^2 is calculated according to the following formula:

$$S^2 = [1/(N-1)][\Sigma R(X_{ij})^2 - (N(N+1)^2/4)]$$

In order to simplify the graphical representation not all significant differences are represented. As an alternative, only major increases and decreases are shown. Linear regression analysis was used to examine relationships between plasma hormones, gonadotropin receptors and *StAR* mRNA levels, and the relationship between gonadotropin receptors and *StAR* mRNA levels. Analysis were performed by combining data over the entire reproductive cycle and also in the different reproductive stages separately. The data are presented as the mean plus/minus the standard error of the mean (SEM). The level of statistical significance was set at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1 Molecular characterization of a European sea bass follicle-stimulating hormone receptor gene: cDNA cloning, expression analysis, and functional activity

It is well established that the follicle-stimulating hormone (FSH), secreted by the pituitary gland, plays 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). In the testis, FSH determines Sertoli cell proliferation and attends germ cell maturation (Allan and Handelsman, 2005). FSH is a member of the glycoprotein hormone family, which also includes the luteinizing hormone (LH), thyroid stimulating hormone (TSH) and the chorionic gonadotropin (CG), present only in primates and equines (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. 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) and include, in addition to the FSHR, the TSH receptor which binds TSH; and the LH receptor which binds LH and CG. They constitute the subfamily of glycoprotein hormone receptors, themselves members of the wider leucine-rich repeat containing GPCR (LGR) family. The members of this family are characterized by a large extracellular 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 helixes and a C-terminal intracellular tail (Hsu et al., 2000).

The *FSHR* gene consists of ten exons spanning a DNA region of 84 kb in the rat (*Rattus norvegicus*) (Heckert et al., 1992) and 54 kb in the human (*Homo sapiens*) genome (Gromoll et al., 1996). The first nine exons encode the extracellular domain of the receptor, while exon 10 encodes the transmembrane and intracellular portions of the protein. The existence of several alternatively spliced *FSHR* transcripts, both in the ovary and in the testis, has been demonstrated in various animal species (reviewed in O'Shaughnessy et al., 1996; Simoni et al., 2002), suggesting that this is a common event.

These *FSHR* variants are generated by exon skipping or usage of alternative splice donor or acceptor sites (Gromoll et al., 1992). This results in mRNA molecules in which the open reading frame (ORF) is usually preserved, and thus code for potentially functional receptor molecules.

Evidence for the presence of gonadotropin receptors in fish was first demonstrated more than ten years ago, by binding studies of coho salmon (*Oncorhynchus kisutch*) FSH and LH to membranes of isolated granulosa cells and theca-interstitial layers of coho salmon ovary (Yan et al., 199; Miwa et al., 1994). The isolation of the cDNA from these receptors remained elusive for years. Finally, the cloning of two types of gonadotropin receptors from the ovaries of amago salmon (*Oncorhynchus rhodurus*) was reported (Oba et al., 1999a; Oba et al., 1999b), making possible the beginning of a new understanding of fish glycoprotein hormone receptors.

Apart from LH and CG that are able to activate the same receptor, *i.e.* the LHR, in primates and equids, the normal interactions between the glycoprotein hormone receptors and their corresponding ligands, are highly specific, with very few cases of cross activity (Themmen and Huhtaniemi, 2000). In contrast to what can be observed in mammalian glycoprotein hormone receptors, their fish counterparts display a less discriminatory selectivity. *In vitro* experiments with African catfish (*Clarias gariepinus*), channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) FSHRs suggest that these are not selective for FSH, as LH can also activate them, whereas, LH receptors are highly selective for homologous LH (Bogerd et al., 2001; Kumar et al., 2001a; Kumar et al., 2001b; Vischer and Bogerd, 2003a; Vischer et al., 2003b; 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 (*Oncorhynchus keta*) FSH but not by LH, whereas *LHR* transfected cells were highly responsive to LH and in a lesser extend to FSH.

This different ligand selectivity could imply that teleosts glycoprotein hormone receptors actions are not totally overlapping those of their mam-

malian counterparts. Moreover, FSHR functions can differ among fish, a highly diverse group of vertebrates. Although the cloning of a *FSHR* has been reported in two species of Perciformes, Nile tilapia (*Oreochromis niloticus*) and gilthead seabream (*Sparus aurata*) (Oba et al., 2001; Wong et al., 2004), there is no information regarding the functional characterization of this receptor in this linage of fish.

To bring more knowledge on fish FSHRs, in the present section we report the isolation and functional characterization of a cDNA coding for a sea bass *FSHR* (*sbsFSHR*). The tissue and cell type distribution of its mRNA were also examined. In addition, two alternately spliced *FSHR* mRNAs, coding for putative variants of the *sbsFSHR*, and a partial analysis of the gene structure are described.

Cloning and sequence analysis of a *sbsFSHR* cDNA

Two degenerate oligonucleotides (fshr 6 and fshr 2) were designed based on published fish and mammalian *FSHR* sequences and corresponding to a highly conserved and intronless region of the transmembrane domain. They were used as primers in a PCR reaction using sea bass gDNA as template. A 330 bp product was amplified, and subsequently subcloned and sequenced. The obtained sequence displayed the highest identity to other *FSHR*s. A *sbsFSHR* specific primer (fshr 9), designed based on the sequence of the amplified product, was used on a PCR reaction in combination with a degenerated primer designed on the extracellular domain (fshr 8). Testis cDNA was used as template. The amplified fragment of about 1.1 Kb, was subcloned, sequenced and used as a probe to screen a cDNA testicular library. A high number of positive clones was obtained. PCR and sequencing analysis of the 5' region of these clones showed that eight of them were full length and contained the ATG initiation codon. One of them, designated pSK-sbsFSHR, was chosen for *in vivo* excision and sequencing. The pSK-sbsFSHR phagemid contained a 3134 bp fragment (**Fig. 3.1**), consisting of an ORF of 2109 nucleotides that was flanked by leader and trailer sequences of 206 and 819 nucleotides respectively. This ORF encoded a protein of 702 amino acids of which the first 20 amino acids were predicted to constitute the putative signal peptide (**Fig. 3.1**). The mature protein displayed typical features of members of the glycoprotein hormone receptor family. The predicted extracellular domain contains 377 amino acids, including the signal peptide, followed by a putative

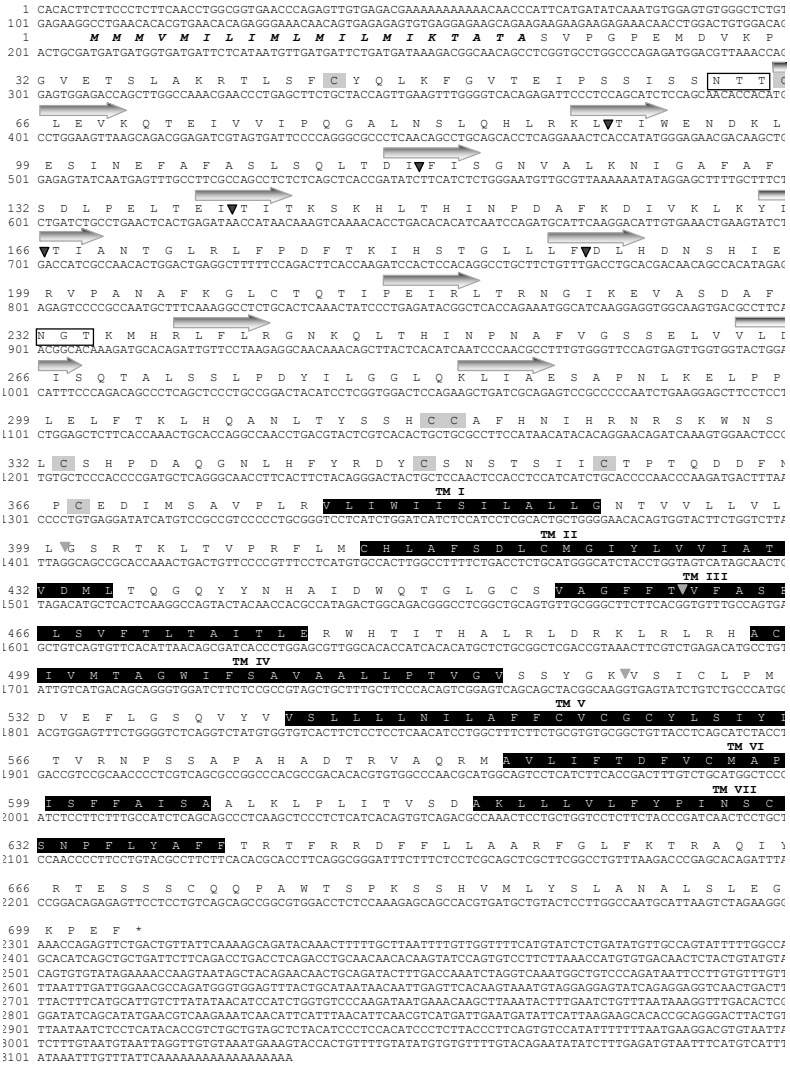

Figure 3.1 Nucleotide and deduced amino acid sequence of the *sbsFSHR* cDNA. Numbers on the left refer to position of the amino acid (top) and the nucleotide residues (bottom). Amino acid numbering begins with the proposed initial methionine. The predicted signal peptide is indicated in bold italics. 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 helixes is shown as black boxes. The positions of the amplified introns are indicated by black (extracellular domain) and grey (transmembrane domain) triangles. The nucleotide sequence has been submitted to the GenBank™ and is available under the accession number AY642113.

seven transmembrane 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 transmembrane domain and the intracellular loop between transmembrane helixe I and II, whereas the least conserved areas are the extracellular domain (**Fig. 3.2**) and the intracellular loop between transmembrane helixe V and helixe VI. The ClustalX alignment also revealed the presence of specific signature sequences (*e.g.* ^{316}CCAF, ^{364}FNPCED, ^{477}ERW, ^{590}FTD, ^{634}NPFLY), highly conserved in glycoprotein hormone receptors (Vassart et al., 2004) (**Fig. 3.2**).

The extracellular domain. 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 extracellular domain of the sbsFSHR (**Fig. 3.2**). 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), could be easily identified in the C-terminal region of the extracellular 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 ^{62}NTT, ^{232}NGT, ^{309}NLT, ^{325}NRS and ^{351}NST (**Fig. 3.1**). In other fish species, these potential sites are also present, but only the first two are conserved in the hFSHR (**Fig. 3.2**) (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. 3.1**). The areas flanking the LRR region of the sbsFSHR contain two and six cysteines, which could represent the N- and C-terminal cysteine-rich clusters respectively.

Transmembrane and intracellular C-terminal domains. The transmembrane domain of the sbsFSHR fulfils all the structural determinants described for the rhosopsin family of GPCR (Baldwin et al., 1997). It consists

of 263 amino acids, and includes seven stretches of 21-23 predominantly hydrophobic residues predicted to form α-helixes and connected by three intracellular and three extracellular loops (**Fig. 3.1**). The first and second extracellular loops of this domain contain two highly conserved cysteines (Cys^{453} and Cys^{528}), predicted to form an intramolecular disulfide bound,

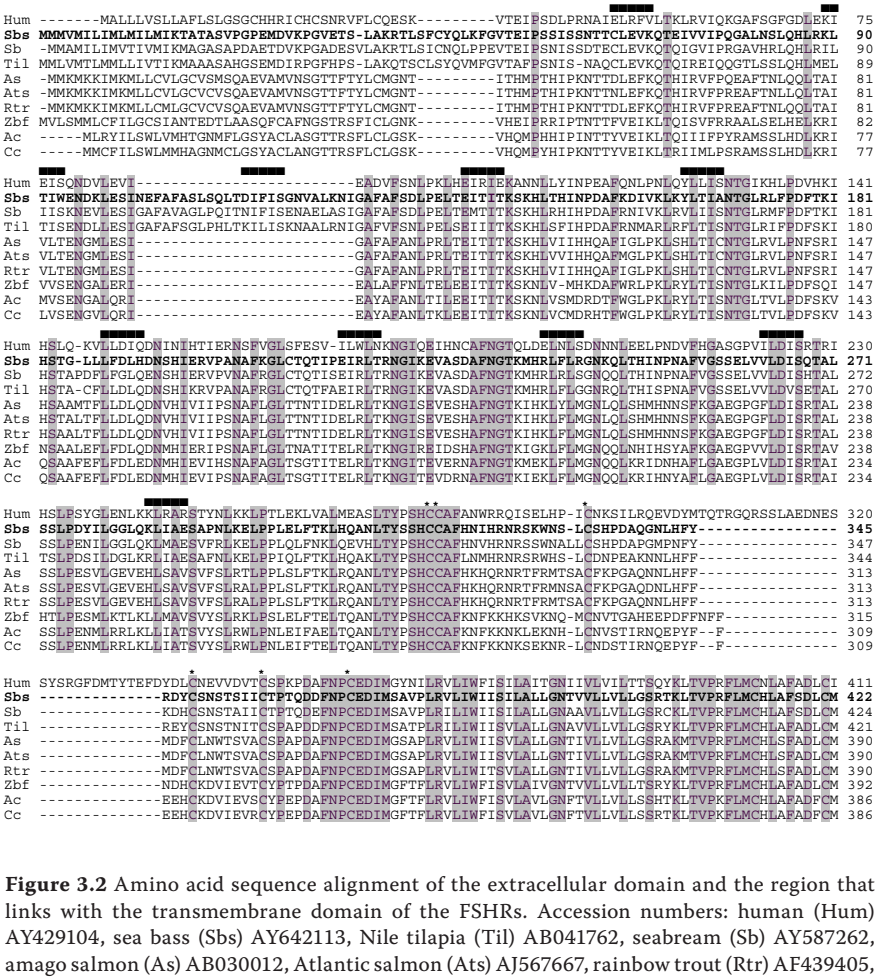

Figure 3.2 Amino acid sequence alignment of the extracellular domain and the region that links with the transmembrane domain of the FSHRs. Accession numbers: human (Hum) AY429104, sea bass (Sbs) AY642113, Nile tilapia (Til) AB041762, seabream (Sb) AY587262, amago salmon (As) AB030012, Atlantic salmon (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.

which constrains the protein (Gudermann et al., 1995). The conserved ERW motif can be found at the bottom of the predicted transmembrane helixe III, and contains Arg^{478} and Glu^{477}, that would form an ionic lock with Asp^{578} at the cytoplasmic end of the transmembrane helixe VI. Activation of the receptor involves the disruption of this ionic lock, which causes a crucial movement of these two α-helixes. The NPxxY motif is also present, and includes Asn^{633} one of the most conserved residues in rhodopsin-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 (Asp^{592} and Asp^{419}) (Vassart et al., 2004). Phosphorylation site predictions identified two potential phosphorylation sites, Thr^{566} and Ser^{571}, in the third intracellular loop and 5 potential phosphorylation sites, Thr^{643}, Ser^{669}, Ser^{671}, Ser^{679} and Ser^{683}, in the intracellular C-terminal domain. From them, Thr^{566} and Thr^{643} are potencial phosphorylation sites for protein kinase C, while Ser^{571} and Ser^{683} are potential sites for protein kinase A phosphorylation.

Phylogenetic analysis of the *sbsFSHR*

The phylogenetic relationship of the *sbsFSHR* with other members of the glycoprotein hormone receptors family was investigated by performing a phylogenetic analysis using the Neighbour-Joining method and complete amino acid sequences (**Fig. 3.21**). In the resulting tree topology, the bootstrap values obtained indicate the presence of three main clades, the *FSHR*, *LHR*, and *TSHR* lineages. The *sbsFSHR* was found to cluster with a high bootstrap value with the rest of *FSHRs* used in the analysis.

Partial analysis of the *sbsFSHR* gene

To further confirm that the isolated cDNA corresponds to the sbsFSHR gene we investigated whether or not it contains the LHR specific intron. Among mammalian glycoprotein hormone receptors genes, LHRs are unique in having an extra intron in the C- terminal region of the extracellular domain (LHR intron 10). Sea bass gDNA and sbsFSHR cDNA were used as templates for PCR amplification with primers fshr 23 and fshr 24 (**Table 2.1**). Both PCR amplicons presented the same size, demonstrating the absence of an intron in this position in the sbsFSHR gene (data not shown).

In addition, the eventual presence of introns within the *sbsFSHR* transmembrane domain coding region was also examined. Based on the posi-

tions of introns 12 and 14 in the *Drosophila melanogaster LGR-1* transmembrane domain (Hauser et al., 1997), two sets of primers were used; the first one (primers fshr 25 and fshr 26; **Table 2.1**) for PCR amplification between nucleotides 1339 and 1621 and the second set (primers fshr 9 and fshr 19; **Table 2.1**) between nucleotides 1598 and 1804. The fragments amplified from gDNA had a bigger size than those amplified using *sbsFSHR* cDNA as template (data not shown). Those fragments were subcloned and sequenced, showing that the transmembrane domain coding region of the *sbsFSHR* has three introns of 677, 83 and 756 bp, which positions are homologous to *Drosophila melanogaster LGR-1* introns 12, 13 and 14, respectively. The first is in "phase 1" while the second and third introns are in "phase 0". Their positions are indicated in (**Fig. 3.1**).

As mentioned before, an insertion coding for a putative extra LRR of 25 amino acids was found in the LRR region. In rat and human FSHRs, each LRR coding motif is interrupted by an intron, thus, to know the genomic structure of the *sbsFSHR* in this region a PCR was performed with primers fshr 26B and fshr 12 (**Table 2.1**), which amplify the region across putative exons 2 to 6. Amplification revealed the presence of introns in the homologous positions to both rat and human *FSHR* genes (Heckert et al., 1992; Gromoll et al., 1996). In addition, an extra 406 bp intron was found within the coding sequence of the 25-amino acid insertion described above, which

Table 3.1 Relatedness of sea bass and human FSHR exons

Sea Bass		Human[a]	
Exon number	Size (bp)[b]	Exon number	Size (bp)
1	*405*	1	251
2	*72*	2	72
3	**75**		
4	**75**	3	**75**
5	**75**	4	**75**
6	72	5	72
7	*81*	6	78
8	*69*	7	69
9	*75*	8	75
10	*186*	9	186
11	*221*	10	>1234
12	374		
13	1353		

[a] The human *FSHR* structure is taken from Gromoll *et al.* 1996.

[b] Numbers in italics correspond to *sbsFSHR* exon sizes deduced from the human *FSHR*, as the corresponding introns were not amplified in this work.

Numbers in bold highlight the existence of an extra 75bp exon in sea bass

corresponds to nucleotide position 478 in the *sbsFSHR* cDNA (**Fig. 3.1**). This new intron is flanked by 72 bp and 75 bp exons. When comparing the sea bass and human *FSHR* genes (**Table 3.1**), striking similarities are disclosed with respect to their exon size in the LRR area. Their sizes follow a similar pattern in both genes, which enabled us to identify the presence of an extra 75 bp exon in the *sbsFSHR*. The GT/AG consensus sequence for donor and acceptor splice sites is conserved in all amplified introns. Besides, all LRR coding exons belong to the 2,2 group (they are flanked by introns of "phase 2"). The amino acid present at nearly each exon/intron junction in the extracellular domain, is rather a leucine, isoleucine or phenylalanine (**Fig. 3.1**).

Expression analysis

The tissue expression pattern of the *sbsFSHR* was analyzed by RT-PCR (**Fig. 3.3, A**). *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. 3.3, A**). Consistent intensity of the *sbs18S rRNA* amplicon proved uniformity in the template cDNA among the reactions (**Fig. 3.3, A**).

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 alternative spliced *sbsFSHR* transcript (**Fig. 3.3, B**).

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 previtellogenetic oocytes (**Fig. 3.4, A**). Positive signals were also detected in the follicular cells of oocytes in early and late stages of the vitellogenesis process (**Fig. 3.4, B, 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).

Alternative splicing of the *sbsFSHR* mRNA

Several alternatively spliced *FSHR* mRNAs coding for different truncated variants of the FSHR have been described in the literature. Most of these transcripts are the result of exon removal, particularly exons coding for the

extracellular domain. Aiming to explore the possible existence of alternatively spliced transcripts of the *sbsFSHR*, we amplified by PCR the exons that code for the extracellular domain, using cDNA from mature fish testis as template. The fshr 24B and fshr 28 primers placed respectively in exon 1 and exon 11 were used. Apart from the expected 902 bp fragment, two additional smaller products were amplified. Sequence analysis confirmed that both were the result of alternative splicing events. Splicing variant 1, with an approximate size of 150 bp, consisted of exon 1 followed by exon 11 that codes for the C-terminal region of the extracellular domain and the transmembrane helix I, and would originate a protein isoform lacking the LRRs

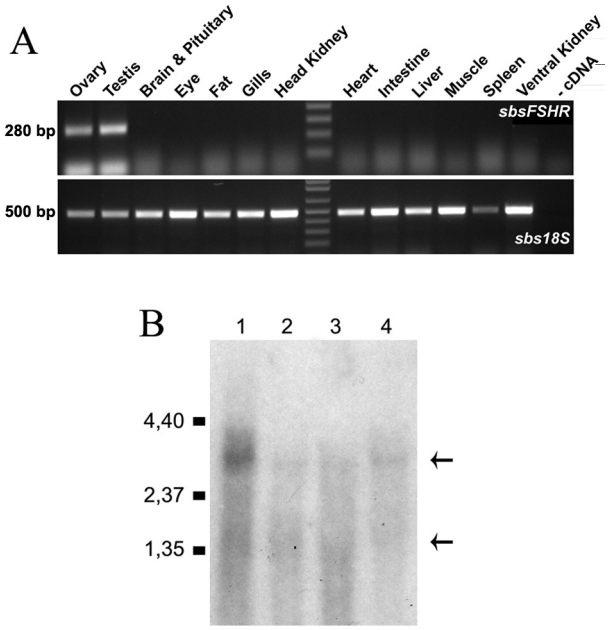

Figure 3.3 (**A**) Analysis of *sbsFSHR* expression in adult fish tissues by RT-PCR. Specific primers encoding a segment of the transmembrane domain were used for cDNA amplification. The presence of an intron in this area of the gene guarantees that amplification comes exclusively from the mRNA. The integrity of the RNAs was verified by uniform amplification of the *sbs18S rRNA* transcript. (**B**) *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. The numbers on the left correspond to the localization of size marker RNAs (in Kb). Two mRNAs of about 1.8 and 3 Kb were detected in both ovary and testis. Their position is indicated by arrows.

and the first two cysteines of the C-terminal cysteine cluster. The 480 bp splicing variant 2 contained the first three exons of the *sbsFSHR*, continuing along with exons 10 and 11 (**Fig. 3.5**). This splicing variant would give rise to an isoform including the N-terminal cysteine cluster and two LRRs.

Activation of the *sbsFSHR* by gonadotropins

To test the functionality of the isolated sbsFSHR we expressed its cDNA in HEK293 cells. It has been described (Tao et al., 2000) that the expression of a high number of FSHRs 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* cDNA. 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. The activation of the FSHR 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

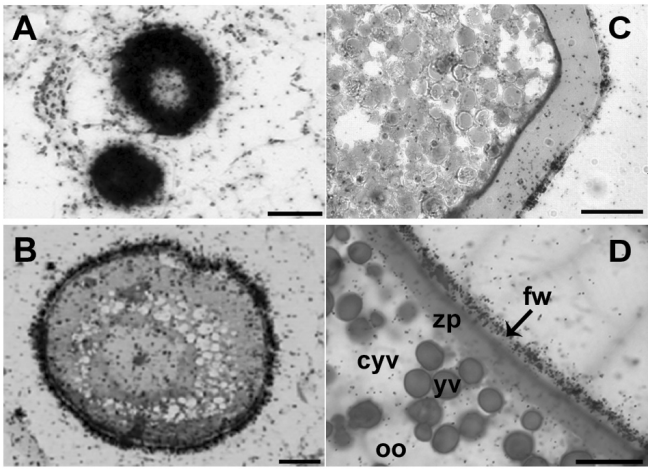

Figure 3.4 *In situ* hybridization of *sbsFSHR* antisense riboprobe in ovary sections of sexually mature females. Expression of *sbsFSHR* (dark grains) was observed on the follicular wall of oocytes in previtellogenesis (**A**), in cortical alveoli stage (**B**) and in later stages of vitellogenesis (**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**, ooplasma). Scale bar: 50 µm.

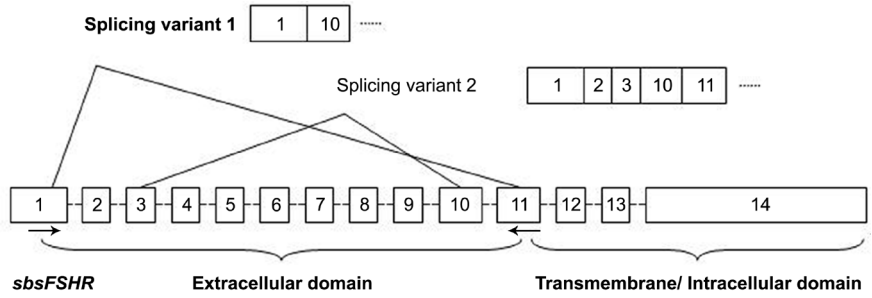

Figure 3.5 Schematic diagram of two alternative-splicing forms of the *sbsFSHR*. Exons are numbered 1 to 13. These two *sbsFSHR* transcripts were identified by RT-PCR using primers fshr 24B and fshr 28, here represented by arrows underneath the exons.

pCRE-luc plasmid constitutes an indirect measurement of the increase of intracellular cAMP.

The double transfectants were stimulated with different amounts (0-5 µg/ml) of bovine FSH (bFSH) and bovine LH (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 µg/ml) (**Fig. 3.6, B**). In a time course experiment we show that maximum luciferase activity is obtained 6 hours after bFSH stimulation and

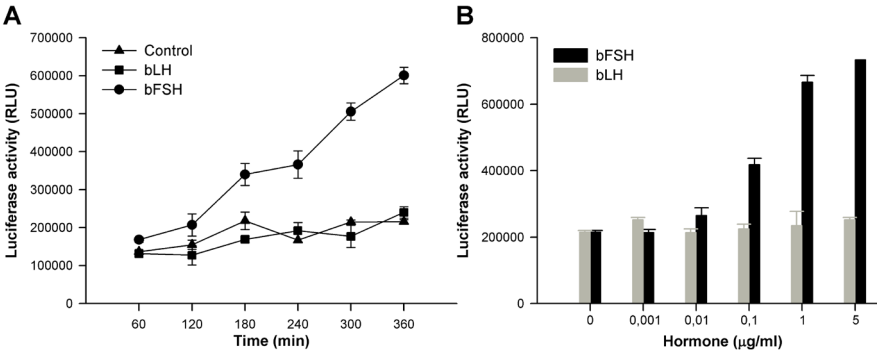

Figure 3.6 Functional analysis of sbsFSHR expressed in HEK293 cells. A stable clone containing the *sbsFSHR* was transiently transfected with the reporter plasmid pCRE-Luc. **A**) Temporal luciferase activity in response to 1 µg/ml of bFSH, bLH or untreated (Control). **B**) Luciferase activity after 6-h treatment with different dosis of bFSH and bLH.

no increase in luciferase activity is obtained in those cells treated with bLH (**Fig. 3.6, A**). Besides, when these cells were stimulated with conditioned medium from CHO-K1 stable clones producing recombinant sbsFSH, an increase in the intracellular cAMP was observed compared to the cells treated with CHO-K1 wild type medium. On the other hand, conditioned medium of CHO-K1 stable clones producing recombinant sbsLH was unable to stimulate the sbsFSHR (**Fig. 3.7**). All together these data show that the sbsFSHR is specifically stimulated by FSH but not by LH.

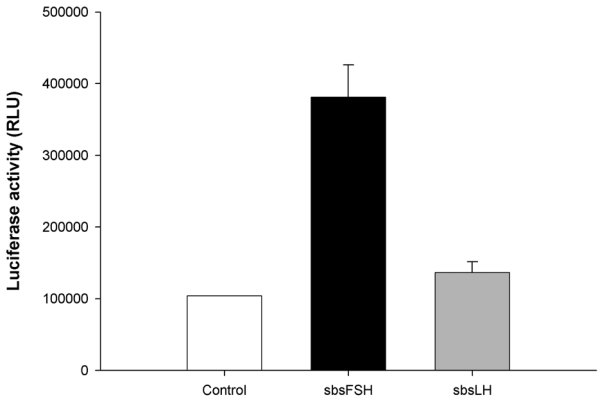

Figure 3.7 Functional analysis of sbsFSHR expressed in HEK293 cells. Luciferase activity after 5-h treatment with a single concentration of conditioned medium of cultured CHO-K1 wild type cells (Control) or stable clones producing recombinant sea bass LH and FSH.

Discussion

This section describes the isolation of a cDNA coding for a sea bass FSH receptor by screening a sea bass cDNA testicular library. Analysis of its deduced amino acid sequence revealed that this receptor is highly identical to other vertebrate FSHRs. Hallmarks of the glycoprotein hormone receptors subfamily were identified in the sbsFSHR: a rhodopsin-like seven transmembrane domain connected to a large extracellular domain that includes a LRR region flanked by N-terminal and C-terminal cysteine-rich clusters. Despite these overall structural similarities, important differences were observed. Unlike hFSHR, and many other LRR-containing proteins that have

four cysteines in their N-terminal cysteine-rich flanking region, the sbsFSHR has only two cysteines (Cys^{45} and Cys^{65}). Similar scenarios are found in FSHRs of other fish species (Oba et al., 1999; Bogerd et al., 2001; Kumar et al., 2001b; Laan et al., 2002). Moreover, the sbsFSHR, as well as Nile tilapia and seabream FSHRs, have an insertion of nine amino acids in the N-terminal cysteine-rich region, precisely where one of these cysteines is found (**Fig. 3.2**). Recently, the crystal structure of the human FSH (hFSH) in complex with a fragment of the hFSHR extracellular domain ($hFSHR_{1-250}$) has been reported (Fan and Hendrickson, 2005). In that paper, the existence of two disulphide bridges between the four cysteines of the hFSHR N-terminal cysteine-rich region is described. In addition, it is also suggested that the N-terminal cysteine cluster is required for proper folding. Considering the different spacing and number of cysteines between human and sea bass FSHRs, the only covalent link made by the two cysteines in the N-terminal cysteine-rich region of the sbsFSHR, would fold polypeptide chains in a different way that the one described for the human receptor.

The N-terminal cysteine-rich region is followed by the LRRs. A LRR domain is constituted of β-strand/α-helix structural units connected by a turn. These units are arranged in a way that all the β-strands and the α-helixes are parallel to a common axis, resulting in a horseshoe-shaped molecule with curved parallel β-sheet lining the inner circumference of the horseshoe and the helixes flanking its outer circumference (Kobe and Kajava, 2001). LRRs have been found in the primary structures of a large number of proteins and are usually involved in protein-protein interactions (Kajava, 1998). The Pfam database identified nine LRRs in the sbsFSHR. Eight of them correspond with repeats of the hFSHR, and the additional one is encoded by the 25-amino acid insertion described previously (**Fig. 3.2**). The presence of this extra LRR in the sbsFSHR, could indicate that the binding domain in this receptor might have the same configuration, but distinct curvature and size, which could influence hormone recognition. Besides, sequence comparisons with the hFSHR point to the presence of a second LRR in the last exon of the extracellular domain of the sbsFSHR, summing a total of ten LRRs. Fan and Hendrickson (2005) have recently shown that the hFSHR has an additional imperfect LRR placed in the N-terminal cysteine-rich region (LRRNT/LRR1) that contributes to the ligand binding domain. Nevertheless, in the sbsFSHR a LRR in that area is difficult to predict due to the high sequence divergence with the hFSHR.

