UNIVERSITAT POLITÈCNICA DE VALÈNCIA

CSIC
Consejo Superior de Investigaciones Científicas
INSTITUTO DE ACUICULTURA
DE TORRE DE LA SAL

CARACTERIZACIÓN BIOLÓGICA DE LAS GONADOTROFINAS DE LUBINA (*Dicentrarchus labrax*) Y DESARROLLO DE HERRAMIENTAS BIOTECNOLÓGICAS E INMUNOLÓGICAS PARA SU ESTUDIO

Memoria presentada por Gregorio Molés Miró para optar al grado de Doctor.

Fdo.
Gregorio Molés Miró

Tesis Doctoral dirigida por la Dra. Silvia Zanuy Doste.

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La Dra. Silvia Zanuy Doste, Profesora de Investigación del CSIC en el Departamento de Fisiología de Peces y Biotecnología del Instituto de Acuicultura Torre la Sal, hace constar que:

Gregorio Molés Miró, licenciado en Ciencias Biológicas, ha realizado bajo su dirección en el Instituto de Acuicultura de Torre la Sal (CSIC) el trabajo de investigación recogido en esta memoria que lleva por título: "Caracterización biológica de las gonadotrofinas de Lubina (*Dicentrarchus labrax*) y desarrollo de herramientas biotecnológicas e inmunológicas para su estudio", para optar al grado de doctor por la Universidad Politécnica de Valencia.

Torre de la Sal,

Fdo.

Dra. Silvia Zanuy Doste

Caracterización biológica de las gonadotrofinas de lubina (*Dicentrarchus labrax*) y desarrollo de herramientas biotecnológicas e inmunológicas para su estudio

Indice

Abreviaturas y Acrónimos

11-KT	11-ketotestosterona
3β-HDS	3β-hidroxiesteroide deshidrogenasa/Δ5-Δ4-isomerasa
11β-HDS	11β-hidroxiesteroide deshidrogenasa
17,20βP	17α,20β-dihidroxi-4-pregnen-3-ona
17β-HDS	17β-hidroxiesteroide deshidrogenasa
20βS	17α,20β,21-trihidroxi-4-pregnen-3-ona
ADN	ácido desoxirribonucleico
ADNc	ADN complementario
Ac	anticuerpo
Ag	antígeno
AG	aparato de Golgi
AMPc	monofosfato de adenosina cíclico
ARN	ácido ribonucleico
Arg (R)	arginina
ASi	ácido siálico
Asn (N)	asparragina
Asp (D)	ácido aspártico
ATP	trifosfato de adenosina
ARNm	ácido ribonucleico mensajero
αL	lazo horquilla β subunidad-α
B:I	relación entre bioactividad e inmunoactividad
βL	lazo horquilla β subunidad-β
CG	gonadotropina corionica placentaria
CGPs	células germinales primordiales
CHO	ovario de hámster chino
CHG	cerebro-hipófisis-gónada
CRE	elemento de respuesta a AMPc
CSIC	Consejo Superior de Investigaciones Científicas
CTP	péptido carboxi-terminal
Cys (C)	cisteína
CV	coeficiente de variación

dph	días post-eclosión
E2	17β-estradiol
ELISA	ensayo por inmunoabsorción ligado a enzimas
FSH	hormona estimulante del folículo
FSHR	receptor de FSH
FSHβ	subunidad FSHβ
Fuc	fucosa
GABA	ácido gamma-amino butírico
Gal	galactosa
GFRP	Grupo Fisiología de la Reproducción de Peces
Gln (Q)	glutamina
Glu (E)	ácido glutámico
Gly (G)	glycina
GnRH	hormona liberadora de gonadotrofinas
GnRHR	receptor de GnRH
GPα	subunidad α de FSH, LH o TSH (también Cga)
GpHRs	receptores de hormonas glicoproteicas
GPCR	receptor acoplado a proteína G
GTH I	gonadotrofina I o FSH
GTH II	gonadotrofina II o LH
GTHRs	receptores de gonadotrofinas (FSHR y LHR)
GV	Generalitat Valenciana
HEK	riñón embrionario humano
His (H)	histidina
IATS	Instituto de Acuicultura de Torre de la Sal
IP3	inositol trifosfato
I3P	beca predoctoral de investigación con interés para el sector industrial
kDa	kilodalton
Leu (L)	leucina

LH	hormona luteinizante
LHR	receptor de LH
LHβ	subunidad LHβ
LRR	repeticiones ricas en leucina
Lys (K)	lisina
Man	manosa
MAPK	proteína quinasa activada por mitógeno
MEC	Ministerio de Educación y Ciencia
Met (M)	metionina
MIS	esteroides inductores de la maduración
NAG	N-acetilglucosamina
NPY	neuropeptido Y
P45011β	P450 11β-hidroxilasa
P450arom	citocromo P450 aromatasa, gonadal (CYP19A1) y cerebral (CYP19A2)
P450c17	citocromo P450 17α-hidroxilasa/17,20-liasa (CYP17)
P450scc	citocromo P450 de escisión de la cadena lateral de colesterol (CYP11A)
PAGE	electroforesis en gel de poliacrilamida
PCR	reacción en cadena de la polimerasa
Phe (F)	fenilalanina
PDP	*pars distalis proximal*
PDR	*pars distalis rostral*
PI	*pars intermedia*
PKA	proteína quinasa A
PKB	proteína quinasa B
PNGase F	péptido *N*-glicosidasa F
Pro (P)	prolina
Proteína G	proteína heterotrimerica que se une a nucleótidos de guanina
RIA	radioinmunoensayo
RER	retículo endoplasmático rugoso
scGTHs	GTHs de cadena única

SDS	dodecilsulfato sódico
Ser (S)	serina
Sf	Spodoptera frugiperda
SGK	proteína quinasa inducida por suero y glucocorticoides
StAR	proteína reguladora de la respuesta esteroidogénica aguda
T	testosterona
TSH	hormona estimulante del tiroides o tirotrofina
TSHR	receptor de TSH
Tyr (Y)	tirosina
UAB	Universitat Autònoma de Barcelona
UB	Universitat de Barcelona
Val (V)	valina
VG	vesícula germinal
VTG	vitelogenina

Abbreviations and Acronyms

2-ME	2-mercaptoethanol
11-KT	11-ketotestosterone
Ala (A)	alanine
ANOVA	analysis of variance
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartic acid
ATP	adenosine triphosphate
atre	atresia
AU	absorbance unit
AUC	area under the concentration-time curve
B0	maximun binding
Bi	sample binding
B:I	biological to immunological ratio
BLAST	basic local alignment search tool

BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
bp	base pair
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic-monophosphate
cDNA	complementary deoxyribonucleic acid
CG	chorionic gonadotropin
CHO	chinese hamster ovary
Cl	clearance rate
CoA	coenzyme A
Con A	concanavalin A
CRE	cAMP response element
CTP	carboxy-terminal peptide
Cys (C)	cysteine
CV	coefficients of variation
DMEM	dulbecco modified eagle's medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
dph	days post-hatching
DTT	dithiothreitol
E2	17β-estradiol
E-AChE	estradiol acetylcholinesterase conjugate
EC50	half maximal effective concentration
E. coli	Escherichia coli
EDTA	ethylendiamin-tetra-acetate
EEC	European Economic Community
e.g.	*exempli gratia* (for example)
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EU	European Union
E.U.	Ellman Units
evtg	early vitellogenesis

FBS	foetal bovine serum
FSH	follicle-stimulating hormone
FSHR	FSH receptor
FSHβ	FSHβ-subunit
g	relative centrifugal force
g	gram
G418	aminoglycoside antibiotic
GAR	Goat anti-rabit antibodies
G protein	heterotrimeric guanine nucleotide-binding protein
Gln (Q)	glutamine
Glu (E)	glutamic acid
Gly (G)	glycine
GnRH	gonadotropin-releasing hormone
GnRHR	GnRH receptor
GPα	glycoprotein α-subunit (also Cga)
GSI	gonadosomatic index
GTH I	gonadotropin I
GTH II	gonadotropin II
h	hour
HEK	human embryonic kidney
His (H)	histidine
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
i.e.	*id est* (that is)
IgG	immunoglobulin G
Ile (I)	isoleucine
IMAC	immobilized metal affinity chromatography
IU	international unit
kV	kilovolts
kDa	kilodalton
L	liter
LL	continuos light

Leu (L)	leucine
LH	luteinizing hormone
LHR	LH receptor
LHβ	LHβ-subunit
LUC	Luciferase
lvtg	late vitellogenesis
Lys (K)	lysine
M	molecular weight marker
mAb	monoclonal antibodies
MALDI	matrix-assisted laser desorption/ionization
mat	maturation
MD	minimal dextrose medium
Met (M)	methionine
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
µg	microgram
µl	microliter
µM	micromolar
µm	micrometer
N	normality
n	number of observations
ng	nanogram
NGS	normal goat serum
nm	nanometer
NP	natural photoperiod
NSB	non-specific binding
ORF	open reading frame
ovul	ovulation
P	probality value

P450arom	cytochrome P450 aromatase (CYP19)
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Tween 20
PCR	polymerase chain reaction
pfu	plaque-forming unit
pg	picogram
pH	negative log of hydrogen ion concentration
Phe (F)	phenylalanine
PI	*pars intermedia*
Pit	pituitary
PMSF	phenylmethylsulfonyl fluoride
PNGase F	peptide-*N*-glycosidase F
POA	preoptic area
PPD	*proximal pars distalis*
prevtg	previtellogenesis
Pro (P)	proline
PVDF	Polyvinylidene difluoride
pvtg	postvitellogenesis
R	correlation coefficient
RIA	radioimmunoassays
RLU	relative light units
RNA	ribonucleic acid
rp	reversed-phase
rpm	revolutions per minute
s	second
sbs	sea bass
scGTHs	single chain gonadotropins
Sf	*Spodoptera frugiperda*
SBR	Sea Bass Ringer
SD	standard deviation
SDP	sex differentiation period
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Ser (S)	serine

T	testosterone
TBS-T	Tris-Buffered Saline-Tween 20
TFA	trifluoroacetic acid
TMB	tetramethylbenzidine
TOF	time-of-flight mass spectrometer
Tris	tris-(hydroxymethyl)-aminomethane
TSH	thyroid-stimulating hormone
Tyr (Y)	tyrosine
U	units
UV	ultraviolet
v	volume
Val (V)	valine
wt	weight
YPD	yeast extract peptone dextrose medium

Capítulo 1. INTRODUCCIÓN GENERAL

Introducción

Los peces teleósteos representan el grupo más grande y diverso de vertebrados (más de 29.000 especies). Se caracterizan principalmente por tener esqueleto óseo y habitar una gran diversidad de biotopos. Para asegurar el éxito reproductivo han desarrollado adaptaciones al medio, generando gran diversidad de estrategias reproductivas con diferentes grados de especialización que van desde la fertilización externa (la mayoría de teleósteos) a la interna. Los peces teleósteos son predominantemente gonocoristas aunque es frecuente encontrar especies hermafroditas, sobre todo en el orden Perciformes. En general, estas diferencias provocan que el inicio de la pubertad, los ritmos de gametogénesis y la propia reproducción sean extremadamente variables entre especies (Vizziano et al., 2008).

La reproducción es un proceso estacional que tiene lugar como consecuencia de la integración de la información ambiental, captada por los sistemas sensoriales, y su transducción en una cascada hormonal que desencadena la producción de gametos en el momento más favorable para la supervivencia de la progenie en un determinado ecosistema. Desde un punto de vista práctico, el control de la reproducción de un organismo es importante y limitante para su producción en cautividad. En ocasiones, la reproducción es un proceso aleatorio y poco controlado, en parte, como consecuencia de alteraciones de las condiciones ambientales en las que se mantienen los animales en cultivo. Estas alteraciones afectan a la evolución normal del proceso reproductivo porque perturban el sistema endocrino a distintos niveles. Así, para poder controlar eficazmente la reproducción de un organismo es necesario conocer profundamente los procesos básicos que regulan la alternancia de su ciclo reproductor y su puesta en funcionamiento. Por lo tanto, dilucidar los eventos endocrinos y neuroendocrinos que regulan la reproducción es crucial para el desarrollo de la acuicultura.

La lubina europea

La lubina, *Dicentrarchus labrax*, es un teleósteo marino de gran interés económico en acuicultura. Pertenece al orden Perciformes y a la familia Moronidae. Se distribuye principalmente por el mar Mediterráneo y las costas del océano Atlántico, desde el norte de Noruega hasta Marruecos. Vive en un amplio rango de ambientes gracias a su capacidad eurihalina (3-38‰) y euriterma (2-32°C) (Carrillo et al., 1995; Pickett y Pawson, 1994).

La lubina es una especie gonocorista, con sexos separados, pero carece de caracteres sexuales externos para identificarlos. No obstante, normalmente, las hembras alcanzan mayor tamaño que los machos. Se reproduce en invierno (Diciembre-Marzo) y la mayoría de los machos alcanzan la primera maduración sexual al segundo año de vida mientras que las hembras maduran un año después. A igual edad, el retraso de la maduración sexual en las hembras favorece un mayor crecimiento corporal ya que los machos tienen que destinar parte de la energía consumida en el desarrollo de las gónadas. Las hembras de lubina presentan un desarrollo ovárico de tipo síncrono por grupos, de manera que producen 3-4 puestas consecutivas durante el periodo de puesta (1-2 meses) (Carrillo et al., 1995). Los huevos son pelágicos y transparentes, con 1 o 2 gotas de grasa. El desarrollo embrionario dura unos 4 días a 15°C y el desarrollo larvario unos 40 días a 19°C. Los juveniles habitan en aguas costeras y estuarios donde crecen y progresan hacia la pubertad (Barnabé, 1991; Carrillo et al., 1995; Pickett y Pawson, 1994).

La reproducción en teleósteos

La reproducción sexual es uno de los procesos biológicos más relevantes que ocurren en la Naturaleza. Se basa en la creación de un nuevo organismo a partir de la fusión de dos gametos de sexo opuesto. Este proceso permite la recombinación del ADN parental, aumentando la diversidad genética de la descendencia. La producción de gametos o gametogénesis tiene lugar en las gónadas mediante un proceso de meiosis. En general, en peces, el ciclo reproductor se divide en dos fases, una fase inicial de crecimiento y desarrollo gonadal, y una segunda fase de maduración de los gametos.

Gametogénesis en machos

Morfología del testículo

En la mayoría de los casos, los testículos de teleósteos son dos órganos alargados separados por un septo y unidos a la pared dorsal de la cavidad corporal. De la superficie medio dorsal posterior de cada testículo sale el espermiducto que desemboca en la papila urogenital (Nagahama, 1983). Internamente, en los testículos se distingue un compartimento germinal y otro intersticial, ambos separados por una membrana basal. El compartimento germinal está compuesto por células germinales y células somáticas asociadas o células de Sertoli, mientras que el compartimento intersticial, lo constituyen células especializadas o células de Leydig, tejido conectivo, vasos sanguíneos y vasos linfáticos (**Fig. 1**). En los teleósteos se han descrito dos tipos de estructura testicular, la tubular y la lobular, en función de la morfología del compartimento germinal y de la distribución de las células germinales en su interior (Grier, 1993). El tipo tubular está formado por un

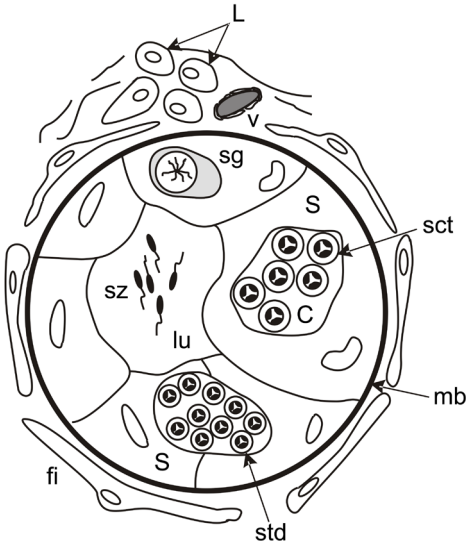

Figura 1. Representación esquemática de un lóbulo testicular durante la espermatogénesis (sección transversal). Las células de Leydig (L) y los vasos sanguíneos (v) se localizan en el compartimento intersticial, entre lóbulos adyacentes. En el compartimento germinal, las células de Sertoli (S) nutren a las células germinales en desarrollo durante las diferentes fases de la espermatogénesis. Espermatogonias (sg), espermatocitos (sct), espermátidas (std), espermatozoides (sz), cistes o espermatocistes (C), lumen lobular (lu), membrana basal (mb), fibroblastos (fi). Adaptado de Billard et al. (1982).

conjunto de túbulos que se extienden desde la periferia del testículo hasta el centro del mismo donde convergen en un gran conducto. Esta estructura es típica de especies con fecundación interna. El testículo de tipo lobular es el más común entre los teleósteos Perciformes y está compuesto por numerosos lóbulos ramificados y anastomosados entre si. En cada uno de estos lóbulos, las células de Sertoli engloban clones de células germinales en el mismo estado de desarrollo (Billard et al., 1982; Pudney, 1995) formando las unidades funcionales del testículo donde tiene lugar la espermatogénesis, los espermatocistes. El espermatociste más simple lo constituye una sola célula germinal o espermatogonia rodeada por una o dos células de Sertoli que se encargan de suministrar el entorno necesario para su supervivencia, proliferación y diferenciación. Durante la espermatogénesis, la célula germinal sufre una serie de divisiones mitóticas y meióticas hasta dar lugar a los espermatozoides. En el compartimiento intersticial, situado entre los lóbulos testiculares, se encuentran las células de Leydig que producen los esteroides necesarios para que tenga lugar la gametogénesis (Nagahama, 1994).