The publication of Fan and Hendrickson (2005) has also revealed how the hFSH binds into the inner face of the curved extracellular domain of the hFSHR making contacts with all 10 β-strands of the LRRs. The receptor β-strands interact with the C-terminal segments of both FSHα and β-subunits and with their *L*2 loops. Besides, residues with complementary charges contribute to direct interactions at the hormone/receptor interface. In the model proposed for hFSH/hFSHR interaction, almost all residues buried at the receptor/ligand interface by FSHβ alone, or by both FSHα and FSHβ, are not conserved in the sbsFSHR. We could only identify one region in the sbsFSHR, between amino acids 163 and 171, which has a high degree of conservation regarding residues that could participate in the receptor/ligand interface. This region corresponds to sbsFSHR LRR 5 and, following the model proposed for the hFSHR, the conserved amino acids in this repeat are mostly residues that make contact only with FSHα. The FSHα-subunit residues that interact with the receptor lie in the *L*2 loop and the C-terminus and the majority of them are conserved in sea bass FSHα (Mateos et al., 2003). One of the key features located in the hFSH/hFSHR interface is an aromatic ring interaction between Tyr^{88} of hFSHα and Tyr^{124} of hFSHR. These two residues are conserved in their sea bass counterparts, corresponding to Tyr^{90} and Tyr^{164} respectively. In those cases where residues from the human receptor or α-subunit are different in their sea bass equivalents, their substitutes would still allow the same kind of interaction, both polar and hydrophobic. This is the case of Leu^{48}, one of the hFSHα residues that interacts with the receptor in this region which is substituted by Thr^{51} in sea bass; this change would still allow hydrogen bonding with sbsFSHR Asn^{169} and hydrophobic contacts with Thr^{170} and Gly^{171}. Exceptions include the substitution of the Ser^{43} in the hFSHα by Ala^{46} in sea bass. This change, although conservative with respect to the amino acid size, would preclude the formation of hydrogen bonds. Nevertheless, an alanine in an equivalent position is also present in other fish species.

Although collective interfaces may contribute to the specificity of binding, three potential determinants of specificity have been identified. They are substantially buried sites of the hFSHR that vary among human glycoprotein hormone receptors and make contact with hFSH residues that are different among their counterpart hormones (Fan and Hendrickson, 2005). These are hFSHR residues Leu^{55}, Lys^{179} and the combination of Glu^{76} and Arg^{101}. In the hFSHR, Leu^{55} makes hydrophobic contacts with Arg^{42} in the

α-subunit, and Leu^{99} and Tyr^{103} in the FSHβ-subunit. In the sbsFSHR, Gln^{70} occupies the position corresponding to hFSHR Leu^{55}, which would also allow hydrophobic interactions with Lys^{45} in the sea bass α-subunit and Gly^{94} and Ser^{98} in the FSHβ-subunit (Mateos et al., 2003). Another specificity pocket described by Fan and Hendrickson contains the side chain of Lys^{179} in the hFSHR which makes hydrogen bonds with Ser^{89} and Asp^{90} in the FSHβ-subunit. On the molecular surface of sea bass FSHβ-subunit, the position of the human Ser^{89} is occupied by Thr^{84}; both are residues with aliphatic side chains containing a hydroxyl group, and human Asp^{90} is substituted by Glu^{85} both negatively charged. Thus, these sea bass FSH residues could host the side chain of sbsFSHR Arg^{220}, a polar hydrophilic positively charged residue found in the same position that Lys^{179} in the hFSHR. The sides of this channel are formed by the basic residues Lys^{54} and Lys^{93} of sea bass FSHα (Lys^{51} and Lys^{91} in hFSHα, respectively), which would make salt bridges with two conserved acidic residues in the sbsFSHR (Asp^{190} and Asp^{193}). The third specificity determinant involves residues that are not conserved in the sea bass sequences, and is based on polar interactions between Arg^{97} and Val^{96} from the hFSHβ with the hFSHR residues Glu^{76} and Arg^{101} respectively. These two residues are structurally adjacent in the human receptor, however, in the sbsFSHR the 25-amino acid insertion coding for a putative extra LRR would originate an extra turn in this region, spatially separating the equivalent sea bass residues, and thus preventing the formation of equivalent bonds. Even with this modification in the structure of the LRR domain, the sbsFSHR still responds to a mammalian FSH, suggesting that this receptor/hormone contact is not essential in receptor activation. The fact that this extra turn does not affect FSH binding can be explained by the model proposed by Moyle et al. (2005). These authors have proposed that LH and FSH make contacts with opposite regions of the LRR domain of their respective receptors. Accordingly, FSH would make contacts with the C-terminal end of the LRR domain, what rules out a relevant role for Glu^{76} and Arg^{101} that are located in the N-terminal turns.

Downstream of the LRR region of the sbsFSHR, in the C-terminal cysteine-rich region, the highly conserved signature SHCCAF could be identified. This segment has an important role in receptor activation, as mutation of the serine present in this signature (Ser^{314} in sbsFSHR) leads to constitutive activation of all three mammalian glycoprotein hormone receptors (Nakabayashi et al., 2000). The sbsFSHR has six conserved cysteines

in this extracellular C-terminal region (Cys residues on positions 316, 317, 333, 349, 357 and 367). These cysteines are present in all the fish and mammalian FSHRs cloned until the moment. As this domain is not included in the crystal structure of the hFSH-$FSHR_{1-250}$ complex, direct evidence of specific contacts involving residues of this region is not available. Nevertheless, different studies have highlighted its importance in ligand binding and signalling (Kene et al., 2005; Moyle et al., 2005). In particular, major ligand binding sites have been found in hFSHR between the third and fourth cysteine of this domain. Interestingly this ligand binding sites do not exist in fish receptors, due to a 30 amino acid deletion (**Fig. 3.2**). Although a complete receptor structure is needed to fully understand the functional role of the extracellular region, the described deletion in the C-terminal cysteine-rich domain, together with the distinct features found in the N-terminal domain, could anticipate the existence of differences in hormone binding between fish and mammalian FSHRs. In the FSHβ-subunit, the seat-belt residues between Cys^{11-12} are extremely important for FSH binding and activity, both in fish (Vischer et al., 2004) and mammals (Moyle et al., 2005). However, in sea bass, as in other perciform and salmonid species (Mateos et al., 2003; Swanson et al., 2003), the spatial organization of these residues is different from the one that exists in mammals. In the hFSHβ, as well as in other tetrapods, Cys^{12} is bound to Cys^{3}, placing the C-terminal residues of the seat-belt (between Cys^{11-12}) near β1 and β3 loops. In some fish species the conserved Cys^{3} is missing. Instead, they contain a cysteine in the very N-terminus, which bonds with Cys^{12}, placing the tail of the seat-belt far from β1 and β3 loops. This results in a different spatial location of residues that have been shown to be important for ligand specificity and binding and should have implications in the ligand binding mode of fish FSHRs. Despite all the differences described both in the receptors and ligands, we, in this paper, and other authors (Bogerd et al., 2001; Kumar et al., 2001; Laan et al., 2002; Kwok et al., 2005) have shown that fish FSHRs can bind and be activated by mammalian FSH, what suggests that amino acids in this area can be important but not fundamental for ligand binding. Besides, this is consistent with the recently shown existence of multiple FSH selective determinants in the hFSHR (Vischer et al., 2006).

In contrast to mammalian FSHRs, where the transmembrane and intracellular domains are encoded by a single exon, partial analysis of the *sbsFSHR* gene enabled the identification of three introns in its transmembrane

domain coding region. A similar intron distribution exists in the fugu (*Takifugu rubripes*) FSHR (EnsEMBL release 42; FUGU4:scaffold_7634), while in the amago salmon and Nile tilapia *FSHR* (type I receptor) transmembrane coding regions only two introns were described (Oba et al., 1999b; Oba et al., 2001). In a similar way, the transmembrane regions of the glycoprotein hormone receptors evolutionary-related genes fly LGR-1 (Hauser et al., 1997) and nematode LGR (Kudo et al., 2000) are encoded by 6 and 4 exons respectively. It was generally accepted that the mammalian glycoprotein hormone receptors genes were originated from the recombination of an intron-less DNA sequence coding for the transmembrane domain of a GPCR and a DNA sequence containing several introns and exons, coding for the LRRs of the receptor extracellular domain (Heckert et al., 1992). However, the presence of introns in the transmembrane-coding regions of fish and invertebrate receptors suggests another scenarios, either a decrease in the number of introns, due to intron loss in the course of eukaryote evolution (Gilbert et al., 1986; Eriksen et al., 2000; Kudo et al., 2000; Nishi et al., 2000; Oba et al., 2001), or the generation of intronless transmembrane domains by reverse transcription and integration of the mRNA (Brosius, 1999; Roy and Gilbert, 2005).

In the portion of the *sbsFSHR* gene corresponding to the extracellular domain, we found introns in the homologous positions of introns 2, 3, 4 and 5 of both rat and human *FSHR* genes (Heckert et al., 1992; Gromoll et al., 1996). In addition, an extra intron was found between putative introns 2 and 3. This new intron splits in two the sequence coding for the 25-amino acid insertion described before, originating a new 75 bp exon (**Table 3.1**). There is a remarkable relationship between the intron-exon structure and the LRR motif in sea bass and other FSHRs (reviewed in Simoni et al., 1997). Every internal exon is interrupted by an intron in a homologous position of the highly conserved LRR β-strand pattern X-L-X-L-X. Besides, all introns are in phase 2, as also seen for human and rat *FSHR*s. Hence, at the amino acid level, the insertion or deletion of an exon would not disrupt the LRRs encoded by neighbouring exons. Recently, a mechanism of evolution, through rapid duplication of the LRR modules and their subsequent divergence, was proposed for the ribonuclease inhibitor (RI) (Haigis et al., 2002), the first LRR-containing protein to be crystallized. An accumulation of functional units as the driving force for exon duplication was also proposed. The mechanism of evolution proposed for the RI could also be applied to the evolu-

tion of other LRR-containing proteins, as it is the case of the sbsFSHR. The phasing and position of the introns in the *sbsFSHR* gene are consistent with exon amplification during evolution (Fedorov et al., 1998), associated with the duplication of a functional domain. Based on this mechanism of evolution, the presence of an extra 75 bp exon in the LRR coding region of the *sbsFSHR*, could be the result of an internal exon duplication. Moreover, this extra exon does not exist in *FSHR*s from salmonids, zebrafish and catfish, and so its duplication would have occurred after the diversification of the Euteleostei, but before the divergence of the Perciformes and Tetraodontiformes lineages, as it also exists in the fugu and medaka (*Oryzias latipes*) *FSHRs* (EnsEMBL release 42; FUGU4: scaffold_7634 and medaka chromosome 19 11454591:11468192) (**Fig. 3.8**).

sbsFSHR mRNA was only detected in sea bass testis and ovary. None of the somatic tissues analyzed showed expression of the receptor, what makes sbsFSHR gonad specific. This is consistent with the *FSHR* expression patterns found in other fish species (Oba et al., 1999b; Bogerd et al., 2001), however low levels of extragonadal expression have been found in kidney in zebrafish and spleen in channel catfish (Kumar et al., 2001b; Kwok et al., 2005). To investigate the size of the *sbsFSHR* mRNA, a *Northern* hybridization analysis was performed. Consistent with the full length cDNA sequence, a transcript of approximately 3 kb was detected. An additional signal of 1.8 kb was also observed. The mechanisms underlying the generation of different *FSHR* transcripts might be related either to the use of different transcriptional start sites or polyadenylation sites, or to alternative splicing processes. In this case and based on the size of the signal, the most probable mechanism responsible for the generation of the 1.8 kb transcript should be a splicing mechanism.

The description of alternately spliced *FSHR* mRNA transcripts, coding for different truncated variants of the FSHR in a wide range of species, led us to investigate the occurrence of splicing events in the *sbsFSHR* gene by RT-PCR. Since introns are mostly in the region coding for the extracellular domain, most of the described splicing isoforms correspond to transcripts with modifications in this region. We cloned two alternative spliced forms of the primary transcript of the *sbsFSHR*. While splicing variant 1 would originate a protein without LRR motifs, splicing variant 2 would encode a protein containing the N-terminal cysteine cluster and two LRRs. It is noteworthy saying that the primers we used may not detect all the differently spliced transcripts, such as a transcript lacking exons coding for the

transmembrane and intracellular domains, similar to that isolated from the ovine testis (Yarney et al., 1997).

Both *sbsFSHR* splicing variants were originated by exon skipping, like the alternative splicing variants already described for the *hFSHR* (Kraaij et

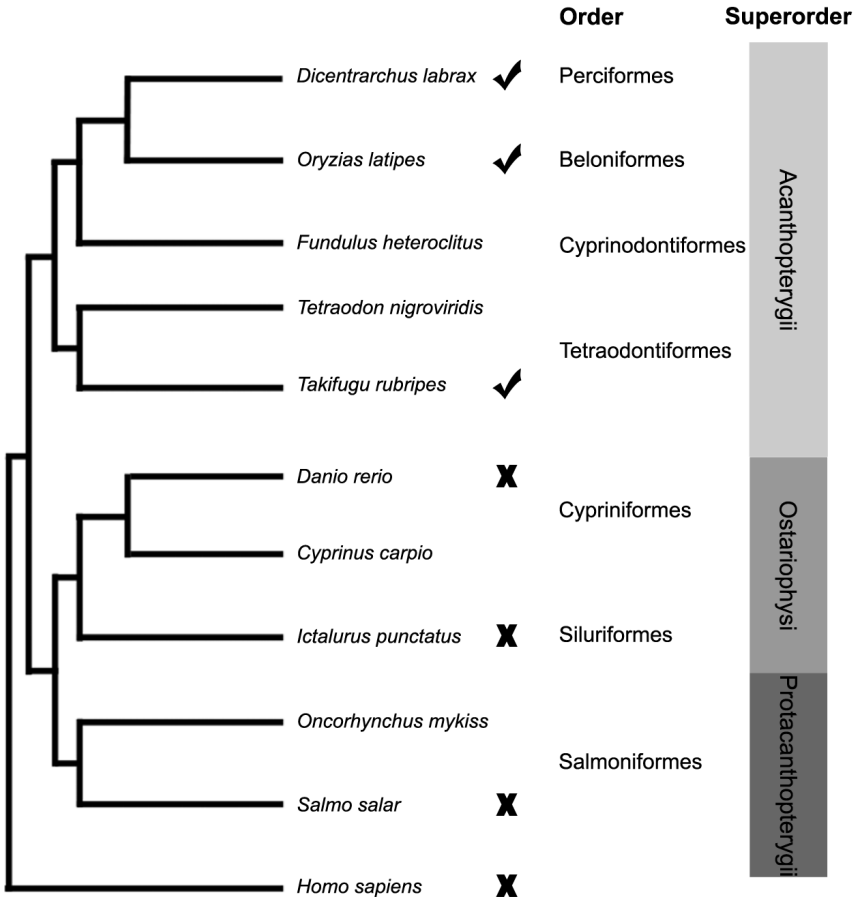

Figure 3.8 Relationships between ten Euteleostei fish species, including the European sea bass (modified from Steinke et al. 2006). Checkmarked fish have an exon at the homologous position of the additional exon found in the extracellular domain of the sbsFSHR. These fish are grouped in the Euteleostei superorder of the Acanthopterygii. The fish with a cross do not have this exon and they are grouped in the other two superorder of the Euteleostei: the Ostariophysi and the Protacanthopterygii. If the presence of this additional exon is a result of internal exon duplication as a mechanism of evolution, this event could have occurred before the divergence of the Perciformes, Beloniformes and Tetraodontiformes since the analyzed fish from these three orders have this extra exon.

al., 1998; Song et al., 2002). As all introns are in the same phase, the reading frame is not disrupted and thus would not generate truncated receptor forms. This leads to potentially functional sbsFSHR isoforms. It has been speculated that such isoforms, if present at the cell surface, could modulate FSH action by altering hormone binding affinity or by subtracting the hormone from binding to the full length receptor. The sbsFSHR variants reported in this work would probably be unable to bind FSH as they lack exons coding for the binding region. From studies performed on FSHR variants, it appears that two criteria are necessary for the existence of the FSHR on the cell surface: *N*-glycosylation of Asn^{174} for proper folding and the presence of a putative transmembrane segment at the carboxyl terminal (revised in Peterson et al., 2000). The *sbsFSHR* variants amplified in this study only fulfil the second criteria. Although the mentioned site for *N*-glycosylation is conserved in the sbsFSHR (^{232}NGT), the exon 8 where it is placed, has been excluded from both *sbsFSHR* variants. This could indicate that these two sbsFSHR variants would not be found on the cell surface. Misfolded GPCRs that fail to reach the cell surface are thought to be trapped within the cell and it has been hypothesize that they could function in a dominant negative manner and hinder cell surface expression of the corresponding wild type receptors, most likely through association in the endoplasmic reticulum, as it occurs with the common rat and human LHR extracellular splice variants (Apaja et al 2006; Minegishi et al., 2007). However, the expression level of these *sbsFSHR* splicing variants is low, since they were not detected in the *FSHR Northern blot* analysis (**Fig. 3.3**) and the amplification signal obtained by PCR was lower than that of the full length product (data not shown). In any case, the detection at the protein level of these receptor forms, once antibodies are available, could help to elucidate whether these *sbsFSHR* splicing variants have a functional role.

In situ hybridization pointed to a strong expression of *sbsFSHR* in previtelogenic oocytes and in follicular cells in early stages of vitellogenesis. In the Nile tilapia ovary, strong signals for *FSHR* (type I receptor) were observed in granulosa cells of vitellogenic follicles (Oba et al., 2001). On the other hand, expression analysis by RT-PCR in isolated zebrafish follicles showed a steady increase of *FSHR* expression throughout vitellogenesis (Kwok et al., 2005). The physiological role of FSH in the different fish species is not well defined. In salmonids, the FSH profile in plasma suggests its implication in early stages of gonadal development (Prat et al., 1996), which is in agreement with the

described function of FSH in mammals. 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 *FSHβ* expression are observed at the final stages of gamete maturation, what does not support a clear role of FSH in early stages of gonadal development. However, the follicle-stage dependent expression obtained for the *sbsFSHR* strongly suggests a role for this receptor in oocyte growth, in accordance with what has been described for salmonids and mammals. This mismatch between the expression of *FSHβ* and *sbsFSHR* could be explained by the spawning strategy of sea bass. The sea bass is a group-synchronous spawner (*i.e.* it spawns three to four discrete clutches in quick succession) and the recruitment of the successive clutches occurs from oocytes at various stages of the secondary growth phase (Mayer et al., 1990; Alvariño et al., 1992). 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.

Our results demonstrate that the sbsFSHR shares similar signalling properties with its fish an mammalian counterparts, by acting via a cAMP-mediated signal transduction pathway (Oba et al., 1999b; Bogerd et al., 2001; Kumar et al., 2001b; Laan et al., 2002; Dias et al., 2002). Though promiscuous hormone binding has been reported in fish FSHRs (Vischer et al., 2003b; Kumar et al., 2001b; Kwok et al., 2005), challenging sbsFSHR-stably-expressing HEK293 cells with bFSH and bLH, revealed that the sbsFSHR displays ligand selectivity as it is only activated by bFSH, even in the presence of pharmacologic doses of bLH. Nevertheless, definitive conclusions, regarding hormone-binding specificity of the sbsFSHR, require the availability of native or recombinant sea bass FSH and LH, as heterologous gonadotropins can exert unpredictable receptor activation (Licht et al., 1979; Kwok et al., 2005). However, the same results were obtained when recombinant sea bass FSH and LH were used. Ligand-induced cAMP production was only achieved when using recombinant sea bass FSH.

In conclusion, we have isolated and characterized a sea bass FSHR. The discovery of an extra LRR in the extracellular domain of this receptor advances a curvature and size of the binding domain distinct from the hFSHR, which could influence hormone recognition. *sbsFSHR* is exclusively expressed in gonadal tissues. The expression in ovarian follicles is consistent with a role in controlling the earlier stages of folliclegenesis. Only bFSH could activate the sbsFSHR, foreseeing ligand selectivity for this percirform

fish FSHR. Analysis of sbsFSHR spatio/temporal expression and its correlation with the sea bass reproductive stage, together with the study of the signalling pathways activated by the sbsFSHR could help us to understand the molecular mechanisms that implicate FSH in the regulation of sea bass gonadal function.

3.2 Molecular characterization of a European sea bass luteinizing hormone receptor gene: cDNA cloning, gene structure, expression analysis and functional activity.

The luteinizing hormone (LH) gonadotropin is a member of the glycoprotein hormone family and has a central role in reproductive physiology (Pierce and Parsons, 1981). In females, LH promotes follicular maturation, ovulation and the synthesis of ovarian steroid hormones. In males, LH supports Leydig cell functions and stimulates the synthesis of androgens, regulating the final stages of spermatogenesis. 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 chorionic gonadotropin (CG), a placental hormone essential for the maintenance of pregnancy, structurally similar to LH (Bousfield et al., 1994). When activated by these hormones, the LHR couples to a number of G proteins and it stimulates the cAMP and inositol phosphate signalling cascades (reviewed in Ascoli et al., 2002). The cAMP pathway appears to be the principal mediator of ovulation and steroid biosynthesis, while the biological consequences of the stimulation of the inositol phosphate cascade are not yet clearly understood (reviewed in ref. Themmen and Huhtaniemi, 2000; Ascoli et al., 2002; Vassart et al., 2004).

The LHR, the follicle-stimulating hormone and the thyroid-stimulating hormone receptors (FSHR and TSHR, respectively) are members of a growing subfamily of G-protein coupled receptors (GPCRs) known as the leucine-rich-repeat containing GPCR subfamily (LGR) (Hsu et al., 2002). They are characterized by the presence of a relatively large extracellular domain containing several leucine rich repeats (LRRs) that are flanked by cysteine-rich clusters. This region is followed by a typical rhodopsin-like seven transmembrane domain, connected to an intracellular domain that interacts with cytoplasmic proteins. Among the different members of the LGR family, the highly similar LHR, FSHR and TSHR constitute the subfamily of glycoprotein hormone receptors (glycoprotein hormone receptors) (Hsu et al., 2000).

The genomic organization of the human (*Homo sapiens*) (Atger et al., 1995) and rat (*Rattus norvegicus*) *LHR* genes (Tsai-Morris et al., 1991, Koo et al., 1991) is very similar. These TATA-less genes are about 80 Kb in size

and each consists of eleven exons and ten introns. Exons 1 to 10 code for the N-terminal cysteine-rich cluster, all the LRRs and part of the cysteine-rich cluster present at the C-terminus of the extracellular domain. The rest of the extracellular domain, the entire heptahelical transmembrane domain and the short intracellular tail of the *LHR*, are encoded by exon 11. Their transcription can be initiated from multiple sites present 50 to 450 nucleotides upstream from the translation start site (Dufau, 1998; Tsai-Morris et al., 1991; Koo et al., 1991). As a result, and also due to differences in polyadenylation and alternative splicing, multiple *LHR* transcripts are processed (reviewed in Ascoli et al., 2002). A number of recent studies performed in mammals have reported the presence of *LHR* transcripts in a variety of extragonadal tissues, in addition to gonads (Rao, 2001), suggesting that its ligand could be pleiotropic rather than a gonadal-specific hormone. Neither the functional role, nor the regulation of extragonadal *LHR* expression are yet completely understood (Apaja et al., 2005; Pakarainen et al., 2005).

As in higher vertebrates, teleosts have two types of gonadotropins, FSH and LH, which coordinately regulate ovarian and testicular physiology. In the mid-nineties, binding studies of coho salmon (*Oncorhynchus kistuch*) FSH and LH to membranes of isolated granulosa cells and theca-interstitial layers of salmon ovary, revealed for the first time, information regarding the existence of gonadotropin receptors in fish (Yan et al., 1992, Miwa et al., 1994). A few years later, the cloning of two types of gonadotropin receptors from the ovaries of amago salmon (*Oncorhynchus rhodurus*) was reported (Oba et al., 1999a,b), thereby providing new experimental tools to understand fish glycoprotein hormone receptors biology.

Ligand specificity of the mammalian glycoprotein hormone receptors is well defined, with no cross-stimulation occurring under physiological conditions. Conversely, promiscuous activation of fish glycoprotein hormone receptors has been described. *In vitro* bioassays of African catfish (*Clarias gariepinus*), channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) recombinant receptors, suggests that FSHRs are not selective for FSH, although FSH is slightly more potent than LH as a ligand; whereas, LHRs are highly selective for LH (Bogerd et al., 2001; Vischer and Bogerd, 2003a; Vischer et al, 2003b; Kumar et al., 2001a,b; Kwok et al., 2005). Similar results were previously observed in coho salmon, as the type I receptor, GTHRI (*i.e.*, the putative FSHR), present in both the theca and granulosa cells, could not discriminate between FSH and LH, while the type II receptor, GTHRII

(*i.e.*, the putative LHR), located only in granulosa cells, was highly selective for LH (Yan et al., 1992, Miwa et al., 1994). Nevertheless, these data are in contrast with the ones obtained in other fish species. Challenging the amago salmon FSHR with chum salmon FSH or LH, resulted in a specific activation, as no effects were observed when using chum salmon LH. However, LHR transfected cells were highly responsive to LH and, in a lower magnitude, also to FSH (Oba et al., 1999a,b).

In the previous section 3.1, the isolation of a cDNA coding for a sea bass *FSHR* (*sbsFSHR*) is described. Its functional properties were examined by developing heterologous clones stably expressing this gene. In this cell line, intracellular cAMP levels were significantly increased in response to bovine FSH, but not bovine LH. Understanding the mechanisms involved in ligand specificity, binding affinity and pathways activated by LH binding to its receptor is of particular importance, considering the functional implications of this hormone in reproduction. The discrepancy in ligand specificity among glycoprotein hormone receptors in teleost species contrasts with what is known in mammals, and reinforces the need of learning more from fish, the most diverse group of vertebrates with dissimilar modes of gonadal development and reproductive strategies.

In this section, we describe the cloning of a *sea bass LHR* (*sbsLHR*) cDNA and the functional characterization of its coding protein. The genomic organization of the *sbsLHR* gene and the tissue distribution of its mRNA were also examined. Lastly, we describe the isolation of three variants with different 3' untranslated regions.

Cloning and sequence analysis of a *sbsLHR* cDNA

Sea bass gDNA was used as template in a PCR reaction with two degenerate oligonucleotides (fshr 1 and fshr 2). They were designed based on a highly conserved and intronless region of the transmembrane domain in fish *LHR* sequences. A 680 bp product was generated, and subsequently subcloned and sequenced. The obtained sequence showed a high identity to other *LHRs*. Screening of a cDNA testicular library using this fragment as a probe rendered a single specific clone containing 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 the information obtained from the genomic sequence of the *sbsLHR* (see

below), a specific primer (lhr 30) 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. 3.9**.

Typical attributes of members of the glycoprotein hormone receptor subfamily could be identified in the mature protein. The large extracellular amino-terminal domain consists of 388 amino acids, including the signal peptide. Searches in the Pfam database and alignments with other glycoprotein hormone receptors 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 and six in a C-terminal group. The predicted transmembrane domain, of 261 amino acids, includes 7 stretches of hydrophobic residues, typical of all the members of the rhodopsin-like GPCR family. The intracellular C-terminal domain consists of 72 amino acids and has two highly conserved contiguous cysteines (Cys^{668} and Cys^{669}). Predictions for *N*-glycosylation sites identified three potential motifs in the sbsLHR extracellular domain at positions ^{42}NVT, ^{103}NLS, and ^{199}NGT (**Fig. 3.9**). NetPhosK 1.0 prediction server found one site, Thr^{590}, in the third intracellular loop of the transmembrane, and six sites, Thr^{652}, Ser^{671}, Ser^{674}, Ser^{683}, Ser^{693} and Ser^{711} 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 extracellular domains are 28% identical, while their transmembrane 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, made clear that the most divergent areas are the C-terminus of the extracellular domain and the intracellular domain (data not shown). The ClustalX alignment also revealed the presence of specific

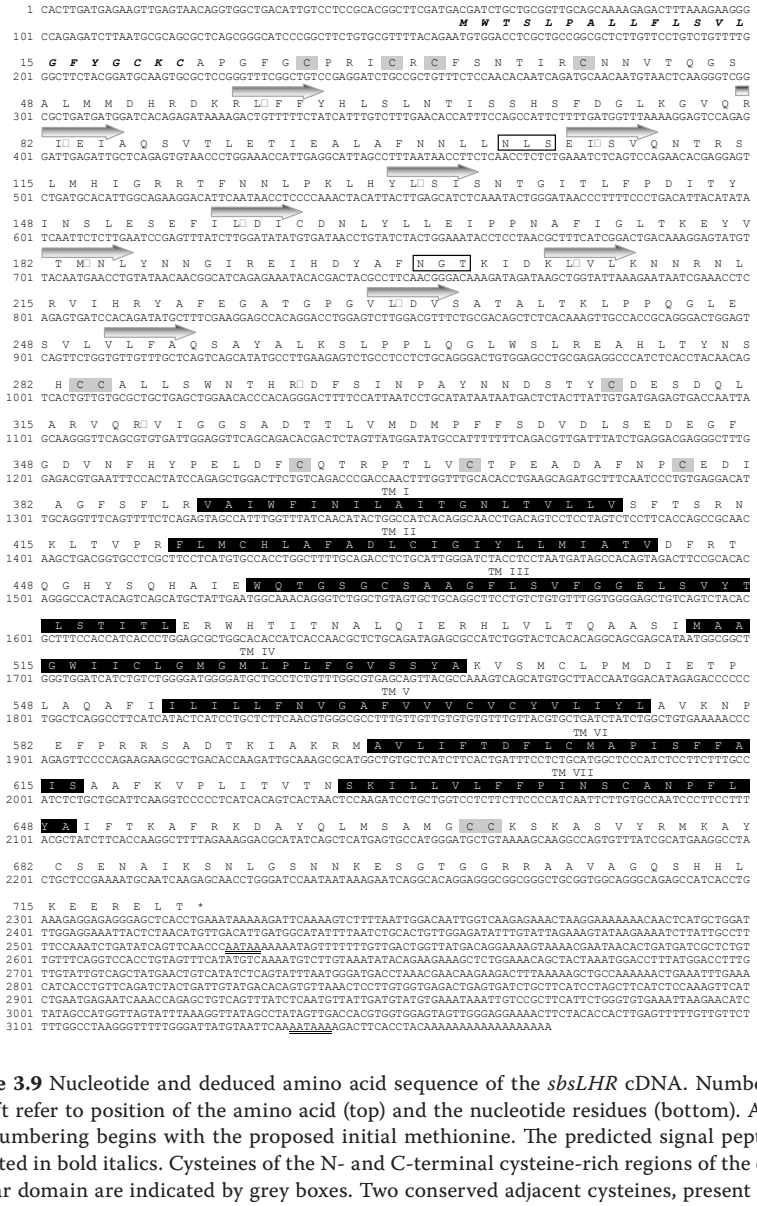

Figure 3.9 Nucleotide and deduced amino acid sequence of the *sbsLHR* cDNA. Numbers on the left refer to position of the amino acid (top) and the nucleotide residues (bottom). Amino acid numbering begins with the proposed initial methionine. The predicted signal peptide is indicated in bold italics. 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 positions of the introns are indicated by black triangles. Consensus sites for polyadenylation are double underlined. The nucleotide sequence has been submitted to the GenBank™ and is available under the accession number AY642114.

signature sequences (*e.g.* ^{375}FNPCEDIMSA, ^{488}ERW, ^{601}FTD, ^{644}NPFLY) highly conserved among glycoprotein hormone receptors (Vassart et al., 2004).

Phylogenetic analysis of the *sbsLHR*

The evolutionary relationship of the *sbsLHR* to other members of the glycoprotein hormone receptors family was inferred by performing a phylogenetic analysis by the Neighbour-Joining method. The topology of the resulting rooted tree (**Fig. 3.21**) shows three main groups: *LHR*, *FSHR* and *TSHR* lineages. As awaited from their close phylogenetic relationships, the *sbsLHR* was found to cluster with the sea bream *LHR*, within the *LHR* group.

Genomic structure of the *sbsLHR* gene

The genomic organization of the *sbsLHR* gene was determined by the combined results of gDNA PCR amplification and GenomeWalker-PCR. PCR amplification on sea bass gDNA with primers lhr 6 and lhr 12 (annealing, respectively, to putative exons 9 and 11, according to mammalian *LHR* genes) yielded a ~ 990 bp product. Sequence analysis of this PCR product showed that in this region the *sbsLHR* contains two introns, corresponding to putative intron 9 and the *LHR* specific intron 10. The sequence data gathered from sea bass gDNA PCR amplifications, using the primers sets fshr 1-fshr 2 and lhr 6-lhr 12, indicate that the *sbsLHR* has no introns in the region coding for the transmembrane domain.

In order to obtain further 5' upstream sequence of the *sbsLHR* gene, a specific antisense primer (lhr 21), corresponding to the C-terminal end of the extracellular domain, was used in a PCR reaction on sea bass gDNA, in combination with a degenerated sense primer (lhr 19) based on a highly conserved region in *LHR* extracellular domains. The amplified fragment comprises *sbsLHR* from putative exons 7 to 9. Three rounds of PCR genome walking were required to obtain the complete sequence of the *sbsLHR* gene. It contains eleven exons and ten introns and spans more than 7.1 Kb of the sea bass genome (**Fig. 3.90** and **Fig. 3.10**). In addition, sequence analysis demonstrated GT-AG consensus motifs at each intron/exon splice junction (**Table 3.2**). Introns interrupt codons between the second and third nucleotide meaning that they are in "phase 2". The extracellular domain is encoded by exons 1 to 10 and by the first 216 nucleotides of exon 11. The remaining sequence of exon 11, codes for the transmembrane and intracellular domains (**Fig. 3.10**). The amino acid present at nearly each intron/exon junction in the extracel-

lular domain is leucine, isoleucine or phenylalanine (**Fig. 3.9**).

Expression analysis

The expression of the *sbsLHR* was analyzed by RT-PCR in different tissues of adult animals. The *sbsLHR* showed the highest expression in gonadal tissues. 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. 3.11**, A, upper panel). Sequencing results from two randomly selected RT-PCR products (liver and gills) confirmed the authenticity of the extragonadal amplicons. The amount of RNA used in each reaction was verified by uniform amplification of the *sbs18S rRNA* transcript (**Fig. 3.11**, A, lower panel).

3' RACE led to the amplification of two additional *sbsLHR* transcripts which differ in the length of their 3'UTR. The smallest one contains a 274 bp 3'UTR while the other transcript has a 355 bp 3'UTR. These 3' UTRs are smaller than the corresponding region in the cDNA isolated from the library, whose size is 850 bp. These three different mRNA transcripts result from the use of alternative polyadenylation signals, present in the pre-RNA molecule of the *sbsLHR*. The consensus polyadenylation sites for the transcripts with the larger (850 bp) and smaller (274 bp) 3'UTRs are indicated in

Table 3.2 *sbsLHR* intron-exon boundaries

	5' donor	Intron size (bp)	3' acceptor	
exon 1 (>331 bp)	**AA/AGA/CT**gtaagt	…~2000…	tttcag**G/TTT/TTC**	exon 2
exon 2 (72 bp)	**AG/AGG/AT**gtaggt	…359…	acacag**T/GAG/ATT**	exon 3
exon 3 (75 bp)	**CT/GAA/AT**gtaagt	…96…	ttgcag**C/TCA/GTC**	exon 4
exon 4 (75 bp)	**AT/TAC/TT**gtaaga	…305…	taacag**G/AGC/ATC**	exon 5
exon 5 (75 bp)	**TT/ATC/TT**gtaagt	…93…	ttacag**G/GAT/ATA**	exon 6
exon 6 (78 bp)	**TT/ACA/AT**gtaagt	…107 …	ttgcag**G/AAC/CTG**	exon 7
exon 7 (69 bp)	**AT/AAG/CT**gtattt	…104…	tttcag**G/GTA/TTA**	exon 8
exon 8 (75 bp)	**GA/GTC/TT**gtaagt	…422…	tcccag**G/GAC/GTT**	exon 9
exon 9 (186 bp)	**CC/CAC/AG**gtgact	…162…	ctgcag**G/GAC/TTT**	exon 10
exon 10 (69 bp)	**TA/GCA/AG**gtaaaa	…284…	aaacag**G/GTT/CAG**	exon 11 (>2065 bp)

All exon sequences are represented by boldface uppercase letters, and intron sequences are represented by lower case letters.

Fig. 3.9. Regarding the transcript with a 355 bp 3'UTR, there is not a clear polyadenylation consensus sequence located upstream of its poly $(A)^+$ tail. Although the intensity of the bands corresponding to the different 3' UTR transcripts was similar, further studies are needed to elucidate their relative level of expression, and also whether this is dependent on the reproductive stage or sex.

In a *Northern blot* analysis of poly $(A)^+$ RNA from sea bass ovary, a faint and diffuse hybridization signal, of approximately 3 kb, was detected, which could contain *sbsLHR* transcripts slightly different in size, like the polyadenylated variants detected by 3'RACE (**Fig. 3.11**, B). No signal could be detected in *Northern blot* when total RNA was used (data not shown).

Activation of the sbsLHR by gonadotropins

To assess the functionality of the sbsLHR, we developed stable transfected HEK293 cells expressing the *sbsLHR* and the luciferase based reporter con-

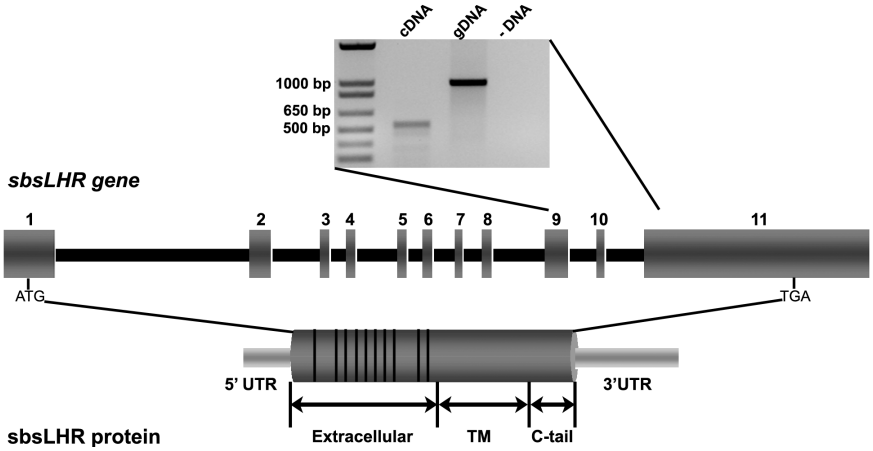

Figure 3.10 Schematic diagram of the *sbsLHR* gene structure. Grey boxes correspond to exons and they are numbered from 1 to 11. Black lines represent introns. Size proportions between exons and introns are maintained. Translational start and stop sites are indicated by the ATG and TGA codons. Among mammalian glycoprotein hormone receptor genes, the *LHRs* are unique in having an extra intron on the C-terminal region of the extracellular domain (intron 10). A PCR analysis of sea bass gDNA and *sbsLHR* cDNA, using primers lhr 6 and lhr 12 is shown. It yielded two products with different sizes, demonstrating the presence of the specific intron 10 in the *sbsLHR* gene. The extracellular domain is encoded by exons 1 to 10 and by the N-terminal region of exon 11 whereas the transmembrane and intracellular domains are encoded by the remaining sequence of exon 11.

struct pCRE-luc (LHR-LUC10 cells). The activation of other LHRs results in the activation of the cAMP route (reviewed in Means et al., 1980), finally leading 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

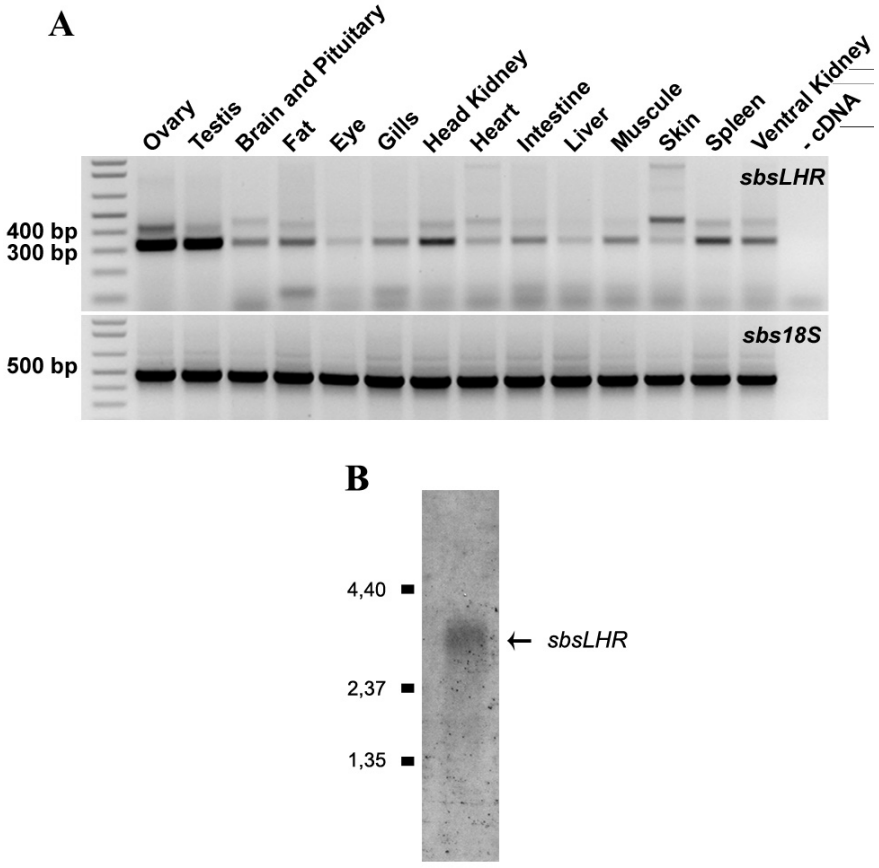

Figure 3.11 Analysis of *sbsLHR* expression in adult fish tissues by RT-PCR. Specific primers encoding a segment of the extracellular domain were used for cDNA amplification. The presence of four introns in this area of the gene guarantees that amplification comes exclusively from the mRNA. The integrity of the RNAs was verified by uniform amplification of the *sbs18S rRNA* transcript. **B**) *Northern blot* analysis. Poly $(A)^+$ RNA from the ovary of 2 adult animals was probed with the complete *sbsLHR* cDNA. The numbers on the left relate to the localization of size marker RNAs (in Kb). One mRNA of about 3 Kb was detected. Its position is indicated by an arrow.

increase in intracellular cAMP.

LHR-LUC10 cells were stimulated with different amounts (0-5 μg/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. 3.12**, A). 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. 3.12**, B). On the other hand, conditioned medium of CHO-K1 stable clones producing recombinant sbsLH was effective in stimulating the sbsLHR (**Fig. 3.12**, B).

Discussion

This section describes the cloning of a cDNA from sea bass testis that codes for a LHR. sbsLHR contains all the structural features that characterize vertebrate glycoprotein hormone receptors such as, an extracellular N-terminal domain that represents more than half of the total length of the

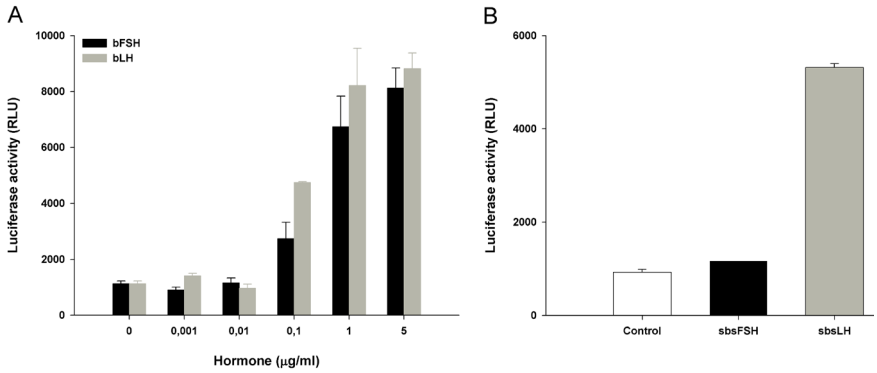

Figure 3.12 Functional analysis of sbsLHR expressed in HEK293 cells. A double stable clone expressing the *sbsLHR* and the reporter construct pCRE-Luc was developed. **A**) Luciferase activity after 5-h treatment with different doses of bFSH and bLH. **B**) Luciferase activity after 5-h treatment with a single concentration of conditioned medium of cultured CHO-K1 wild type cells (Control) or stable clones producing recombinant sea bass LH and FSH.

protein, seven transmembrane stretches of largely hydrophobic amino acids connected by three intracellular loops and three extracellular loops, and an intracellular C-terminal tail.

Analysis of the sbsLHR extracellular domain sequence allowed us to identify nine putative LRR motifs spanning residues 55 to 271. They are placed in the corresponding positions of the nine LRRs described for the hLHR. LRRs are structural units, each consisting of a short β-strand and an α-helix almost parallel to each other. These structural units are arranged in such a way that all the β-strands and the α-helixes are parallel to a common axis, resulting in a horseshoe-like curved molecule, in which all β-strands line the inner, whereas the α-helixes line in the outer part of the shoe (Kobe & Kajava 2001). LRR motifs have been identified in a variety of proteins which are involved in protein-protein interactions. Structural predictions for the human LHR (hLHR), and functional analysis of naturally occurring loss-of-function mutants, have led to the proposal of the LRRs as the main hormone-binding site (reviewed by Ascoli et al., 2002; Puett et al., 2005). Recently, the crystal structure of a complex between the human FSH (hFSH) and the first 250 amino acids of the extracellular domain of the hFSHR (FSH-$FSHR_{1-250}$) was solved (Fan and Hendrickson, 2005). This work provided the first direct evidence for the implication of the hFSHR LRRs in recognition of specificity. The mode of binding seen for the FSH-$FSHR_{1-250}$ complex was proposed to apply to other glycoprotein hormone receptors and their respective ligands (Fan and Hendrickson, 2005). Besides, it has been suggested that residues outside the LRRs of the human and rat LHRs are also involved in ligand binding (Galet and Ascoli, 2005). Six ionizable amino acids of the rat LHR (rLHR) extracellular domain (Glu^{132}, Asp^{135}, Lys^{158}, Lys^{183}, Glu^{184} and Asp^{206}) found to be involved, either directly or indirectly, in ligand binding (Bhowmick et al., 1999) are conserved in the sbsLHR, or substituted by amino acids with similar size, shape and chemical composition. These are sbsLHR Asp^{158}, Asp^{161}, Asn^{184}, Lys^{209}, Asn^{210} and Asp^{232}, respectively. Other residues described to be involved in ligand binding are also conserved in the sbsLHR. One of them is Ile^{118} in the third LRR of the sbsLHR (Ile^{114} in the hLHR). This isoleucine was reported to be essential for ligand binding, after the identification of a natural occurring mutation in a patient with Leydig Cell Hypoplasia, which results in the substitution of Ile^{114} for a phenylalanine (Leung et al., 2006). Accordingly, previous studies had shown that the amino acids that form the third LRR β-strand of the hLHR act as human LH-selective

key determinants, in particular Asn^{104}, which is also conserved in the sbsLHR (Asn ^{111}) (Vischer et al., 2003c).

The LRR domain of the sbsLHR is bordered by two conserved cysteine-rich regions: N-terminal (Cys residues at positions 27, 31, 33 and 40) and C-terminal (Cys residues at positions 283, 284, 308, 360,368 and 378). In the rLHR, the cysteines of the N-terminal cysteine-rich region were shown to be essential for hormone binding activity, while the cysteines of the C-terminal cluster would be essential for receptor membrane insertion (Zhang et al., 1996). Besides the above mentioned cysteines, the extracellular domains of rat and human LHRs have two additional cysteines (Cys^{109} and Cys^{134} in the rLHR). They are localized in two adjacent β-strands of the LRRs (Tsai-Morris et al., 1991) and are predicted to form a disulfide bond (Zhang et al., 1996). They have been shown to be important for receptor cell surface expression and also for hormone binding (Zhang et al., 1996). Nevertheless, rLHR Cys^{109} is conserved neither in the sbsLHR, nor in the rest of fish LHRs (Oba et al., 1999a; Kumar et al., 2001b; Vischer and Bogerd, 2003a; Kwok et al., 2005). In its place, a serine is present (Ser^{133}), which would prevent the formation of the proposed disulfide bridge. Even so, the sbsLHR, like other studied fish LHRs, can be activated by mammalian LH, indicating that its presence is not strictly necessary for the functionality of these receptors.

The extracellular domain of the sbsLHR has three consensus sites for *N*-linked glycosylation, compared with the six consensus sites present in mammals. Only two of them, the second and third, are conserved between mammalian and sea bass LHRs. According to the mass spectrometric analysis of porcine (*Sus scrofa*) LHR, at least five of the potential sites for mammalian LHR carbohydrate attachment are used (Vu-Hai *et al.,* 2000). But, due to discrepancies in the results reported by different groups (reviewed by Ascoli et al., 2002), it is not clear whether the two *N*-glycosylation sites conserved in the sbsLHR correspond to any of the ones used in mammals.

The sbsLHR has a characteristic rhodopsin-like hydrophobic core of seven transmembrane helixes. Several key residues of the hLHR transmembrane domain (Asn^{355}, Asp^{383}, Ser^{431}, Asp^{556}, Asn^{593}, Ser^{594} and Asn^{597}) are completely conserved in the sbsLHR (Asn^{402}, Asp^{430}, Ser^{478}, Asp^{603}, Asn^{640}, Ser^{641} and Asn^{644}). It has been suggested that they are involved in receptor activation and conformation, by forming a network of hydrogen-bond interactions (Puett et al., 2005). Following the model proposed by Puett et al. (2005), specific interhelical hydrogen-bond interactions between these residues would include

(transmembrane helix number-amino acid number): I-Asn^{402} with II-Asp^{430} and VII-Asn^{644}, II-Asp^{430} with VII-Ser^{641}, III-Ser^{478} with VII-Asn^{640}, and VI-Asp^{603} with VII-Asn^{640}. Moreover, residues that are highly conserved in the GPCR family, were also found in the sbsLHR, *e.g.* Arg^{489} located immediately downstream of transmembrane helix III (in the ERW motif) and also known to participate in charge reinforced hydrogen-bonding with the adjacent Glu^{488} and Asp^{589} from the transmembrane helix VI (Puett et al., 2005).

The short intracellular tail of the sbsLHR, like all the available fish LHR sequences, has two highly conserved adjacent cysteines, Cys^{668} and Cys^{669}, whose rat counterparts (Cys^{621} and Cys^{622}) are palmitoylated (Kawate and Menon, 1994). Palmitoylation is a well conserved post-translational modification among members of the GPCR family (Qanbar and Bouvier, 2003) and in the hLHR it has been shown to play an important role in post-endocytic processing, promoting receptor recycling (Munshi et al., 2005). Most of the internalized agonist-GPCRs complexes are sorted into endosomes and quickly recycled back to the plasma membrane, as it is the case of the hLHR. However, other receptors as the rodent or porcine LHRs are instead directed to lysosomes where they are degraded (reviewed in Ascoli et al., 2002). This difference in the rLHR post-endocytotic processing has been attributed to the presence and/or absence of specific structural motifs (Kishi and Ascoli 2000; Kishi et al., 2001). Among them, the presence of Val^{661} in the rLHR instead of the leucine found in a homologous position in the hLHR, and the absence of a cysteine at the end of the intracellular tail of the rLHR (Galet et al., 2004). The sbsLHR has a valine (Val^{707}) at the homologous position of Val^{661} in the rLHR, and lacks a cysteine at the C-terminus of the intracellular domain, suggesting that the sbsLHR, like the rLHR, could be directed to a degradation pathway. Lysosomal degradation of internalized GPCRs is thought to be involved in the acute termination of signalling and it also contributes to a more prolonged attenuation of signalling because it results in a net loss of cell surface receptors (Galet et al., 2004). The physiological interpretation of this post-endocytotic processing, in the context of sea bass reproduction, could be linked to the proposed functions of LH in this fish species, which are restricted to the induction of final gonadal maturation and ovulation/spermiation (Navas et al., 1998).

The gDNA corresponding to the *sbsLHR* was isolated and its structural organization studied. The knowledge of the genomic structure of the sbs*LHR* is valuable to understand its functional domains, relationships with

other *LHR*s and other hormone receptors, expression mechanisms and evolution, as this is the first time that the entire genomic organization of a fish *LHR* is described in detail.

The coding region of the *sbsLHR* gene spans over ~ 6 kb and its general organization follows the one described for the human and rat *LHR*s (Atger et al., 1995, Koo et al., 1991, Tsai-Morris et al., 1991). The sea bass gene consists of eleven exons and ten introns. *sbsLHR* exons 2 to 9 are identical in size to their rat and human orthologs and code for the nine LRRs. Exon 10 is only 12 bp smaller and codes for part of the extracellular C-terminal cysteine cluster. The repeated LRR motifs within the extracellular domain of the sbsLHR are represented at the genomic level by the regular insertion of introns, at approximately 70 bp intervals, between exons 2 and 8. The transmembrane and intracellular domains are encoded by the last exon, which also codes for the extracellular domain C-terminus.

In tetrapod glycoprotein hormone receptors, the transmembrane and intracellular domains are encoded by a single exon, namely exon 10 in the *FSHR* and *TSHR* subfamilies (Heckert et al., 1992, Gross et al., 1991) and exon 11 in the *LHR* subfamily (Atger et al., 1995). Like in tetrapods, *sbsLHR* gene is intronless in this region. Nonetheless, *sbsFSHR* has three introns in the transmembrane coding region (section 3.1). A similar intron distribution was described for the Nile tilapia (*Oreochromis niloticus*) *LHR* and *FSHR* transmembrane coding regions (Oba et a., 2001). However, both receptors in amago salmon, as well as other related invertebrate *LGR* genes, contain introns in their transmembrane coding regions (Oba et al., 1999a,b; Kudo et al., 2000; Hauser et al., 1997). Introns are proposed to have been both lost and gained during the course of evolution. Nevertheless, recent estimations indicate that a decrease in intron number during evolution seems to be a more common event (Roy and Gilbert, 2006). In addition, there is a tendency for concerted loss of adjacent introns along the gene, which occurred preferentially in the 3'portions of genes by a mechanism of reverse-transcription (Roy and Gilbert, 2005). The absence of introns in the transmembrane domain of the *sbsLHR*, compared to the presence of three introns in the same domain of *sbsFSHR*, suggests that *sbsLHR* could have been under stronger evolutionary forces responsible for the loss of introns. This lack of introns in the transmembrane is also in accordance with the higher degree of sequence and structure conservation between sea bass and mammalian *LHR* genes compared to the *FSHR* ones.

The ten introns of the *sbsLHR* vary greatly in their sizes, ranging from 93 bp for intron 5 to 2 kb for intron 1. In general, they are smaller than the ones present in the rat and human *LHR*s. However, their position is conserved with the exception of intron 10 whose position is 12 nucleotides upstream in sea bass, resulting in a smaller exon 10 with respect to the *hLHR*.

In sea bass, as in other fish species, the *LHR* is mainly expressed in gonads. In addition, transcripts of this gene have been found in other sea bass tissues, which is in accordance with the results reported for 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, 2003a; Kwok et al., 2005). In the sea bass, the head kidney presented one of the strongest amplification signals among the non-gonadal tissues analyzed. In teleosts, the head kidney is formed by a variety of tissues organized diffusely, and includes the corticosteroidogenic interrenal tissue, the homologue to the adrenocortical tissue in amniotes. Two different salmon gonadotropin preparations were described to be extremely corticotropic and to stimulate androstenedione secretion by the interrenal of coho salmon (Oncorhynchus kisutch) (Schreck et al., 1989). Besides, the expression of the *LHR* in African catfish was higher in head kidney than in gonads, when analyzed by quantitative real time PCR (Vischer and Bogerd, 2003a). Despite many reports documenting the presence of LHR transcripts and/or protein in diverse extragonadal tissues, the physiological significance of these findings remains poorly understood due to the absence of *in vivo* data on their functionality (Pakarainen et al., 2005).

Three different *sbsLHR* cDNAs with 3' UTR lengths of 274 bp, 355 bp and 850 bp were obtained. They reflect the use of different polyadenylation sites. Similarly, in the rat ovary, four *LHR* transcripts, with 3'UTRs ranging from 3500 bp to 800 bp, have been identified (Wang et al., 1991; Lu et al., 1993; Lapolt et al., 1990; McFarland et al., 1989). Although many features of an mRNA can contribute to its translation, most control elements are located within the untranslated regions. 3' UTR-mediated translational control has been associated with the regulation of subcellular localization, stability of transcripts and translational efficiency. In line with these observations, it has been shown that the 3500 bp long 3' UTR of the *rLHR* transcript contains *cis*–elements, which are involved in the decrease of the number of expressed receptors on the surface of HEK293T transfected cells (Lu and Menon, 1996). It is difficult to speculate if this translational control mechanism could also occur with sbsLHR, however, a computational analysis of a large

UTR database (Mignone et al., 2005) suggests that the length of 3' UTRs increases with evolutionary age and, possibly, with organism complexity. According to this observation, the *sbsLHR* 3' UTRs amplified by us are smaller than those described for the *rLHR*. Pairwise and multiple alignments of fish and mammalian 3' UTR sequences could be extremely helpful to search for potential regions involved in translational regulation.

In mammals, promiscuous stimulation among glycoprotein hormone-receptor couples is avoided by the presence of specificity determinants. Conversely, the functional studies conducted 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 and Bogerd, 2003a; Vischer et al., 2003b; Kumar et al., 2001a,b; Basu and Bhattacharya, 2002; Kwok et al., 2005). We had previously concluded that the sbsFSHR displays ligand selectivity as it is only activated by bFSH but not bLH, even in the presence of pharmacologic doses (section 3.1). In the present study, the effects of the heterologous hormones bFSH and bLH were also tested for the sbsLHR. Challenging sbsLHR HEK293 cells with different doses of bLH resulted in an increase of intracellular cAMP in a dose-dependent manner. However, similar results were obtained when bFSH was used. These results are in agreement with those obtained in zebrafish and amago salmon, where both, bLH and bFSH, could activate their LHRs, while the FSHRs were only responsive to bFSH (Kwok et al., 2005, Oba et al., 1999a,b). It is noteworthy to mention that other mammalian hormones such as human FSH, LH and CG, can act in a different way in other fish receptors. For example, studies conducted in the channel catfish showed that hFSH preferentially stimulated FSHR with little effect on LHR, although hCG could activate both receptors (Kumar et al., 2001a,b). However, in the African catfish, hFSH stimulated both FSHR and LHR, whereas human LH and CG activated only LHR (Bogerd et al., 2001; Vischer and Bogerd, 2003a).

Despite the promiscuous activation of the sbsLHR by bovine gonadotropins, challenging the sbsLHR with recombinant sea bass FSH and LH resulted in an increase of intracellular cAMP only when recombinant sbsLH was used. This divergent response of the sbsLHR to bFSH and sbsFSH is probably based in sequence differences in the β-subunit of the respective hormones. It has been shown that in these subunits, residues between conserved $Cys^{10\text{-}11}$ (small seat-belt loop) are important for LHR binding, while residues between $Cys^{11\text{-}12}$ (carboxy terminal seat-belt) are important for FSHR binding. The sequence

between Cys^{10-11} contains positively charged residues in mammalian LHs and negatively charged in mammalian FSHs. Fish β-subunits contain also an specificity determinant in the Cys^{10-11} region, however it does not exist such a clear charge difference (Vischer et al., 2004). Both sea bass gonadotropins and bFSH have a clear negative net charge in this area, while bLH contains one positively charged residue. Thus, net charges in this region do not seem to play a role in the promiscuous activation of the sbsLHR. The sequence between Cys^{11-12} is not conserved among the bovine and sea bass gonadotropins and does not show any differential feature for sbsFSH. However, the spatial position of this region is predicted to be different for sbsFSH compared to the other three gonadotropins. In the β-subunits of the bovine gonadotropins and sbsLH Cys^{12} is predicted to bind to Cys^{3}, placing the C-terminal residues of the seat-belt near β loops 1 and 3 (β*L*1 and β*L*3). However, in sbsFSH, as in other perciform and salmonid species, Cys^{3} is lacking and thus Cys^{12} would bind to a cysteine present in the very N-terminus, placing the tail of the seat-belt far from the β*L*1 and β*L*3 (see **Fig. 1.2**). This difference could denote a different binding mode in the gonadotropin receptors of these fish species. Considering the whole sequence of the bovine and sea bass gonadotropins, there are several features which are more similar among the bovine gonadotropins plus sbsLH and different from sbsFSH, and thus, could contribute to the ability of bFSH to activate sbsLHR. On one hand, bFSH β-subunit sequence, as a whole, is more similar to sbsLH than to sbsFSH, being more evident between Cys^{1-2} and Cys^{6-7}. Moreover, between Cys^{5-6} sbsLH is more similar to bFSH than to bLH. This region corresponds to β*L*2 that has been shown to interact with the receptor in the crystal structure of the complex hFSH/hFSHR-ectodomain (Fan and Hendrickson, 2005). However, it has been demonstrated that this loop can be interchanged between hFSH and hCG without affecting the ability to activate LHR (Campbell et al., 1991). Regarding the number of residues, sbsFSH has five amino acids less than the other three gonadotropins between conserved Cys^{6-8}. This results in a shorter β*L*3 for sbsFSH than for the other gonadotropins. Considering the model proposed by Moyle et al., this fact could influence hormone binding, as they suggest that the tips of β*L*1 and β*L*3 in the hCG contact the receptor (Moyle et al., 2004).

The results included in this section, together with the ones obtained for the sbsFSHR (section 3.1), only activated by recombinant sbsFSH, suggest that in the sea bass, the interactions between gonadotropins and their corresponding receptors are specific. In these experiments a single hormone-

dose was used, thus more experiments with different hormone doses are needed to further assess this specificity. Specific interactions between ligand and corresponding receptors have been recently suggested also in the rainbow trout (Sambroni et al., 2007). *In vitro* functional studies have demonstrated that purified rainbow trout FSH (rtFSH) activates the rainbow trout FSHR, but also the rainbow trout LHR (rtLHR) although high doses of rtFSH are required to activate the rtLHR, leading the authors to question the physiological relevance of this interaction (Sambroni et al., 2007).