Espermatogénesis

La espermatogénesis es un proceso altamente organizado y coordinado en el cual células madre espermatogoniales (diploides) proliferan y se diferencian para dar lugar a espermatozoides maduros (haploides). Durante este proceso pueden distinguirse cinco fases principales (**Fig. 2**): 1) renovación de espermatogonias, 2) proliferación espermatogonial "hacia la meiosis", 3) meiosis, 4) espermiogénesis y 5) maduración del esperma (Zanuy et al., 2009).

Tras la diferenciación de células germinales primordiales (CGPs) en células madre espermatogoniales o espermatogonias A, se produce una renovación por mitosis dando lugar a espermatogonias similares. Posteriormente, durante la proliferación espermatogonial, las mitosis ya no dan lugar a espermatogonias A y cada generación de espermatogonias se distingue de la anterior por un conjunto de características morfológicas, como el incremento de la heterocromatina y la disminución del tamaño celular (Zanuy et al., 2009). La última generación de espermatogonias, denominadas espermatogonias B, entra en mitosis para dar lugar a los espermatocitos primarios. Estos experimentan la primera división meiótica, dando lugar a los espermatocitos secundarios de vida media muy corta, que después de la segunda división meiótica se convierten en espermátidas. Posteriormente, tras una

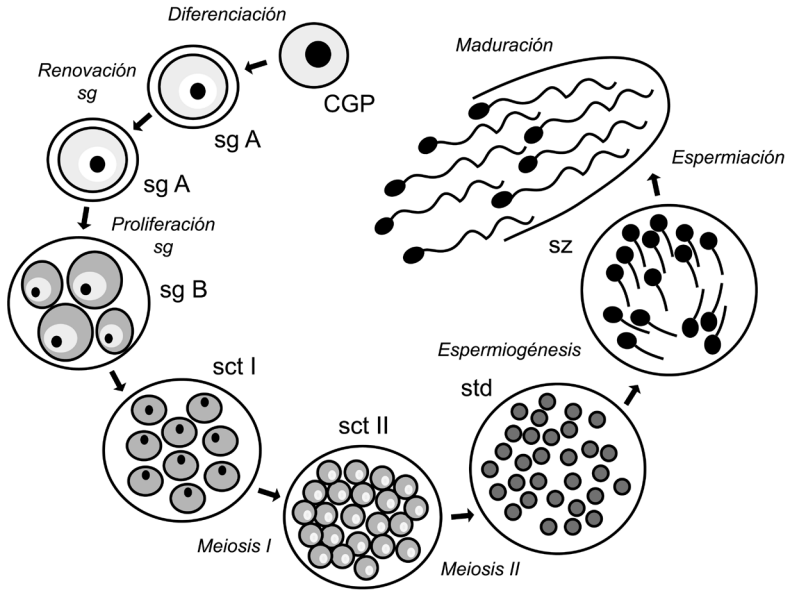

Figura 2. Representación esquemática del proceso de espermatogénesis en teleósteos. Célula germinal primordial (CGP), espermatogonia A (sg A), espermatogonia B (sg B), espermatocitos I y II (sct I y II), espermátidas (std), espermatozoides (sz).

serie de cambios morfológicos las espermátidas sufren una reducción del volumen nuclear y celular, convirtiéndose en espermatozoides (espermiogénesis) (Schulz et al., 2010; Zanuy et al., 2009). Por último, los espermatocistes se abren mediante ruptura de las células de Sertoli y los espermatozoides son liberados (espermiación) a la luz lobular y de allí al espermiducto, donde adquieren motilidad (maduración) y capacidad de fertilización (Schulz et al., 2010).

Gametogénesis en hembras

Morfología del ovario

El ovario de los teleósteos, a diferencia del de los mamíferos, muestra diferencias inter-específicas, reflejando un amplio rango de patrones o estrategias reproductivas. Sin embargo, las estructuras fundamentales, como la morfología de las células germinales y los elementos somáticos que constituyen el tejido gonadal, son similares (Nagahama, 1983). En general, los ovarios son órganos pares, alargados y situados dorsalmente en la cavidad

abdominal (Jalabert, 2005). Internamente, el ovario está formado por un tejido de soporte o estroma ovárico compuesto por fibras de colágeno, envuelto por una capa muscular lisa, tejido vascular y tejido nervioso. En la zona central está la cavidad ovárica, hacia la que se proyectan multitud de pliegues del estroma formando las láminas o lamelas ováricas, en las que se asienta el epitelio germinativo ovárico, formado por células germinales y epiteliales somáticas (Grier y Lo Nostro, 2000). La parte posterior de cada ovario se conecta mediante un oviducto con la papila o poro genital por el que se liberan los oocitos (Jalabert, 2005). Cada oocito está delimitado por una envoltura denominada corion o zona radiata que a su vez está rodeada de una capa de células somáticas epiteliales especializadas o células de la granulosa. Estas células secretan una membrana basal y alrededor de ella se organiza otra fina monocapa de células somáticas llamadas células de la teca, fibroblastos y vasos sanguíneos (**Fig. 3**). El oocito y sus correspondientes envolturas foliculares (células de la granulosa y de la teca) forman el folículo y constituye la unidad ovárica básica (Yaron y Levavi-Sivan, 2006; Zanuy et al., 2009).

Atendiendo al ritmo de desarrollo de los oocitos se han definido tres tipos principales de ovarios (Wallace y Selman, 1981):

a) Ovarios con desarrollo síncrono. En este tipo de ovario todos los oocitos se desarrollan y ovulan al mismo tiempo. Es propio de especies que po-

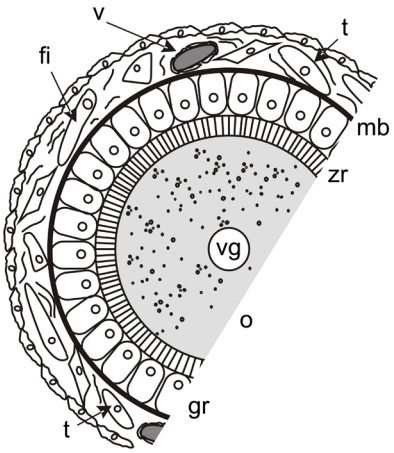

Figura 3. Representación esquemática de un folículo ovárico en vitelogénesis temprana. Vasos sanguíneos (v), fibroblastos (fi), células de la teca (t), membrana basal (mb), células de la granulosa (gr), zona radiata (zr), oocito (o), vesícula germinal o núcleo (vg), Adaptado de Nagahama (1983).

nen un vez en la vida y después mueren, como los salmones del Pacífico (*Oncorhynchus spp.*).

b) Ovarios con desarrollo síncrono por grupos. En este caso el ovario posee al menos dos grupos de oocitos en distinto estado de desarrollo. Es propio de especies de puesta estacional y se pueden dividir en dos subgrupos, especies de puesta única o puesta múltiple. En especies de puesta única el grupo de oocitos más avanzado se desarrolla de forma simultanea, produciendo una sola puesta al año como la trucha arcoiris (*Oncorhynchus mikiss*). Sin embargo, las especies de puesta múltiple repiten este proceso varias veces durante la época de puesta, como el caso de la lubina (*Dicentrarchus labrax*) que pone 3-4 veces por temporada.

c) Ovarios con desarrollo asíncrono. En este caso el ovario posee oocitos en todos los estados de desarrollo. Es característico de especies que ponen en repetidas ocasiones a lo largo de un dilatado periodo anual de puesta, como la medaka (*Oryzias latipes*) y el pez rojo o carpin (*Carassius auratus*.).

Oogénesis

La oogénesis es el proceso de formación de gametos femeninos. Un proceso en el que a partir de oogonias se desarrollan óvulos listos para ser fecundados. En peces, durante la oogénesis se pueden distinguir cinco fases principales: 1) proliferación oogonial, 2) crecimiento primario, 3) crecimiento secundario, 4) maduración y 5) ovulación (Le Menn et al., 2007; Planas y Swanson, 2008).

Después de la diferenciación sexual, en el ovario, las CGPs se van diferenciando en oogonias. Posteriormente, al comienzo o durante cada ciclo reproductivo, tiene lugar una fase de proliferación oogonial que tras varias divisiones mitóticas, inician su primera división meiótica, que queda detenida en profase I, dando lugar a oocitos primarios que se aíslan y rodean de una capa plana de células foliculares (células de la granulosa y de la teca). Durante la profase I el núcleo de los oocitos pasa a través de cinco estados sucesivos: leptoteno, zigoteno, paquiteno, diploteno y diacinesis. *El crecimiento primario* del oocito tiene lugar durante los cuatro primeros estados de profase I e incluye la *previtelogénesis* que tiene lugar en el estado de diploteno (Le Menn et al., 2007). El crecimiento primario se caracteriza por la condensación, sinapsis y recombinación de cromosomas homólogos y la formación de las capas foliculares que rodean el oocito. El oocito aumenta extraordinariamente de tamaño y experimenta una serie de transforma-

ciones que afectan al núcleo, nucleolo y citoplasma. Durante esta fase se expresan importantes genes necesarios para el desarrollo de la oogénesis, produciéndose una abundante síntesis de ARNm. Aparecen numerosos nucleolos en la periferia del núcleo (estado perinucleolar) y se sintetizan abundantes glicoproteínas que son incorporadas a los alvéolos corticales (estado de alveolo cortical). La profase I se para en el estado de diploteno durante un tiempo determinado que en los teleósteos puede variar entre unos pocos días y algunos meses. Durante esta parada meiótica tiene lugar la fase de *crecimiento secundario*, también conocida como *vitelogénesis*. Esta fase de crecimiento se caracteriza por la acumulación en el oocito de reservas nutricionales (gránulos de vitelo), vitaminas y hormonas que servirán para que culminen con éxito el desarrollo embrionario y larvario (Le Menn et al., 2007; Lubzens et al., 2010; Zanuy et al., 2009). La vitelogénesis implica la síntesis hepática de vitelogenina (VTG), y su incorporación en los oocitos en crecimiento. La VTG es una gran fosfolipoglicoproteína precursora de las proteínas del vitelo y de su correcta síntesis, acumulación y procesado depende la calidad del huevo y viabilidad de la larva (Lubzens et al., 2010).
Finalmente el oocito entra en fase de *maduración*, proceso durante el cual se completa la primera división meiótica dando lugar al oocito secundario que progresa hasta metafase de la segunda división meiótica (metafase II). Durante este proceso, se produce condensación de los cromosomas y el núcleo del oocito (vesícula germinal o VG), que estaba inicialmente localizado en el centro del citoplasma, migra hacia el polo animal donde se disgrega. Después de este evento, denominado rotura de la vesícula germinal, tiene lugar la extrusión del primer corpúsculo polar (**Fig. 4**). Durante la maduración también se observan varios cambios en el citoplasma, como la fusión de gránulos de vitelo, clarificación del citoplasma y, en especies marinas, el aumento del tamaño del oocito por hidratación (Cerdà, 2009). Tras el proceso de hidratación, la envoltura folicular se rompe y el oocito fertilizable u ovulo es liberado al oviducto (*ovulación*). Por último, tras la fertilización finaliza la segunda división meiótica, detenida en metafase II.

Si durante la oogénesis, los oocitos no se desarrollan correctamente entran en atresia, proceso por el que los oocitos degeneran y son reabsorbidos por el ovario.

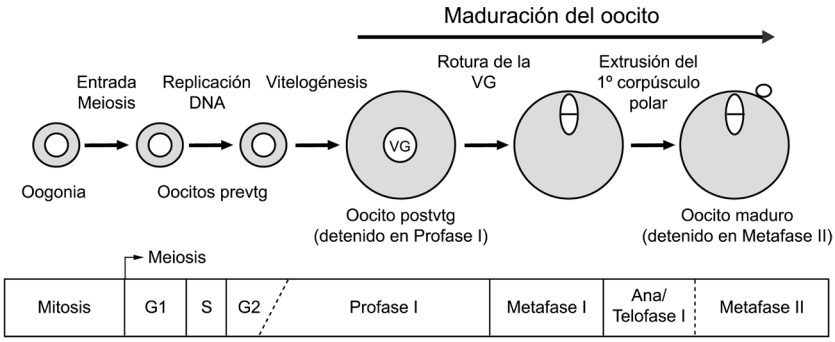

Figura 4. Representación esquemática del crecimiento y maduración del oocito. Los oocitos, producidos por la entrada en meiosis de oogonias, crecen de tamaño principalmente por la acumulación de vitelogenina (vitelogénesis). En respuesta a la estimulación hormonal, los oocitos detenidos en profase I (inmaduros) prosiguen la meiosis hasta metafase II (maduros). Vesícula germinal o núcleo (VG), previtelogénicos (prevtg), postvitelogénicos (postvtg). Adaptado de Suwa y Yamashita (2007).

Control endocrino de la reproducción en teleósteos

La reproducción en peces, como en otros vertebrados, está controlada por una red neuro-endocrina constituida por el cerebro, la glándula hipofisiaria y las gónadas. Esta red se denominada comúnmente eje cerebro-hipófisis-gónada (eje CHG) (Fink, 2000) y es responsable de la cascada hormonal que regula el proceso reproductor. Con el fin de asegurar el éxito reproductivo y evitar condiciones ambientales inapropiadas, el cerebro integra los diferentes estímulos, externos e internos, y los traduce en señales neuroendocrinas que regulan el eje CHG. En líneas generales, el proceso se inicia con la síntesis cerebral del neuropéptido hormona liberadora de gonadotrofinas (GnRH), que induce la síntesis y secreción hipofisaria de dos gonadotrofinas (GTHs), la hormona folículo estimulante (FSH) y la hormona luteinizante (LH). Posteriormente, las GTHs llegan a las gónadas a través del torrente sanguíneo, donde actúan sobre el desarrollo gonadal y la gametogénesis mediante la estimulación de la síntesis de esteroides sexuales. A su vez, los esteroides gonadales actúan sobre la síntesis y liberación de GTHs, ejerciendo un feedback positivo o negativo según el estado de desarrollo gonadal, bien

sea directamente sobre las células hipofisarias o indirectamente en ciertos núcleos hipotalámicos (Levavi-Sivan et al., 2010) (**Fig. 5**).

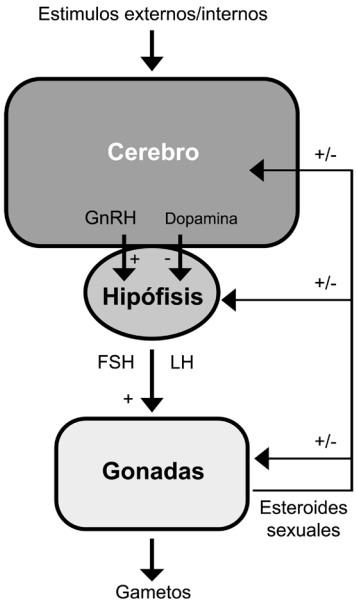

Figura 5. Representación esquemática del eje CHG en teleósteos. El símbolo + indica estimulación mientras que – representa inhibición.

Cerebro

Al cerebro se le ha atribuido un papel mediador de la influencia del medio ambiente sobre el proceso reproductor. Los estímulos ambientales son captados por los órganos sensoriales y trasmitidos al sistema nervioso central que responde mediante la liberación de determinados neuropéptidos y neurotransmisores. En los últimos años, el número de factores neuroendocrinos relacionados con el control del proceso reproductivo de los peces ha ido creciendo progresivamente. Entre ellos, la GnRH es considerada el principal factor cerebral implicado en la estimulación de la síntesis y secreción de las GTHs hipofisarias (Kah et al., 2007; Zohar et al., 2010). No obstante, existen otros factores cerebrales que modulan directa o indirectamente su liberación, como el neuropéptido Y (NPY), el acido gamma-amino butírico (GABA), la dopamina o las Kisspeptinas (Zohar et al., 2010).

Hormona liberadora de gonadotrofinas (GnRH)

La GnRH es un decapéptido producido y secretado por neuronas localizadas en diferentes regiones del cerebro. En la década de los 70 se aisló por primera vez en el hipotálamo de mamíferos y se denominó de forma genérica GnRH por su capacidad de estimular la secreción de FSH y LH (Burgus et al., 1971; Matsuo et al., 1971). En realidad, el sistema GnRH es una familia de neuropéptidos y hasta la fecha, se han identificado un total de 26 isoformas con estructura molecular similar, 15 en vertebrados (**Tabla 1**) y 11 en invertebrados (Kavanaugh et al., 2008). Tradicionalmente las isoformas de GnRH han recibido el nombre de las especies en las que fueron descritas por primera vez, si bien pueden estar presentes en diferentes especies. La mayoría de vertebrados expresan dos o tres formas de GnRH, aunque en zonas diferentes y aparentemente ejerciendo funciones distintas (Kah et al., 2007; Muñoz-Cueto, 2009). Para evitar confusiones, se propuso una nueva nomenclatura atendiendo a criterios filogenéticos y neuroanatómicos y las diferentes formas de GnRH se agruparon en tres tipos, GnRH-1, GnRH-2 y GnRH-3 (Fernald y White, 1999). Posteriormente, Silver et al. (2004) sugirieron un cuarto grupo (GnRH-4).