In aquaculture, mammalian hormone preparations have being used pharmacologically to overcome reproduction related dysfunctions caused by confinement. The results obtained herein, together with those from other groups (Oba et al., 1999a,b; Bogerd et al., 2001; Vischer and Bogerd, 2003a; Kumar et al., 2001a,b; Basu and Bhattacharya, 2002; Kwok et al., 2005), emphasize the problematic use of heterologous gonadotropins, considering the complexity arising from cross activation of the gonadotropin receptors by such heterologous hormones in various fish species.

In conclusion, this section reports the isolation and characterization of a sea bass LHR. This, together with section 3.1 reporting the cloning of a sea bass FSHR, completes, for the first time, the characterization of both gonadotropin receptors in a Perciform species. It is also the first report describing the complete genomic organization of a fish *LHR* gene. The molecular and functional information gathered in this section, supported by the analysis of the *sbsLHR* spatio/temporal expression and its association with sea bass reproductive stage, will contribute to the knowledge on the specific roles of LH in the regulation of sea bass reproduction.

3.3 Seasonal changes in gonadal expression of gonadotropin receptors and steroidogenic acute regulatory protein and their relationship with plasma sexual hormones in the European sea bass

In mammals, gametogenesis is regulated by the interplay of extragonadal (systemic) and intragonadal factors, and the importance of each type of regulation varies depending on the developmental stage of the gonad. Similar systems exist in teleost fish (Patiño and Sullivan, 2002; Schulz and Miura, 2002). The pituitary-derived gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) are primary mediators of gametogenesis and gonadal steroid production. While the structural duality of the gonadotropins has been confirmed in fish (Yaron et al., 2003), their functional duality is largely unknown (Swanson et al., 2003). In salmonids, complementary functions of the gonadotropins were suggested by assessment of their transcript and plasma levels. FSH seems to be involved in the initiation and early stages of gametogenesis, while LH stimulates the final maturation and spermiation/ovulation (Weil et al., 1995; Prat et al., 1996; Gómez et al., 1999). However, evaluation of the bioactivities of fish gonadotropins revealed a functional overlap regarding sex steroid production. Data obtained with female salmonids indicate that increased FSH levels in the blood induce follicular production of 17β-estradiol (E2) which, in turn, stimulates hepatic vitellogenesis (Patiño and Sullivan, 2002). However, in the red seabream (*Pagrus major*), ovarian E2 production may be regulated by LH (Okuzawa, 2002). Both FSH and LH stimulated the *in vitro* production of E2 by vitellogenic ovarian tissue in the chum salmon (*Oncorhynchus keta*) (Suzuki et al., 1988), coho salmon (*Oncorhynchus kisutch*) (Planas et al., 2000), common carp (*Cyprinus carpio carpio*) (Van Der Kraak et al., 1992) and tuna (*Thunnus obesus*) (Okada et al., 1994). In male salmonids, FSH and LH are equipotent in stimulating the *in vitro* production of the androgens, 11-ketotestosterone (11-KT) and testosterone (T). However, LH is more potent in stimulating the production of maturation-inducing steroids (MIS) at final maturation and spawning in both female and male (Planas and Swanson, 1995).

The gonadotropins act on the gonads via activation of specific G-protein-coupled receptors, the FSH receptor (FSHR) and the LH receptor (LHR). In coho salmon, two gonadotropin receptors were initially identified by *in vitro* ligand autoradriography on the membrane surface of somatic cells in both testis and ovary. In the testis, the FSHR was observed on Sertoli cells

and the LHR on Leydig cells. In the ovary, the FSHR was localized on both granulosa and theca cells and the LHR on granulosa cells, depending on the stage of ovarian development (Yan et al., 1992; Miwa et al., 1994). The dichotomy of these receptors in teleosts was first confirmed through the cloning of two gonadotropin receptor cDNAs from another salmonid species, the amago salmon (*Oncorhynchus rhodurus*) (Oba et al., 1999a,b) and later in several other fish taxa (Bogerd et al., 2001; Oba et al., 2001; Kumar et al., 2001a,b; Laan et al., 2002; Vischer and Bogerd, 2003a; Wong et al., 2004; Kwok et al., 2005; Maugars and Schmitz, 2006). Studies on the functional activity of these receptors have led to the assumption that fish gonadotropin receptors are less discriminatory regarding ligand binding specificity. So far, information concerning the seasonal expression profile of fish gonadotropin receptors genes is limited to a small number of studies (Rahman et al., 2003; Kumar and Trant 2004; Bobe et al., 2004; Kwok et al., 2005; Kusakabe et al., 2006; Campbell et al., 2006).

In teleosts, final gamete maturation is initiated by a rapid shift from estrogen/ androgen synthesis to MIS synthesis (Nagahama, 1994). This steroidogenic shift is typically accompanied by an increase in the rate of steroid synthesis. The rate-limiting step in steroid biosynthesis is the transport of cholesterol into the mitochondria, where it is converted by the cytochrome P450-associated side chain cleavage enzyme (P450scc) into pregnenolone, the starting compound for the synthesis of all other steroid hormones (Payne and Hales, 2004). This transport is mediated by the steroidogenic acute regulatory (StAR) protein (Manna and Stocco, 2005). In mammals, *StAR* expression is positively regulated by trophic hormones such as FSH and LH in granulosa cells (Balasubramanian et al., 1997; Sekar et al., 2000) and by LH or its analogue human chorionic gonadotropin (hCG) in Leydig cells (Manna et al., 1999).

In sea bass (*Dicentrarchus labrax*), the duality in gonadotropin hormones and cognate receptors has already been demonstrated (Mateos et al., 2003; sections 3.1 and 3.2). Contrary to what was described for salmonids (revised in Swanson et al., 2003), the analysis of gonadotropin subunits mRNA levels during the reproductive cycle of male sea bass suggests the involvement of both hormones in the control of all stages of gonadal development (Mateos et al., 2003). In sea bass, reproduction is repeated once a year during winter. The females present a group-synchronous type of ovarian development, producing 3-4 consecutive spawns during a 1-2

months spawning period (Asturiano et al., 2000; Carrillo et al., 1995). Most of the available information regarding physiological aspects of fish gonadatropins refers to salmonid species which are annual-spawning teleosts, whose germ cells develop in a synchronous fashion.

The sea bass is highly appreciated in aquaculture. It is also increasingly being used as a perciform fish model for studies on the endocrine control of reproduction. Gonadal development (Mayer et al., 1988; Zanuy et al., 1999), patterns of vitellogenin secretion (Mañanós et al., 1997) and hormonal profiles (Prat et al., 1990; Mañanós et al., 1997; Navas et al., 1998; Asturiano et al., 2000; Asturiano et al., 2002) have been described for this species throughout its reproductive cycle. Information on the release of LH during the reproductive cycle and its regulatory mechanisms are also available (Mateos et al., 2002; Mateos et al., 2006). Nevertheless, this information is still insufficient to explain the actions of gonadotropins in the reproductive physiology if this species. The primary objective of the study described in this section was to develop quantitative methods for the measurement of mRNA levels of both gonadotropin receptors during the first gonadal maturation in the sea bass. We have also searched for relationships between these profiles, those of *StAR* expression and plasma profiles of essential reproductive hormones.

Cloning of a partial cDNA of *sbsStAR*

A partial cDNA for *sbsStAR* was amplified to allow the design of specific primers and a probe for real-time quantitative RT-PCR assays. For it, two degenerate oligonucleotides, star1 and star2 (see Materials and Methods), were designed based on two conserved regions of largemouth bass (*Micropterus salmoides*) *StAR* cDNA. They were used as primers in a PCR reaction using sea bass head kidney cDNA as template. A 290 bp product was amplified, and subsequently subcloned and sequenced. The obtained sequence displayed a 93.20% identity to the largemouth bass *StAR*. The partial cDNA sequence of *sbsStAR* is available in the GenBank™ data base under the accession no. EF409994.

Gonadal development and changes in gonadosomatic index (GSI)

Representative sections of sea bass testes and ovaries showing the morphological characteristics of each developmental stage are illustrated in **Fig. 3.13** and **Fig. 3.14**. Changes in GSI values during this study are shown in **Fig. 3.15**. In both males and females, these values were low during the sum-

mer and early fall (July-October), when testes were at the immature and early recrudescence spermatogenesis stages (**Fig. 3.13**, **A** and **B**) and ovaries at the previtellogenic and beginning of vitellogenic stages (**Fig. 3.14**, **A** and **B**). In males, the GSI (**Fig. 3.15**, **A**) started to increase in October to reach high values in December, remaining high during mid and late recrudescence (**Fig. 3.13**, **C** and **D**) and full spermiation stages (**Fig. 3.13**, **E**). Then, they gradually declined, starting in March to attain GSI values lower than one in April, at the post-spawning stage (**Fig.3.15**, **F**). In females, GSI (**Fig.**

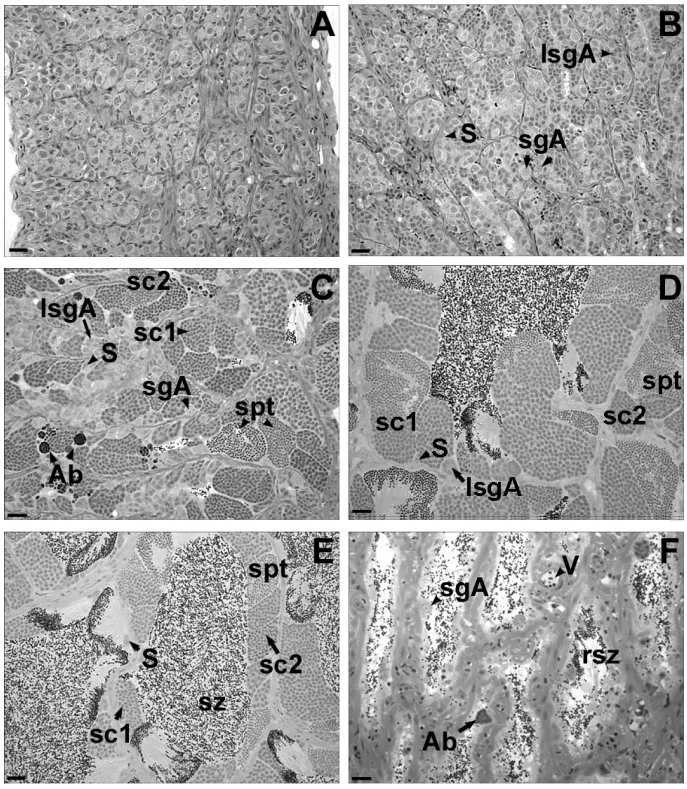

Figure 3.13 Representative stained sections for each of the six stages of sea bass testicular development showing seasonal changes in cell components. (**A**) stage I, immature; (**B**) stage II, early recrudescence; (**C**) stage III, mid recrudescence; (**D**) stage IV, late recrudescence; (**E**) stage V, full spermiating testis; (**F**) stage VI, post-spawning. sgA, primary spermatogonia; lsgA, late A spermatogonia ; S, Sertoli cell; sc1, primary spermatocytes; sc2, secondary spermatocytes; spt, spermatids; sz, spermatozoa; Ab, apoptotic body; V, blood vessel; rsz, residual spermatozoa. Bars, 20 μm.

3.15, **B**) rapidly increased from November on, during active vitellogenesis and post-vitellogenesis (**Fig. 3.14**, **C** and **D**) until it peaked in February (13.157% ± 1.91), during the maturation-ovulation gonadal stage (**Fig. 3.14**, **E**). A progressive decrease of the GSI was then observed from March onwards, concurrent with the follicular atresia (**Fig. 3.14**, **F**), until low values were reached, similar to the ones observed during previtellogenesis.

Seasonal changes in hormone plasma levels

Plasma 11-KT levels in male started to increase in November and rapidly peaked in December (P=0.045) during late recrudescence. These high levels

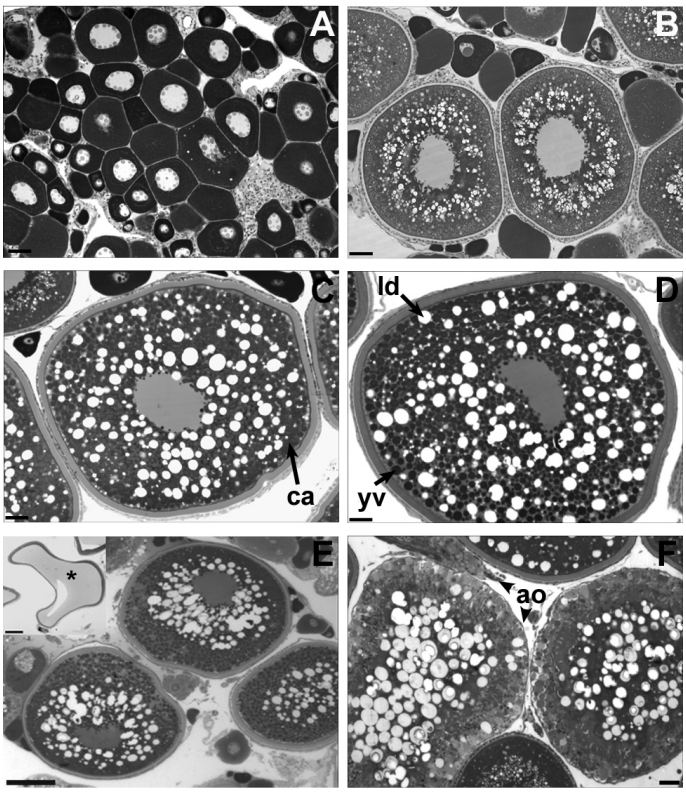

Figure 3.14 Representative stained sections for each of the five stages of sea bass ovarian development showing seasonal changes in cell components. (**A**) previtellogenesis; (**B**-**C**) vitellogenesis; (**D**) post-vitellogenesis; (**E**) maturation, ovulation (labelled by * on the inset); (**F**) atresia. ca, cortical alveoli; ld, lipid droplet; yv, yolk vesicle; ao, atretic oocytes. Bars, 50 μm. Bar in the inset of panel E, 200 μm.

were maintained during January and significantly dropped in February when testis were still in the full spermiation stage (P=0.001) (**Fig. 3.16**, **A**). During the immature stage, plasma LH levels showed a significant elevation in September (P=0.009) followed by an immediate and significant drop in October. Then levels gradually increased, reaching its highest values in February, during the full spermiation stage (**Fig. 3.16**, **B**).

In females, E2 levels gradually increased during vitellogenesis. They peaked at the end of this stage and then they slightly but significantly decreased in January (P=0.046), remaining unchanged during the maturation-ovulation stage until April, when a significant decline was recorded (P<0.001). In June levels were again similar to the ones observed in July due to a significant increase of the values (P<0.001) (**Fig. 3.16**, **C**). During previtellogenesis and vitellogenesis female plasma LH values remained low. Levels started to increase during post-vitellogenesis and peaked in April, at the end of the maturation-ovulation stage. The levels then decreased during atresia to reach significantly (P< 0.001) low levels in June (**Fig. 3.16**, **D**).

Seasonal changes in *18S rRNA* and *Ef1-alpha* expression levels

The seasonal changes in the expression of the reference genes, *18S rRNA* and

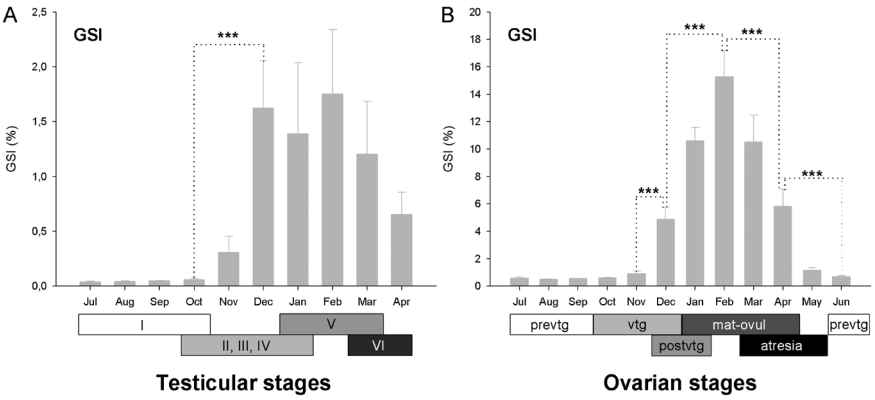

Figure 3.15 Changes in the gonadosomatic index (GSI; gonad weight/body weight x 100) in male (**A**) and female (**B**) sea bass, during the sampling period. Values represent the mean ± SEM (n = 5 fish/month). The histological stages of gonadal development from the samples (see first section of Materials and Methods) are represented by horizontal bars below each group of graphs. Different significance levels are indicated with *, ** and *** for P≤0.05, P≤0.01 and P≤0.001, respectively.

Ef1-alpha, during gonadal development in both sea bass male and female are presented in **Fig. 3.17**. During the sampling period these genes changed significantly ($P \leq 0.001$), in both sexes.

In males, the *18S rRNA* expression levels changed significantly during the sampling period. Nevertheless, the difference between the highest (September) and lowest (April) level was only slightly bigger than threefold (**Fig. 3.17**, **A**). On the other hand, *Ef1-alpha* levels were more than fourteen times higher in September, October and November than in April (**Fig. 3.17**, **B**). In females, *18S rRNA* levels (measured using total RNA) in December were approximately thirty five times higher than the levels in the first third of the study, and they returned to low levels at the end of the sampling period (**Fig.**

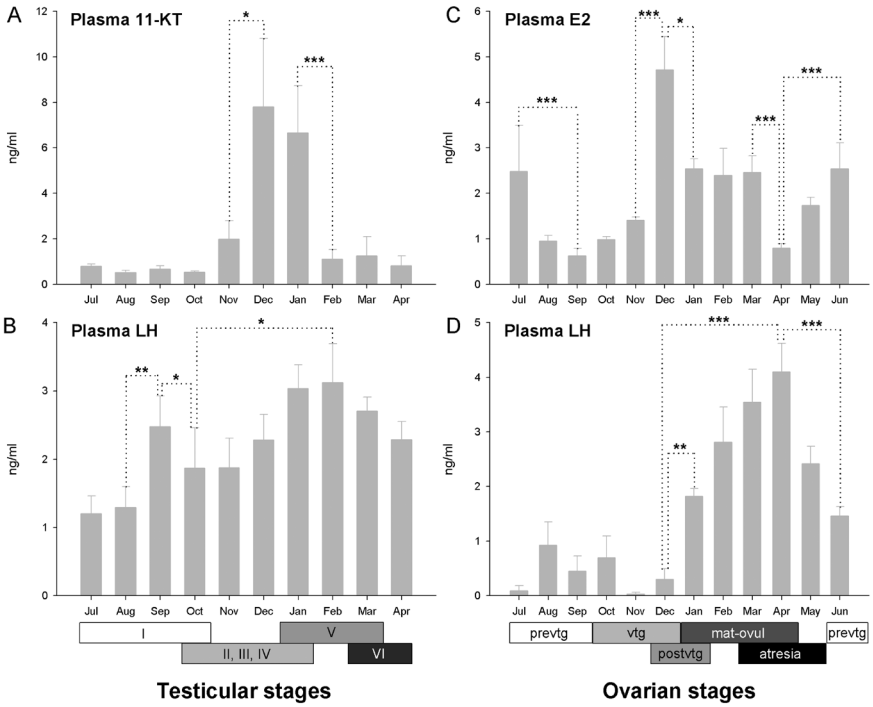

Figure 3.16 Changes in plasma levels of 11-KT (**A**), LH (**B** and **D**) and E2 (**C**) in male and female sea bass during their first gonadal recrudescence. Data are shown as the mean ± SEM (n = 5 fish/month). The histological stages of gonadal development from the samples (see Materials and Methods) are represented by horizontal bars below each group of graphs. Different significance levels are indicated with *, ** and *** for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

3.17, **C**). Although with a lower magnitude, *Ef1-alpha* expression levels also changed during the female study being three to fivefold higher in the first third of the sampling period than in the second third (**Fig. 3.17**, **D**).

Seasonal changes in *FSHR*, *LHR* and *StAR* expression levels

Changes in gonadal expression of the three genes of interest during a complete reproductive cycle were first examined using nonnormalized arbitrary input amounts (**Fig. 3.18**). When normalized to *18S rRNA* (males) and adjusted *Ef1-alpha* (females), the expression patterns of all genes were similar to those of nonormalized values (**Fig. 3.19**). To avoid repeating information, only results from normalized values are described below.

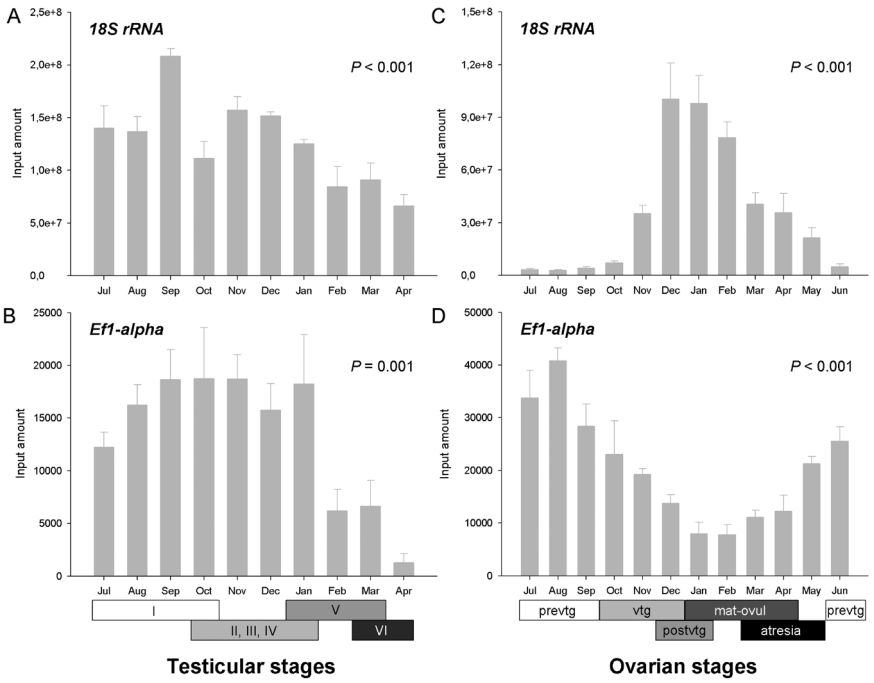

Figure 3.17 Changes in the amount of *sbs18S rRNA* and *Ef1-alpha* mRNAs in male testes (**A**, **B**) and female ovaries (**C**, **D**) sampled during their first gonadal recrudescence. Values represent the mean ± SEM (n = 5 fish/month). The histological stages of gonadal development from the samples (see Materials and Methods) are represented by horizontal bars below each group of graphs. One-way ANOVA was performed. The P value is indicated in each graphic.

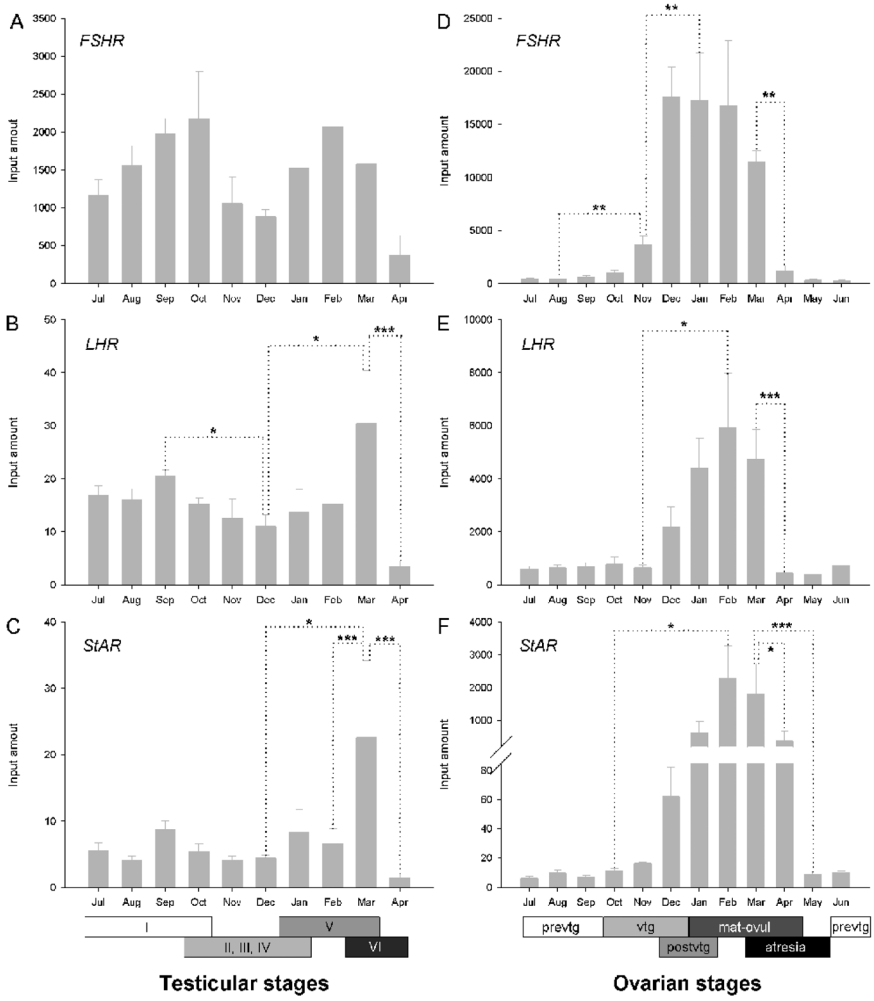

Figure 3.18 Sea bass *FSHR*, *LHR* and *StAR* non-normalized expression values in males (**A, B, C**) and females (**D, E, F**) sampled during their first gonadal recrudescence. Values represent the amount of target gene as determined by RT-PCR and are expressed as the mean ± SEM (n = 5 fish/month). The histological stages of gonadal development from the samples (see Materials and Methods) are represented by horizontal bars below each group of graphs. Male *sbsFSHR* levels showed no significant differences between sampling points (P = 0.085). Different significance levels are indicated with *, ** and *** for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

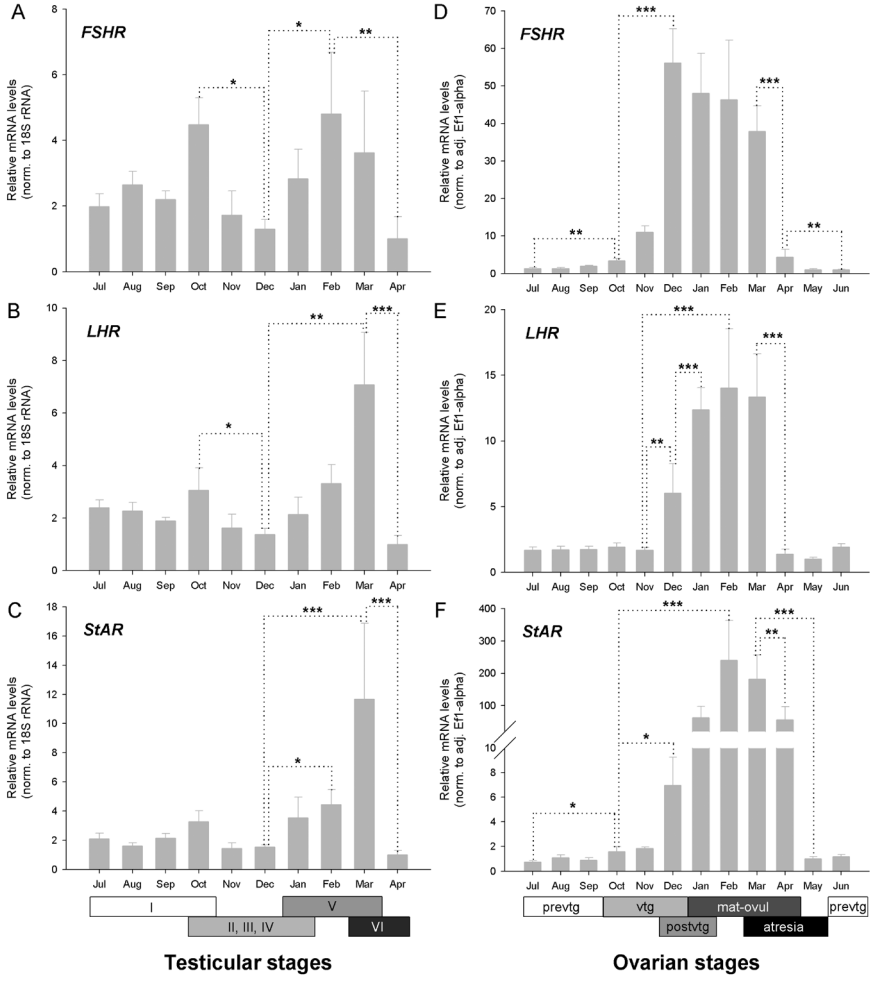

Figure 3.19 Relative changes in expression of *FSHR*, *LHR* and *StAR* in male (**A, B, C**) and female (**D, E, F**) sea bass, sampled during their first gonadal recrudescence. Male expression values are normalized to *sbs18S rRNA* and expressed as a proportion of the mean value in April, set as 1. Female expression values are normalized to *sbsEf1-alpha* which was adjusted to compensate for changes in expression across the reproductive cycle, and expressed as a proportion of the mean value in May, set as 1. Values represent the mean ± SEM (n = 5 fish/month). Statistically significant differences are indicated. The histological stages of gonadal development from the samples (see Materials and Methods) are represented by horizontal bars below each group of graphs. Different significance levels are indicated with *, ** and *** for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively.

Males The *FSHR* expression profile across the male reproductive cycle was bimodal (**Fig. 3.19**, **A**). During the immature stage, the levels progressively increased peaking in October, in the early recrudescence stage. Differences between the values in the initial sampling and October were not statistically different. This peak of expression was followed by a decrease during mid recrudescence (P=0.016) until the beginning of the full spermiating stage when a new elevation of the values occurred, followed by a second decline, reaching the lowest levels in April (P=0.002), during the post-spawning. The expression patterns of *LHR* and *StAR* genes were identical to each other (**Fig. 3.19**, **B** and **C**). A slight and not significant increase was first observed in October, during the immature stage. Levels slender decreased during the early and mid recrudescence stage. With the beginning of full spermiation stage (January), levels started to increase to reach the highest values in March. Expression then decreased to the lowest levels during the post-spawning stage.

Females *FSHR* expression (**Fig. 3.19**, **D**) was low during previtellogenesis (July-September). In October, coinciding with the beginning of vitellogenesis, a slight increase of the expression was observed and values were significantly different form the ones registered in July (P<0.01). Then, during post-vitellogenesis, a boost of expression of approximately seventeen times led values to their highest. Expression levels remained high during the maturation-ovulation stage until April, when they significantly decreased (P<0.001) to values similar to the ones in October. The lowest levels were observed in June, at the beginning of a new cycle. *LHR* expression (**Fig. 3.19**, **E**) remained low and unchanged during previtellogenesis and vitellogenesis until December, when a significant increased was observed (P=0.005). Values continued to rise until they peaked in February during the maturation-ovulation stage, which corresponds to an eightfold expression increment (P<0.001). The expression sharply decreased in April to reach levels similar to the ones of the previtellogenic stage (P<0.001). As in males, the expression pattern of *StAR* in females (**Fig. 3.19**, **F**) was similar to the *LHR* one. Expression remained low during previtellogenesis and vitellogenesis until December, when it started to increase to reach the highest levels in February during maturation-ovulation. In this case, the expression increment was of one hundred and thirty fold (P<0.01). In May, during the atresia stage, levels were low again.

Regression analysis

Linear regression analysis of gene expression during the entire male repruductive cycle (**Table 3.3**, A) identified a significant and moderately strong positive relationship between changes in *StAR* expression and transcript levels of *FSHR* and *LHR*. Relative changes in *LHR* expression were weakly, but significantly positively correlated to *FSHR* transcript levels. During the recrudescence stage, the expression of *FSHR* was negatively and weakly correlated to the plasma levels of 11-KT. Other correlations were not significant.

In females, and regarding the complete cycle (**Table 3.3**, **B**), changes in *LHR* expression were strongly and significantly positively correlated to *FSHR* transcript levels. Changes in *StAR* expression were significantly positively correlated to those of *LHR*. A significant positive correlation, though relatively weak, was also found between changes in *StAR* and *FSHR* expression. Other significant but weak positive correlations include changes in plasma E2 levels and *FSHR* expression and plasma LH levels and changes in the expression of *LHR* and *StAR* genes. During vitellogenesis, levels of E2 were weakly and positively correlated to changes in *LHR* and *StAR* expression.

Table 3.3 Linear regression analysis among changes in transcript levels of *FSHR*, *LHR* and *StAR* and plasma hormones for sea bass males (**A**) and females (**B**).

A	*LHR*	*StAR*	11-KT	LH
FSHR	[a]*P*<0.01, [b]R=0.44	*P*=0.0001, R=0.56	*P*=0.16, R=-0.21	*P*=0.60, R=-0.08
LHR		*P*<0.0001, R=0.75	*P*=0.21, R=-0.18	*P*=0.92, R=-0.02
StAR			*P*=0.85, R=-0.03	*P*=0.83, R=0.03
11-KT				*P*=0.48,R=0.10
B	*LHR*	*StAR*	E2	LH
FSHR	*P*<0.0001, R=0.84	*P*<0.01, R=0.47	*P*<0.01, R=0.44	*P*=0.4015, R=0.11
LHR		*P*<0.0001, R=0.63	*P*=0.10, R=0.21	*P*<0.05, R=0.28
StAR			*P*=0.93, R=-0.011	*P*=0.0001, R=0.49
E2				*P*=0.72, R=-0.05

[a]Probability value.

[b]Correlation coefficient value.

Discussion

The aims of this study were to investigate the seasonal expression of the sea bass gonadotropin receptor genes during the first gonadal maturation in males and females, and to search for relationships between their expression profiles and that of the *StAR* and plasma profiles of essential reproductive hormones.

Before discussing these results, the subject of the normalization of the real-time RT-PCR assay data, already mentioned in Material and Methods, should be further addressed. Fish gonads change considerably their size and cellular components during the reproductive cycle. This unique characteristic becomes problematic when searching for a constitutively expressed gene that can be used for data normalization. In our experiments, the expression of *sbs18S rRNA* and *sbsEf1-alpha* changed significantly during the sampling period in both male and female (**Fig. 3.19**). Transcripts of sea bass *28S rRNA* and *β-actin* have also been found to significantly change during sea bass ovary follicular growth and maturation (Halm et al., 2008). Even though, testis *sbs18S rRNA* expression values were used for male data normalization since the difference between the highest (September) and lowest (April) level of expression in males was approximately threefold (**Fig. 3.19**, **A**). Regarding females, the analysed reference genes were not suitable for normalization, as they would result in an erroneous alteration of the expression levels. We have then used two alternative methods to quantify seasonal changes in gene expression in female sea bass; nonnormalized expression values and standardization of the expression of a reference gene. In our studies, the expression patterns of the genes of interest normalized to adjusted *Ef1-alpha* values were similar to those of nonnormalized expression, implying that both methods are feasible.

In male sea bass, both gonadotropin receptors show different expression patterns during the reproductive cycle. Levels of *FSHR* mRNA increased progressively during the immature stage and beginning of the recrudescence stage but then, when reached the mid recrudescence stage, they dropped significantly. Once in the spermiating stage, a new increment was observed. In contrast *LHR* mRNA levels were low during the immature and recrudescence stages, but a significant increase was observed at full spermiating stage. Data on the expression of gonadotropin receptors in fish are still very limited. In male yellowtail (*Seriola quinqueradiata*) and rainbow trout

(*Oncorhynchus mykiss*), *FSHR* mRNA levels showed an increase during the early spermatogenesis while at spermiation, transcript levels decreased in yellowtail and fluctuated in rainbow trout (Rahman et al., 2003; Kusakabe et al., 2006). These pattern of expression differ from the one described for sea bass in this work as enhancement of the *FSHR* expression was also observed during the spermiation period. Regarding the FSHR ligand, Mateos et al (2003) reported that in male sea bass, *FSHβ* mRNA levels increased continuously with the progression of gonadal growth, reaching maximum levels at the initiation of the spermiation period, and remaining high during all this period. However, it remains unknown if, in sea bass, the patterns of *FSH* and *FSHR* expression are correlated with the presence of their protein products, since detection methods for them are still not available.

These results suggest that in the sea bass, FSH through its cognate receptor may be implicated in the regulation of processes that occur during the early stages of gonadal development and also of gamete maturation and spermiation. The specific role of FSH in testicular function is still unclear even for mammals, but functions such as stimulation of Sertoli cell proliferation in the immature testis and maintenance of qualitatively and quantitatively normal spermatogenesis, through indirect effects mediated by Sertoli cells, have been proposed (revised by Petersen and Söder, 2006). In coho salmon males, FSHR was observed by autoradiography on Sertoli cells throughout spermatogenesis (Miwa et al., 1994) and Schulz et al. (2003) reported that FSH stimulates proliferation of Sertoli cells in African catfish. A recent study has demonstrated that in maturating and adult testis from African catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*), Sertoli cell proliferation occurs primarily during spermatogonial proliferation, remarkably increasing from primary spermatogonia to spermatocyte cysts stages in the Nile tilapia. In both African catfish and Nile tilapia, Sertoli cell proliferation is strongly reduced when germ cells have progressed into meiosis, and stops in postmeiotic cysts. At the beginning of spermiogenesis there is a dilution of Sertoli cells due to the expansion of cyst volume and the stabilization of Sertoli cell number per cyst. However, during the spermiogenic process there is a striking reduction of cyst volume in Nile tilapia testis (Schulz et al., 2005). Assuming an analogous behavior for sea bass Sertoli cells proliferation, we could expect that the progressive increase of *sbsFSHR* expression during stages I and II of the sea bass testicular development could be related with a proliferation of Sertoli cells, and that the observed decrease of expression

in stages III and IV is the result of a dilution of somatic cells with respect to germ cells, rather than a reduction in *sbsFSHR* transcripts. During the spermiation stage this dilution effect is no longer observed, resulting in a second increase in the expression levels. Nevertheless, it is also possible that the decline in the expression during mid recrudescence would be the result of a transient transcription downregulation to prevent Sertoli cell overstimulation by FSH (Themmen et al., 1991). Then, the observed enhancement of expression during spermiation could be due to an upregulation of *sbsFSHR* expression, and/or connected with a new Sertoli cell proliferation needed for the maintenance of spermatogenesis in several clutches of gametes present in the testis, since spermiation is associated with the degeneration of at least some of the Sertoli cells (Billard, 1986; Prisco et al., 2003). In fact, in the testes of male sea bass, groups of immature spermatozoa remain together with maturing or mature gametes and spermatogenesis occurs in the spawning season simultaneously with spermiation.

The temporal profile described for *sbsLHR* expression in testis (**Fig. 3.19**, **B**) is consistent with data from maturating rainbow trout and yellowtail males, showing maximum receptor mRNA levels during the spermiation stage (Rahman et al., 2003; Kusakabe et al., 2006). However, in rainbow trout and yellowtail these maximum levels were achieved after a steady increase in receptor expression, as testicular maturation advanced, while in sea bass the receptor mRNA levels were maintained almost constant until the end of the recrudescence stage. Nevertheless, a small increase in the expression of the *sbsLHR* was seen in October, which could be related to the peak of expression of the *sbsFSHR* observed in the same month, since it is known that in mammals FSH stimulates the expression of the *LHR* (Richards 1994). However, the physiological significance of a rise in *sbsLHR* expression at this stage of testicular development is unclear. Analysis of sea bass plasma LH levels (**Fig. 3.16**, **B**) showed an increase of this hormone during spermatogenesis reaching the highest levels during the spermiation stage which is in agreement with the expression profiles of sea bass *LHβ* (Mateos et al., 2003) and *LHR* (**Fig. 3.19**, **B**). These results support the already suggested role of LH as being mainly involved in the regulation of the final stages of fish gamete maturation and spermiation (revised in Swanson et al. 2003).

In male teleosts, 11-KT is the major produced androgen (Borg 1994) and is considered to play an important role in spermatogenesis (Cavaco et al., 1998). In addition, steroids have feedback actions on gonadotropin produc-

tion and release (revised in Yaron et al., 2003). The profile of 11-KT presented in this study (**Fig. 3.16**, **A**) is similar to the ones obtained in previous works in sea bass (Rodriguez et al., 2000), with levels increasing during mid recrudescence, and dropping once spermiation begins, which confirms the already suggested important role of this hormone in the stimulation of spermatogenesis in sea bass (Rodriguez et al., 2000). In fish, very little information is available on the specific roles of FSH and LH in regulating androgen production by the testis. In salmon, FSH and LH were equipotent in stimulating the production of 11-KT, T, and the MIS 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) by testicular tissue in stage IV of spermatogenesis. However, LH is more potent in stimulating 17,20βP production at final maturation and spawning, in both males and females (Planas and Swanson 1995). In red seabream, both FSH and LH stimulated the production of 11-KT in sliced testis of animals in the spawning season (Kagawa et al., 1998). In this study, we did not find any linear relationship between sea bass gonadotropin receptors mRNA levels and the 11-KT profile in plasma (**Table 3.3**). Further studies will be needed to understand the actions of gonadotropins during the spermatogenic process of sea bass. Apparently, the signalling pathways activated by both FSH and 11-KT lead to the production of factors that are required for germ cell maturation into spermatozoa. Only by identifying those signalling pathways we will be able to understand the independent, overlapping or synergistic actions of these two hormones.

Interestingly, the quantification of *StAR* transcripts in sea bass testis (**Fig. 3.19**, **C**) revealed a profile identical to the one observed for the *LHR* (**Fig. 3.19**, **B**), what was supported by a significant linear relationship between both gene mRNA levels (R=0.75, $P<0.0001$; **Table 3.3**), which was higher than that between *FSHR* and *StAR* expression (R=0.56, $P=0.0001$; **Table 3.3**). In rainbow trout males, the expression profile of *StAR* was also correlated with *LHR* and *FSHR* mRNA levels (Kusakabe et al., 2006). The acute, steroidogenic effect of LH in mammalian Leydig cells is based on an increased availability of cholesterol for the mitochondrial P450scc. This is achieved via induction of *StAR* (Stocco et al.,2005). Our results indicate that a similar regulation may occur in the sea bass testis.

Contrary to what was found for males, in sea bass females both gonadotropin receptors follow a similar expression pattern (**Fig. 3.19**). Expression of these genes was strongly positively correlated (R=0.84, $P<0.0001$; **Table 3.3**). Before yolk incorporation, during primary growth and early stages of

secondary growth phase (previtellogenesis), both receptors are expressed at extremely low levels in sea bass ovary. In early vitellogenesis (October), the expression levels of *FSHR* slightly increased while *LHR* mRNA levels remained unchanged. Recent work in channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) has suggested that an increase in ovarian *FSHR* expression and pituitary *FSHβ* occurs prior to vitellogenesis, coinciding with the accumulation of cortical alveoli, and this upregulation continues through vitellogenesis (Kumar and Trant 2004; So et al., 2005; Kwok et al., 2005). However, in coho salmon, during the same period of gonadal development, levels of *FSHR* expression did not increase in parallel with pituitary and plasma FSH levels (Campbell et al., 2006). In most teleost species, deposition of cortical alveoli in the ooplasm of oocytes occurs before yolk incorporation. Sea bass is an exception, since the formation of this type of inclusions occurs after the beginning of vitellogenesis (Mayer et al., 1988). Thus, in sea bass ovary, the rise in *FSHR* expression may also be connected with the accumulation of cortical alveoli. Increases in *sbsLHR* mRNA levels were only observed when postvitellogenesis began (December). At that stage, *sbsFSHR* mRNA levels were already at their maximum. During the maturation-ovulation period, expression levels of both receptors remained elevated, returning to their basal levels only after spawning.

As in mammals, fish gonadotropins are thought to promote follicular steroid production and oocyte growth; however, the relative roles of each hormone in vitellogenesis and oocyte maturation are not fully understood and may diverge among species (Swanson et al., 2003; Yaron et al., 2003). Studies on female salmonids, which have a synchronous type of oocyte development, suggest that secondary oocyte growth is regulated primarily by FSH, whereas LH plays a major role in regulating final oocyte maturation. Nonetheless, the observed expression pattern of *sbsFSHR* (**Fig. 3.19**, **D**) involves this receptor (and FSH) also in processes occurring after secondary oocyte growth. In a target gene expression profile study on rainbow trout ovary, an increased expression of *FSHR* in females exhibiting high maturational competence was observed (Bobe et al., 2004). This expression remained elevated during oocyte maturation, which is consistent with the rise in FSH circulating levels observed in rainbow trout before oocyte maturation (Breton et al., 1998). Based on these data the authors suggested a role for FSHR in oocyte maturation and, possibly, in ovulation. Regarding sea bass, we consider that the observed high expression level of *FSHR* during

maturation could be connected with oocyte growth and is explained by the reproductive strategy of this species. Sea bass ovary exhibits a group-synchronous type of development and contains clutches of oocyte populations at various stages of secondary growth that are successively recruited (Mayer et al., 1990; Asturiano et al., 2000). Therefore, the expression of any gene measured at the ovary level reflects the average of the existing follicles, including that of growing oocytes that would still express *FSHR*. This idea is supported by a previous *in situ* hybridization study on post-vitellogenic sea bass ovary, which showed a strong expression of *FSHR only* in the follicular cells of previtellogenic and early vitellogenic oocytes (section 3.1).

The profile of E2 plasma levels observed in this study (**Fig. 3.16**, **C**) is similar to previous works (Prat et al., 1990; Mañanós et al., 1997), with a single annual peak at late vitellogenesis (December), and constant high levels during the whole maturation and ovulation period. E2 is known to stimulate the hepatic synthesis of vitellogenin, which is then progressively incorporated into the growing oocytes during the period of gametogenesis (Mañanós et al., 1994). The maintenance of constant high E2 levels during the entire maturation and ovulation stage has been attributed to a prolongation of the vitellogenic process, as vitellogenic oocytes are also present during this stage (Mañanós et al., 1997). In mammals, the actions of FSH are intimately related to the actions of E2 in folliculogenesis. Accordingly, FSH stimulates the *in vitro* production of E2 in cultured sea bass ovaries (Molés et al., 2007) and in salmonid fish it was established that FSH alters ovarian P-450 aromatase expression and activity (Montserrat et al., 2004). Linear regression analysis identified a positive relationship between *sbs-FSHR* mRNA levels and E2 plasma profile, although this correlation was moderately weak (R=0.44, $P<0.01$; **Table 3.3**). There are evidences showing that intragonadal systems involved in the regulation of gametogenesis in teleost fish, such as growth hormone-insulin growth factor, epidermal growth factor and transforming growth factor systems, can be modulated by sex steroids in a stage dependent fashion (Gioacchini et al., 2005). By studying the signalling crosstalk between FSH and these other systems we may understand how FSH and E2 interact to regulate sea bass ovarian function. In mammalian ovaries, the expression of the *LHR* is induced by FSH, estrogens and growth factors in granulosa cells of preovulatory follicles (Dufau, 1998). It is interesting to note that in the sea bass ovary, the expression levels of the *LHR* remained basal until *FSHR* expression and

E2 plasma levels were high (**Fig. 3.16** and **3.19**), indicating that a similar induction mechanism could occur during late vitellogenesis and post-vitellogenesis in sea bass.

In this study, a significant elevation of *sbsStAR* expression was first observed at the end of vitellogenesis and beginning of postvitellogenesis. The highest expression values were observed at the maturation-ovulation stage (**Fig. 3.19, F**). The progestagens 17,20βP and 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) have been proven to be the sea bass MIS (Asturiano et al., 2000). Maximum plasma concentrations of 17,20βP were observed during postvitellogenesis, immediately before the maturation stage, while the highest plasma levels of 20βS coincided with final maturation (Asturiano et al., 2002). The shift from estrogen to MIS synthesis needs both the partial reworking of the steroidogenic pathway and the rapid delivery of cholesterol substrate. As already mentioned, the rapid changes in plasma sex steroid concentrations during maturation in most teleost species suggest the involvement of the StAR protein. The expression profile of *sbsStAR* obtained in this study suggests that StAR mediates the rapid delivery of sterol substrate for the synthesis of ovarian MIS. As described for sea bass males, there was a significant linear relationship between sea bass *LHR* and *StAR* mRNA levels in females (R=0.63, $P<0.0001$), which was higher than that of *FSHR* and *StAR* mRNA (R=0.47, $P=0.01$). *sbsStAR* transcript levels were also correlated with plasma LH levels (R=0.49, P=0.0001) in females. In mammals and birds, LH administration increases *StAR* promoter activity (Stocco et al., 2005; Nakao et al. 2007). In sea bass, exposure of follicle-enclosed-oocytes to hCG induces oocyte maturation and synthesis of MIS (Sorbera et al., 1999).Taken together, this data could imply that in sea bass ovary, LH regulates *StAR*s expression and that high levels of *StAR* expression are associated with MIS production.

In both sexes, expression levels of *sbsFSHR* were higher than *sbsLHR* ones. This is easily observed in the graphical representation using input amount (**Fig. 3.18**). Since we used total RNA in the male study and poly $(A)^+$ enriched RNA in the female analysis, comparison of expression levels between sexes would not be accurate. Even though, it is interesting to note that expression levels in males are much higher than in females which is in accordance with previous RT-PCR assays (sections 3.1 and 3.2).

In summary, the present section provides information on changes in the expression of the sea bass gonadotropin receptors, in relation to changes in

StAR expression and important reproductive hormones during the first gonadal recrudescence in male and female sea bass. In both sexes, the expression of all genes changed throughout the reproductive cycle. While in male sea bass, both gonadotropin receptors show different expression patterns, in females their expression patterns were somehow similar. Expression of the *sbsFSHR* was connected with early stages of gonadal development, but increased expression was also observed during the spermiation period in males and the maturation-ovulation period in females, suggesting that this receptor may also be involved in the control of processes occurring during these stages of gonadal development. The expression profile of the *sbsLHR*, in both sexes, supports the already suggested involvement of LH in the regulation of the final stages of fish gamete maturation and ovulation/spermiation. *sbsStAR* expression was strongly correlated with *sbsLHR* expression. In males, no linear relationship was found between sea bass gonadotropin receptors mRNA levels and the 11-KT profile in plasma. In females, *sbsFSHR* mRNA levels were only moderately correlated to the E2 plasma profile. Further studies will be needed to understand how gonadotropins and sex steroids interact to regulate sea bass reproduction.

3.4 Molecular characterization of a European sea bass thyrotropin receptor and gonadal gene expression profile throughout a reproductive cycle

The thyroid stimulating hormone (TSH) is a glycoprotein synthesized and secreted from thyrotroph cells of the anterior pituitary gland (Pierce and Parsons, 1981). Binding of TSH to its specific receptor, the TSHR, transmits the major trophic stimulus to the thyroid gland, as well as the necessary signals for the production and release of thyroid hormones (Vassart and Dumont, 1992). For these reasons, the TSHR can be regarded as the major regulator of thyroid function.

The TSHR, together with the luteinizing hormone receptor (LHR) and the follicle stimulating hormone receptor (FSHR) constitute the glycoprotein hormone receptor family, a subclass of the large rhodopsin-like G protein-coupled receptors (GPCRs) family (Vassart et al., 2004). All three receptors share close sequence similarity, and are characterized by a large extracellular amino-terminal domain, responsible for hormone recognition and binding via leucine-rich repeats (LRR). This is followed by a serpentine portion with seven transmembrane helixes, responsible for signal transduction, and connected to a short intracellular segment that interacts with cytoplasmic proteins (Vassart et al., 2004).

Despite this overall similarity, the TSHR is distinct from the other glycoprotein hormone receptors in several potentially important aspects. The TSHR has two unique stretches of amino acids, one of 8 residues close to the amino terminus of the receptor and one of 50 residues toward the carboxyl end of the extracellular domain (Parmentier et al., 1989; Nagayama et al., 1989; Misrahi et al., 1990). For this reason, the TSHR is the largest of the glycoprotein hormone receptors. Also unique to the TSHR is a posttranslational cleavage phenomenon, which involves removal of the 50- amino acid insert, leaving most of the extracellular domain (A or α subunit) tethered to the membrane-bound portion of the receptor (B or β subunit) by disulfide bonds (de Bernard et al., 1999).

After interaction between ligand and receptor, the signal passes the extracellular and transmembrane domains to induce an activated conformation of the receptor, which allows activation of G proteins. The best known signalling pathway of TSH in the thyroid gland is one that involves the increase of intracellular cAMP production (Wilson et al., 1968). However, TSH has also been

shown to stimulate the mitogen-activated protein kinase and/ or to produce inositol phosphate in the thyroid (Field et al., 1987; Saunier et al., 1995).

Not much is known about TSHR function in fish, where thyroid follicles are diffusely dispersed within the connective tissue that surrounds the ventral aorta and other basibranchial areas (Gorbman, 1969), instead of being encapsulated into a singular gland as it is in mammals. Valuable insight into teleost TSHR function was provided with the isolation of the cDNAs encoding this receptor in a few number of fish species (Oba et al., 2000; Kumar et al., 2000; Vischer and Bogerd, 2003b; Goto-Kazeto et al., 2003). Whereas the expression of the two *TSHR*s cloned from the amago salmon (*Oncorhynchus rhodurus*) is limited to the thyroid (Oba et al., 2000), those of striped bass (*Morone saxatilis*) and catfish (*Clarias gariepinus* and *Ictalurus punctatus*) are found in many other tissues (Kumar et al., 2000; Vischer and Bogerd, 2003b; Goto-Kazeto et al., 2003). In mammals, the presence of TSHR has been described in numerous cells and organs other than the thyroid, including lymphocytes, thymus, pituitary, testis, kidney, brain, adipose/fibroblast, heart and bone (reviewed in Davis and Latif, 2002). Several biological roles for TSH have thus been speculated. However, for none of the proposed functions has TSH been shown to be either necessary or sufficient. An exception is the evidence for direct effects of TSH on the regulation of mice skeletal remodelling, mediated via the TSHR (Abe et al., 2003).

In fish, TSH not only regulates growth, development and overall metabolism but also modulates some specific physiological processes such as osmoregulation, migratory movements (Swanson et al., 1987) and larval metamorphosis (Inui et al., 1989). Apart from the referred essential functions, a direct role of this glycoprotein hormone on fish gonadal physiology has been recently suggested, after the molecular cloning of *TSHR*s from the gonads of three fish species (Kumar et al., 2000; Vischer and Bogerd, 2003b; Goto-Kazeto et al., 2003). Nevertheless, there is - to our knowledge - no information on the regulation or function of the TSHR in the gonads of any fish species.

This section reports the cloning of a cDNA obtained from the gonads of the European sea bass (*Dicentrarchus labrax*) that codes for a TSHR (sb-sTSHR). The tissue distribution of its mRNA and the identification of two variants with different 3' untranslated regions (UTRs) are also described. Finally, aiming to investigate the hypothetical role of this receptor in the European sea bass reproductive function, changes in ovary and testis *TSHR* expression, associated with the female and male reproductive cycles, were also measured using real-time PCR.

Cloning and sequence analysis of a *sbsTSHR* cDNA

Two degenerate oligonucleotides, fshr1 and fshr2 (**Table 2.1**), were designed for a highly conserved region of the transmembrane domain of fish and mammalian glycoprotein hormone receptors. They were used as primers in a PCR reaction using sea bass testis cDNA as template. A 680 bp product was amplified, and subsequently subcloned and sequenced. The obtained sequence displayed the highest identity to other *TSHR*s. A specific reverse primer (tshr8), designed based on the sequence of the amplified product was used on a PCR reaction in combination with a degenerated forward primer designed on the extracellular domain (tshr7). Testis cDNA was used as template. The amplified fragment of about 1054 bp, was subcloned, sequenced and used as a probe to screen a sea bass cDNA testicular library. A single specific clone containing a cDNA insert of 2069 bp was isolated. Sequence analysis revealed that this clone did not contain the full length *sbsTSHR* cDNA, as it was lacking the complete sequence coding for the extracellular domain. Using the sea bass cDNA testicular library as template, a PCR reaction was performed where a *sbsTSHR* specific reverse primer (tshr9), located on the extracellular domain of the *sbsTSHR*, was used in combination with the SKL primer which anneals to the multiple cloning site of the UNI-ZAP XR vector, thus obtaining the whole 5' end of this cDNA. Based on the sequence of this PCR product, a specific forward primer (tshr17) annealing to the 5' UTR of *sbsTSHR* gene was designed. It was used in combination with the primer tshr8 for amplification of testis cDNA in order to obtain a fragment overlapping with the cDNA sequence obtained from the library. The complete *sbsTSHR* cDNA sequence has a length of 3587 bp. It consists of an open reading frame of 2340 bp that codes for a 779 amino acid polypeptide, flanked by 5' and 3' UTRs of 227 bp and 1020 bp, respectively. The first 23 amino acids were predicted to constitute the putative signal peptide. The complete nucleotide and deduced amino acid sequence are shown in **Fig. 3.20**.

Typical attributes of members of the glycoprotein hormone receptors subfamily could be identified in the mature protein. The large extracellular amino-terminal domain consists of 427 amino acids, including the signal peptide. Searches in the Pfam database and further comparison with the only available crystal structure of a glycoprotein hormone receptor, the human FSHR (Protein Data Bank accession code 1XWD), allowed us to identify eleven β-strands of imperfect LRRs within this domain (**Fig. 3.20**). These LRRs are flanked by ten conserved cysteines, four of them in an N-terminal

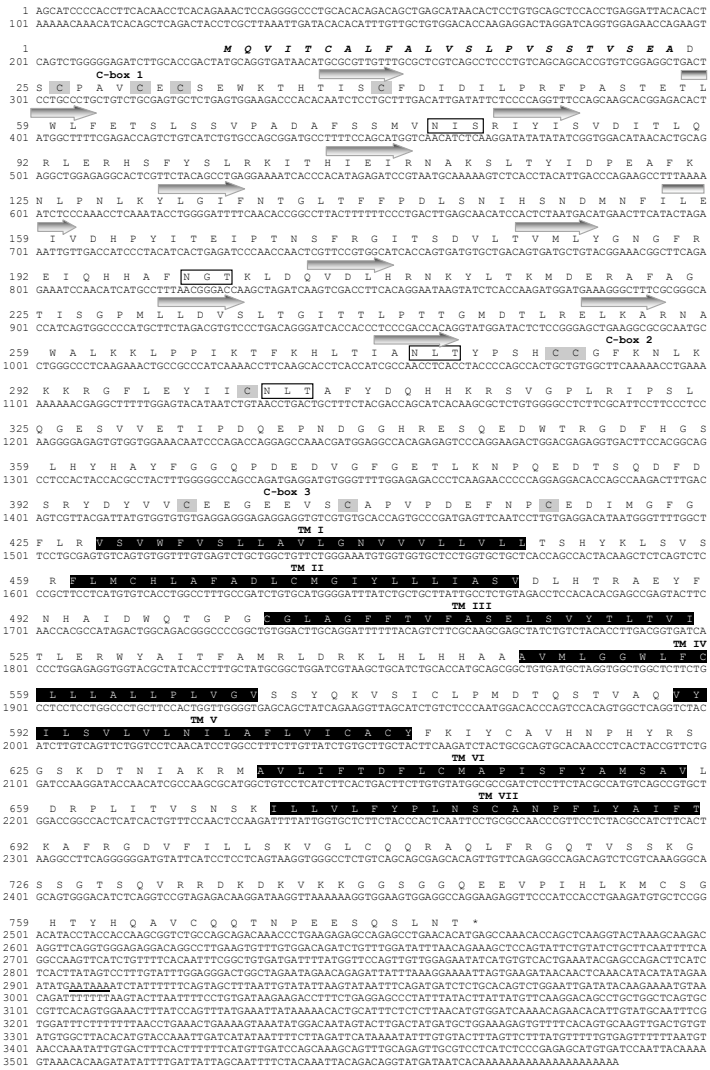

Figure 3.20 Nucleotide and deduced amino acid sequence of the *sbsTSHR* cDNA. Numbers on the left refer to position of the amino acid (top) and the nucleotide residues (bottom). Amino acid numbering begins with the proposed initial methionine. The predicted signal peptide is indicated in bold italics. Cysteines of the N-terminal cysteine-box 1, cysteine-box 2 and cysteine-box 3 of the extracellular domain are indicated by grey boxes. The eleven β-strand motifs (consensus sequence: X-L-X-L-X) of the LRRs, found by Pfam Blast and sequence alignments, are shown as arrows. Four potential N-linked glycosylation sites are indicated by open boxes. Only the first, second and fourth are conserved in the hTSHR. The position of the seven predicted transmembrane domains is shown as black boxes. Consensus site for polyadenylation is double underlined. The nucleotide sequence has been submitted to the GenBank and is available under the accession number DQ386646.

cluster (C-box1), three in a central cluster (C-box2) and the other three in a C-terminal cluster (C-box3). The predicted transmembrane domain, of 264 amino acids, includes 7 stretches of hydrophobic residues, typical of all the members of the rhodopsin-like GPCR family. The intracellular C-terminal domain consists of 88 amino acids. Predictions for N-glycosylation sites identified four potential motifs in the sbsTSHR extracellular domain at positions ^{78}NIS, ^{199}NGT, ^{277}NLT and ^{303}NLT (**Fig. 3.20**). Kinase-specific phosphorylation site predictions identified in the intracellular C-terminal domain of the sbsTSHR, three potential phosphorylation sites for protein kinase C (Thr^{719}, Ser^{721} and Ser^{722}) and one for protein kinase A (Ser^{743}).

The sbsTSHR protein has the highest identity to TSHRs of other fish species (98.2%-61.4%), followed by mammalian TSHRs (57.8%-56%), chicken TSHRs (57.4%-55.1%), mammalian LHRs (43.7%-42.7%), chicken LHR (42.9%), reptile FSHRs (42.8%-37.8%), mammalian FSHRs (42.4%-40.9%), fish LHRs (41.1%-38.1%) and fish FSHRs (40.5%-37.1%). The overall amino acid identity between the sbsTSHR and the other two members of the sea bass glycoprotein hormone receptors (section 3.1 and 3.2) is 39.3% respect to the sbsFSHR and 39.7% for the sbsLHR. Regarding protein domains, the extracellular domain of sbsTSHR is only 25% and 31% identical to the homologous domains of sbsFSHR and sbsLHR respectively, while sbsTSHR transmembrane domain is 67% and 62% identical to the homologous domains of sbsFSHR and sbsLHR. Alignment of the sbsTSHR amino acid sequence with other TSHRs, made clear that the most divergent areas are the C-terminus of the extracellular and intracellular domains (data not shown). The ClustalX alignment also revealed the presence of specific signature sequences (*e.g.* ^{279}[T/S]YPSHCC[G/A]F, ^{414}FNPCEDIMG, ^{527}[E/D]R[W/Y], ^{640}FTD, ^{683}NPFLY) highly conserved among glycoprotein hormone receptors (Vassart et al., 2004).