El tipo GnRH-1 engloba todas las formas hipofisiotróficas de GnRH de gnatóstomos (vertebrados con mandíbula), que son la mayoría de las formas descritas. Se expresan principalmente en neuronas del hipotálamo y del área preóptica de vertebrados, desde donde envían proyecciones axonales a la hipófisis. El tipo GnRH-2 se corresponde con la GnRH II de pollo (cGnRH-II) y se expresa de forma conservada en el sinencéfalo/mesencéfalo de todos los vertebrados, desde peces hasta mamíferos. No hay evidencias claras sobre la función de GnRH-2, aunque se le atribuye acciones neuromoduladoras y/o conductuales (Muñoz-Cueto, 2009). Recientemente se ha descrito otra forma de GnRH en lamprea (lGnRH-II) que deriva de una forma ancestral común a cGnRH-II, pero se expresa en el área preóptica e hipotálamo y se le atribuye una acción estimuladora de la hipófisis (Kavanaugh et al., 2008). El tercer tipo, GnRH-3, se corresponde exclusivamente con la forma GnRH de salmón (sGnRH), y hasta la fecha sólo se ha identificado de forma concluyente en el cerebro anterior de peces. Como en el caso del tipo I, en numerosas especies de peces, las neuronas secretoras de GnRH-3 envían fibras nerviosas a la hipófisis. Su función no está del todo clara, pero se piensa que podría participar junto al tipo GnRH-1 en la regulación de la síntesis de

Tabla 1. Estructura primaria de las 15 isoformas de GnRH conocidas en vertebrados. Se muestran las sustituciones respecto de la primera secuencia GnRH descrita en mamíferos.

GnRH	tipo	1	2	3	4	5	6	7	8	9	10	Referencia
Mamífero (mGnRH)	I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH2	Matsuo et al., 1971
Pollo I (cGnRH-I)	I	---	---	---	---	---	---	---	Gln	---	---	Miyamoto et al., 1982
Rana (frGnRH)	I	---	---	---	---	---	---	---	Trp	---	---	Yoo et al., 2000
Dorada (sbGnRH)	I	---	---	---	---	---	---	---	Ser	---	---	Powell et al., 1994
Salmón (sGnRH)	III	---	---	---	---	---	---	Trp	Leu	---	---	Sherwood et al., 1983
Cobaya (gpGnRH)	I	---	Tyr	---	---	---	---	Val	---	---	---	Jiménez-Liñán et al., 1997
Corégono (wfGnRH)	I	---	---	---	---	---	---	Met	Asn	---	---	Adams et al., 2002
Medaka (mdGnRH)	I	---	---	---	---	Phe	---	---	Ser	---	---	Okubo et al., 2000
Pez gato (cfGnRH)	I	---	---	---	---	His	---	---	Asn	---	---	Ngamvongchon et al., 1992
Arenque (hgGnRH)	I	---	---	---	---	His	---	---	Ser	---	---	Carosfeld et al., 2000
Tiburón (dfGnRH)	I	---	---	---	---	His	---	Trp	Leu	---	---	Lovejoy et al., 1992
Pollo II (cGnRH-II)	II	---	---	---	---	His	---	Trp	Tyr	---	---	Miyamoto et al., 1984
Lamprea II	II	---	---	---	---	His	---	Trp	Phe	---	---	Kavanaugh et al., 2008
Lamprea III (lGnRH-III)	IV	---	---	---	---	His	Asp	Trp	Lys	---	---	Shower et al., 1993
Lamprea I (lGnRH-I)	IV	---	---	Tyr	---	Leu	Glu	Trp	Lys	---	---	Sherwood et al., 1986

Modificado de Kah et al., 2007 y Kavanaugh et al., 2008.

GTHs y en la conducta reproductiva junto al tipo GnRH-2 (Muñoz-Cueto, 2009). Por último, el tipo GnRH-4 parece exclusivo de los agnatos (vertebrados sin mandíbula) y engloba a dos de las tres GnRHs descritas en lamprea (lGnRH-I y lGnRH-III). Se expresan en el área preóptica e hipotálamo y se les atribuye una acción hipofisiotrófica (Kavanaugh et al., 2008; Silver et al., 2004).

Estudios realizados en Perciformes, como la lubina, han puesto de manifiesto que expresan tres formas de GnRH, sbGnRH (GnRH-1), cGnRH-II (GnRH-2), y sGnRH (GnRH-3) (Muñoz-Cueto, 2009). Aunque las tres formas tienen una clara acción estimuladora sobre la liberación de GTHs hipofisarias (Zohar et al., 1995), estudios inmunohistoquímicos realizados en la lubina demuestran que las neuronas productoras de GnRH-1 y GnRH-3 inervan directamente la hipófisis, mientras que las de GnRH-2 no envían fibras nerviosas a la misma. No obstante, como en otros Perciformes, la inervación GnRH-1 es la más abundante en la hipófisis y la principal forma hipofisiotrófica, implicada en la regulación de la síntesis y secreción de GTHs (Gonzalez-Martinez et al., 2002a).

Receptores de GnRH (GnRHRs)

Las GnRHs ejercen su acción tras unirse a receptores específicos (GnRHR) presentes en las membranas de las células diana. Los GnRHR pertenecen a la superfamilia de receptores acoplados a proteínas G y poseen una sola cadena polipeptídica con siete dominios transmembrana separados por giros hidrófilos extra e intra-celulares de extensión variable (Sealfon et al., 1997). La interacción de GnRH con su receptor desencadena una cascada de reacciones intracelulares que conduce a la producción de segundos mensajeros. En los últimos años se han clonado y caracterizado distintos receptores de GnRH presentes en diferentes especies de peces (Kah et al., 2007; Lethimonier et al., 2004). Los primeros receptores fueron detectados en la hipófisis, aunque también se han encontrado en tejidos extrahipofisarios como el cerebro, las gónadas, el hígado o el riñón, reforzando la idea de que las GnRHs no solo ejercen acciones reproductivas a nivel de la hipófisis, sino que pueden actuar como neurotransmisores y/o neuromoduladores en el cerebro, y como factores autocrinos o paracrinos en las gónadas (Muñoz-Cueto, 2009). En la lubina se han caracterizado 5 tipos de receptores (Gonzalez-Martinez et al., 2004; Moncaut et al., 2005), de los cuales solo el tipo dlGnRHRII-1a, también denominado dlGnRHR-2A, está altamente expre-

sado en las células gonadotropas de la hipófisis y presenta afinidad por las formas sbGnRH y sGnRH (Gonzalez-Martinez et al., 2004; Kah et al., 2007). Además, este receptor dlGnRHRII-1a muestra diferencias en su expresión durante el ciclo reproductor (Gonzalez-Martinez et al., 2004), lo que sugiere un papel relevante en el control de la secreción de GTHs.

Hipófisis

La hipófisis o pituitaria es una pequeña glándula endocrina adosada a la parte ventral del cerebro de todos los vertebrados. En ella se sintetizan importantes hormonas implicadas en la regulación de la mayoría de los procesos biológicos del organismo. La hipófisis consta de una porción nerviosa, la neurohipófisis, formada por axones de neuronas que se proyectan desde el cerebro y una porción endocrina, la adenohipófisis, constituida por células secretoras no nerviosas. A su vez, en teleósteos, la adenohipófisis puede dividirse en tres regiones, la *pars distalis rostral* (PDR), la *pars distalis proximal* (PDP) y la *pars intermedia* (PI). En general, en la PDR se localizan las células lactotropas y corticotropas; en la PDP las células somatotropas, tirotropas y gonadotropas; y en la PI se localizan las células melanotropas, somatolactotropas y en ocasiones, en los bordes, también gonadotropas (Weltzien et al., 2004). A diferencia de los vertebrados superiores, diversos estudios inmunohistoquímicos han demostrado que en los peces, la FSH y la LH se producen en células gonadotropas diferentes (Levavi-Sivan et al., 2010).

Gonadotrofinas (GTHs)

Durante muchos años se pensó que en la hipófisis de los peces se producía una sola GTH, a la que se le atribuía la regulación de todo el proceso reproductor (Burzawa-Gerard y Fontaine, 1972). A mediados de la década de los 80, Idler y Ng (1983) propusieron un sistema dual para las GTHs de los teleósteos que no quedó totalmente establecido hasta la identificación de dos GTHs diferentes en la hipófisis de salmón chum (*Onchorhynchus keta*), GTH-I y GTH-II (Kawauchi et al., 1986). Desde entonces, aunque se ha demostrado la existencia de dos GTHs en numerosas especies de teleósteos, solo se han aislado y caracterizado en un reducido número de especies (**Tabla 2**).

A finales de los 90, en base a las secuencias de nucleótidos que codifican las GTHs y sus predicciones aminoacídicas, se estableció la homología entre

Tabla 2. Especies de teleósteos en las cuales se han purificado las gonadotrofinas y/o sus subunidades.

Especie	Nombre común	Gonadotrofina	Peso molecular	Referencia
Oncorhynchus keta	Salmón chum	GPα, FSHβ y LHβ FSH y LH	22, 17 y 18 Kda 50 y 36 KDa	Suzuki et al., 1988a,b
Oncorhynchus kisutch	Salmón coho	GPα, FSHβ y LHβ FSH y LH	20-24, 19 y 22 Kda 43 y 39 KDa	Swanson et al., 1991
Cyprinus carpio	Carpa común	FSH y LH	45 y 35 KDa	Van Der Kraak et al., 1992
Katsuwonus plelamis	Bonito	FSH y LH	39 y 30 KDa	Koide et al., 1993
Pagrus major	Dorada japonesa	FSH y LH	32 y 38 KDa	Tanaka et al., 1993
Micropogonias undulatus	Corvina	GPα, FSHβ y LHβ FSH y LH	17, 19 y 20.5 KDa	Copeland y Thomas,1993
Thunnus obesus	Patudo	FSH y LH	40.5 y 27 KDa	Okada et al., 1994
Seriola dumerilii	Pez limón	GPα, FSHβ y LHβ FSH y LH	17, 28 y 21.5 KDa 47 y 29 KDa	García-Hernandez et al., 1997
Oncorhynchus mykiss	Trucha arcoiris	FSH y LH	45 y 35 KDa	Govoroun et al., 1997 Santos et al., 2001
Morone saxatilis	Lubina estriada	GPα, LHβ y LH	14.8, 20.4 y 34.5 KDa	Mañanós et al., 1997
Fundulus heteroclitus	Fúndulo	GPα, FSHβ y LHβ FSH y LH	22-23, 18 y 21 KDa	Shimizu y Yamashita, 2002
Hippoglossus hippoglossus	Fletan	GPα, FSHβ y LHβ FSH y LH	19, 25 y 24 KDa 33 y 32 KDa	Weltzien et al., 2003
Anguilla japonica	Anguila japonesa	GPα, FSHβ y FSH	19, 17-21 y 33 KDa	Kamei et al., 2005
Dicentrarchus labrax	Lubina	GPα, LHβ y LH	12, 22 y 31 KDa	Mateos et al., 2006

Modificado de Zanuy et al., 2009.

la GTH-I y la GTH-II de peces con la FSH y la LH de tetrápodos, respectivamente (Li y Ford, 1998; Querat et al., 2000).

Estructura de las gonadotrofinas

Las GTHs forman parte, junto con la gonadotrofina coriónica placentaria (CG) y la tirotrofina hipofisaria (TSH), de una familia de hormonas glicoproteicas evolutivamente conservadas. Estas hormonas son heterodímeros relacionados estructuralmente, compuestos por la asociación no covalente de una subunidad α (GPα; también denominada Cga) común dentro de una misma especie y una subunidad β especifica que determina la actividad biológica y la especificidad de la hormona (Bousfield et al., 1994; Pierce y Parsons, 1981). Recientemente se ha incorporado a este grupo de glicoproteínas un nuevo miembro, la tiroestimulina (Nakabayashi et al., 2002) con la particularidad de que no comparte la misma subunidad α que el resto y cuya síntesis se ha detectado en numerosos tejidos aparte de la hipófisis. Las subunidades gonadotropas (GPα, FSHβ y LHβ) están codificadas por genes parálogos, descendientes de un gen ancestral común (Li and Ford, 1998). La dimerización y glicosilación es un requisito imprescindible para que las GTHs alcancen su actividad biológica completa, de manera que las subunidades por si mismas son biológicamente inactivas (Pierce y Parsons, 1981).
Las secuencias de aminoácidos de las subunidades α y β conocidas contienen una proporción alta de residuos de cisteína (Cys) localizados en posiciones conservadas. Estos residuos de Cys, que generalmente son 10 en la subunidad α y 12 en las subunidades β, forman 5 y 6 puentes disulfuro intracatenarios, respectivamente (Hearn y Gomme, 2000) (**Fig. 6**). La determinación de la estructura cristalina de la CG y la FSH humana mediante difracción de rayos X (Fan y Hendrickson, 2005; Fox et al., 2001; Lapthorn et al., 1994) ha permitido conocer mejor su estructura. A pesar de las considerables diferencias en la secuencia de aminoácidos, las subunidades α y β comparten una organización estructural similar. Modelos de estructura terciaria de las subunidades gonadotropas de peces sugieren que como en mamíferos, estas subunidades pertenecen a una superfamilia de proteínas caracterizadas por la presencia de un nudo de Cys, "cystine knot", formado por tres puentes disulfuro intracatenarios entre seis Cys que es crítico para el correcto plegamiento y dimerización de las subunidades (Hearn y Gomme, 2000). Desde el nudo de Cys salen tres lazos de horquilla β que se extienden dos hacia un lado de la molécula (L1 y L3; **Fig. 6**) y un tercero en sentido opuesto (L2; **Fig.**

6). Cuando se forma el heterodímero, las dos subunidades se asocian en un posicionamiento del tipo "cabeza-cola", formando una estructura alargada con los lazos βL2, αL1 y αL3 hacia un extremo y los lazos αL2, βL1 y βL3 hacia el opuesto (**Fig. 6**). La unión entre las dos subunidades se realiza de forma no covalente y se estabiliza mediante un lazo del extremo C-terminal de la subunidad β que envuelve a αL2 como un "cinturón de seguridad" y se ancla a βL1 por un puente disulfuro entre las Cys conservadas 3 y 12 (**Fig. 6**). Además de estabilizar el heterodímero, esta zona "cinturón de seguridad",

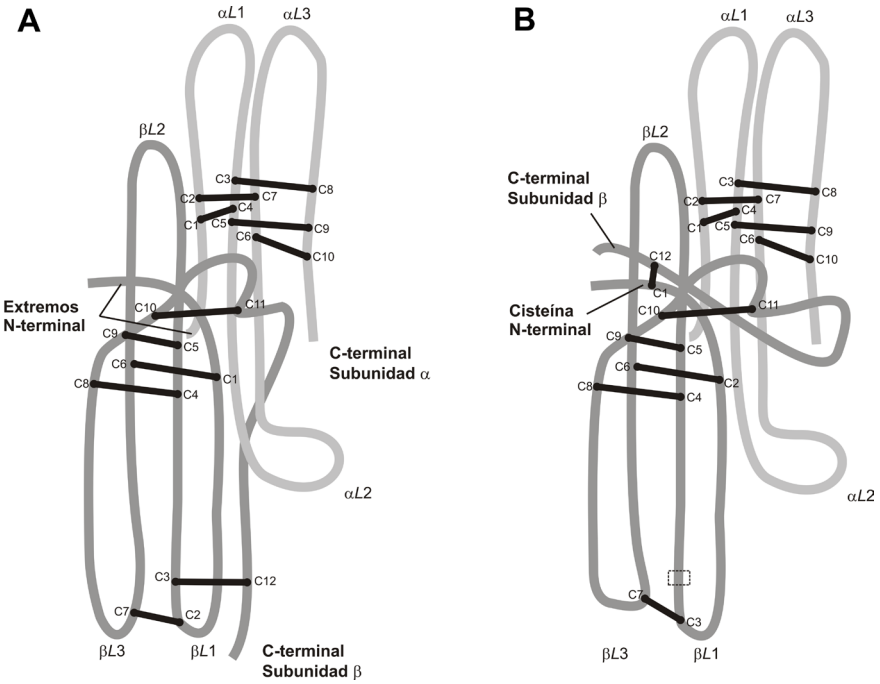

Figura 6. Representación esquemática de la estructura tridimensional de dos patrones de plegamiento de hormonas glicoproteicas encontrados en vertebrados. Los heterodímeros están compuestos por una subunidad α (gris claro) y una subunidad β (gris oscuro) que se alinean en un posicionamiento "cabeza-cola". Cada subunidad contiene un nudo central de cisteínas que divide el polipéptido en tres lazos de horquilla (ver texto). La asociación no covalente del heterodímero es estabilizada por un lazo de la subunidad β denominado "cinturón de seguridad" que se envuelve alrededor de αL2 y se ancla a βL1. (A) Patrón de plegamiento presente en las glicoproteínas de tetrápodos. (B) Patrón de plegamiento de algunas FSH de peces teleósteos. En algunas subunidades β FSH de peces falta la tercera cisteína conservada en βL1 (rectángulo discontinuo abierto), y se ha sugerido que sus "cinturones de seguridad" se anclan a una cisteína del extremo N-terminal. Esto se traduce en una marcada diferencia en la ubicación espacial de los residuos entre la undécima y duodécima cisteínas de la subunidad β. Adaptado de Rocha (2008).

parece que también esta implicada en la especificidad de unión al receptor y transducción de la señal (Moyle et al., 1994).