Phylogenetic analysis of the *sbsTSHR*

The evolutionary relationship of the *sbsTSHR* to other members of the glycoprotein hormone receptors family was inferred by performing a phylogenetic analysis by the Neighbour-Joining method. The topology of the resulting rooted tree (**Fig. 3.21**) shows three main clades: *TSHR*, *FSHR* and *LHR* lineages. The *FSHR* and *TSHR* clades are grouped with a bootstrap value of 100, while the *LHR* group is in a different branch in 90% of the replicates. This analysis suggests that the *LHR* lineage may have individualized before the split of the

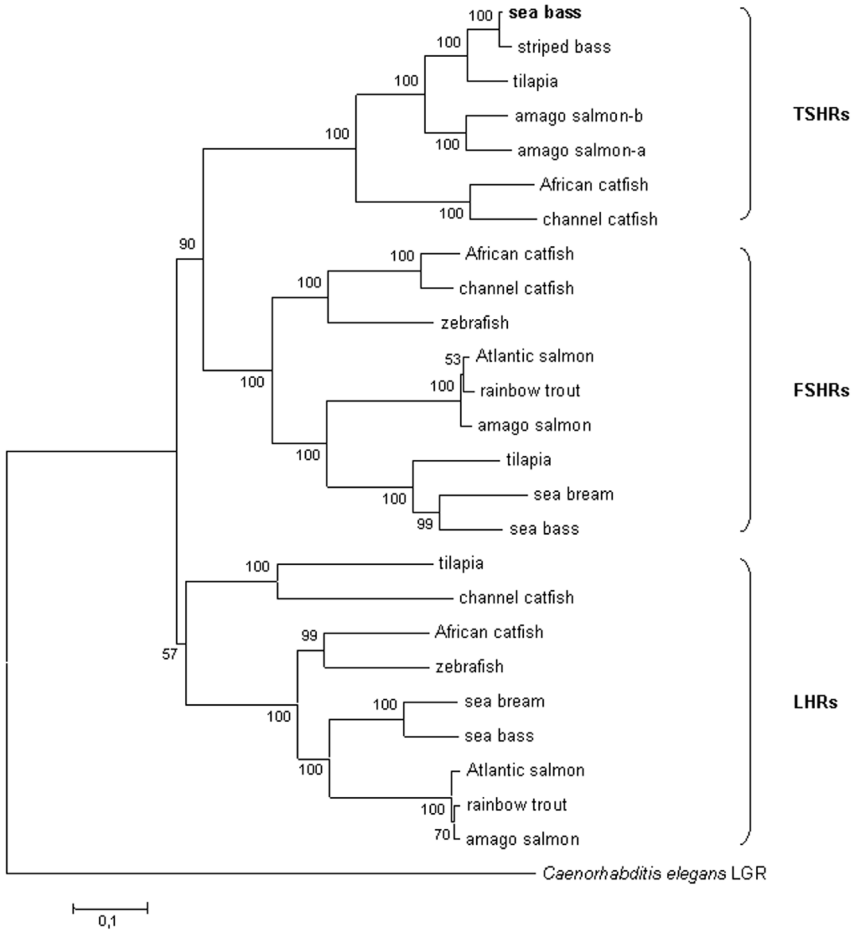

Figure 3.21 Phylogenetic tree of fish glycoprotein hormone receptors inferred from the Neighbor-Joining method. Accession numbers: sbsTSHR (DQ386646), striped sea bass TSHR (AF239761), Nile tilapia TSHR (AB047390), amago salmon TSHRb (AB030955), amago salmon TSHRa (AB030954), African catfish TSHR (AY129556), channel catfish TSHR (AY533543), African catfish FSHR (AJ012647), channel catfish FSHR (AF285182), zebrafish FSHR (AY278107), Atlantic salmon FSHR (AJ567667), rainbow trout FSHR (AF439405), amago salmon FSHR (AB030012), Nile tilapia FSHR (AB041762), gilthead seabream FSHR (AY587262), sbsFSHR (AY642113), Nile tilapia LHR (AB041763), channel catfish LHR (AF285181), African catfish LHR (AF324540), zebrafish LHR (AY424302), gilthead seabream LHR (AY587261), sbsLHR (AY642114), Atlantic salmon LHR (AJ579790), rainbow trout LHR (AF439404), amago salmon LHR (AB030005). The LGR sequence from *Caenorhabditis elegans* (AF224743) was used as the outgroup. Bootstrap values (in %) from 1000 replicates are indicated for each tree node.

FSHR and *TSHR* lineages. The *sbsFSHR*, *sbsLHR* and *sbsTSHR* were found to cluster within the *FSHR*, *LHR* and *TSHR* groups, respectively. As awaited from their close phylogenetic relationships (Nelson, 1994), *sbsFSHR* and *sbsLHR* were found to cluster with sea bream and Nile tilapia FSH and LH receptors. The *sbsTSHR* was found to cluster with striped bass and Nile tilapia *TSHRs*.

Expression analysis

The expression of the *sbsTSHR* was analyzed by RT-PCR in different tissues of adult animals. The results showed that *sbsTSHR* is highly expressed in testis and with a lower magnitude in the ovary. This transcript could also be detected in several somatic tissues from which, brain/ pituitary, heart and kidney presented the strongest amplification signal (**Fig. 3.22**, upper panel). The uniform amplification of the *sbs18S rRNA* transcript confirmed equal amount of RNA used in each reaction (**Fig. 3.22**, lower panel).

3' RACE led to the amplification of a *sbsTSHR* mRNA with a trailer sequence of 382 bp. This 3' UTR is shorter than the corresponding region in the cDNA isolated from the library, whose size is 1020 bp. These two different transcripts result from the use of alternative polyadenylation signals present in the pre-RNA molecule of the *sbsTSHR*. The consensus polyadenylation site for the transcript with the shortest 3'UTR is indicated in **Fig. 3.20**. Regarding the other transcript, there is not a clear consensus polyadenylation sequence located upstream of its poly $(A)^+$ tail.

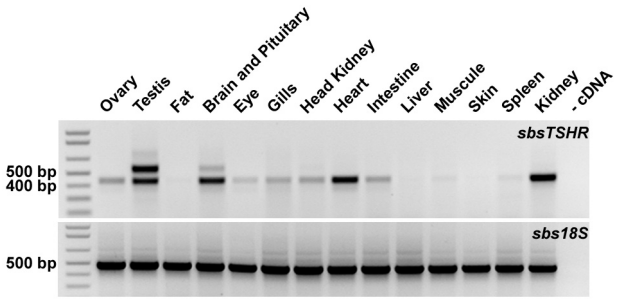

Figure 3.22 Analysis of *sbsTSHR* expression in adult fish tissues by RT-PCR. Specific primers encoding a segment of the extracellular domain were used for cDNA amplification. The presence of four introns in this area of the gene guarantees that amplification comes exclusively from the mRNA. The integrity of the RNAs was verified by uniform amplification of the *sbs18S rRNA* transcript.

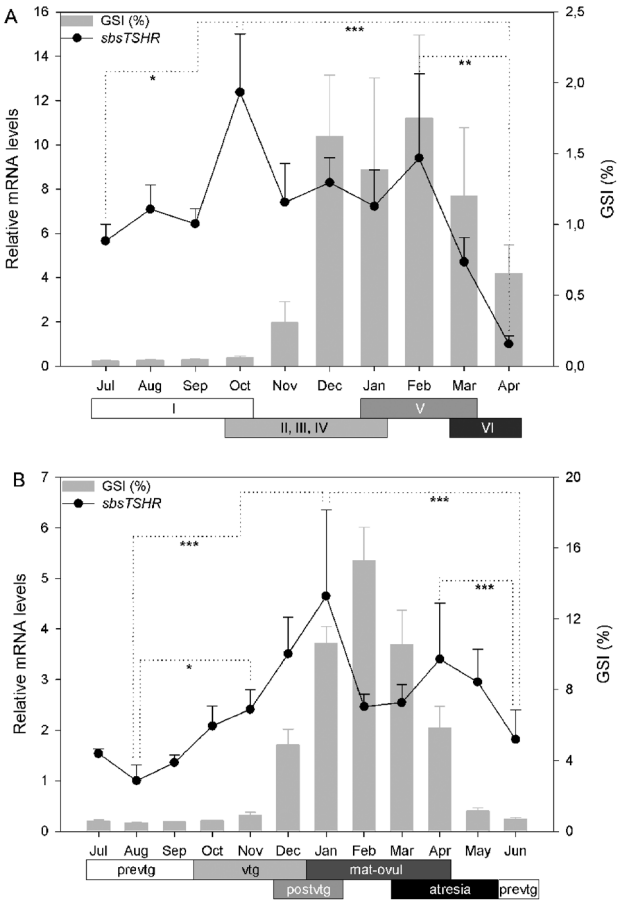

Figure 3.23 Relative changes in gonadal *sbsTSHR* expression of male and female sea bass, sampled during their first gonadal recrudescence. (**A**) Changes in male gonadosomatic index (GSI; gonad weight/body weight x 100, vertical grey bars) and testis *sbsTSHR mRNA* (black line plot). Levels are expressed as a proportion of the mean value in April, set as 1. Data are presented as mean ± SEM (n = 5 fish/month except in October, November and April where n = 4 fish/month). One-way ANOVA was performed (P= 0.026). Significant differences identified by ANOVA and Holm-Sidak test are indicated. (**B**) Changes in female gonadosomatic index (vertical grey bars) and ovary sbsTSHR mRNA (black line plot). Levels are expressed as a proportion of the mean value in August, set as 1. Data are presented as mean ± SEM (n = 5 fish/month). One-way ANOVA was performed (P< 0.001). Significant differences identified by the Holm-Sidak test are indicated with *, ** and *** for P≤0.05, P≤0.01 and P≤0.001, respectively. The histological stages of gonadal development from the samples (see Histological analysis in section 2) are represented by horizontal bars below each graph.

Seasonal changes in *sbsTSHR* mRNA levels in female and male sea bass during the first ovarian and testicular recrudescence

Representative stained sections for each of the stages of sea bass testicular and ovarian development, showing seasonal changes in cell components have already been reported in section 3.3 (**Fig. 3.13** and **3.14**). Data collected on the GSI of the fish were described in detail in section 3.3 and are shown in this section in **Fig. 3.23**. Briefly, in both male and female, the GSI values were low during the summer and early fall (July-October). In males, the GSI (**Fig. 3.23**, A) started to increase in October to reach high levels in December remaining high during the recrudescence (**Fig. 3.13**, C and D) and full spermiation stages (**Fig. 3.14**, E). In females, the GSI (**Fig. 3.23**, B) rapidly increased from November on until it peaked in February, during the maturation-ovulation gonadal stage (**Fig. 3.14**, E). A progressive decrease of the GSI was then observed in both sexes from March onwards until low values were reached again.

In males, levels of *sbsTSHR* mRNA increased approximately twofold (P=0.014) between the immature stage and the beginning of the recrudescence stage. The levels remained high during mid and late recrudescence, started to decline at the end of the full spermiation stage, and reached the lowest values in the post-spawning stage (**Fig. 3.23**, A). This represents a twelve fold decrease from October to April (P<0.001).

In females, *sbsTSHR* mRNA levels were low during previtellogenesis (July-September). From then on, *sbsTSHR* expression increased progressively during active vitellogenesis and post-vitellogenesis, to peak in January at the end of post-vitellogenesis, beginning of maturation-ovulation period. This corresponds to a fourfold expression increment (P=0.001). *sbsTSHR* transcript levels remained high during all the maturation-ovulation stage, although a non significant decline was observed in February-March. The levels then decreased during the atresia stage to reach significantly (P<0.001) low levels in June.

Discussion

The present study describes the cloning of a cDNA from the European sea bass testis that codes for a TSHR. The isolated sbsTSHR contains all the structural features that characterize vertebrate glycoprotein hormone receptors such as, an extracellular N-terminal domain that represents more than half of the total length of the protein, seven transmembrane stretches of largely hydrophobic

amino acids connected by three intracellular and three extracellular loops, and an intracellular C-terminal tail. The higher sequence identity observed between sbsTSHR and mammalian TSHRs (57.8%-56%) when compared with the corresponding values obtained for sbsLHR (49%-48%) and sbsFSHR (46%-45%) (sections 3.1 and 3.2) points to a stronger structural preservation of the *TSHR* gene during evolution compared with the other two family members.

Similar to all other TSHRs, the extracellular domain of sbsTSHR is composed of: i) a signal peptide, ii) an N-terminal cysteine-rich region, commonly termed C-box1 (sbsTSHR Ser^{25}-Thr^{37}), iii) a motif composed of repeated LRRs (sbsTSHR His^{38}-Tyr^{280}) followed by the hinge region (sbsTSHR Pro^{281}- Leu^{426}) that can further be subdivided into iv) a central cysteine box named C-box2 (sbsTSHR Pro^{281}- Gly^{317}), v) the TSHR specific segment that in mammals is approximately 50 amino acids long although in the sea bass, this segment is larger due to an insertion of 8 amino acids (sbsTSHR Pro^{318}-Phe^{375}) and vi) a C-box3 (sbsTSHR Gly^{376}-Leu^{426}) positioned close to the transmembrane helix 1.

Based on sequence analysis, and considering the molecular model proposed for the human TSHR (hTSHR) (Kleinau et al., 2004), the sbsTSHR, like the mammalian (Parmentier et al., 1989; Nagayama et al., 1989; Misrahi et al., 1990) and fish TSHRs (Oba et al., 2000; Kumar et al., 2000; Vischer and Bogerd, 2003b; Goto-Kazeto et al., 2003), has nine typical LRRs (LRRI-IX) plus one additional (LRR0) at the N-terminus and another one at the C-terminal side (LRRX), summing a total of eleven LRRs (**Fig. 3.20**). Cys^{42} contributes to the formation of the LRR0, making the C-box1 an integral structural part of the LRR domain, while LRRX would be attached back-to-back to C-box2, very likely via a short turn/loop. LRRs are structural units, consisting of alternating parallel-arranged short β-strands and α-helixes. Structural determinants present in the LRRs of glycoprotein hormone receptors have been shown to be involved in ligand recognition specificity, mainly by establishing specific hormone-receptor electrostatic interactions (Kajava et al., 1995; Smits et al., 2003; Fan and Hendrickson, 2005).

There are ten conserved cysteine residues present in the extracellular domain of the sbsTSHR (Cys 26, 30, 32, 42, 284, 285, 302, 399,407 and 417), which are clustered into the three regions, C-box1, 2 and 3 refereed previously. Besides the above mentioned cysteines, the extracellular domains of primate TSHRs have one additional cysteine (Cys^{176} in the hTSHR) localized in the fifth LRR (Nagayama et al., 1989; Misrahi et al., 1990). In the sbsTSHR, like in all known fish TSHR sequences, this cysteine is substituted

by a threonine (sbsTSHR Thr^{177}). Cysteines from C-box1 were first thought not to be important for binding, since mutation of the first three in the hTSHR, did not affect TSH binding (Kosugi et al., 1992). Nevertheless, a disulfide-bonded loop involving these cysteines has been recently shown to be required in the formation of an epitope for the binding of human TSH autoantibodies, the cause of Graves' hyperthyroidism (Chen et al., 2001). Moreover, the fourth cysteine of this cluster (Cys^{42} in sbsTSHR) was found to be crucial in contributing to high affinity TSH binding and necessary for receptor trafficking to the cell surface (Chen et al., 2001), which is in agreement with the molecular model proposed for the hTSHR by Kleinau and co-authors (Kleinau et al., 2004).

The cysteines of C-box2 (sbsTSHR Cys 284, 285 and 302) and C-box3 (sbsTSHR Cys 399,407 and 417) interact via disulfide bonds (Rapoport, et al., 1998; Mueller et al., 2006) and it is known that mutations disrupting these disulfide bridges lead to constitutive activation (Ho et al., 2001; Ho et al., 2005). In addition, it has been postulated that portions of C-box2 and C-box3 are localized in close spatial proximity, and play a pivotal role as a common unit in transferring the signal from the LRR binding domain to the transmembrane domain (Kleinau et al., 2004; Mueller et al., 2006). The C-box2 residues predicted to interact with C-box3 belong to the highly conserved amino acid motif TYPSHCC (residues T^{279}-C^{285} in the sbsTSHR) and surrounding areas, while the C-box3 would contribute to the formation of the tightly packed signalling interface with residues of the also conserved motif DEFNPCED (residues D^{412}-D^{419} in the sbsTSHR). In the hTSHR, substitution of Ser^{281} (Ser^{282} in the sbsTSHR) in the conserved motif TYPSHCC by threonine or asparagine has been identified as an activating mutation *in vivo* (Duprez et al., 1997). This residue is conserved in all available fish TSHRs, except in striped bass, which has an arginine at the homologous position (striped bass TSHR Arg^{282}) (Kumar et al., 2000). This amino acid substitution was proposed to be one of the reasons for the very high constitutive activity observed for the striped bass TSHR (Farid et al., 2004). Nevertheless, substitution of Ser^{281} by an arginine in the hTSHR was recently reported to elicit a loss of basal and TSH-induced cAMP production, possibly due to strongly impaired cell surface expression of the receptor (Jaeschke et al., 2006). These results contrast with the ones reported for the recombinant striped bass TSHR that was activated when stimulated with bovine TSH (Kumar et al., 2000).

The extracellular domain of the sbsTSHR has four consensus sites for N-

linked glycosylation, compared with the six consensus sites present in the hTSHR. Only three of them, the first, second and the fourth, are conserved between human and sea bass TSHRs. All six potential N-linked glycosylation sites on the hTSHR extracellular domain were shown to be actually glycosylated in the context of a mammalian cell line (Nagayama et al., 2000). Although a decrease in the number of carbohydrate moieties affects cell surface expression of the hTSHR, there is no evidence that it impairs TSH-binding affinity (Nagayama et al., 2000).

Transcripts of the *sbsTSHR* were found to be widespread distributed on sea bass tissues. Among the analyzed tissues, the gonads, kidney, brain/pituitary and heart presented the strongest amplification signals (**Fig. 3.22**, upper panel). The heart includes the ventral aorta, site of thyroid follicles localization in bony fish. These results are in accordance with the ones reported for mammals (reviewed in Davis and Latif, 2002). Abundant extrathyroidal *TSHR* expression was also reported for the striped bass (Kumar et al., 2000), with transcripts being found in gonads while weak expressions was detected in the forebrain and muscle. In African catfish, abundant *TSHR* mRNA levels were reported in the cerebellum, brain and ovary. Lower levels were seen in testis, ventral aorta and pituitary and scarce expression in other tissues analyzed (Vischer and Bogerd, 2003b). Similar results were reported for the channel catfish (Goto-Kazeto et al., 2003). Nonetheless, transcripts of the Amago salmon *TSHRs* (*asTSHR-a* and *asTSHR-b*) are exclusively observed in the basibranchial region, with no amplification signal detected in gonads, liver, kidney or brain (Oba et al., 2000). Although the analysis of *sbsTSHR* tissue distribution should be viewed as qualitative not quantitative, the expression of the *sbsTSHR* in testis seems to be much higher than in ovary. Data from striped bass (Kumar et al., 2000) appear to be similar to that from sea bass. These sex-related differences could indicate that the TSH, acting through its gonadal receptor could be regulating the reproductive function differently in female and male sea bass. Conversely, tissue expression analysis of the African catfish *TSHR* revealed higher transcript levels in ovary (Vischer and Bogerd, 2003b). Considering that the gonads from the animals used in the African catfish analysis and here in the sea bass were in similar developmental stages (two post-vitellogenic females and two full spermiating males), these results suggest that the regulation of gonadal function by this hormone could also be dissimilar between fish species. Another interesting difference in the pattern of the *TSHR* expression is the abundance of *sbsTSHR* tran-

scripts found in sea bass kidney while weak or even absence of expression was reported for the other fish species (Oba et al., 2000; Kumar et al., 2000; Vischer and Bogerd, 2003b). The brain tissue is another example of dissimilar *TSHR* expression pattern between fish species (Oba et al., 2000; Kumar et al., 2000; Vischer and Bogerd, 2003b). Though mRNA expression does not always reflect protein levels and thus a biological function, these data suggest that the TSHR could be implicated in additional, hitherto unknown, physiological processes that could be differently regulated in each fish species.

Two different *sbsTSHR* cDNAs with 3' UTRs of 382 bp and 1020 bp were obtained. They reflect the use of different polyadenylation sites. Similarly, in the human thyroid gland three full length *hTSHR* transcripts with 3' UTRs ranging from 950 to 1941 bp have been identified (Kakinuma and Nagayama, 2002). The longest one appears to be a predominant transcript. Though many features of an mRNA can contribute to its translation, most control elements are located within the UTRs. It is widely accepted that 3' UTRs play crucial roles in transcript cleavage, polyadenylation and nuclear export, and in stability of transcripts and translational efficiency. In line with these observations, it has been shown that a 4 kb *hTSHR* transcript, with an approximately 1600 bp long 3'UTR, contains unknown elements which are involved in the decrease of the number of receptors on the surface of mammalian transfected cells. The authors suggested that the binding of trans-acting proteins to specific recognition sequences present in the 3'UTR of the *hTSHR* could be the reason for such decreased expression (Kakinuma et al., 1996).

The idea that TSH, acting through its gonadal receptor, could be regulating sea bass reproductive function differently in female and male lead us to investigate the seasonal changes in *sbsTSHR* mRNA levels in the gonads of both sexes during the first ovarian and testicular recrudescence. To our knowledge, this is the first study that provides data on the gonadal expression profile of the *TSHR* during the reproductive cycle of any vertebrate.

The ovarian expression of the *sbsTSHR* showed its lowest levels during previtellogenesis, increasing continuously during the progression of vitellogenesis, following the profile of the GSI increase. Maximum levels were reached immediately prior to spawning. Despite of the tendency of *sbsTSHR* mRNA to peak in the beginning of the maturation-ovulation stage, transcript levels remained high during all the spawning season, declining to lower levels only at the end of the reproductive cycle. These results suggest

that, at least in this species, TSHR could participate in active vitellogenesis and in the regulation of gamete maturation and ovulation. The *sbsTSHR* expression levels in males followed a profile somewhat different from the one observed in females. Whereas in the ovaries, the levels of *sbsTSHR* mRNA progressively increased during vitellogenesis, in the testes a significant increase occurring during the immature stage resulted in maximal levels to be reached at the beginning of the gonadal recrudescence. These values were maintained during the progression of gonadal recrudescence and spermiation period, declining to minimal levels only after the spermiation. These results suggest that in males, the TSHR would be involved in the regulation of processes that occur during the early stages of the gonadal development and also of gamete maturation and spermiation. The approximately twelvefold difference between maximal and minimal *sbsTSHR* mRNA levels could indicate that in the testis, the transcription of this gene is highly regulated.

With the available data, there are indications that point to a role of TSH in the vertebrate gonads. As early as 1978, it was known that in the mammalian testis a protein exists that binds thyroid stimulators (Davies et al., 1978). Further support, to the hypothesis that TSH could have a direct role in mammalian reproductive function, was brought by studies on rat (*Rattus norvegicus*) Sertoli cells-enriched cultures. Sertoli cells were then proposed to be the primary testicular site of TSH action (Hutson and Stocco, 1981). It has also been demonstrated that *TSHR* knockout mice, which are severely hypothyroid, are infertile (Marians et al., 2002). However, TSH and/or its cognate receptor have never been shown to be necessary for mammalian reproductive function.

Regarding teleosts, *in situ* hybridization studies of striped bass gonads confirmed the presence of *TSHR* transcripts in both testis and ovary but contrary to what was reported in mammals, striped bass *TSHR* was found to be transcribed in the ooplasm of maturing oocytes and spermatogenic cysts and not in the follicular cells (Kumar et al., 2000). The same cellular localization was described for the gonadal expression of *TSHβ* in orange-spotted and red-spotted groupers (Wang et al., 2004). Interestingly, *TSHβ* transcripts were reported to be more abundant in the testes than in ovaries and in gonads during sex reversal of both species (Wang et al., 2004).

TSH is known to have an important role in thyroid follicular survival, preventing apoptosis by promoting cell-matrix adhesion and cell cycle progression partly via the cAMP pathway (Li et al., 1999; Saavedra et al., 2002).

Apoptosis has been shown to be involved in the homeostasis of endocrine tissue function as well as in the regression due to hormone deprivation (Chinnaiyan and Dixit 1996). These phenomena have been observed in various endocrine organs, *e.g.* apoptotic cell death associated with the initiation of ovarian follicular atresia (Hughes et al., 1991) or the maintenance of the correct ratio between Sertoli cells and gametes during normal spermatogenesis (Lee et al., 1997). During the teleost spawning season, many thousands of gamete-forming cells within the gonads are recruited into the pool of cells that will mature and be spawned. In sea bass females, this recruitment occurs in a group synchronous fashion, resulting in 3-4 individual spawning episodes during 1-2 months period (Asturiano et al., 2000). In the ovary of female sea bass, successive clutches of cells are recruited from a heterogeneous population of oocytes at various stages of secondary growth phase (Alvariño et al., 1992). Males are actively spermiating during 3-5 months, with successive batches of spermatogenic growth and spermiation (Carrillo et al., 1995). In the testis of male sea bass, groups of immature spermatozoa remain together with maturing or mature gametes and spermatogenesis occurs in the spawning season simultaneously with spermiation. Considering the rather long and cyclic pattern of gamete development in the sea bass it is possible that the TSH acting through its gonadal receptor could be involved in the survival of the recruited pool of germ cells and ultimately contribute to the optimization of fecundity.

In conclusion, this section reports the isolation and characterization of a gonadal cDNA coding for a sea bass TSHR. This is the first report describing the gonadal seasonal changes of *sbsTSHR* transcripts in any vertebrate. The expression profile in female and male sea bass during the first ovarian and testicular recrudescence indicates that this receptor could be involved in the sea bass gamete physiology.

4. CONCLUSIONS

Conclusions

First: The cDNAs coding for sea bass FSH, LH and TSH receptors have been isolated and characterized. Their coding proteins contain all the structural features that characterize vertebrate glycoprotein hormone receptors, such as: a large extracellular N-terminal domain that includes a LRR region flanked by N-terminal and C-terminal cysteine-rich clusters, a rhodopsin-like seven transmembrane domain, and a short intracellular C-terminal tail.

Second: The mature proteins of the sbsLHR and sbsTSHR are highly conserved when compared to other fish or mammalian receptors; however, the sbsFSHR contains some remarkable differences. Among them, a distinct extracellular N-terminal cysteine-rich domain as regards to its length and cysteine number and the presence of an extra LRR, advancing a curvature and size of the binding domain distinct from human FSHR, which could influence the mode how the hormone is recognized by the receptor.

Third: The *sbsFSHR* is exclusively expressed in gonadal tissues, specifically in the follicular wall of previtellogenic and early-vitellogenic follicles. On the contrary, *sbsLHR* and *sbsTSHR* mRNAs were found to be widely distributed in sea bass somatic tissues resembling their mammalian counterparts and other *LGRs*.

Fourth: The *sbsFSHR* gene has an extra exon in the LRRs region of the extracellualr domain, whose existence could be the result of an internal exon duplication ocurring before the divergence of the Perciformes, Beloniformes and Tetraodontiformes lineages. Like in tetrapods, *sbsLHR* gene is intronless in the transmembrane coding region. Nonetheless, *sbsFSHR* has three introns in this region. Together, this information suggests a higher rate of diversification of the *FSHR* during vertebrate evolution compared to the *LHR*.

Fifth: *sbsFSH*R mRNAs include alternatively spliced transcripts originated by removal of exons from the extracellular domain. Likewise, *sbsLHR* and *sbsTSHR* mRNAs contain transcripts that differ in the length of their 3'UTRs and reflect the use of alternative polyadenylation cleavage sites. Their functional significance, if any, remains unknown.

Sixth: Recombinant sbsFSHR is specifically stimulated by bFSH, while sbsLHR is activated by both bLH and bFSH. Nevertheless, specific stimulation is observed when recombinant sea bass gonadotropins are used.

Seventh: In both sexes, the expression of the sea bass gonadotropin receptors and *StAR* changes throughout the reproductive cycle. While in male sea bass, both *sbsFSHR* and *sbsLHR* show different expression patterns, in females their expression patterns are strongly correlated. No strong relationship was found between sea bass gonadotropin receptors expression and plasma levels of important sex steroids.

Eight: Expression of the *sbsFSHR* is connected with early stages of gonadal development, but it is also highly expressed during the spermiation period in males and maturation-ovulation period in females. This expression profile suggests that the sbsFSHR may also be involved in the control of processes occurring during later stages of gametogenesis. Nevertheless, this profile is also in agreement with a cyclic pattern of gamete development that characterizes the group-synchronous type of gonadal development seen in sea bass.

Ninth: The expression profile of the *sbsLHR*, in both sexes, supports the already suggested involvement of LH in the regulation of the final stages of fish gamete maturation and ovulation/ spermiation.

Tenth: The expression profile of *StAR* suggests that StAR could be important for the synthesis of gonadal MIS, by mediating the rapid delivery of sterol substrate. Moreover, the significant linear relationship between sea bass *LHR* and *StAR* mRNA levels observed in both sexes, suggests that LH may induce *StAR* expression.

Eleventh: The seasonal changes in gonadal expression of a *TSHR* are reported for the first time in any vertebrate, and indicate a regulated role of this receptor in sea bass gonad physiology. The expression of *sbsTSHR* in females suggests that it could participate in active vitellogenesis and in the regulation of gamete maturation and ovulation, whereas in males, the TSHR would be involved in the regulation of processes that occur during the early stages of gonadal development and also gamete maturation and spermiation.

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Summary

In vertebrates, the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are the main regulators of gametogenesis and gonadal hormone production. To exert their biological actions, gonadotropins must bind to, and activate specific cell-surface receptors. The primary aim of the studies underlying this thesis was to investigate the role of gonadotropins in the sea bass (*Dicentrarchus labrax*) reproductive function by studying their cognate receptors. Two cDNAs encoding a FSH receptor (*sbsFSHR*) and a LH receptor (*sbsLHR*) were cloned and characterized. Their gene structure, expression, and functional activity were evaluated. Sea bass FSHR and LHR 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. *sbsLHR* is more conserved than *sbsFSHR* when comparing their genomic primary structures with other vertebrate gonadotropin receptors, particularly in the extracellular domain. Furthermore, two alternately spliced variants of the *sbsFSHR* were amplified. Both correspond to shorter transcripts originated by removal of exons from the extracellular domain. 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 both sea bass FSHR and LHR was observed when recombinant sea bass gonadotropins were used advancing specific interactions between sea bass gonadotropin receptors and their corresponding ligands. This behaviour resembles the one described for mammalian gonadotropins but differs from studies in other teleost where promiscuous activation was observed. The expression profiles of sea bass gonadotropin receptors were investigated in the gonads of both male and female during a complete reproductive cycle, in parallel with the expression of steroidogenic acute regulatory protein (*StAR*) gene and reproductive hormones. While in male sea bass,

both gonadotropin receptors show different expression patterns, in females their expression patterns were somehow similar. Expression of the *sbsFSHR* was connected with early stages of gonadal development, but increased expression was also observed during the spermiation period in males and the maturation-ovulation period in female, suggesting that this receptor may also be involved in the control of processes occurring during these stages of gonadal development. The expression profile of the *sbsLHR*, in both sexes, supports the already suggested involvement of LH in the regulation of the final stages of fish gamete maturation and ovulation/spermiation. *sbsStAR* expression was strongly correlated with *sbsLHR* expression. In addition, the expression profile obtained for *sbsStAR* suggests that this protein mediates the rapid delivery of sterol substrate for the synthesis of gonadal maturation inducing steroids. No strong relationship between gonadotropin receptor expression and plasma levels of the analysed sex steroids was seen. Further studies will be needed to understand how gonadotropins and sex steroids interact to regulate sea bass reproduction.