Análisis filogenéticos de subunidades gonadotropas de peces han mostrado que la subunidad α es la más conservada entre las diferentes especies y que la subunidad FSHβ ha evolucionado a un mayor ritmo que la LHβ (Querat et al., 2004). Al contrario que en la FSHβ, en las subunidades LHβ las posiciones de los 12 residuos de Cys y el único sitio potencial de *N*-glicosilación, están estrictamente conservados. Lo mismo sucede con las regiones consideradas importantes para la interacción con el receptor como la zona "cinturón de seguridad" (Swanson et al., 2003). Sin embargo, en la mayoría de teleósteos, excepto en los más primitivos, la FSHβ se desvía de la estructura básica de tetrápodos con 12 residuos Cys conservados y dos sitios potenciales de *N*-glicosilación. En la FSHβ de Salmónidos, Perciformes y Pleuronectiformes falta la tercera Cys conservada y el segundo sitio de *N*-glicosilación y tiene una Cys adicional cerca del extremo N-terminal (**Fig. 6**) que altera la conformación del "cinturón de seguridad". Dado la importancia en las interacciones con el receptor y en la estabilidad del heterodímero, la variación estructural de esta región entre las FSHβ de algunas especies de peces podría dar lugar a diferencias considerables entre ellas. Además, la perdida del segundo sitio de *N*-glicosilación podría afectar a su potencia biológica ya que los oligosacáridos alteran la vida media de las glicoproteínas y en algunos casos las interacciones con el receptor (Swanson et al., 2003).

Los pesos moleculares de las GTHs, estimados en diferentes especies de teleósteos, oscilan entre 12-28 KDa para las subunidades y 27-50 KDa para los dímeros (Zanuy et al., 2009) (**Tabla 2**). La porción glicosídica contribuye entre un 20 y un 40% de la masa total de las GTHs y desempeña un importante papel en muchas de las características funcionales de las mismas. Los oligosacáridos influyen en el correcto plegamiento de las subunidades, en el ensamblaje del dímero, en el almacenamiento intracelular y secreción, en la unión a su receptor, transducción de la señal y en la vida media de la hormona (Hearn y Gomme, 2000; Ulloa-Aguirre et al., 2003). El proceso de *N*-glicosilación empieza en el Retículo Endoplasmático Rugoso (RER) y termina en el Aparato de Golgi (AG), donde finalmente se obtiene la proteína madura con las modificaciones necesarias para ser secretada al torrente sanguíneo. Los oligosacáridos se unen a las GTHs mediante enlaces *N*-glicosídicos con residuos de Asparagina (Asn) en determinadas secuencias

potenciales de glicosilación (Asparagina-X-Serina/Treonina, donde X puede ser cualquier aminoácido excepto Prolina). La composición de los oligosacáridos en las hormonas glicoproteicas es altamente variable y depende en gran parte del tipo celular que las sintetiza. Además, dentro de una misma glicoproteína, puede encontrarse diferentes grados de glicosilación, constituyendo esta la principal base química para la formación de isoformas. En general, las *N*-glicosilaciones pueden agruparse en tres grandes categorías o subtipos: 1) subtipo de alto contenido en manosa, 2) subtipo complejo y 3) subtipo híbrido (**Fig.7**).

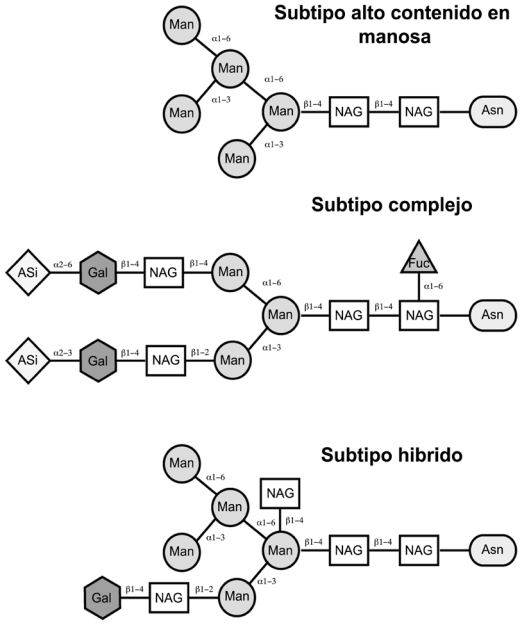

Figura 7. Estructura general de los tres principales subtipos de oligosacáridos encontrados en las isoformas de las hormonas glicoproteicas: subtipo alto contenido en manosa, subtipo complejo y subtipo hibrido. Los oligosacáridos se unen a determinadas aminoácidos de Asparragina (Asn) de la proteína y están compuestos por diversos residuos de azúcar: manosa (Man), N-acetilglucosamina (NAG), fucosa (Fuc) galactosa (Gal) y acido siálico (ASi). Adaptado de Hearn y Gomme (2000), y Gates et al., (2004).

Función de las gonadotrofinas

Las GTHs son esenciales en el control de la reproducción de todos los vertebrados. Como se comento anteriormente, se sintetizan en las células gonadotropas de la adenohipófisis, que en los peces estan situadas en la PDP y en ocasiones, en el borde de la PI. Una vez liberadas al torrente sanguíneo actúan sobre las gónadas a través receptores específicos, regulando la gametogénesis a través de su mediación en la producción de esteroides sexuales. A diferencia de los tetrápodos, donde está bien establecido el papel de cada GTH, en la mayoría de teleósteos no existe una información clara sobre sus diferentes funciones. En general, en peces existen pocos estudios donde se compara directamente la actividad de FSH y de LH debido a la ausencia de herramientas apropiadas para su estudio, principalmente de ensayos para determinar los niveles de FSH. A pesar de la reconocida importancia de ambas GTHs en la reproducción, falta información en procesos críticos como la diferenciación sexual, pubertad o gametogénesis. El acceso a fracciones puras y bioactivas de GTHs es fundamental para desarrollar ensayos y estudiar sus funciones en cada especie o grupo de especies. En las ultimas dos décadas se han desarrollado numerosos inmunoensayos para determinar LH en diferentes especies de peces, por contra, para FSH únicamente se han desarrollado en unas pocas, principalmente Salmónidos (**Tabla 5**). De los resultados obtenidos a partir de los inmunoensayos disponibles en estas especies (**Tabla 3**), se desprende que en salmónidos la FSH regula las fases iniciales de crecimiento y desarrollo gonadal (vitelogénesis e inicio espermatogénesis) mientras que la LH controla los procesos finales de maduración de los gametos, la ovulación en hembras y la espermiación en machos (Levavi-Sivan et al., 2010). En tilapia (*Oreochromis niloticus*) – la única especie no salmónido en la que se dispone de inmunoensayo para FSH – se ha observado un aumento de los niveles de ambas GTHs durante la vitelogénesis, sugiriendo que en este Perciforme la LH podría jugar un papel no solo durante la maduración de los oocitos sino también durante la vitelogénesis (Aizen et al., 2007). Por otro lado, estudios *in vitro* han demostrado que ambas GTHs estimulan la producción testicular de 11-ketotestosterona (11KT) durante el proceso de espermatogénesis en Salmónidos (Planas y Swanson, 1995) y Perciformes (Kagawa et al., 1998b), así como la producción de 17β-estradiol (E2) en ovarios vitelogénicos de Cipriniformes, Perciformes y Salmónidos (Okada et al., 1994; Planas et al., 2000; Suzuki et al., 1988c;

Tabla 3. Valores plasmáticos de GTHs medidos en algunas especies de teleósteos.

		Machos (≈ ng/ml)			Hembras (≈ ng/ml)					
Especie	**GTH**	*Inmad.*	*Rec. Test.*	*Esperm.*	*Inmad.*	*Vtg*	*Mad.*	*Ovul.*	*Postovul.*	**Referencia**
Oncorhynchus keta	FSH	---	---	---	---	---	---	40	---	Suzuki et al., 1988d
	LH	---	---	---	---	---	---	70	---	
Oncorhynchus rhodurus	FSH	---	---	---	---	---	---	2	---	Suzuki et al., 1988d
	LH	---	---	---	---	---	---	18	---	
Oncorhynchus mykiss	FSH	< 2	5	---	< 2	8	---	---	---	Suzuki et al., 1988d
	LH	< 2	1.5	---	< 2	2	---	---	---	
Oncorhynchus mykiss	FSH	< 2.5	5-6	3-4	< 2.5	17	< 7	34	---	Prat et al., 1996
	LH	< 0.3	< 0.3	3	< 0.3	< 0.3	2	70	---	
Oncorhynchus mykiss	FSH	---	---	---	8	17	10	8	> 25	Breton et al., 1998
	LH	---	---	---	< 0.5	1.5	21	13	4.5	
Oncorhynchus mykiss	FSH	2	9	4	3.5	15	3-8	---	15	Gomez et al., 1999
	LH	N.D.	< 0.5	0.8	N.D.	< 0.1	2.5-6	---	15	
Oncorhynchus mykiss	FSH	---	---	---	3	11	---	32	---	Santos et al., 2001
Oncorhynchus kisutch	FSH	< 2	50	20	< 2	30	---	10-15	---	Swanson, 1991
	LH	< 1	< 1	5-12	< 1	< 1	---	15-40	---	
Morone saxatilis	LH	---	---	5	---	1	4.5	---	3	Mylonas et al., 2001
Oreochromis niloticus	FSH	---	---	---	---	5-6	---	5-6	---	Aizen et al., 2007
	LH	---	---	---	---	5-6	---	8-10	---	
Dicentrarchus labrax	LH	1.2	2.5	2.5	< 0.5	1	3	3	2.5	Rocha et al., 2009

Inmaduros (Inmad.); Recrudescencia testicular (Rec. Test.); Espermiación (Esperm.); Vitelogénesis (Vtg); Maduración (Mad.); Ovulación (Ovul.); Post-ovulación (Postovul.); No detectable (N.D.).

Van der Kraak et al., 1992). Sin embargo, estos estudios mostraron que la potencia esteroidogénica de la LH excede a la de FSH a medida que avanza la espermatogénesis y la maduración de los oocitos. Este incremento de la potencia de LH respecto FSH coincide con la aparición del receptor de LH en el testículo, la espermiación, el cambio en la esteroidogénesis ovárica de E2 a 17α,20β-dihidroxi-4-pregnen-3-ona (17,20βP) y con la rotura de la vesícula germinal (Swanson et al., 2003). La única función descrita hasta el momento exclusiva de la FSH es su participación en la incorporación de vitelogenina en los oocitos (Tyler et al., 1997; Tyler et al., 1991). Mas recientemente, se ha demostrado también que la FSH regula la expresión de ciertos genes como la conexina (cx34.3), clusterina 1 (clu1), fibronectina (fn) y decorina (dcn), los cuales podrían estar involucrados en la comunicación celular, diferenciación de las células foliculares y remodelado de la estructura del folículo ovárico (Luckenbach et al., 2011).

En la última década, la biología molecular ha abierto nuevas posibilidades en el estudio de las funciones de las GTHs. La disponibilidad de sus secuencias codificantes ha permitido desarrollar métodos de análisis de expresión génica que ofrecen información indirecta sobre la síntesis GTHs mediante la medición de la actividad transcripcional (ARNm) de los genes que las codifican. Aunque los niveles de ARNm no reflejan necesariamente los niveles de hormona circulante, en Salmónidos los perfiles de expresión génica de ambas hormonas en la hipófisis se han correlacionado con sus niveles plasmáticos a lo largo del ciclo reproductor (Gomez et al., 1999), incrementando los niveles de ARNm de FSHβ antes que los de LHβ. En peces no Salmónidos como la anguila japonesa (*Anguilla japonica*), el esturión del Danubio (*Acipenser gueldenstaedtii*), el congrio del Pacífico (*Conger myriaster*) y la tilapia se han encontrado resultados similares, con altos niveles de expresión de FSHβ en estados tempranos de la gametogénesis y una expresión predominante de LHβ en fases finales de maduración, ovulación y espermiación. Sin embargo, en otras especies con desarrollo gonadal asíncrono o de puestas múltiples como el pez rojo (*Carassius auratus*), la dorada japonesa (*Pagrus major*), la lubina, la lubina estriada (*Morone saxatilis*) y el falso halibut del Japón (*Paralichthys olivaceus*), se ha observado una activa expresión de la subunidad FSHβ durante la fase de maduración gonadal (Levavi-Sivan et al., 2010). Además, en algunas especies como el pez rojo y la lubina los perfiles de expresión de ambas subunidades, LHβ y FSHβ, fluctúan de manera similar (Mateos et al., 2003; Sohn et al., 1999). Es posible que esta fluctuación

paralela de ambas GTHs en peces de puesta múltiple, refleje una regulación diferente por cada GTH de las distintas generaciones de oocitos presentes al mismo tiempo en el ovario (Levavi-Sivan et al., 2010).

Además de regular los procesos de gametogénesis en individuos adultos, la detección temprana de GTHs también sugiere que podrían participar en procesos tan importantes como la diferenciación sexual (Devlin y Nagahama, 2002). Apenas unos pocos estudios se han centrado en este aspecto pero de ellos se desprende que las GTHs y en particular la FSH, juegan un papel critico en la diferenciación sexual, tanto en especies gonocoristas como en hermafroditas (Kobayashi et al., 2010b; Pandolfi et al., 2006; Yamaguchi et al., 2007). En este sentido, mediante técnicas inmunohistoquímicas se ha visto que las poblaciones hipofisarias de células secretoras de FSH aparecen antes de la diferenciación sexual mientras que las de LH surgen varios días después de iniciado el proceso o justo al final (Feist y Schreck, 1996; Magliulo-Cepriano et al., 1994; Pandolfi et al., 2006; Saga et al., 1993).

Clonación y producción de gonadotrofinas recombinantes

Desde la década de los 80 hasta la fecha se han aislado y caracterizado las secuencias codificantes de las subunidades gonadotropas en 56 especies de peces, representadas en al menos 14 ordenes de teleósteos (Levavi-Sivan et al., 2010). La clonación de las subunidades gonadotropas ha permitido comparar las secuencias de nucleótidos y de aminoácidos en diversos vertebrados, demostrando que la GTH I y GTH II de peces son homologas a la FSH y LH de tetrápodos (Querat et al., 2000). Además, la disponibilidad de estas secuencias ha supuesto un gran avance en el estudio del proceso reproductor de los teleósteos, permitiendo caracterizar estos genes y desarrollar herramientas moleculares para estudiar los perfiles de expresión en determinados momentos del proceso reproductor. Por otra parte, la clonación de las tres subunidades gonadotropas ha abierto la posibilidad de usar técnicas de ADN recombinante para producir FSH y LH de diversas especies. Durante muchos años, las GTHs de peces se han obtenido mediante purificación a partir de hipófisis, un proceso altamente costoso que no siempre llega a buen termino. En la última década, la producción de GTHs recombinantes se muestra como una alternativa excelente a las hormonas nativas ya que pueden ser producidas continua y selectivamente, asegurando su disponibilidad y evitando la contaminación cruzada con otras glicoproteínas hipofisarias. En líneas generales, la metodología consiste en la inserción de

los ADN codificantes de las subunidades gonadotropas en vectores de expresión (plásmidos o virus) para transformar o transfectar diferentes líneas celulares u organismos heterólogos, donde, bajo el control de promotores fuertes, se expresan las GTHs en grandes cantidades. Esta metodología permite el suministro continuado de GTHs y elimina la necesidad de sacrificar cientos o miles de animales para purificar las hormonas nativas.

Para la producción de GTHs recombinantes se han usado diferentes sistemas de expresión, tanto procariotas como eucariotas. Los sistemas procariotas tienen la ventaja de su fácil manejo y bajo coste, pero presentan el inconveniente de carecer de los orgánulos celulares necesarios para procesar correctamente proteínas complejas como las GTHs. Por el contrario, los sistemas eucariotas poseen la maquinaria celular adecuada para realizar las modificaciones post-traduccionales necesarias y asegurar la completa bioactividad de estas glicoproteínas. Desde que se empezó a usar esta metodología en peces, se han producido varias subunidades libres y dimeros GTHs recombinantes de diferentes especies en distintos sistemas de expresión (**Tabla 4**). La producción de dímeros recombinantes se ha logrado de varias formas, mediante co-transfección de dos vectores de expresión, cada uno con el ADN codificante de una subunidad, por transfección con un único vector con ambos ADNs o mediante transfección con un vector con ambos ADN codificantes unidos que da lugar a proteínas de fusión o de cadena única (scGTHs).

Entre los sistemas de expresión eucariotas más usados para la producción de GTHs de peces se encuentran: - líneas celulares de la palomilla del maíz (*Spodoptera frugiperda*) (Sf9, Sf21) o larvas enteras del gusano de seda (*Bombyx mori*), infectadas con baculovirus modificados, - líneas celulares de mamíferos, como las de ovario de hamster chino (CHO) transfectadas con plásmidos, o - cultivos de la levadura *Pichia pastoris* (GS115, KM71) transformados con plásmidos. Todos estos sistemas de expresión han dado lugar a GTHs de peces biológicamente activas, aunque cada uno con unas ventajas e inconvenientes. La producción de subunidades libres y dímeros recombinantes tienen diversas aplicaciones, entre las que destacan: - su uso en estudios básicos de estructura y función, - el uso de FSHβ y LHβ como antígenos para la producción de anticuerpos específicos, esenciales para el desarrollo de inmunoensayos, y - la administración in vivo en aplicaciones biotecnológicas o terapéuticas (Levavi-Sivan et al., 2010).

Tabla 4. Producción de gonadotrofinas recombinantes de peces en diferentes sistemas de expresión.

Especie	Nombre común	Sistema expresión	Línea celular/ organismo	Gonadotrofina	Referencia
Oncorhynchus tschawytscha	Salmón	Bacterias	*Escherichia coli*	LH	Hew et al., 1989
Cyprinus carpio	Carpa común	Baculovirus	Sf9	GPα	Huang et al., 1991
Sparus aurata	Dorada	Baculovirus	Sf9	FSH y LH	Meiri et al., 2000
Morone saxatilis	Lubina estriada	Células mamíferos	CHO	FSH y LH	Blaise et al., 2000
Clarias gariepinus	Pez gato	Amebas	*Dictyostelium discoideum*	FSH y LH	Vischer et al., 2003
Ictalurus punctatus	Azul	Células de insecto	*Drosophila* S2	FSH y LH	Zmora et al., 2003, 2007
Carassius auratus	Pez rojo	Baculovirus	*Bombyx mori*	FSH y LH	Kobayashi et al., 2003 Kobayashi et al., 2006
Anguilla japonica	Anguila japonesa	Levaduras	*Pichia pastoris*	scFSH	Kamei et al., 2003
Carassius auratus	Pez rojo	Transgenicos	Embriones trucha arcoiris	scFSH y scLH	Morita et al., 2003 Morita et al., 2004
Oreochromis niloticus	Tilapia	Levaduras	*Pichia pastoris*	scFSH y scLH	Kasuto y Levavi-Sivan, 2005 Aizen et al., 2007a
Danio rerio	Pez cebra	Células mamíferos	CHO	scFSH y scLH	So et al., 2005
Brachymystax lenok	Trucha de Manchuria	Células mamíferos	CHO	scFSH y scLH	Choi et al., 2005
Epinephelus coioides	Mero	Baculovirus	Sf9	LH	Cui et al., 2007
Brachymystax lenok	Trucha de Manchuria	Baculovirus	*Bombyx mori*	scFSH y scLH	Ko et al., 2007
Anguilla japonica	Anguila japonesa	Células de insecto	*Drosophila* S2	FSH y LH	Kazeto et al., 2008
Danio rerio	Pez cebra	Levaduras	*Pichia pastoris*	FSH / scFSH	Yu et al., 2010
Anguilla japonica	Anguila japonesa	Baculovirus	*Bombyx mori*	scFSH y scLH	Kobayashi et al., 2010a

sc: hormona recombinante de cadena única. Modificado de Zanuy et al., 2009.

Receptores de GTHs (GTHRs)

Los receptores de FSH y LH (FSHR y LHR) junto con el de TSH (TSHR) constituyen una subfamilia de receptores de hormonas glicoproteicas (GpHRs) dentro de la gran familia de receptores acoplados a proteínas G (GPCRs) (Vassart et al., 2004). En peces, los GTHRs se expresan predominantemente en las gónadas, concretamente en la superficie de las membranas de las células foliculares de la teca (FSHR) y de la granulosa (FSHR y LHR), en las células de Leyding (FSHR y LHR) y en las de Sertoli (FSHR). Sin embargo, también se ha descrito la presencia de transcritos de FSHR y LHR en numerosos tejidos extragonadales como el riñón y el cerebro, si bien se desconoce su significado biológico (Levavi-Sivan et al., 2010).

Estructura

Los GTHRs están constituidos por una sola cadena proteica en la que se pueden identificar un gran dominio extracelular N-terminal, una región transmembrana con siete hélices alfa y un dominio intracelular C-terminal. Una de las principales características de los GTHRs es su gran dominio extracelular que constituye más de la mitad del receptor y esta involucrado con el reconocimiento y afinidad de unión al ligando (Ascoli et al., 2002; Dias et al., 2002). El dominio extracelular de los GTHRs tiene una porción central, tradicionalmente reconocida como la zona de unión al ligando, que contiene diez repeticiones ricas en leucina (LRR), flanqueadas por dos regiones ricas en cisteínas que protegen el centro hidrofóbico de las LRR. Además del papel protector, se cree que estas regiones ricas en cisteínas también podrían estar involucradas en el reconocimiento y unión al ligando, así como en la transducción de la señal (Fan y Hendrickson, 2005; Vassart et al., 2004).

A pesar de la elevada similitud estructural entre los GTHRs de los peces y los mamíferos, se pueden observar algunas diferencias importantes, sobre todo en los FSHRs. Contrariamente a lo observado en los FSHRs y LHRs de los mamíferos y en otras proteínas con dominios LRR, los FSHRs de los peces presentan notables diferencias en la región rica en cisteínas que bordea el extremo N-terminal del dominio LRR (Maugars y Schmitz, 2006; Oba et al., 1999a; Oba et al., 1999b; Rocha et al., 2007; Sambroni et al., 2007). Además, hay una delección de 30 aminoácidos en la región que bordea el extremo C-terminal del dominio LRR. Por otra parte, en los FSHRs de los peces Perciformes descritos hasta el momento existe una inserción de 25 aminoácidos, en la porción central del dominio extracelular, la cual

representa una LRR extra y sugiere una diferente curvatura y longitud de esta región. Se desconocen algunas de las implicaciones funcionales de las variaciones encontradas pero, considerando las diferencias existentes tanto en la distribución espacial de las LRR como en el número de los residuos de cisteínas, cabría suponer un plegamiento de las cadenas polipeptídicas de los FSHRs de peces distinto al descrito para humanos que potencialmente afectaría al modo de unión al ligando. A pesar de las diferencias estructurales mencionadas, la mayoría de los FSHRs de peces responden a las FSH de mamíferos (Levavi-Sivan et al., 2010; Zanuy et al., 2009). Respecto al LHR, no se dispone de la estructura cristalina pero se sabe que los aminoácidos implicados en la unión y especificidad del ligando de LHR de mamíferos están bien conservados en los LHR de peces (Rocha et al., 2007; Vischer y Bogerd, 2003).

El dominio transmembrana de los GTHRs es la parte más conservada y es la responsable de la activación del receptor y transducción de la señal. Por el contrario, el dominio intracelular muestra una alta variabilidad de secuencia entre los GTHRs de peces. Esta región contiene residuos involucrados en el acoplamiento a proteínas G, internalización del receptor y unión a diferentes moléculas de señalización intracelular (Ulloa-Aguirre et al., 2007).

Especificidad

Los GTHRs de mamíferos son altamente específicos con sus ligandos, mostrando menos de 0,1% de actividad cruzada (Braun et al., 1991; Moyle et al., 1994), de manera que la actividad de cada GTH está dirigida solo a aquellas células que expresan su receptor. Sin embargo, en peces teleósteos la especificidad de los GTHRs parece ser menos evidente (Bogerd et al., 2005). A pesar de la conservación estructural entre los GTHRs de peces, algunos estudios *in vitro* han revelado reconocimientos promiscuos de FSH y LH dependiendo de la especie y el origen (homólogas o heterólogas) (Ko et al., 2007; Kwok et al., 2005; Oba et al., 1999a; Oba et al., 1999b; Rocha et al., 2007; Sambroni et al., 2007; So et al., 2005; Vischer et al., 2003).

Transducción de la señal

Según el modelo clásico de transducción de la señal de los GPCR, la activación del receptor conlleva la activación de proteínas G localizadas en la cara interna de la membrana plasmática que a su vez activan otras mo-

léculas efectoras. La ruta de señalización intracelular mejor caracterizada y probablemente la más importante es la de la proteína quinasa A (PKA) mediada por AMP cíclico (AMPc). La activación de la proteína G estimula la adenilato ciclasa que cataliza la conversión de ATP a AMPc, el principal segundo mensajero de la acción de hormonas glicoproteicas (Zhang et al., 1991). El aumento de la concentración del AMPc intracelular libera la subunidad catalítica de la PKA permitiendo la fosforilación de numerosas proteínas celulares, entre ellas una clase de factores de transcripción que se unen a secuencias especificas conocidas como elementos de respuesta a AMPc (CREs). La fosforilación activa estos factores de trascripción y estos regulan la expresión de determinados genes, principalmente de enzimas implicados en la síntesis de esteroides. Además de la PKA se han identificado otras rutas de señalización que también son activadas por los GTHRs como las rutas MAPK, PKB, SGK o IP3/Ca2+/calmodulina/fosfatasa (Dias et al., 2010) cuyo significado fisiológico sigue bajo investigación.

Gónadas

Las gónadas son importantes órganos endocrinos destinados a producir gametos y especializados en la síntesis de esteroides sexuales. Estos esteroides juegan un papel esencial en los sistemas endocrinos de todos los vertebrados, con acciones que van desde la regulación del balance hídrico y mineral, la modulación de la respuesta inmunitaria, hasta la regulación de procesos implicados en la reproducción como la gametogénesis (Pankhurst, 2008).

Esteroidogénesis

Los esteroides sexuales, andrógenos, estrógenos y progestágenos, se sintetizan a partir del colesterol y su producción esta bajo el control de las GTHs. La esteroidogénesis ocurre en la mitocondria y en el retículo endoplasmático liso, donde una compleja cascada de enzimas oxidativos convierte el colesterol en diferentes tipos de esteroides. En particular, dentro de la mitocondria el colesterol es transformado en pregnenolona que es el precursor para la síntesis de todas las hormonas esteroideas (**Fig. 8**).

En líneas generales, durante las fases iniciales de la gametogénesis en machos, la FSH estimula la síntesis de andrógenos en las células de Leyding, principalmente de Testosterona (T) y 11-Ketotestosterona (11-KT), los cua-

les regulan procesos de proliferación espermatogonial y espermiogénesis (Schulz et al., 2010). Posteriormente, durante la espermiación, se produce un cambio en la actividad esteroidogénica del testículo y la acción de la LH provoca un incremento en la síntesis de progestagenos o esteroides inductores de maduración (MIS), 17α,20β-dihidroxi-4-pregnen-3-ona (17,20βP) o 17α,20β,21-trihidroxi-4-pregnen-3-ona (20βS), esenciales para la espermiación y la maduración de los espermatozoides (Fostier et al., 1983; Schulz et al., 2010). En hembras, la esteroidogénesis ovárica se desarrolla en la ma-

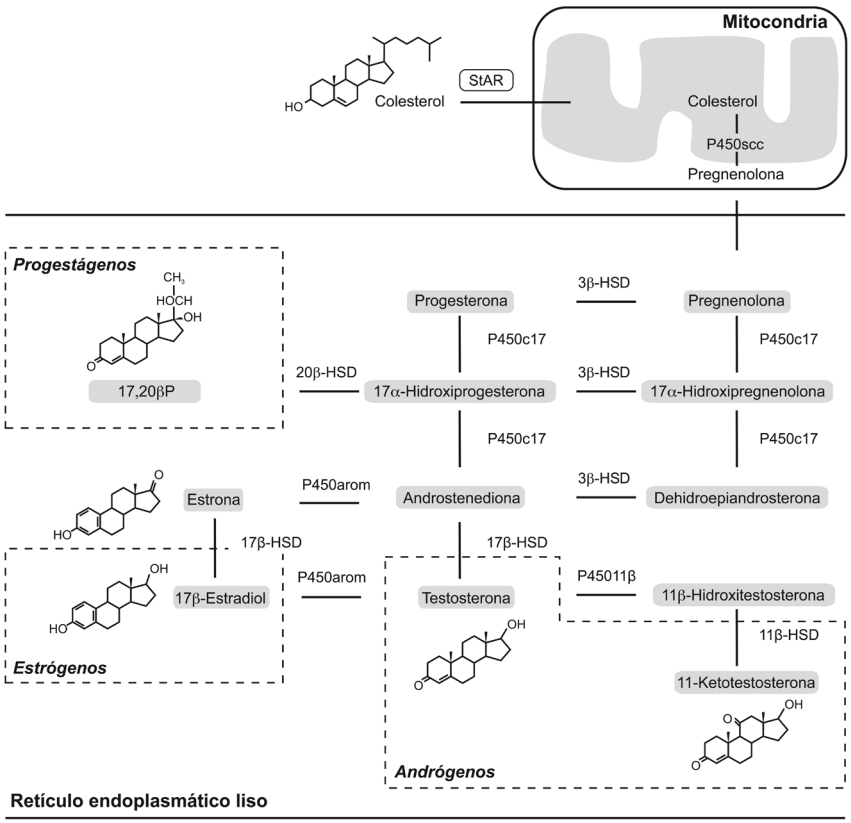

Figura 8. Ruta de biosíntesis de esteroides gonadales en peces teleósteos. Proteína reguladora de la respuesta esteroidogénica aguda (StAR); enzima P450 de la escisión de la cadena lateral de colesterol (P450scc); 17α-hidroxilasa/17,20-liasa (P450c17); 3β-hidroxiesteroide deshidrogenasa/Δ5-Δ4-isomerasa (3β-HSD); 11β-hidroxiesteroide deshidrogenasa (11β-HSD); 17β-hidroxiesteroide deshidrogenasa (17β-HSD); 20β- hidroxiesteroide deshidrogenasa (20β-HSD); P450 aromatasa (P450arom); P450 11β-hidroxilasa (P45011β); 17α,20β-hidroxi-4-pregnen-3-ona (17,20βP). Las áreas con líneas discontinuas delimitan los esteroides predominantes en plasma de teleósteos. Modificado de Rocha (2008)

yoría de especies según el modelo de dos células ("two-cell type model") propuesto por Nagahama (1994). En este modelo, las células foliculares de la teca sintetizan andrógenos precursores que son transportados a las células de la granulosa, donde son transformados en estrógenos o progestágenos. Durante la vitelogénesis, bajo el control principal de la FSH, las células de la teca sintetizan T que es convertida a E2 en las células de la granulosa mediante la enzima citocromo P-450 aromatasa (P-450arom), también conocida como CYP19A1 (Montserrat et al., 2004; Senthilkumaran et al., 2004). El E2 producido estimula la síntesis hepática de VTG que es transportada al ovario para ser incorporada a los oocitos en crecimiento (Nagahama, 1994). En la lubina, los niveles plasmáticos de T y E2 aumentan en paralelo conforme avanza el crecimiento oocitario y disminuyen durante la época de puesta, describiendo un perfil plasmático similar al de VTG (Asturiano et al., 2000; Asturiano et al., 2002; Prat et al., 1990). Una vez completada la vitelogénesis, antes de la maduración del oocito, la LH induce un giro en la actividad esteroidogénica del ovario. Se produce un cambio en la síntesis de esteroides precursores, de T a 17α-hidroxiprogesterona, y en la activación de enzimas esteroidogénicas, de P450arom (síntesis de E2) a 20β-HSD para la producción de MIS (17, 20βP o 20βS) que regula la maduración del oocito (Nagahama y Yamashita, 2008). En especies de puesta múltiple como la lubina, se ha visto que después de cada oleada de progestágenos se produce un nuevo cambio en la esteroidogénesis gonadal provocando una nueva elevación de los niveles plasmáticos de T y E2. Este patrón se repite varias veces dependiendo del numero de ovulaciones en las hembra y espermiaciones en los machos (Asturiano et al., 2002) y responde a la presencia de grupos de gametos en diferente estados de desarrollo.

Como se menciono anteriormente, existe una comunicación permanente entre las diferentes partes del eje CHG. Este dialogo permite que la actividad de los diferentes componentes del eje este sincronizada a lo largo de la vida del animal, lo cual es crucial para coordinar adecuadamente las respuestas fisiológicas. Los esteroides sexuales son particularmente importantes ya que el cerebro y la hipófisis los usan como indicadores del estatus sexual. Modulan la actividad de los sistemas neuronales, afectando a la expresión de neuropéptidos y neurotransmisores, así como sus correspondientes receptores en el cerebro y la hipófisis, y la expresión hipofisaria de GTHs (Levavi-Sivan et al., 2010; Zohar et al., 2010).

Métodos de cuantificación de GTHs

En las últimas décadas se han desarrollado diferentes tipos de ensayos para medir GTHs en diferentes especies de animales. En líneas generales, estos métodos se pueden agrupar en ensayos que estiman un número de moléculas o su masa mediante la unión específica a anticuerpos (inmunoensayos) y en ensayos que determinan una respuesta biológica de un sistema vivo ante la estimulación de FSH o LH (bioensayos *in vitro*).

Inmunoensayos

Los inmunoensayos están basados en la formación de complejos específicos antígeno-anticuerpo (Ag-Ac). Los primeros inmunoensayos que se desarrollaron para determinar GTHs, fueron radioinmunoensayos (RIA) basados en el uso de radioisótopos asociados a los complejos Ag-Ac. Aunque actualmente todavía siguen en uso, los RIAs se ha sustituido ampliamente por ensayos colorimétricos como los de inmunoabsorción ligados a enzimas, también conocidos como ELISAs. En líneas generales, tanto RIAs como ELISAs, se basan en la unión de las GTHs a anticuerpos específicos contra la subunidad FSHβ o LHβ, estableciéndose una reacción competitiva de naturaleza físico-química entre moléculas (GTHs) marcadas, de concentración conocida y moléculas no marcadas de concentración desconocida.