Recent studies reported abundant expression of *TSHR* transcripts in both the ovary and testis of some fish species. To help elucidate the physiological role that TSHR may have in the gonadal function of sea bass, a cDNA encoding a TSHR was isolated from the gonads of this fish species. The mature protein displays typical features of the members of the glycoprotein hormone receptor family. By RT-PCR analysis we demonstrate the extra-thyroidal expression of *sbsTSHR* in numerous tissues of the sea bass. Seasonal changes in *sbsTSHR* mRNA levels in female and male sea bass during the first ovarian and testicular recrudescence suggest that in females the TSHR could participate in active vitellogenesis and in the regulation of gamete maturation and ovulation, whereas in males, the TSHR would be involved in the regulation of processes that occur during the early stages of the gonadal development and also of gamete maturation and spermiation.

Resumen en castellano

Introducción

El proceso reproductor de los teleósteos se basa en una cadena temporal de acontecimientos complejos que ocurren en el eje cerebro-hipófisis-gónadas, en el cual, las hormonas gonadotropas juegan un papel fundamental, regulando los procesos de gametogenesis y esteroidogenesis gonadal. En peces, como en vertebrados superiores, se han aislado y caracterizado dos gonadotrofinas hipofisarias, la hormona estimulante de los folículos (FSH) y la hormona luteinizante (LH) (Li y Ford, 1998; Swanson et al., 2003). Ambas hormonas, junto con la hormona estimulante del tiroides (TSH), forman una familia de hormonas glicoproteicas químicamente relacionadas. Cada miembro de la familia presenta una estructura heterodimérica compuesta por una subunidad-α común unida, de forma no covalente, a una subunidad-β específica (Pierce y Parsons, 1981). Se conoce muy poco acerca de la participación de las gonadotrofinas, sobre todo de la FSH, en la reproducción de los teleósteos ya que sólo se dispone de ensayos para su medición solamente en unas pocas especies de salmónidos (Prat et al., 1996; Gomez et al., 1999; Davies et al., 1999; Santos et al., 2001). En estas especies se ha visto que los niveles de FSH en plasma son altos durante los estados tempranos de la gametogénesis y disminuyen en el momento en que se inician las fases finales de la gametogénesis, coincidiendo con la elevación de los niveles de LH (Suzuki et al., 1988a; Prat el al., 1996; Breton et al., 1998). Ambas gonadotrofinas son equipotentes en la estimulación de la producción de 17β-estradiol (E2) pero la LH es más potente que la FSH en la estimulación del esteroide inductor de la maduración MIS (17α,20β-P; 17α,20β-dihydroxy-4-pregnen-3-one) (Suzuki et al., 1988b). Esto sugiere que la FSH juega un papel importante en la vitelogénesis y en las etapas más tempranas de la espermatogénesis y la LH en la maduración y la ovulación de los oocitos y en la espermiación y maduración espermática.

Las gonadotrofinas ejercen su acción uniéndose a receptores de membrana específicos localizados en la superficie de las células diana. En vertebrados superiores se han descrito dos tipos de receptores específicos para las gonadotrofinas uno que liga la FSH (FSHR) y otro que liga la LH (LHR). El *FSHR* se expresa solamente en las células de la granulosa en el ovario y en las células de Sertoli en el testículo. El *LHR* se expresa mayormente en las células

de la teca y de la granulosa de los folículos preovulatorios y en las células de Leydig en los testículos (Dias et al., 2002; Ascoli et al., 2002).

Los receptores de las gonadotrofinas, FSHR y LHR, junto con el receptor de la TSH (TSHR), constituyen la familia de los receptores de las hormonas glicoproteicas, una subclase de la gran superfamilia de receptores acoplados a proteínas G (GPCRs) que actúan como transductores de señales a través de la membrana celular (Vassart et al., 2004). Los tres receptores de glicoproteínas están constituidos por una sola cadena proteica en la que se pueden identificar tres dominios: un largo dominio N-terminal expuesto en la cara extracelular de la membrana, una región de plegamiento con 7 hélices α transmembranales conectadas por 3 asas intracelulares y 3 asas extracelulares y un dominio intracelular C-terminal. Una de las principales características de los receptores de glicoproteinas es la existencia de un dominio extracellular, compuesto por series de repeticiones ricas en leucinas (LRR), flanqueado por regiones ricas en cisteínas. Este dominio constituye más de la mitad de la proteína y contribuye al reconocimiento y la especificidad de unión de la hormona al receptor (Dias et al., 2002; Ascoli et al., 2002). La unión de las gonadotrofinas a sus correspondientes receptores produce micro-agregaciones y modificaciones en la estructura del receptor, en particular en el acoplamiento de su región intracelular a proteínas G. Estas proteínas G reaccionan con una serie de factores citoplasmáticos que dan lugar a la activación en cadena de distintas rutas intracelulares. En los mamíferos, su ruta principal de señalización es la ruta de la proteina quinasa A (PKA) mediada por un aumento de AMP ciclíco (cAMP) intracelular (Richards, 1994).

Los genes de los receptores de las gonadotrofinas de mamíferos son genes de copia única y comparten una elevada similitud estructural. Los genes del *FSHR* están constituidos por diez exones y nueve intrones mientras que los genes del *LHR* tienen once exones (Tsai-Morris, 1991; Koo et al., 1991; Heckert et al., 1992; Atger et al., 1995; Gromoll et al., 1996). El exón adicional que presentan los *LHR*s respecto a los *FSHR*s está insertado entre el exón nueve y diez. Los exones 2-8, que codifican LRRs, tienen tamaños y secuencias muy similares. Cada uno de estos exones está interrumpido por un intrón en una posición homóloga de cada motivo LRR. En comparación con cada uno de los exones 2-8, el exón 9 les duplica en tamaño y codifica dos LRRs consecutivas. El último exón de cada gen de estos receptores codifica una pequeña fracción del dominio extracelular y la totalidad de los dominio transmembrana e intracelular.

En peces, los primeros estudios sobre receptores de las gonadotrofinas no dejaban clara su dualidad (ver Bieniarz y Kime, 1986; Breton et al., 1986; Kanamori et al., 1987, Kanamori y Nagahama, 1988). Los estudios de unión al ligando realizados por Yan et al. (1992) y Miwa et al. (1994) en el salmón plateado (*Oncorhynchus kisutch*), demostraron por primera vez que, en la superficie de las células somáticas de ovarios y testículos de los peces teleósteos existían dos tipos de receptores de gonadotrofinas. En el ovario, el FSHR se detectó en las células de la teca, en las células de granulosa y en el tejido conectivo intersticial, mientras que el LHR se detectó en las células de la granulosa. En el testículo, el FSHR se localizó en las células de Sertoli y el LHR en las células de Leydig. Posteriormente, y por primera vez en los teleósteos, Oba et al. (1999a,b) lograron aislar y caracterizar funcionalmente los cDNAs completos que codifican tanto el LHR como el FSHR a partir de ovario y testículo de salmón "amago" (*Oncorhynchus rhodurus*), demostrando definitivamente la dualidad de los receptores de gonadotrofinas, al menos en los salmónidos. En la actualidad, la existencia de estos dos tipos de receptores se ha demostrado también en peces de otros taxa, mediante el aislamiento de sus respectivos cDNAs codificantes (Oba et al., 2001; Wong et al., 2004; Kumar et al., 2001a,b; Bogerd et al., 2001; Vischer y Bogerd, 2003a; Laan et al., 2002; Kwok et al., 2005; Bobe et al., 2003; Maugars y Schmitz, 2006).

Los receptores de gonadotrofinas de los mamíferos responden de una manera muy específica a sus respectivos ligandos, existiendo menos de un 0,1% de actividad cruzada (Braun et al., 1991; Moyle et al., 1994). Por el contrario, la dualidad funcional y especificidad de los receptores de la FSH y de la LH parece ser menos aparente en los peces teleósteos. Los ensayos realizado *in vitro* con receptores recombinantes de dos especies de pez gato, *Clarias gariepinus* y *Ictalurus punctatus*, y del pez cebra *(Danio rerio)*, sugieren que los FSHRs no son selectivos para la FSH, aunque la FSH es ligeramente más potente que la LH como ligando, mientras que los LHRs son altamente selectivos para la LH (Bogerd et al., 2001; Vischer y Bogerd, 2003a; Vischer et al., 2003; Kumar et al., 2001a,b; Kwok et al., 2005; So et al., 2005). En el salmón plateado se habían obtenido previamente resultados similares, ya que el FSHR distinguía entre la FSH y la LH, mientras que el LHR sólo unía la LH (Yan et al., 1992; Miwa et al., 1994). No obstante, estos datos contrastan con los resultados obtenidos en otras especies de peces salmónidos. La estimulación del FSHR de salmón "amago" con FSH y LH de salmón "chum"

(*Oncorhynchus keta*), mostró que este receptor se activaba específicamente con FSH pero no con LH. Sin embargo, células transfectadas con el LHR respondieron con una elevada sensibilidad a la LH y, con menor magnitud, también a la FSH (Oba et al., 1999a,b).

La lubina es un modelo consolidado de investigación en fisiología de la reproducción en peces (Zanuy et al., 2001). Teniendo en cuenta la importancia de las gonadotrofinas en la regulación fisiológica de la reproducción, y con objeto de comprender mejor su papel en este proceso, parecía conveniente estudiar en profundidad sus receptores específicos, sobre los cuales no existía ningún estudio, no sólo en esta especie, si no en ninguna otra del grupo de los Perciformes.

Objetivos de esta tesis doctoral

El objetivo general de esta tesis doctoral fue profundizar en el conocimiento del papel de las gonadotrofinas en la función reproductora de un teleósteo marino, la lubina (*Dicentrarchus labrax*), mediante el estudio de sus receptores. Los objetivos específicos fueron las siguientes:

- Investigar las características moleculares estructurales del FSHR y el LHR de lubina
- Determinar la conservación estructural de sus genes codificantes
- Identificar los lugares de expresión de estos genes
- Evaluar la especificidad de unión al ligando de los receptores de gonadotrofinas de la lubina
- Estudiar cómo varia la expresión de los genes de los receptores de las gonadotrofinas durante el ciclo reproductivo de la lubina
- Investigar cómo se relaciona la expresión de estos genes con el crecimiento gonadal, con el perfil temporal de hormonas sexuales en plasma y con la expresión de la proteína responsable de la respuesta aguda de la esteroidogénesis (*StAR*)
- Investigar si la TSH, actuando a través de su receptor gonadal, podría participar en la regulación de la función reproductora

Resultados

3.1 Caracterización molecular del receptor de la hormona estimulante de los folículos de la lubina Europea: clonación del cDNA, análisis de la expresión y actividad funcional

En este apartado se describe la clonación de un cDNA de testículo de lubina de 3134 pb que codifica un polipéptido de 702 aminoácidos, el cual corresponde al FSHR de lubina (sbsFSHR). En la proteína madura del sbsFSHR se identificaron ciertas características propias de los receptores de las gonadotropinas (Vassart et al., 2004), incluyendo un largo dominio extracelular N-terminal y un dominio transmembranal típico de los GPCRs. Sin embargo, el sbsFSHR presenta claras diferencias estructurales al compararlo con sus homólogos en otros peces y en mamíferos (**Fig. R.1**). Entre ellas cabe destacar que la región N-terminal rica en cisteínas es distinta en tamaño y número de residuos de cisteína. En los FSHRs de mamíferos la región N-terminal rica en cisteínas contiene cuatro residuos de cisteína. En el sbsFSHR

Figura R.1 Alineamiento múltiple de las secuencias de amino ácidos de FSHRs mostrando la región N-terminal rica en cisteínas y parte de la región LRR del dominio extracelular. Números de acceso: humano (Hum) AY429104, lubina (Sbs) AY642113, tilapia del Nilo (Til) AB041762, dorada (Sb) AY587262, salmón "amago" (As) AB030012, salmón Atlántico (Ats) AJ567667, trucha arco-iris (Rtr) AF439405, pez cebra (Zbf) AY278107, pez gato Africano (Ac) AJ012647, pez gato (Cc) AF285182. Los números a la derecha se refieren a la posición de los amino ácidos en la proteína. La secuencia del sbsFSHR está destacada en negrita. Aquellos residuos conservados en todas las secuencias se encuentran en un recuadro gris. Las barras negras encima de la secuencia indican el motivo (X-L-X-L-X) de la hoja-β de las LRRs. Se indica la posición de los intrones amplificados en esta región.

esta región tiene solamente dos cisteínas. Además, en el sbsFSHR existe una inserción de nueve aminoácidos en esta región que incluye uno de los dos residuos de cisteína. A continuación de esta región N-terminal se encuentra el dominio con LRRs. En general, las LRRs están implicadas en interacciones proteína-proteína, y están formadas por unidades estructurales del tipo hoja β corta/ hélice α conectadas entre si. En el dominio extracelular del sbsFSHR se identificaron nueve motivos LRR, que coinciden con las repeticiones descritas en el FSHR humano (Fan y Hendrickson, 2005). No obstante, el sbsFSHR posee una LRR adicional codificada por una inserción de 25 aminoácidos. La presencia de esta LRR extranumeraria podría indicar que el dominio de unión al ligando de este receptor, a pesar de presentar una misma configuración, tiene un tamaño y curvatura distintos a los descritos para el FSHR humano, lo que podría afectar a la unión de la hormona. En la región C-terminal, que se sigue al dominio LRR, se identificaron seis cisteínas conservadas, pero sus posiciones espaciales son distintas a las existentes en mamíferos, debido a una deleción de 30 aminoácidos.

Por otra parte se realizó un análisis filogenético usando el método de Neighbor-Joining para inferir la relación evolutiva del *sbsFSHR* con otros miembros de la familia de los receptores de las hormonas glicoproteicas. En este análisis, el *sbsFSHR* se agrupa con los receptores de su propio linaje, presentando una relación más cercana con los *FSHRs* de tilapia (*Oreochromis niloticus*) y de dorada (*Sparus aurata*). El análisis parcial de la estructura del *sbsFSHR* reveló que este gen no posee el intron específico de los *LHRs*. Además, se detectó la presencia de tres intrones en el dominio transmembranal, así como la existencia de un intron en la inserción que codifica 25 aminoacidos y que correspondería a una LRR adicional. Esto da lugar a la existencia de un exon adicional en el dominio extracelular del *sbsFSHR* al compararlo con el gen *FSHR* humano (**Fig. R.1**). El análisis de expresión del *sbsFSHR* mediante RT-PCR mostró que este gen se expresa exclusivamente en las gónadas (**Fig. R.2, A**). Además, el análisis *Northern blot* de RNA total y poly $(A)^+$ de testículos y ovarios de lubina con sondas específicas para el *sbsFSHR* mostró dos señales débiles de hibridación (**Fig. R.2, C**). Una de 3 kb, que corresponde al tamaño del RNA mensajero (mRNA) completo, y otra de tamaño inferior que podría corresponder a una forma de procesamiento alternativo del *sbsFSHR*. Con el objetivo de establecer la localización celular y el tipo de oocitos en los que se expresa el *sbsFSHR*, se llevaron a cabo hibridaciones *in situ* en secciones de ovario de hembras maduras. Empleando

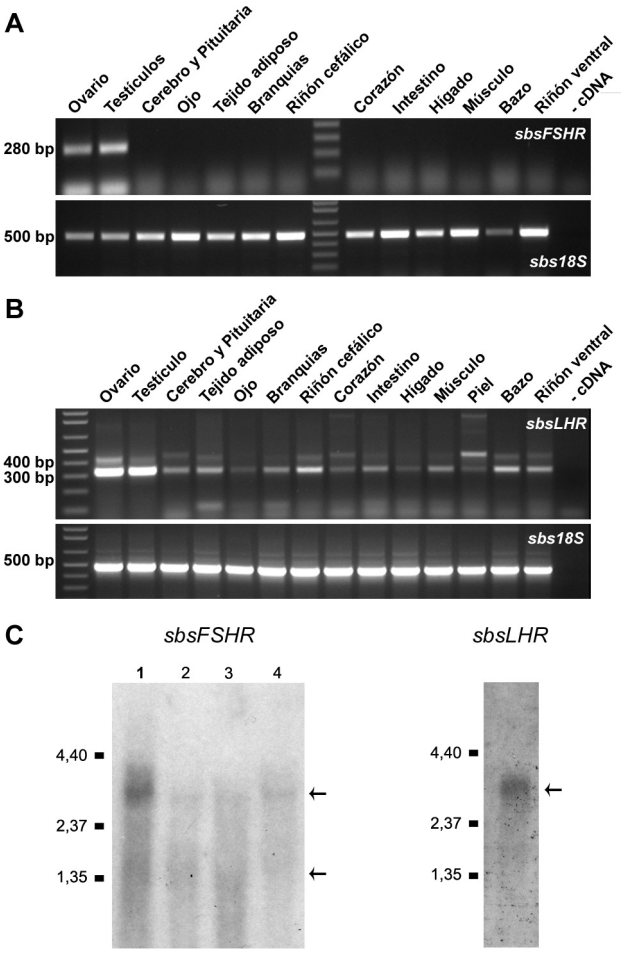

Figura R.2 Análisis de la expresión del *sbsFSHR* (**A**) y del *sbsLHR* (**B**) en tejidos de peces adultos mediante RT-PCR (transcripción inversa seguida de amplificación cíclica con polimerasa). Utilizando cebadores específicos, se amplificaron segmentos correspondientes a los dominios transmembranal y extracelular de los cDNAs del *sbsFSHR* y del *sbsLHR*, respectivamente. La presencia de intrones en estas regiones garantiza la amplificación exclusivamente del cDNA. La integridad de los RNAs utilizados se verificó mediante amplificación uniforme del transcrito del *18S rRNA* de lubina. (**C**) Análisis *Northern blot*. Hibridación de RNA poly $(A)^+$ de ovario de lubina (calle 1) y RNA total de testículo (calles 2 y 3) y ovario (calle 4) de dos animales adultos con el cDNA completo del *sbsFSHR*, usado como sonda (panel de la izquierda). Hibridación de RNA poly $(A)^+$ de ovario con el cDNA completo del *sbsLHR*, usado como sonda (panel de la derecha), el RNA en esta membrana es idéntico al de la calle 1 en el panel de la izquierda. El tiempo de exposición de esta membrana fue bastante más largo que para el *sbsFSHR*. Las flechas indican la posición de dos mRNAs del *sbsFSHR* de 1,8 y 3 kb, y un transcrito del *sbsLHR* de aproximadamente 3 kb.

una sonda antisentido, se observó una expresión fuerte en los oocitos en previtelogénesis. La señal de hibridación también se detectó en las células foliculares de oocitos en estadios tempranos y tardíos del proceso de vitelogénesis, lo cual es concordante con un posible papel del sbsFSHR en etapas tempranas del desarrollo de los foliculos. En otros trabajos se ha descrito la existencia de mRNAs del *FSHR* de distintos tamaños, como resultado de un procesamiento alternativo. Se especula que este tipo de isoformas podrían modificar la acción del FSHR interfiriendo en su expresión, en el procesamiento intracelular del receptor nativo ó alterando la afinidad de unión de la hormona al receptor. Para detectar la posible existencia de formas alternativas de expresión para el *sbsFSHR*, se amplificaron, mediante RT-PCR, los exones que codifican su dominio extracelular. Se obtuvieron varios productos de PCR que corresponden al cDNA completo y a dos variantes resultantes del procesamiento alternativo de su mRNA. Ambas formas son transcritos más cortos que tienen su origen en la eliminación de exones.

Para ensayar la funcionalidad del sbsFSHR se expresó su cDNA en células de riñón embrionario humano (HEK293). Se ha descrito que la expresión de un número elevado de receptores por célula puede dar lugar a un aumento del nivel basal de activación del receptor (Tao et al., 2000). Con el objetivo de expresar una cantidad reducida de receptores por célula, se generaron clones HEK293 conteniendo el cDNA del *sbsFSHR* integrado en su genoma. Sobre

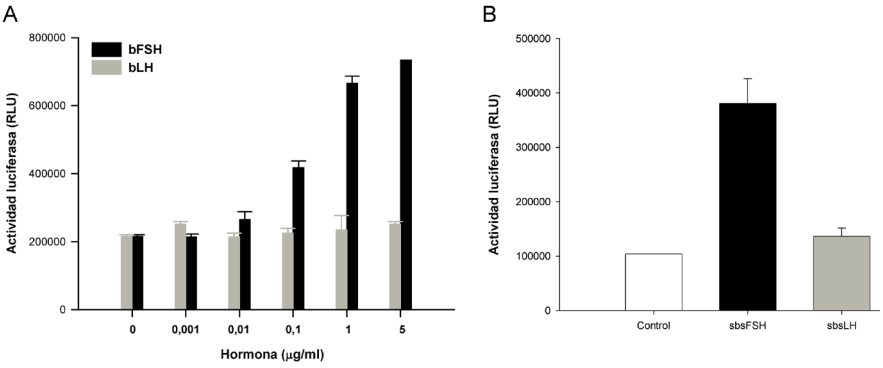

Figura R.3 Análisis funcional del sbsFSHR. Sobre un clón HEK293 conteniendo el cDNA del *sbsFSHR* integrado en su genoma, se efectuaron transfecciones transitorias del plásmido pCRE-Luc. (**A**) Actividad luciferasa después de un tratamiento de 6 horas con diferentes dosis de bFSH y bLH. (**B**) Actividad luciferasa después de un tratamiento de 5 horas con una concentración única de medio condicionado de células de ovario de hámster Chino (CHO-K1) (Control) o de células CHO-K1 que producen de forma estable FSH y LH recombinantes de lubina.

un clon que presentaba un nivel bajo de expresión del receptor, se efectuaron transfecciones transitorias del plásmido pCRE-luc que contiene el gen de la luciferasa bajo el control de un promotor dependiente de factores de transcripción que se unen a sitios CRE (Cyclic AMP Responsive Element). La estimulación de los receptores de gonadotrofinas da lugar a un aumento del cAMP intracelular que finalmente conduce a la expresión de genes que contienen sitios de unión CRE en sus promotores. Por lo tanto, un incremento de la actividad luciferasa como resultado de su expresión en el plásmido pCRE-luc, constituye una medida indirecta del incremento de cAMP intracelular. Las células HEK293 que coexpresaban el *sbsFSHR* y pCRE-luc se estimularon con diferentes cantidades de FSH y LH bovinas (bFSH y bLH). Los resultados obtenidos demuestran que el sbsFSHR se activa en presencia de bFSH pero no en presencia de bLH (**Fig. R.3, A**). La estimulación de esas mismas células con FSH y LH recombinantes de lubina mostró los mismos resultados (**Fig. R.3, B**), indicando que el sbsFSHR es selectivo para FSH, a diferencia del comportamiento de este mismo receptor en otras especies de teleósteos (Kumar et al., 2001a; Bogerd et al., 2001; So et al., 2005). Los resultados presentados en este apartado confirman que se ha clonado un cDNA a partir de tejido gonadal de lubina que codifica un FSHR funcional.

3.2 Caracterización molecular del receptor de la hormona luteinizante de la lubina Europea: clonación del cDNA, estructura del gen, análisis de la expresión y actividad funcional

Este apartado describe la clonación de un cDNA de 3172 nucleótidos que codifica un LHR de lubina (sbsLHR). En la proteína madura de 700 aminoácidos se identificaron caracteristicas estructurales de los miembros de la familia de los receptores de las hormonas glicoproteicas, incluyendo un largo dominio extracelular N-terminal compuesto por nueve LRR flanqueadas por regiones ricas en cisteínas, seguido por un dominio transmembrana compuesto por 7 segmentos de residuos esencialmente hidrofóbicos y un corto dominio intracelular C-terminal (Ascoli et al., 2002). El sbsLHR presenta un grado de identidad muy alto con las secuencias proteicas de LHRs de otras especies de peces (86-45,5%). Estos valores se reducen cuando se compara al sbsLHR con las secuencias proteicas de LHRs de mamíferos, oscilando entre 49-48%.

La relación evolutiva del *sbsLHR* con otros miembros de la familia de los receptores de las hormonas glicoproteicas se dedujó mediante un análisis filogenético usando el método de Neighbor-Joining. En ese análisis el sbsLHR se agrupa con los receptores de su respectivo linaje.

El estudio de la estructura del gen *sbsLHR* reveló la presencia de once exones y 10 intrones, incluyendo el intron específico de los *LHRs*. Los intrones del *sbsLHR* son, de manera general, más pequeños que los del *LHR* de rata y humano, aunque sus posiciones están conservadas; con la excepción del intrón 10 que se encuentra desviado 12 nucleótidos en el receptor de lubina. Los dominios transmembranal e intracelular del *sbsLHR* están codificados por un mismo exón. En conjunto, estos resultados sugieren que, durante la evolución de los receptores de gonadotrofinas de los vertebrados, la estructura del *sbsLHR* se ha conservado más que la del gen *sbsFSHR*, particularmente en el dominio extracelular. El análisis de la distribución tisular del mRNA de *sbsLHR* mediante RT-PCR, demostró que la expresión de este gen no es específica de las gónadas, ya que su mRNA se encuentra ampliamente distribuido por los tejidos somáticos de la lubina, aunque a niveles inferiores (**Fig. R.2, B**). Este patrón de expresión es semejante a lo encontrado en mamíferos y otras especies de peces (Rao, 2001; Oba et al., 1999a; Kumar et al., 2001b; Vischer y Bogerd, 2003a; Kwok et al., 2005). Haciendo uso de la técnica de 3'RACE se han identificado tres mRNAs del *sbsLHR* que difieren

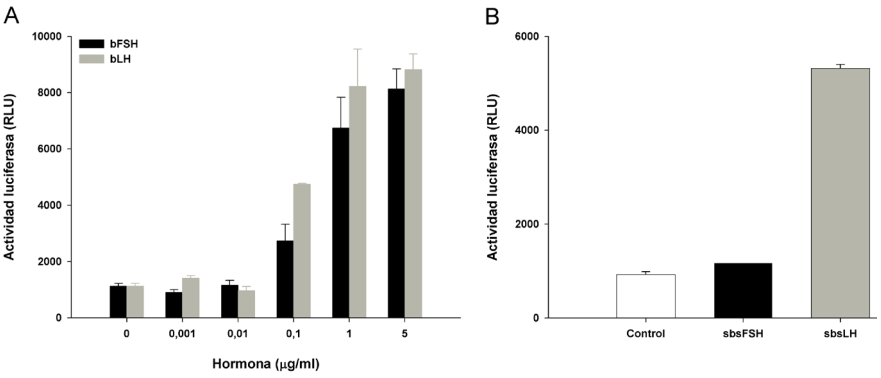

Figura R.4 Análisis funcional del sbsLHR. Se desarrollaron clones de células HEK293 que contienen de manera estable el cDNA del *sbsLHR* y el plásmido pCRE-luc. (**A**) Actividad luciferasa después de un tratamiento de 5 horas con diferentes dosis de bFSH y bLH. (**B**) Actividad luciferasa después de un tratamiento de 5 horas con una concentración única de medio condicionado de células de ovario de hámster Chino (CHO-K1) (Control) o de células CHO-K1 que producen de forma estable FSH y LH recombinantes de lubina.

en el tamaño de sus regiones no traducidas en el extremo 3' (3' UTR). Estos transcritos aparecen como resultado del uso de señales de poliadenilación alternativas. En un análisis *Northern blot* sobre RNA poly $(A)^+$ de ovario de lubina usando una sonda específica para *sbsLHR*, se detectó una señal de hibridación de 3 kb poco nitida, que podría corresponder a varios transcritos del *sbsLHR* que difieren ligeramente en tamaño (**Fig. R.2, C**).

La funcionalidad del sbsLHR se ensayó en clones de células HEK293 que contenían de manera estable el cDNA del *sbsLHR* y el plásmido pCRE-luc. Estas células se estimularon con diferentes cantidades de bFSH y bLH. El tratamiento con bLH originó un aumento de la actividad luciferasa de una forma claramente dosis-dependiente. Igualmente, la bFSH fue capaz de estimular al sbsLHR (**Fig. R.4, A**). Estos resultados son semejantes a los obtenidos con los LHRs de salmón "amago" y pez cebra (Oba te al., 1999a; So et al., 2005). No obstante, cuando se emplearon gonadotrofinas recombinantes de lubina se observó una estimulación específica del sbsLHR (**Fig. R.4, B**). El hecho de que el sbsLHR responda de manera distinta a la FSH de lubina y a la bovina debe estar basado en la existencia de alguna diferencia estructural entre las subunidades β de estas dos hormonas. En conjunto, los estudios de activación de los receptores de gonadotropinas de lubina sugieren que, a diferencia de otros peces, las interacciones entre estos receptores y sus respectivas hormonas son específicas.

Los resultados descritos en este apartado confirman que se ha clonado un cDNA, a partir de tejido gonadal de lubina, que codifica un LHR funcional. Junto con la clonación del *sbsFSHR*, este estudio proporciona una base para estudios futuros sobre la función de los receptores de gonadotrofinas en la regulación de la reproducción de esta especie de pez perciforme.

3.3 Variación estacional de la expresión gonadal de los receptores de las gonadotrofinas y de la proteína responsable de la respuesta aguda de la esteroidogénesis (StAR), y su relación con los perfiles plasmáticos de hormonas sexuales en la lubina Europea

En los mamíferos, la gametogénesis está regulada por la interacción de factores sistémicos y otros propios de la gónada, y la importancia de cada tipo de regulación varía dependiendo del estado de desarrollo de la misma. En los teleósteos existen sistemas similares de regulación. En los vertebrados, las

gonadotrofinas (FSH y LH) son reguladores esenciales de la gametogénesis y de la producción de esteroides gonadales (Chappel y Howles, 1991). Sus acciones están mediadas por la activación de sus respectivos receptores. A pesar de la importancia funcional de estos receptores, en teleósteos se dispone de muy poca información en lo que respecta a su expresión estacional. En este estudio se desarrolló un método de RT-PCR cuantitativo para medir los perfiles de expresión de los receptores de gonadotrofinas durante la primera maduración gonadal de machos y hembras de lubina. Asimismo, se investigaron posibles relaciones entre esos perfiles y el de la expresión de *StAR* y los perfiles plasmáticos de hormonas esenciales en el proceso reproductor, como el E2 en hembras y la 17β-hidroxilo-4-androstene-3,11-diona (11-KT) en machos. La proteína StAR media el transporte de colesterol a la mitocondria. El colesterol es el sustrato primario en la síntesis de todas las hormonas esteroideas. Este transporte es pues un paso limitante en la síntesis de los esteroides sexuales.