Desde finales de los 80 se han desarrollado numerosos inmunoensayos homólogos para medir LH en diferentes especies de peces teleósteos, sin embargo para FSH únicamente se han puesto apunto en tres especies de salmónidos con desarrollo ovárico síncrono, salmón chum (*Oncorhynchus keta*), salmón coho (*Oncorhynchus kisutch*) y trucha arcoiris (*Oncorhynchus mykiss*), y en un perciforme con desarrollo ovárico asíncrono, tilapia (*Oreochromis niloticus*) (**Tabla 5**). Esta situación ha propiciado que existan muchos estudios sobre la actividad y función de la LH y menos sobre la de la FSH. Hecho que ha obstaculizado el esclarecimiento de las funciones de cada GTH en numerosas especies de peces.

Tabla 5. Especies de teleósteos en las que se han desarrollado inmunoensayos para GTHs.

Especie	N. común	Ensayo	GTH	Sensibilidad (ng/ml)	CV Intra- (%)	CV Inter- (%)	Referencia
Hypophthalmichthys molitrix	Carpa plateada	RIA	GTH	0,58	6,8	8,6	Kobayashi et al., 1985
Oncorhynchus tschawytscha	Salmón chinook	RIA	GTH	0,44	6,0	7,9	Kobayashi et al., 1987
Oncorhynchus keta	Salmón chum	RIA	FSH LH	< 2 < 2	3,6 2,5	10,4 9,8	Suzuki et al., 1988
Micropogonias undulatus	Corvina	RIA	GTH	0,05	---	14,5	Copeland y Thomas, 1989
Oncorhynchus kisutch	Salmón coho	RIA	FSH LH	~ 0,28 ~ 0,28	--- ---	--- ---	Swanson et al., 1989, 1991
Carassius auratus	Pez rojo	ELISA *	GTH	0,125	5,0	9,0	Kah et al., 1989
Oncorhynchus mykiss	Trucha arcoíris	ELISA *	LH	0,070	4,2	6,3	Salbert et al.,1990
Sparus aurata	Dorada	RIA	GTH	0,3	10-11	---	Zohar et al., 1990
Pagrus major	Dorada japonesa	RIA	LH	0,78	6-7	11	Tanaka et al., 1993
Oncorhynchus mykiss	Trucha arcoíris	RIA *	FSH LH	2,34 0,26	--- ---	--- ---	Prat et al., 1996
M. saxatilis x M. chrysops	Lubina estriada h.	ELISA	LH	0,156	7-8	8-15	Mañanós et al., 1997
Oncorhynchus mykiss	Trucha arcoíris	RIA	FSH LH	0,87-1,42 0,1-0,2	4,6 5,9	9,8 8,3	Govoroun et al., 1998
Oncorhynchus mykiss	Trucha arcoíris	RIA	FSH	1	---	---	Santos et al., 2001
Seriola dumerilii	Pez limón	RIA	LH	0,25	---	<15	García Hernández et al., 2002
Dicentrarchus labrax	Lubina europea	ELISA	LH	0,65	11,7	11	Mateos et al., 2006
Oreochromis niloticus	Tilapia	ELISA	FSH LH	0,00024 0,0158	8 7,2	12,5 14,8	Aizen et al., 2007

* Ensayos heterólogos.

Bioensayos

Los bioensayos *in vitro* están basados, en respuestas celulares cuantificables a la estimulación con GTHs. Estas respuestas incluyen la producción de AMPc, producción de testosterona por cultivos de células de Leydig, actividad aromatasa o producción del activador de plasminógeno por cultivos de células de Sertoli o células de la granulosa.

Desde la clonación en los 90 de los ADNc de los FSHRs en rata y humanos, ha emergido una nueva generación de bioensayos basados en líneas celulares – como fibroblastos murinos (Ltk-), células embrionarias de riñón humano (HEK 293), células adrenales de ratón (Y-1) o de ovario de hámster chino (CHO) – que expresan los genes de FSHR o LHR de la especie de in-

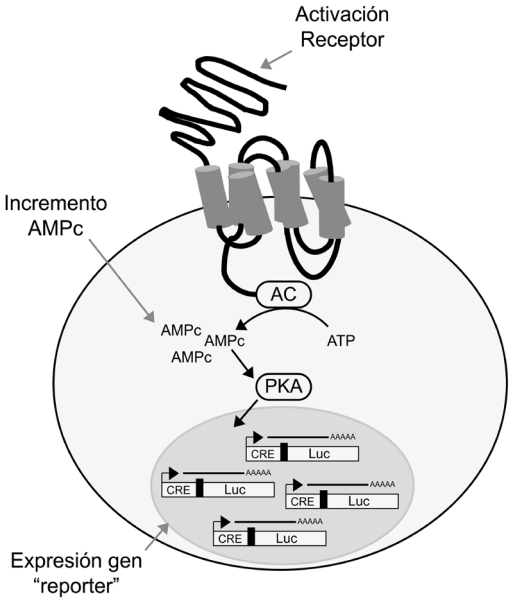

Figura 9. Representación esquemática de la activación de los GTHRs y su medición a través del uso de genes "reporter". Después de transfectar una línea celular con el receptor (FSHR o LHR) y un gen "reporter" como la luciferasa (Luc), la presencia y unión del ligando (FSH o LH) provoca la activación del receptor que estimula la adenilato ciclasa (AC) y cataliza la conversión de ATP a cAMP. El aumento intracelular del cAMP libera la subunidad catalítica de la proteína kinasa A (PKA) permitiendo la fosforilación de factores de transcripción que se unen a secuencias especificas conocidas como elementos de respuesta a cAMP (CRE) y regulan la expresión de determinados genes como el gen "reporter".

terés como herramienta para medir las propias GTHs (Christin-Maitre et al., 2000). Como se vio anteriormente, los receptores de GTHs pertenecen a una familia de receptores acoplados a proteínas G. La unión de las GTHs a su respectivo receptor incrementa la actividad de la adenilato ciclasa y aumenta la producción de AMPc que se puede cuantificar directamente mediante radioinmunoensayos (RIAs) o indirectamente a través del uso de genes "reporter" que contienen en su promotor elementos de respuesta al AMPc (CRE) (**Fig. 9**). Estos bioensayos, desarrollados hasta el momento únicamente en mamíferos, han proporcionado métodos muy sensitivos de estimación de la actividad biológica de las GTHs presentes en diferentes muestras biológicas o en preparaciones puras de FSH y LH (Christin-Maitre et al., 2000; Rose et al., 2000) (**Tabla 6**).

Tabla 6. Especies en las que se han desarrollado bioensayos para GTHs.

Especie	Línea celular	Gen "reporter"	GTHR	Sensibilidad	CV Intra- (%)	CV Inter- (%)	Referencia
Homo Sapiens	HEK 293	Luciferasa	FSHR	---	---	---	Tilly et al., 1992
Homo Sapiens	Y-1	---	FSHR	---	---	---	Kelton et al., 1992
Homo Sapiens	HEK 293	Luciferasa	LHR	0,3 ng/ml	18	13	Jia et al., 1993
Rattus norvegicus	Ltk^-	---	FSHR	0,3 UI/l	5,2	16,2	Gudermann et al., 1994
Homo Sapiens	CHO	Luciferasa	FSHR	< 3 UI/l (1,1ng/ml)	---	---	Albanese et al., 1994
Homo Sapiens	CHO	---	FSHR	6,2 UI/l	7,3	10,3	Tano et al., 1995
Homo Sapiens	CHO	Luciferasa	FSHR	< 4 UI/l	8	16	Cristin-Maitre et al., 1996
Homo Sapiens	CHO	Luciferasa	FSHR	0,010 UI/l	---	---	Kajitani et al., 2008

Capítulo 2. OBJETIVOS

Objetivos

La investigación desarrollada en la presente memoria de Tesis Doctoral se enmarca dentro del área de endocrinología reproductiva de peces. En las dos últimas décadas, en el Grupo de Fisiología de la Reproducción de Peces (GFRP) del Instituto de Acuicultura de Torre la Sal (IATS-CSIC), se ha venido realizando una gran actividad investigadora en relación con la reproducción de la lubina, un Perciforme de gran interés comercial en acuicultura. A pesar de los progresos alcanzados todavía existen algunos vacíos de conocimiento, sobre todo en lo que respecta a las funciones de las GTHs, y más concretamente de la FSH, durante la diferenciación sexual, pubertad y el ciclo reproductor.

En este contexto, **el objetivo general de la presente Tesis Doctoral es contribuir al conocimiento de la función de las GTHs durante el proceso reproductor de la lubina**. Para su consecución se plantearon **cinco grandes objetivos** que se abordaron usando diferentes metodologías:

1. Desarrollar herramientas moleculares para estudiar el perfil de expresión génica de GnRHs, GnRHR y GTHs durante la diferenciación sexual de la lubina y avanzar en el conocimiento del control endocrino de la misma.

- *Desarrollo de PCRs semicuantitativos*
- *Análisis de los perfiles de expresión génica*

2. Aislar y caracterizar bioquímica y funcionalmente la FSH nativa de lubina.

- *Extracción alcohólica de glicoproteínas hipofisarias*
- *Cromatografía de intercambio iónico*
- *Cromatografía de fase reversa*
- *Secuenciación de proteínas*
- *Caracterización bioquímica por secuenciación de aminoácidos, espectrometría de masas, SDS-PAGE y western blot*

- *Caracterización funcional en cultivos in vitro (líneas celulares y tejidos gonadales)*

3. Desarrollar sistemas de producción de gonadotrofinas recombinantes de lubina que permitan disponer de los dímeros y las subunidades de FSH y LH de forma continuada.

- *Puesta a punto de un sistema de expresión Baculovirus en células Sf9*
- *Puesta a punto de un sistema de expresión en células CHO*
- *Puesta a punto de un sistema de expresión en células de levadura*

4. Caracterizar y comparar, bioquimica y funcionalmente, las diferentes GTHs recombinantes producidas (especificidad, biopotencia y vida media).

- *Caracterización bioquímica por SDS-PAGE y western blot*
- *Activación especifica de receptores homólogos de GTHs*
- *Análisis de bioactividad en cultivos in vitro (líneas celulares y tejidos gonadales)*
- *Análisis de vida media en sangre (experimentos in vivo)*

5. Desarrollar métodos para cuantificar FSH y estudiar su papel en el control endocrino de la diferenciación sexual y de la gametogénesis.

- *Producción de anticuerpos policlonales mediante inmunización de conejos*
- *Desarrollo de un inmunoensayo homologo para FSH tipo "dot-blot"*
- *Desarrollo de un inmunoensayo homologo para FSH tipo "ELISA"*
- *Desarrollo de un bioensayo in vitro homologo para FSH usando el FSHR*
- *Estudio inmunohistoquímico de las GTHs en la hipófisis*
- *Análisis de los niveles de GnRHs y GTHs durante el proceso de diferenciación sexual*
- *Análisis de los niveles de FSH durante el proceso de gametogénesis*

El desarrollo de estos objetivos ha dado lugar a resultados que han sido publicados en diferentes revistas científicas. La metodología empleada, los resultados obtenidos y la discusión de cada apartado se presentan en una

compilación de las publicaciones originadas, capítulos 3-7. Finalmente, en el capitulo 8 se incluye una discusión general que aborda conjuntamente todos los resultados de esta Tesis Doctoral.

Nota: Las publicaciones mantienen los criterios de uniformidad de sus respectivas revistas, aunque se han editado para facilitar su lectura y adaptarlos al formato de la presente memoria de Tesis Doctoral.

Capítulo 3:

Temporal profile of brain and pituitary GnRHs, GnRH-R and gonadotropin mRNA expression and content during early development in European sea bass (*Dicentrarchus labrax* L.)

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Abstract

A likely endocrine control mechanism for sexual differentiation in size-graded populations of European sea bass (*Dicentrarchus labrax*) is proposed by evaluating the brain expression and pituitary content of two forms of gonadotropin-releasing hormone (GnRH), namely sea bream (sbGnRH) and salmon (sGnRH), the pituitary expression of one subtype of GnRH receptor (dlGnRH-R-2A) and the three gonadotropin (GtH) subunits, namely glycoprotein α (GPα), follicle-stimulating hormone β (FSHβ) and luteinizing hormone β (LHβ), as well as the pituitary and plasma LH levels between 50 and 300 days post-hatching (dph). Four gradings were conducted between 2 and 8 months after hatching, resulting in a population of large and small individuals, having 96.5% females (female-dominant population) and 69.2% males (male-dominant population), respectively, after the last grading. The onset of gonadal differentiation was different in the two sexes, and coincided with a peak of expression of sbGnRH or sGnRH. Furthermore, the expression of these GnRHs was correlated with the expression of dlGnRH-R-2A. Sex-related differences in the brain and pituitary content of sbGnRH were also found at the time of sexual differentiation. Moreover, the observed sexual dimorphism at the transcriptional or synthesis level of these GnRH forms suggests that a different neuro-hormonal regulation is operating according to sex. At the onset of sex differentiation, FSHβ transcriptional activity reached maximal values, which were maintained until the completion of the process. The present study suggests a role for sbGnRH, sGnRH and the dlGnRH-R-2A during gonadal differentiation, possibly through enhancement of FSHβ gene expression. In males, a different endocrine regulation seems to exist also during spermiogenesis and spermiation, when gene transcription, peptide synthesis and release of LH are of greater importance.

Keywords: GnRHs, GnRH receptor, gonadotropins, sex differentiation, European sea bass

1. Introduction

The study of sex differentiation in fish represents a unique opportunity to understand the plasticity of this process, considering the high diversity and the wide range of aquatic habitats. Since the pioneer publication of Yamamoto (1969), excellent reviews on sex differentiation in fish have demonstrated its importance and interest (Baroiller et al., 1999; Devlin and Nagahama, 2002; Nakamura et al., 1998). The endocrine control of sex differentiation in fish requires a complex interplay between the brain, pituitary and gonads through the production of brain neuropeptides and neurotransmitters, pituitary-derived gonadotropins (GtH) and steroids produced in the gonad and brain. Moreover, a strong interaction with some still weakly defined environmental factors also contributes to activate the brain–pituitary–gonadal axis to trigger the process of sex differentiation in fish. This extraordinary complexity has been an important barrier that restricted such studies to only few species of teleosts, where only partial aspects of the molecular and endocrine regulation of sex differentiation have been examined (Devlin and Nagahama, 2002; Nagahama, 1999). In teleosts, the activation of the brain–pituitary axis may be a key event in the onset of sex differentiation (Baroiller et al., 1999), but what leads to the activation of this axis is not yet well understood.

The European sea bass (*Dicentrarchus labrax*) is a highly valuable fish for European aquaculture. Due to the fact that females grow faster than males and reach marketable size before maturing, it is desirable to produce female monosex populations. As a result, this species has been the object of various studies on the environmental, genetic and hormonal control of sex differentiation (reviewed by Carrillo et al. (1999) and Piferrer et al. (2005)). In European sea bass, as in other perciform fishes, three different GnRH forms are expressed in the brain: sea bream GnRH (sbGnRH) is expressed mainly in the preoptic area (POA) cells, salmon GnRH (sGnRH) mostly in the olfactory bulbs and chicken GnRH-II (cGnRH-II) in the midbrain tegmentum with apparently diverse functions, but not playing a direct role in regulating the pituitary–gonadal axis (González-Martínez et al., 2002a,b). These GnRH systems are already developed by the time the genital crest starts to be evident in this species (González-Martínez et al., 2004), indicating that this machinery is totally formed, well before sex differentiation occurs. It has been stated that in European sea bass, sbGnRH represents

the main hypophysiotrophic hormone (González-Martínez et al., 2002a,b; González-Martínez et al., 2001; Rodríguez et al., 2000). A direct effect on the gonadotropic cells to regulate GtH secretion has been ascribed to sGnRH (González-Martínez et al., 2002b) because sGnRH innervation has been observed in European sea bass pituitary, although it is considerably less than sbGnRH. Nevertheless, both forms peaked at the time of sex differentiation in a population of European sea bass of a mixed and unidentified sex (Rodríguez et al., 2000). However, no specific information is available from populations of fish in which sex is known or could be anticipated with a certain degree of confidence. In the European sea bass, two subtypes of GnRH receptors (GnRH-R) were described (González-Martínez et al., 2004; Lethimonier et al., 2004), whereas recently five GnRH-R subtypes, encoded by five different genes, have been isolated in fish (Moncaut et al., 2005). From these five subtypes, dlGnRH-R-2A is highly expressed in the pituitary LH cells, but also in some FSH cells, and its expression increases as the European sea bass matures sexually (González-Martínez et al., 2004), indicating its importance in reproductive events. Nevertheless, the role of dlGnRH-R-2A during sex differentiation remains to be explored.

Gonadotropin-releasing hormone acts on the pituitary gland to stimulate the synthesis and secretion of GtHs. Today, the existence of two GtHs in the pituitary gland [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] with distinct patterns of expression at different stages of the reproductive cycle has been confirmed in various teleosts, including the European sea bass (reviewed in Yaron et al., 2001, 2003; Mateos et al., 2003). Gonadotropins have been shown to play a critical role in sex differentiation both in gonochoristic and hermaphroditic fishes (reviewed in Devlin and Nagahama, 2002). Most physiological studies have been conducted mainly on LH, while the function of FSH has not been fully addressed, because FSH immunoassays for non-salmonid fishes are not available. Nevertheless, it has been claimed that FSH regulates gametogenesis, since it promotes the production of 17β estradiol (E2) and the incorporation of vitellogenin into the oocytes (Planas et al., 2000; Swanson, 1991). In addition, it appears to stimulate Sertoli cell proliferation and maintenance of quantitatively normal spermatogenesis mediated by these cells (Bogerd et al., 2005). On the other hand, LH is known to stimulate gonadal steroidogenesis in testicular Leydig cells and is involved in oocyte maturation and ovulation, and in spermiation (Prat et al., 1996; Schulz and Miura, 2002). In male European

sea bass, FSH mRNA levels increase continuously with gonadal growth suggesting an important role in the maintenance of spermatogenesis, while LH synthesis and release is maximum during late gametogenesis and spawning (Mateos et al., 2003). Moreover, in European sea bass, LH was more involved with later stages of gonadal development than with sex differentiation (Rodríguez et al., 2000).

It has been shown recently that in European sea bass, there is a relation between growth and phenotypic sex differentiation early in development, i.e. large fish usually differentiate into females and small ones into males (Papadaki et al., 2005). Taking advantage of a male-dominant and a female-dominant population created in the latter study, the objective of the present study was to investigate the possible endocrine control mechanisms of sexual differentiation in European sea bass by evaluating the brain expression and pituitary content of sbGnRH and sGnRH, which are the two hypophysotopic forms of GnRH; the pituitary expression of the gonadotropin-releasing hormone receptor (dlGnRH-R-2A) and the expression of the three GtH subunits, which are glycoprotein alpha subunit (GPα), FSHβ, LHβ, as well as the pituitary and plasma LH levels throughout the period of sexual differentiation of male- and female-dominant populations.

2. Materials and methods

2.1. Fish husbandry and production of female- and male-dominant stocks: fish sampling

The populations used in the present study were those developed by size grading in the study of Papadaki et al. (2005). In brief, hatched larvae and fry obtained from a natural spawning (February 2001) were reared at the Hellenic Centre for Marine Research, Institute of Aquaculture (Crete, Greece), using the "mesocosm" method (Divanach and Kentouri, 2000). Four gradings were conducted between 2 and 8 months after hatching, separating the fish to large and small individuals, approximately in a 50:50 ratio. The first grading was performed at 66 dph using a 3.5-mm bar grader, separating the population to large (L) and small (S) fish. The second grading was done at 123 dph for the S population (6-mm bar grader), separating the fish again to small (SS) and large (SL). The SL fish were discarded. At 143 dph, the L population was similarly graded (8-mm bar grader), but instead the

LL fish were kept and the LS were discarded. Following the same principle, the third grading was performed on 166 and 167 dph for the SS and the LL populations, respectively, and the fourth grading on 222 and 223 dph, for the SSS and LLL populations, respectively. In this way, the L-extreme and S-extreme populations were created, by discarding about 50% of the population at each grading. Examination of gonadal histology of fish from these populations revealed that phenotypic sex could not be determined prior the second grading (123–143 dph), and therefore it is not known what effect the first grading had on the sex ratio of the two populations. After the second grading, the female percentage was already 91% in the L-extreme population, but the male percentage in the S-extreme population was only 50%. With each subsequent grading, the percentage of females in the L-extreme population increased slightly (5%), while that of males in the S-extreme population had a larger increase (20%). At 300 dph, the S- and L-extreme populations consisted of 69.2% males (male-dominant) and 96.5% females (female-dominant), respectively (Papadaki et al., 2005). In the present study, fish were sampled every 50 days between 50 and 300 dph. At 50 dph, samples were taken before the grading procedure started. At 100, 150 and 200 dph, the sex proportion of the two populations was different (Papadaki et al., 2005), but the sex of the individual fish sampled for tissues could not be macroscopically identified. On the 250 and 300 dph sampling, however, the sex of each sampled fish was identified after sacrifice by a tissue squash examined under a microscope, and only females were included in the female-dominant group and only males in the male-dominant one.

Samples of brains and pituitaries of fish from the male- and female-dominant populations (S- and L-extreme populations, respectively) were collected every 50 days between 50 and 300 dph, whereas blood was collected from 150 to 300 dph. Dissection of the brain and pituitary was not possible between 50 and 100 dph due to the small size of the fish; therefore, whole fish heads were collected at these times. Brain and pituitaries (or the whole head when available) were deep frozen immediately in liquid nitrogen and kept at -80°C until analysis. From 150 dph onwards, blood samples were taken from the caudal vasculature using heparinized syringes and centrifuged at 4°C, 6000 rpm for 15min. The separated plasma was aliquoted and kept at -80°C until analysis.

Handling of the fish was done according to the European Union Directive (EEC, 1986) for the protection of animals used for experimental and other

scientific purposes.

2.2. Development of a semiquantitative polymerase chain reaction technique for GnRHs, GtHs and GnRH-R

Semiquantitative polymerase chain reactions (PCRs) were set up to analyze the relative amounts of mRNA for several genes, including the GnRHs (sbGnRH and sGnRH), the *D. labrax* GnRH receptor 2A (dlGnRH-R-2A) and the GtH subunits (GPα, FSHβ and LHβ) in brain and pituitary. PCR amplification of ribosomal 18S was used as a reference for RNA quantity and reverse transcription efficiency, based on the limited variation of 18S expression with the time and stage of the animals. The full-length cDNAs for all these genes were previously cloned (Mateos et al., 2003; Zmora et al., 2002), which allowed us to design the corresponding specific primers for use in PCR (Table 1). To develop the semiquantitative PCR technique, we tested diVerent ranges of MgCl2 (0.75, 1.5 and 3mM) and annealing temperatures (56–58°C) for each set of primers. Once the concentration of the reagents and the optimal annealing temperatures were standardized, a test of cycles (from 12 to 40 cycles) for each gene was performed for several initial concentrations of template (0.2, 0.4, 0.8 and 1.6 µl of cDNA). The optimal number of cycles, which gave an intensity located in the midpoint of the growing phase, was chosen for each gene in order to determine its relative abundance (Table 1). Briefly, total RNA was extracted of individual brains and pituitaries with the Trireagent (Molecular Research Centre, Inc., Cincinnati, OH, USA) and treated with RQ1 DNase (Promega Corp.). Total RNA (1–4 µg) was reverse-transcribed to cDNA in a 20-µl reaction volume using random primers (hexamers, 50 ng) and SuperScript II (200 units) reverse transcriptase (Invitrogen). The PCR amplifications were performed with a thermal cycling, Gene Amp PCR System 2700 (Applied Biosystems), and all were performed with 1 µl of cDNA, 0.5 µM of each specific primer, 0.2 mM dNTPs and 1.5–3mM MgCl2 in a 20-µl of reaction volume. Thermal cycling parameters were: denaturation step of 94°C for 30 s, annealing step of 57°C for 45 s and elongation step of 72°C for 45 s. PCR that contained water in place of cDNA template served as negative controls. At the end, PCR samples were run on 1% agarose gels, containing ethidium bromide and the intensity of the bands analyzed on a densitometer, under UV light. The data were normalized to the transcript abundance of ribosomal 18S;

Table 1. Primers and conditions used in a semiquantitative PCR technique for the GnRHs, the dlGnRH-R-2A and the GtH subunits gene expression analysis.

Primer	Sequence (5'-3')	Amplicon size (bp)	Mg^{2+} (mM)	Cycles
GP-α	F: TGGACTTATCAAACATGGG	245	3	24
	R: GTGGCAGTCTGTGTGGTTTC			
FSH-β	F: GTCATCCCACCAACATCAG	275	3	28
	R: TATGTCTCCAGGAAAGCG			
LH-β	F: GCTCATCAACCAGACAGTG	286	3	38
	R: CAGGCTCTCGAAGGTACAG			
sbGnRH	F: GAGAGAGAAGAATGGCTG	294	1.5	36
	R: GATTTCCGTTCTCCCTGTGAGTG			
sGnRH	F: GTTGTTGGCGTTGGTGG	214	1.5	34
	R: CGGTCAAAATGACTGGAATC			
cGnRH-II	F: CTGTTTGGGCTGCTTCTATGTGT	234	1.5	38
	R: TCACTTCCTCTTCTGGAGCTC			
GnRH-R	F: GAATATCACAGTCCAGTGG	535	1.5	36
	R: CCAGCAGACGATGAAAGAC			
18S	F: TCAAGAACGAAAGTCGGAGG	485	3	18
	R: GGACATCTAAGGGCATCACA			

thus, the results were expressed as the ratio between the optical density of the specific gene and that of 18S.

2.3. GnRH forms immunoassays

Phenolic phases from the RNA extraction of 8–10 individual heads or brains or pituitaries, per sampling point, were extracted for 10 min at 80°C by adding 150 µl of 2 N acetic acid, centrifuged (4°C for 30 min at 10,000g) and supernatants collected, air-dried and reconstituted in assay buffer for analysis. Levels of sbGnRH and sGnRH were measured simultaneously in each reconstituted sample using competitive enzyme-linked immunosorbent assays (EIA), specific for each GnRH form. The sGnRH was determined using an EIA developed for European sea bass (Kah et al., 1994). The EIA for sbGnRH was performed according to Holland et al. (1998). The sGnRH EIA had a sensitivity of 2 pg/well and a cross-reactivity with sbGnRH of 0.005%. The sbGnRH assay had a sensitivity of 6 pg/well and a cross-reactivity with sGnRH of 0.4% (Holland et al., 1998). Protein levels were measured according to Bradford (1976).

2.4. LH immunoassay

Measurement of LH was carried out in pituitaries and plasma of 10–12 fish, per sampling point, by a homologous ELISA (Mateos et al., 2006) based

on the method described for the striped bass Morone saxatilis (Mañanós et al., 1997). The European sea bass LH EIA uses specific polyclonal antibodies against the European sea bass LHβ subunit and European sea bass LH for the standard curve. The sensitivity of the assay was 0.65 ng/ml and intra- and inter-assay coefficients of variation of 11.7% (n = 8) and 11% (n = 10), respectively.

2.5. Data analysis

Differences between the female- and male-dominant populations throughout the experiment, in mRNA expression levels of GnRHs, dlGnRH-R-2A and GtH subunits; brain and pituitary content of the GnRHs; and pituitary and plasma levels of LH were examined using two-way ANOVA, with sex (male- and female-dominant population) and sample time as fixed factors, followed by the Holm-Sidak test. When necessary, normality was ensured using the Kolmogorov–Smirnoff test after logarithmic transformation of data. Barlett's test was used to establish homogeneity of variances. A linear correlation test was also conducted on the relationship between gene expression and brain and pituitary levels of the sbGnRH and sGnRH, dlGnRH-R-2A receptor and the three GtH subunits and pituitary and plasma levels of LH. A Pearson product–moment correlation coefficient was calculated. Statistical analyses were done with a statistical software (Sigmastat v.3.0 SPSS). The level of P at which the results were deemed significance was $P < 0.05$. Results are reported as means ± S.E.M.

3. Results

3.1. Brain sGnRH and sbGnRH gene expression

Brain mRNA levels for sbGnRH and sGnRH showed significant differences, both over time and between male and female-dominant populations. A significant and steady increase in expression of sbGnRH was observed in the male-dominant population from 50 dph onwards, peaking at 200 dph and decreasing thereafter (Fig. 1A). The pattern of sbGnRH gene expression in the female-dominant population was bimodal, peaking both at 150 and 250 dph (Fig. 1B). At 300 dph, the expression of sbGnRH in the female population was significantly higher ($P \leq 0.05$) than in the male one. At 50

and 100 dph, values of sGnRH expression were very low in both populations, with a significant ($P \leq 0.001$) increase occurring at 150 dph (Figs. 1C and D). However, in the male-dominant population, the transcript values of sGnRH continuously increased in the following period until reaching maximum levels at 200 dph (Fig. 1C), whereas in the female-dominant population there were two peaks at 150 and 250 dph (Fig. 1D). Salmon GnRH gene expression in the male-dominant population was significantly higher than in the female-dominant population, both at 100 and 200 dph ($P \leq 0.05$ and $P \leq 0.001$, respectively). A regression correlation between the expression of sbGnRH and sGnRH in the two populations yielded a coefficient of correlation (r) of 0.942 ($P \leq 0.01$) for the male and 0.960 ($P \leq 0.01$) for the female-dominant populations, respectively (Table 2).

3.2. Gene expression of dlGnRH-R-2A in the pituitary

At 50 dph, pituitary dlGnRH-R-2A mRNA in the male-dominant population was very low, increased progressively until 150 dph, and a high and significant ($P \leq 0.001$) surge was observed at 200 dph (Fig. 1E). Thereafter, high levels of expression were maintained until the end of the surveyed period at 300 dph. In the female-dominant population, pituitary dlGnRH-R-2A gene expression was low at 50 dph. A steady increase was observed from that time on to peak at 250 dph, while a significant decrease ($P \leq 0.001$) was observed at 300 dph (Fig. 1F). The relationship between the profiles of expression of pituitary dlGnRH-R-2A and brain sbGnRH and sGnRH was evaluated through a regression line analysis both in the male- ($r = 0.816$, $P \leq 0.05$ and $r = 0.909$, $P \leq 0.05$, respectively) and female-dominant populations ($r = 0.809$, $P \leq 0.05$ and $r = 0.697$, $P \leq 0.1$, respectively) (Table 2).

3.3. Brain levels of sbGnRH and sGnRH

At 50 and 100 dph, the whole-head content of sbGnRH was very low both in the female- and in the male-dominant populations (data not shown). In the male-dominant population, brain content of sbGnRH was low at 150 and 200 dph, increasing significantly ($P \leq 0.001$) at 250 dph and decreasing ($P \leq 0.05$) in the following sampling time (Fig. 2A). At 150 dph, brain sbGnRH content in the female-dominant population was also low, but increased steadily thereafter and peaked at 250 dph (Fig. 2B). The sbGnRH brain con-

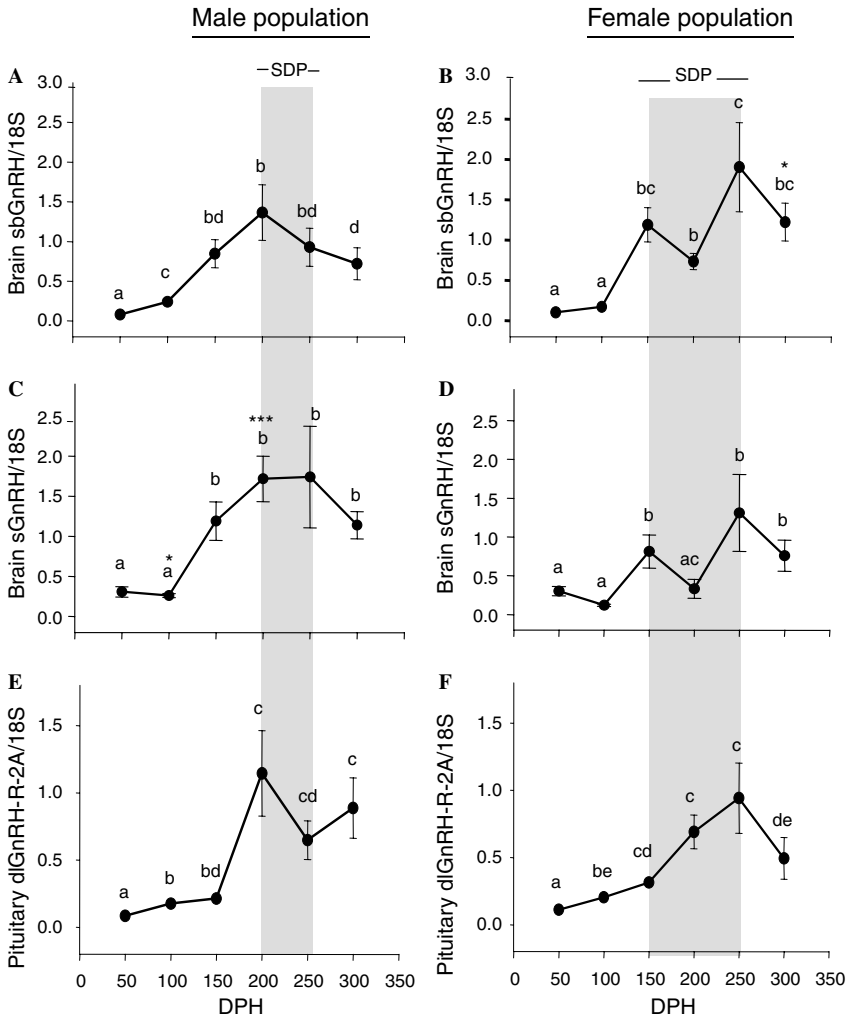

Figure 1. Temporal variations of the levels of the brain sbGnRH mRNA (A and B) and sGnRH mRNA (C and D) and of the pituitary dlGnRH-R-2A mRNA (E and F) in male- and female-dominant populations of European sea bass during the first year of life. Different letters indicate significant differences over the time for female or male populations. Asterisks indicate significant (*P≤0.05; ***P≤0.001) differences between two populations at equivalent dates. Sex differentiation period (SDP) are depicted as a shaded bar in the figure.

tent of the female-dominant population was significantly higher than that of the male-dominant population only at 150 and 200 dph ($P \leq 0.05$ and $P \leq 0.01$, respectively). Similarly, the whole-head content of sGnRH was very low at 50 and 100 dph in both populations (data not shown). A steady increase

was detected from 150 dph onwards, peaking at 250 dph in both populations (Figs. 2C and D).