El nivel de expresión de los genes analizados cambió durante el ciclo reproductor en ambos sexos (**Fig. R.5**). Mientras que en machos, los receptores de gonadotrofinas presentaron patrones de expresión diferentes (**Fig. R.5, A** y **B**), en hembras los perfiles de expresión del *sbsFSHR* y *sbsLHR* eran similares (**Fig. R.5, D** y **E**). La expresión del *sbsFSHR* se asociaba con estadios tempranos del desarrollo gonadal, pero se observan igualmente niveles elevados de expresión durante el periodo de espermiación en machos y maduración-ovulación en hembras (**Fig. R.5, A** y **D**), lo que sugiere que este receptor también estaría implicado en procesos que ocurren durante estos estadios de desarrollo gonadal. No obstante, los niveles elevados de expresión del *sbsFSHR* durante la espermiación en machos y la maduración-ovulación en hembras podrían estar relacionados con la estrategia reproductiva de la lubina, ya que en los machos de lubina, la espermatogénesis ocurre simultáneamente con la espermiación y en los ovarios de las hembras coexisten grupos de oocitos en distintas fases de crecimiento secundario, que se reclutan sucesivamente para su maduración final (Mayer et al., Asturiano et al., 2000). El perfil de expresión del *sbsLHR* en ambos sexos (**Fig. R.5, B** y **E**) indica la implicación de la LH en la regulación de las fases finales de la maduración y la ovulación/ espermiación de los gametos de peces, tal y como se ha sugerido previamente. La expresión de *StAR* está altamente correlacionada con la expresión del *LHR* (**Fig. R.5**). Los valores más altos de expresión de este gen se observaron en las fases de espermiación y maduración-ovulación en machos y hembras respectivamente. Este perfil de expresión podría estar

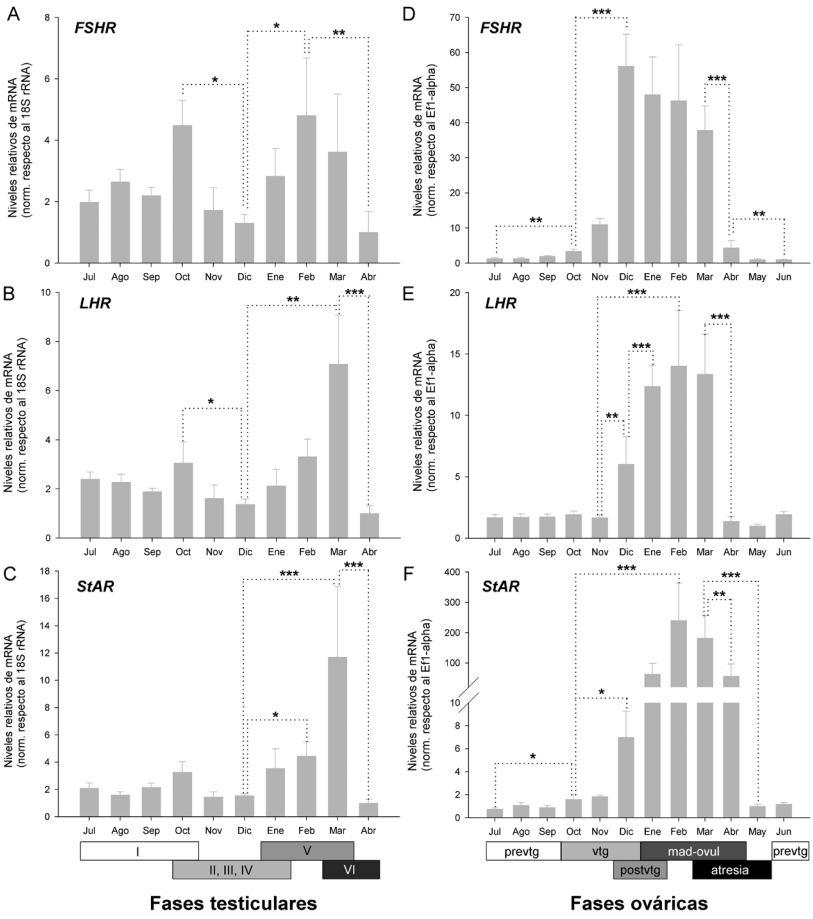

Figura R.5 Variaciones estaciónales de los niveles relativos de la expresión de *FSHR*, *LHR* y *StAR* en machos (**A, B, C**) y hembras (**D, E, F**) de lubina muestreados durante su primera recrudescencia gonadal. Los valores de expresión en machos se han normalizado respecto a la expresión de *18S rRNA* y están representados como una proporción del valor de la media de abril, establecido como 1. Los valores de expresión en hembras se han normalizado respecto a la expresión del gen del factor de elongación 1-alfa (Ef1-alpha) de lubina, ajustado para compensar los cambios de su expresión a lo largo del ciclo reproductor, y representados como una proporción del valor de la media de mayo, fijado como 1. Los datos se han representado como la media ± el error estándar de la media (n = 5 peces/ mes). Las diferencias estadísticamente significativas identificados por ANOVA de una vía y por test de comparación múltiple, están indicados con *, ** y *** para $P \leq 0.05$, $P \leq 0.01$ y $P \leq 0.001$, respectivamente. Las fases histológicas del desarrollo testicular y ovárico, clasificadas según Begtashi et al. (2004) y Asturiano et al. (2000), de las muestras estudiadas están representadas por barras horizontales en la parte inferior de cada gráfica. Fases del desarrollo testicular: fase I, inmaduro; fase II, recrudescencia temprana; fase III, recrudescencia; IV, recrudescencia tardía; fase V, espermiación; VI, postpuesta. Fases del desarrollo ovárico: previtelogenesis (prevtg), vitelogenesis (vtg), post-vitelogenesis (postvtg), maduración-ovulación (mat-ovul) y atresia.

relacionado con la síntesis de esteroides inductores de la maduración, la cual requiere disponibilidad del sustrato colesterol en la mitocondria (Stocco et al., 2005). Sin embargo, no se observa una relación entre la expresión de los receptores de gonadotrofinas y el perfil plasmático de los esteroides analizados, 11-KT en machos y E2 en hembras (**Fig. R.6**). En ambos sexos, los niveles de expresión del *sbsFSHR* son más altos que los del *sbsLHR*. En conjunto estos resultados muestran variaciones en la expresión de los receptores de gonadotrofinas relacionados con el sexo y la estacionalidad, y establecen una relación con la respuesta aguda de la esteroidogénesis y con los niveles plasmáticos de hormonas sexuales. Este estudio constituye un primer paso para, en futuros trabajos, comprender como las gonadotropinas y los esteroides sexuales interactúan regulando la reproducción en lubina.

3.4 Caracterización molecular de un receptor de la hormona estimulante del tiroides de la lubina Europea y variación estacional de su expresión gonadal

La hormona estimulante del tiroides es una glicoproteína sintetizada y secretada por los tirotropos del lóbulo anterior de la glándula pituitaria. Esta

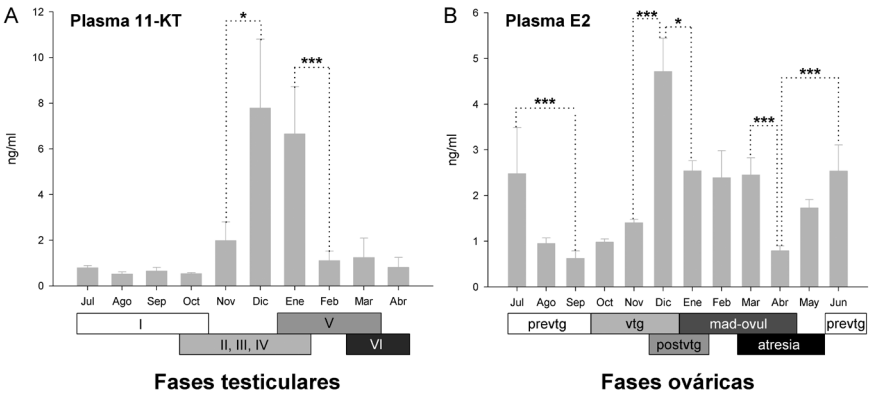

Figura R.6 Cambios en los niveles plasmáticos de 11-KT (**A**) y E2 (**B**), en hembras y machos de lubina durante la primera recrudescencia gonadal. Los datos se han representado como la media ± el error estándar de la media (n = 5 peces/ mes). Las fases histológicas del desarrollo testicular y ovárico están representadas por barras horizontales en la parte inferior de cada grupo de gráficas (Ver leyenda de la Fig. R.5). Los diferentes niveles de significación identificados por ANOVA de una vía y por test de comparación múltiple, están indicados con *, ** y *** para $P \leq 0.05$, $P \leq 0.01$ y $P \leq 0.001$, respectivamente.

hormona actúa uniéndose y activando su receptor específico, el TSHR, induciendo la síntesis y secreción de las hormonas tiroideas (Vassart y Dumont, 1992). Estudios recientes realizados en diversas especies de peces sugieren que la TSH podría estar directamente implicada en la fisiología gonadal (Kumar et al., 2000; Vischer y Bogerd, 2003b; Goto-Kazeto et al., 2003). En este apartado se describe el aislamiento, a partir de gónadas de lubina Europea, de un cDNA de 3587 nucleótidos que codifica un péptido de 779 aminoácidos en el que se identifican atributos estructurales característicos de los miembros de la familia de los receptores de las hormonas glicoproteicas, incluyendo un largo dominio extracelular N-terminal y el dominio transmembranal típico de los GPCRs. Como ocurre en los demás TSHRs, el dominio extracelular del TSHR de lubina (sbsTSHR) posee una región N-terminal rica en cisteínas, seguida de una región compuesta por once LRRs a la que continúa una región bisagra. Ésta última puede a su vez dividirse en una región central de cisteínas, un segmento específico de los TSHRs que en los mamíferos tiene aproximadamente 50 aminoácidos, aunque en la lubina es más largo debido a una inserción de 8 aminoácidos y finalmente un segmento C-terminal rico en cisteínas. El sbsTSHR es muy similar a los TSHRs de otras especies de peces, seguido de los TSHRs de mamíferos y de aves. La identidad de secuencia compartida entre el sbsTSHR y los otros dos miembros de la familia de los receptores de hormonas glicoproteicas de la lubina es de 39,3% con el sbsFSHR y de 39,7% con el sbsLHR. En cuanto a la similitud entre los distintos dominios proteicos, el dominio extracelular del sbsTSHR presenta sólo un 25% y 31% de amino ácidos idénticos a los dominios homólogos del sbsFSHR y sbsLHR respectivamente, mientras que el dominio transmembrana tiene una identidad del 67% y el 62% con los dominios homólogos del sbsFSHR y sbsLHR. La relación evolutiva del *sbsTSHR* con otros miembros de la familia de los receptores de las hormonas glicoproteicas se analizó usando el método de Neighbor-Joining. La topología del árbol obtenido (**Fig. R.7**) muestra tres grupos principales: el del TSHR, FSHR y LHR. En ese análisis el sbsTSHR se agrupa con su linaje respectivo. Los grupos FSHR y TSHR presentan un valor de "bootstrap" de 100 mientras que los LHRs están separados de los restantes receptores de las hormonas glicoproteicas en 90% de los replicados (**Fig. R.7**). El análisis de la expresión del *sbsTSHR* en diferentes tejidos de animales adultos mostró altos niveles de expresión de este gen en los testículos y con menor magnitud en los ovarios. También se detectaron transcritos de este gen en

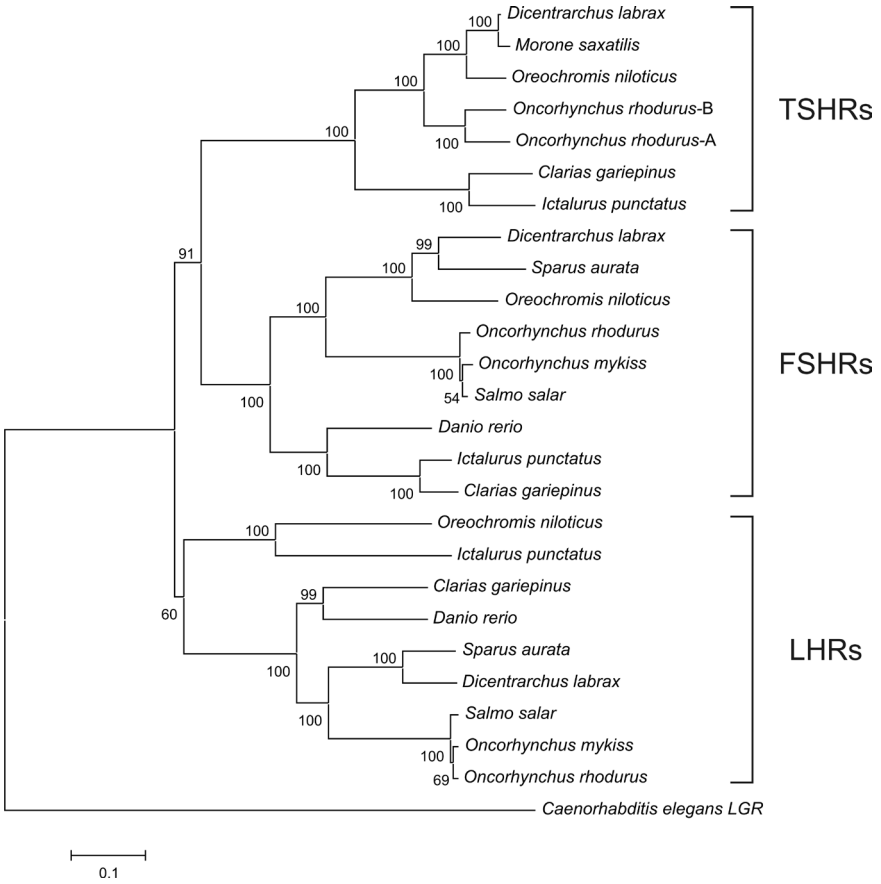

Figura R.7 Árbol filogenético de las secuencias aminoacídicas completas de receptores de hormonas glicoproteicas de peces, inferido según el método de Neighbor-Joining. Números de acceso: lubina (*Dicentrarchus labrax*) TSHR (DQ386646), FSHR (AY642113) y LHR (AY642114); lubina Americana (*Morone saxatilis*) TSHR (AF239761); Tilapia (*Oreochromis niloticus*) TSHR (AB047390), FSHR (AB041762) y LHR (AB041763); Salmón "amago" (*Oncorhynchus rhodurus*) TSHR-B (AB030955), TSHR-A (AB030954), FSHR (AB030012) y LHR (AB030005); Pez gato Africano (*Clarias gariepinus*) TSHR (AY129556), FSHR (AJ012647) y LHR (AF324540); Pez gato (*Ictalurus punctatus*) TSHR (AY533543), FSHR (AF285182) y LHR (AF285181); Pez cebra (*Danio rerio*) FSHR (AY278107) y LHR (AY424302); Salmón del Atlántico (*Salmo salar*) FSHR (AJ567667) y LHR (AJ579790); Trucha arco-iris (*Oncorhynchus mykiss*) FSHR (AF439405) y LHR (AF439404); Dorada (*Sparus aurata*) FSHR (AY587262) y LHR (AY587261). La secuencia del LGR del gusano *Caenorhabditis elegans* se usó como grupo externo. Los valores del "bootstrap" (en %) de 1000 replicas se indican en cada nodo del árbol.

diferentes tejidos somáticos, entre los cuales el cerebro/ pituitaria, corazón y riñón presentaron la señal de amplificación más fuerte (**Fig. R.8**). La expresión extratiroidea del *sbsTSHR* está en consonancia con lo encontrado en mamíferos y en otros peces (Davies et al., 2002; Kumar et al., 2000; Vischer y Bogerd, 2003b; Goto-Kazeto et al., 2003). Además, se amplificaron dos mRNAs que difieren en el tamaño de sus UTRs en 3'. Estos transcritos son resultado del uso de señales de poliadenilación alternativas. El estudio de las variaciones estaciónales de los niveles del mRNA de *sbsTSHR* en machos y hembras de lubina durante la primera recrudescencia gonadal sugiere que, en las hembras el TSHR podría participar en la vitelogénesis activa, en la regulación de la maduración de los gametos y en la ovulación (**Fig. R.9, B**). Por otra parte, en los machos, el TSHR estaría implicado en la regulación de procesos que ocurren durante lo estadios tempranos del desarrollo gonadal y además, en la maduración de los gametos y en la espermiación (**Fig. R.9, A**). Los resultados de este estudio demuestran que se ha clonado un TSHR a partir de tejido gonadal de lubina y proporcionan la base para estudios futuros relacionados con un posible papel del TSHR en la fisiología reproductiva de esta especie.

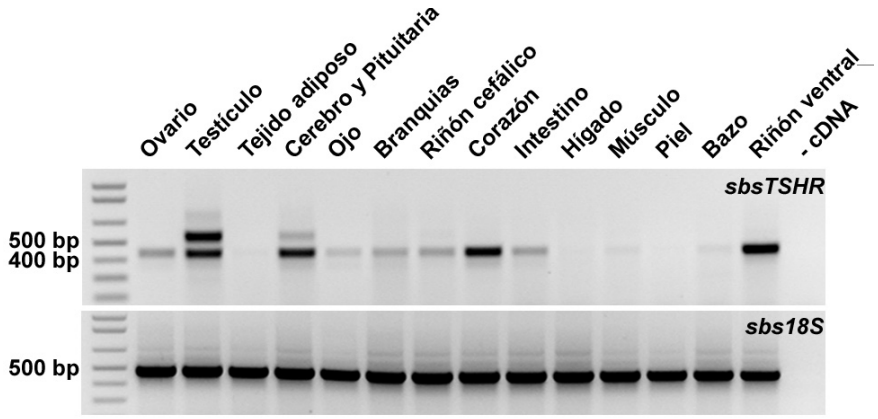

Figura R.8 Análisis de la expresión del *sbsTSHR* en tejidos de peces adultos mediante RT-PCR. En la amplificación del cDNA, se usaron oligonucleótidos específicos correspondientes al dominio extracelular del sbsTSHR. La presencia de cuatro intrones en esta región del gen garantiza que el producto amplificado corresponde exclusivamente a cDNA. La integridad de los RNAs se verificó mediante amplificación uniforme del *18S rRNA* de lubina.

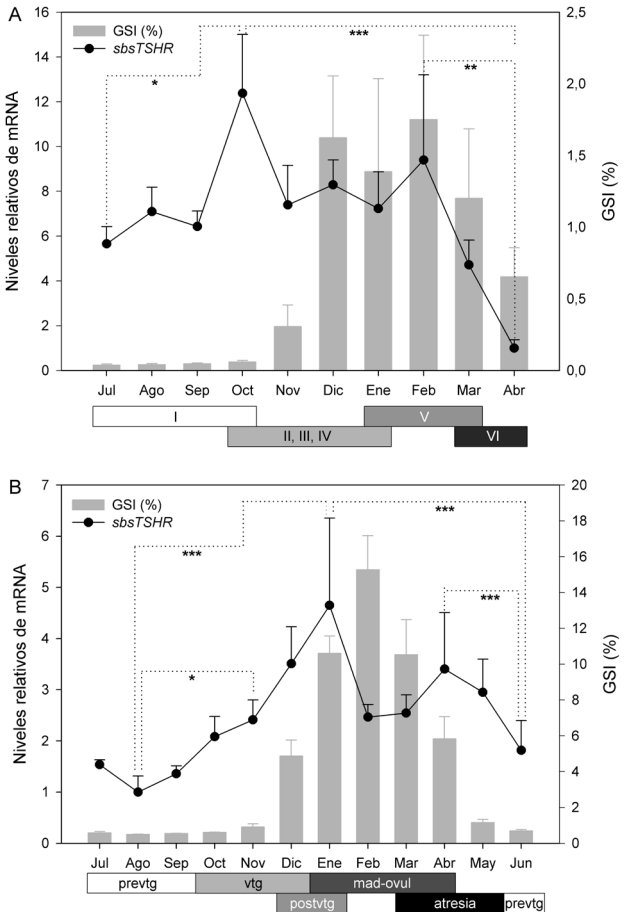

Figura R.9 Variaciones estaciónales de los niveles relativos del mRNA de *sbsTSHR* en machos y hembras de lubina durante la primera recrudescencia gonadal. (**A**) Cambios en el índice gonadosomático (GSI; peso de la gónada/ peso corporal x 100, barras grises verticales) de machos y expresión del sbsTSHR en testículo (línea negra). Los niveles se han expresado como una proporción del valor de la media de abril, establecido como 1. Los datos se han representado como la media ± el error estándar de la media (n = 5 peces/ mes excepto en octubre, noviembre y abril en que n = 4 peces/mes). Se llevó a cabo un análisis de ANOVA de una vía (P = 0,026). (**B**) Cambios en el GSI de hembras (barras grises verticales) y expresión del *sbsTSHR* en ovario (línea negra). Los niveles se han expresado como una proporción del valor de la media de agosto, asignado como 1. Los datos se han representado como la media ± el error estándar de la media (n = 5 peces/ mes). Se llevó a cabo un análisis de ANOVA de una vía (P< 0,001). Los diferentes niveles de significación, identificadas por ANOVA y por el test Holm-Sidak, están indicados con *, ** y *** para P≤0.05, P≤0.01 y P≤0.001, respectivamente. Las fases histológicas del desarrollo testicular y ovárico de las muestras analizadas (ver leyenda de la Fig. R.5) están representadas por barras horizontales en la parte inferior de cada gráfica.

Conclusiones

Primera: Se han aislado y caracterizado los cDNAs que codifican los receptores de FSH, LH y TSH de lubina. Las proteínas codificadas contienen todos las atributos estructurales que caracterizan a los receptores de hormonas glicoproteicas en vertebrados, tales como: Un dominio largo extracelular en el extremo N-terminal que incluye una zona de LRRs flanqueada en sus extremos N-terminal y C-terminal por regiones ricas en cisteínas, un dominio transmembranal del tipo rodopsina, y un cola corta intracelular en su extremo C-terminal.

Segunda: Las proteínas maduras de los receptores sbsLHR y sbsTSHR están muy conservadas respecto a otros receptores de peces y mamíferos; sin embargo, el sbsFSHR contiene varias diferencias destacables. Entre ellas, un dominio N-terminal rico en cisteínas diferente en cuanto a tamaño y número de cisteínas, y la presencia de una LRR adicional, lo que sugiere la existencia de un dominio de unión al ligando con distinta curvatura y tamaño respecto al FSHR humano, que podría influir en el modo en el que el receptor reconoce a la hormona.

Tercera: El *sbsFSHR* se expresa exclusivamente en tejidos gonadales, concretamente en las cubiertas de los folículos en fases previtelogénicas y de vitelogénesis temprana. Por el contrario, los mRNA de los genes *sbsLHR* y *sbsTSHR* se encontraron ampliamente distribuidos en tejidos somáticos de lubina, de manera similar a sus congéneres en mamíferos y a otros LGRs.

Cuarta: El *sbsFSHR* contiene un exón adicional en la región de las LRRs del dominio extracelular, cuya existencia podría ser el resultado de la duplicación de un exón interno, que habría sucedido antes de la diversificación de los linajes de Perciformes, Beloniformes y Tetraodontiformes. El gen *sbsLHR* no contiene intrones en la región codificante del dominio transmembranal, al igual que en tetrápodos. Sin embargo, el *sbsFSHR* contiene tres intrones en esta región. En conjunto, esta información sugiere que el *sbsFSHR* ha sufrido una mayor tasa de diversificación que el *sbsLHR* durante la evolución de los vertebrados.

Quinta: Los mRNAs del *sbsFSHR* incluyen transcritos con procesamiento alternativo, originados por eliminación de exones del dominio extracelular. Así mismo, los mRNAs del *sbsLHR* y el *sbsTSHR* contienen transcritos que difieren en su extremos 3' no traducidos y reflejan el uso de sitios de poliadenilación alternativos. Su significado funcional, en caso de que exista, no se conoce.

Sexta: El sbsFSHR recombinante se estimula específicamente por bFSH, mientras que el sbsLHR se activa por ambas bLH y bFSH. Sin embargo, se observó una estimulación específica cuando se usaron gonadotrofinas recombinantes de lubina.

Séptima: En ambos sexos, la expresión de los receptores de gonadotrofinas de lubina y de *StAR* varía a lo largo del ciclo reproductor. Mientras en machos de lubina ambos, *sbsFSHR* y *sbsLHR*, muestran distintos patrones de expresión, en hembras, sus patrones de expresión están muy correlacionados. No se observó una correlación importante entre la expresión de los receptores de gonadotrofinas de lubina y los niveles plasmáticos de esteroides sexuales importantes.

Octava: La expresión del *sbsFSHR* se relaciona con fases tempranas del desarrollo gonadal, pero este receptor está también muy expresado durante el periodo de espermiación en machos y el de maduración-ovulación en hembras. Este perfil de expresión sugiere que el sbsFSHR podría estar también implicado en el control de procesos que ocurran en etapas más tardías de la gametogénesis. No obstante, este perfil también coincide con el patrón cíclico de desarrollo de los gametos que caracteriza a un desarrollo gonadal del tipo síncrono por grupos, como es el caso de la lubina.

Novena: El perfil de expresión del *sbsLHR*, en ambos sexos, apoya la implicación de la LH en la regulación de las fases finales de la maduración de los gametos y de la ovulación/espermiación en peces.

Décima: El perfil de expresión de *StAR* sugiere que StAR podría ser importante para la síntesis de MIS gonadal, mediando el aporte rápido de sustrato esterol. Es más, la correlación significativa, en ambos sexos, entre los niveles de mRNA de *LHR* y *StAR* en lubina sugiere que la LH podría inducir la expresión de *StAR*.

Undécima: Por primera vez en un vertebrado se describen las variaciones estacionales de expresión de un *TSHR* gonadal, éstas indican que este receptor está regulado ejerciendo un papel en la fisiología gonadal de lubina. La expresión del *sbsTSHR* en hembras sugiere que éste podría participar en la vitelogenesis y en la regulación de la maduración de los gametos y la ovulación, mientras en machos, el TSHR estaría implicado en la regulación de procesos que ocurren durante las primeras etapas del desarrollo gonadal, y también en la maduración de los gametos y la espermiación.

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Resumo em português

Nos vertebrados, as gonadotrofinas pituitárias, a hormona folículo-estimulante (FSH) e a hormona luteinizante (LH), são os principais factores de regulação do processo de gametogénese e da produção de hormonas gonadais. Para exercer as suas funções biológicas, as gonadotrofinas deverão unir-se e activar receptores específicos, presentes na superfície de células alvo. O objectivo genérico da presente tese de doutoramento foi o de investigar o papel das gonadotrofinas na função reprodutora do robalo (*Dicentrarchus labrax*), mediante o estudo dos respectivos receptores. Dois cDNAs que codificam o receptor da FSH (*sbsFSHR*) e o receptor da LH (*sbsLHR*) de robalo foram clonados e caracterizados. A estrutura e expressão dos respectivos genes, bem como a sua actividade funcional, foram estudadas. As proteínas maduras do FSHR e LHR de robalo apresentam características típicas dos membros da família dos receptores das hormonas glicoproteicas, no entanto, o sbsFSHR também contém diferenças consideráveis quando comparado com outros FSHRs de peixes e de mamíferos. Entre elas cabe destacar uma região N-terminal rica em cisteínas, diferente no que respeita ao tamanho e número de resíduos de cisteínas, e a presença de uma repetição rica em leucinas extra numerária. Comparando a estrutura genómica primária dos receptores de gonadotrofinas do robalo e de outros vertebrados, verificamos que durante a evolução, a estrutura do *sbsLHR* se conservou mais do que a do gene do *sbsFSHR*, particularmente no domínio extracelular. Os mRNAs do *sbsFSHR* incluem transcritos com processamento alternativo, originados pela eliminação de exões do domínio extracelular. As análises da expressão revelaram que o *sbsFSHR* se expressa exclusivamente nas gónadas, em concreto, na parede folicular de folículos previtelogénicos e em fases iniciais da vitelogénese. Em contrapartida, o mRNA do *sbsLHR* encontra-se amplamente distribuído pelos tecidos somáticos do robalo. Quando expressado de forma estável num linha celular de mamíferos, o sbsFHSR foi estimulado especificamente pela FSH bovina, enquanto que o sbsLHR foi activado por ambas FSH e LH bovinas. No entanto, observou-se uma estimulação específica de ambos FSHR e LHR do robalo, quando foram usadas gonadotrofinas recombinantes do robalo, sugerindo a existência de interacções específicas entre os receptores de gonadotrofinas do robalo e os seus correspondentes ligandos. Este comportamento assemelha-se ao descrito para os receptores de gonadotrofinas de mamíferos mas difere dos estudos realizados em outras espécies de peixes, em que se observou uma

activação promíscua. Investigaram-se os perfis da expressão dos receptores de gonadotrofinas nas gónadas de machos e fêmeas de robalo, durante um ciclo reprodutor completo, em paralelo com a expressão do gene da proteína responsável pela resposta aguda da esteroidogénese (*StAR*), e importantes hormonas reprodutoras. Enquanto nos machos, os receptores das gonadotrofinas apresentaram perfis de expressão diferentes, nas fêmeas, os perfis destes receptores foram similares. A expressão do *sbsFSHR* relacionou-se com as fases iniciais do desenvolvimento gonadal, no entanto, níveis elevados de expressão foram igualmente observados durante o período de espermiação nos machos e durante o período de maduração-ovulação nas fêmeas, sugerindo um possível envolvimento deste receptor no controle de processos que ocorrem durante estas fases do desenvolvimento gonadal. Os perfis de expressão do *sbsLHR* observados em ambos sexos, são concordantes com o já sugerido papel da LH na regulação das fases finais de maduração dos gâmetas e na espermiação/ ovulação nos peixes. A expressão da *sbsStAR* correlacionou-se de forma elevada, com a expressão do *sbsLHR*. Adicionalmente, o perfil de expressão obtido para a *StAR* sugere que esta proteína poderia mediar a rápida disponibilidade do substrato colesterol na mitocôndria, necessário à síntese dos esteróides indutores da maduração gonadal. Não se observou uma relação forte entre a expressão dos receptores de gonadotrofinas e o nível plasmático dos esteróides sexuais analisados. Serão necessários estudos adicionais para compreender como as gonadotrofinas e os esteróides sexuais interagem, de forma a regular a reprodução do robalo.

Estudos recentes descreveram a presença abundante de transcritos do receptor da hormona estimulante da tiróide (TSHR) nos ovários e testículos de algumas espécies de peixes. Com o intuito de elucidar o papel fisiológico que o TSHR poderá desempenhar na função gonadal do robalo, isolou-se a partir de gónadas de este peixe, um cDNA que codifica um TSHR do robalo (*sbsTSHR*). A proteína madura do sbsTSHR apresenta características típicas dos membros da família dos receptores das glicoproteínas. Mediante análises de RT-PCR, demonstrou-se a expressão extratiroideia do *sbsTSHR* em numerosos tecidos somáticos do robalo. O estudo das variações sazonais dos níveis de expressão do *sbsTSHR* em machos e fêmeas de robalo, durante a primeira recrudescência gonadal, sugere que nas fêmeas, o TSHR poderá participar na vitelogénese activa e na regulação da maduração dos gâmetas e ovulação, enquanto que nos machos, o TSHR estará envolvido na regulação de processos de desenvolvimento da gónada e também na maduração dos gâmetas e na espermiação.