3.4. Pituitary levels of sbGnRH and sGnRH

High levels of sbGnRH pituitary content were found in the male-dominant population at 150 dph (Fig. 2A). A significant decrease ($P \leq 0.001$) was observed at 200 dph, which was maintained almost constant until the end of the study. Similarly, the sbGnRH pituitary content of the female-

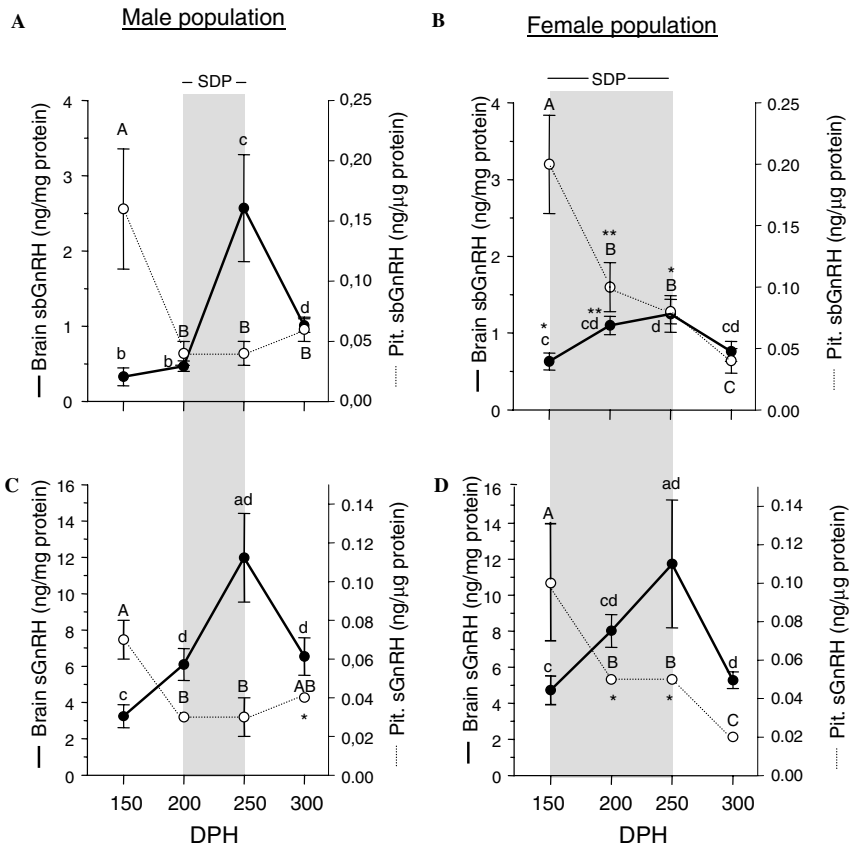

Figure 2. Temporal profiles of brain (–) and pituitary (....) content of sbGnRH (A and B) and sGnRH (C and D) in male- and female-dominant populations of European sea bass during the first year of life. Different lower case and upper case letters indicate significant differences over the time for brain or pituitary GnRH levels, respectively. Asterisks indicate significant (*P≤0.05; **P≤0.01) differences between two populations at equivalent dates. Rest of the legends refer to Fig. 1.

dominant population was highest at 150 dph and decreased significantly ($P \leq 0.05$) thereafter. In addition, at 200 and 250 dph, the pituitary sbGnRH levels of female-dominant population were significantly higher than in the male-dominant population ($P \leq 0.01$ and $P \leq 0.05$ and Figs. 2B and A, respectively). In both populations, the profile of pituitary sGnRH content was very similar to that of sbGnRH in which it exhibited the same significant differences within and between populations (Figs. 2C and D). The only difference was that values at the starting time (150 dph) were about half those of sbGnRH.

3.5. Gene expression of the GtH subunits in the pituitary

Expression of GPα, FSHβ and LHβ was almost undetectable at 50 and 100 dph in both populations (Figs. 3A–D and 4A and B, respectively). In the male-dominant population, the first significant rise ($P \leq 0.001$) of GPα subunit mRNA occurred at 150 dph, peaked further at 200 dph and high levels of expression were maintained thereafter until the completion of the experiment (Fig. 3A). In the female-dominant population, the first significant increase of expression occurred at 150 dph, with high levels at 200 dph, which remained constant thereafter (Fig. 3B). The level of GPα gene expression in the male population was significantly higher ($P \leq 0.01$) than that of

Table 2. Linear regression tests on the relationship between brain and pituitary gene expression levels of sbGnRH and sGnRH, dlGnRH-R-2A and the three GtH subunits (GPα, FSHβ and LHβ) and pituitary and plasma levels of LH.

	Male population			Female population		
	[a]RL	[b]r	P≤	[a]RL	[b]r	P≤
Gene expression/gene expression						
sbGnRH *vs* sGnRH	Y=-0.0244+0.682X	0.942	0.01	Y= 0.0305+1.514X	0.960	0.01
dlGnRH-R-2A *vs* sbGnRH	Y= 0.00158+0.751X	0.816	0.05	Y= 0.134+0.368X	0.809	0.05
dlGnRH-R-2A *vs* sGnRH	Y= -0.00188+0.447X	0.909	0.05	Y= 0.158+0.499X	0.696	0.1
LHβ *vs* GPα	Y=-0.268+1.352X	0.975	0.05	Y= 0.161+2.064X	0.972	0.05
FSHβ *vs* GPα	Y= 0.609+1.058X	0.990	0.01	Y= 0.188+0.427X	0.977	0.05
dlGnRH-R-2A *vs* GPα	Y= 0.0757+0.935X	0.959	0.01	Y= 0.1320+0.866X	0.873	0.05
dlGnRH-R-2A *vs* FSHβ	Y= 0.0892+ 0.5X	0.932	0.001	Y= 0.1240+0.536X	0.843	0.05
dlGnRH-R-2A *vs* LHβ	Y= 0.1300+1.151X	0.892	0.05	Y= 0.1990+0.993X	0.811	0.05
Levels/gene expression						
LH pit *vs* LH βmRNA	Y= 4.638+2.297X	0.885	0.1	Y= 6.396 +2833X	0.973	0.05
Levels/levels						
LH pit *vs* LH plasma	Y= 1.780+1.276X	0.966	0.05	Y= 2.676+0.995X	0.887	0.1

[a] (RL) Regression line. [b] (r) Correlation coefficient.

the female-dominant one at 200 dph. The pattern of variation of FSHβ during the monitoring period was similar to that of GPα (Figs. 3C and D). At 200–300 dph, there was a trend towards higher levels of FSHβ expression in the male- (>1.5 ratio FSHβ/18S) than in the female-dominant (≤1 ratio FSHβ/18S) population, though no significant difference was detected. In the male-dominant population (Fig. 3C) the highest levels of FSHβ transcript appeared at 200 dph, coinciding with the onset of male sexual differentiation, and were maintained until the experiment was terminated. Similar pattern of expression was observed in the female-dominant population (Fig. 3D), where the FSHβ mRNA levels displayed a significant rise (P ≤ 001) at 150 dph, coinciding with the onset of sexual differentiation in females. The expression pattern of LHβ differed from that of FSHβ, in that LHβ exhibited a steady increase from 150 dph, peaking at the end of the experimen-

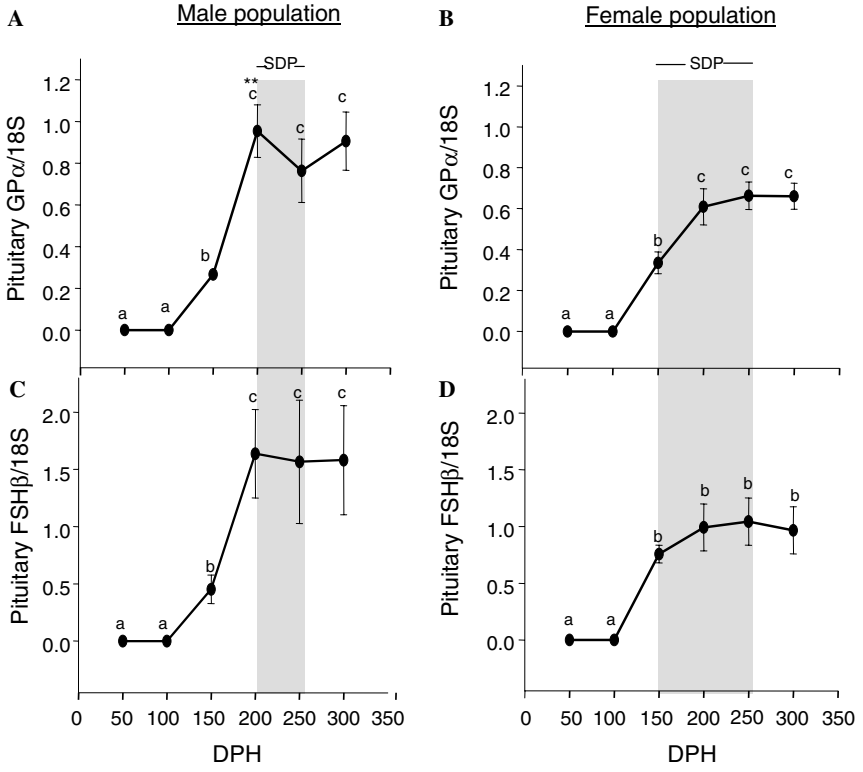

Figure 3. Temporal variations of the levels of pituitary glycoprotein α (GPα) mRNA (A and B) and pituitary FSHβ mRNA (C and D) in male and female populations of European sea bass during the first year of life. Asterisks indicate significant (**P≤0.01) differences between two populations at equivalent dates. Rest of the legends refer to Fig. 1.

tal period in both populations (Figs. 4A and B). At 200–300 dph, the LHβ mRNA levels of the male-dominant population showed a tendency to be higher than those of the female-dominant one. The FSHβ expression ratio was higher than that of the other two subunits (GPα and LHβ) and the lowest magnitude of transcript corresponded to the LHβ gene. A regression analysis indicated that the changes of the expression of LHβ were directly related to those of GPα, in both the male- and female-dominant populations ($r = 0.975$, $P \leq 0.05$ and $r = 0.972$, $P \leq 0.05$, respectively) and the correlation between FSHβ and GPα mRNA was even higher in both populations ($r = 0.990$, $P \leq 0.01$ and $r = 0.977$, $P \leq 0.05$, respectively) (Table 2). Similarly, the changes of expression of dlGnRH-R-2A as a function of the expression of GPα, FSHβ and LHβ were highly correlated in both male- ($r = 0.959$, $P \leq 0.01$; $r = 0.932$, $P \leq 0.01$ and $r = 0.892$, $P \leq 0.05$, respectively) and female-dominant ($r = 0.873$, $P \leq 0.05$; $r = 0.843$, $P \leq 0.05$ and $r = 0.811$, $P \leq 0.05$, respectively) populations (Table 2).

3.6. Pituitary content and plasma levels of LH

At 150 dph, the pituitary levels of LH in the male-dominant population were very low (Fig. 4C). In the following days, a steady and significant increase ($P \leq 0.001$) was observed until 250 dph, followed by a fivefold surge at 300 dph. In the female-dominant population, there was also a continual increase in pituitary LH content, with a sixfold increase at 250 dph and a twofold increase at 300 with respect to the previous one. At 250 dph, pituitary LH content in the female population was significantly higher ($P \leq 0.01$) than that of the male one (Fig. 4D). The correlation between LHβ mRNA levels and pituitary LH content was calculated from the beginning of the sex differentiation period (150 dph) to the end of the experiment, which coincided with the earlier stages of male tentative gonadal development (TGD, i.e. period of early testicular recrudescence or partial spermatogenesis which occurs in prepubescent male European sea bass) (Begtashi et al., 2004). This relationship was estimated separately for male- and female-dominant populations ($r = 0.885$, $P \leq 0.1$ and $r = 0.973$, $P = 0.05$, respectively; Table 2).

The male-dominant population exhibited a steady increase of plasma LH during the experiment, whereas the female-dominant population displayed a significant increase only at 250 dph (Figs. 4E and F). At this time, plasma LH levels of the female-dominant population were significantly higher ($P \leq$

0.05) than those of the male population. However, at 300 dph, plasma LH in the female population was reduced significantly and was lower than that in the male population (Fig. 4F). A correlation between the content of LH in the pituitary and the plasma LH levels of the male- and female-dominant populations (r = 0.966, P ≤ 0.05 and r = 0.887, P = 0.1, respectively) are shown in Table 2.

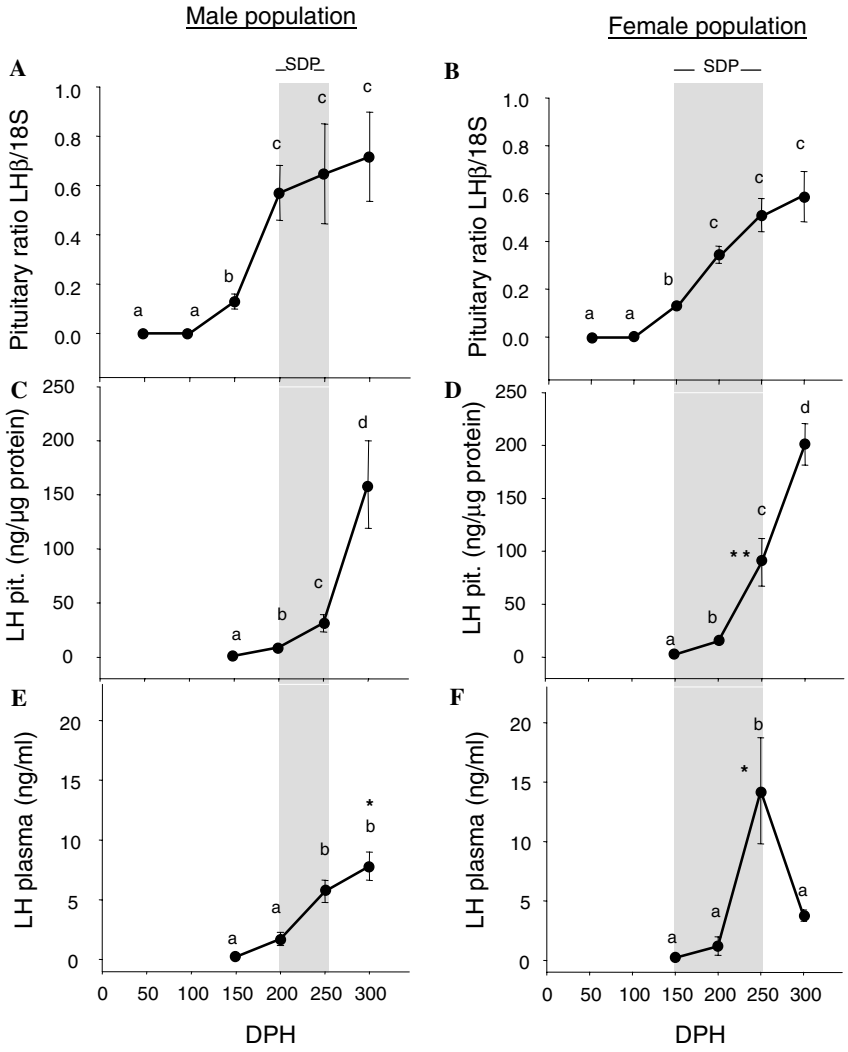

Figure 4. Temporal variation of pituitary LHβ mRNA (A and B), pituitary LH content (C and D) and plasma LH levels (E and F) in male- and female-dominant populations of European sea bass during the first year of life. Asterisks indicate significant (*P≤0.05; **P≤0.01) differences between two populations at equivalent dates. Rest of the legends refer to Fig. 1.

4. Discussion

The objective of this study was to investigate the endocrine control of sexual differentiation in European sea bass, starting early in development (50 dph). To date, size grading is the only non-hormonal method available in the European sea bass for the production of single-sex-dominant populations, and is based on the existence of a strong relation between growth and phenotypic sex differentiation early in development (Blázquez et al., 1999; Saillant et al., 2003). Intensive grading between 2 and 8 months after hatching increased the percentage of females among larger fish up to 96.5%, whereas the percentage of males among smaller fish increased to 69.8% (Papadaki et al., 2005). The first histological signs of gonadal differentiation in female European sea bass took place around 150 dph and in males around 200 dph, whereas complete gonadal differentiation occurred at around 250 dph in both sexes (Papadaki et al., 2005). As sex could not be determined macroscopically at the time of sampling and collection of the various tissues prior to 250 dph, it is likely that some sampled individuals from the female-dominant population were indeed males, whereas some individuals from the male-dominant population were indeed females. Since the female-dominant population consisted of 91% females already after the first grading at 66 dph, then the possibility of sampling a male in this population was negligible (<10%). On the contrary, the possibility of some female individuals contributing to the mean values of the male-dominant population during the sampling times between 100 and 200 dph is substantial. Still, given the creation of an almost monosex female population in the present study, any significant differences between the male- and female-dominant population can be attributed to the male component of the first, thus considering those as "male" values.

The present study shows that both male- and female-dominant populations had a peak of brain sbGnRH gene expression, coinciding with the onset of sex differentiation. In both populations, levels of sGnRH mRNA changed in parallel to those of sbGnRH. In addition, the patterns of sbGnRH and sGnRH gene expression were unimodal in the male-dominant population and bimodal in the female one. These results suggest that the onset of sex differentiation, as well as its accomplishment, requires high sbGnRH and sGnRH transcriptional activity, with differences due to age and sex. Of the three forms of GnRH present in the brain of the European

sea bass, sbGnRH is considered as the hypophysotropic one and its role as the natural releaser of LH has been clearly demonstrated (Forniés et al., 2002; González-Martínez et al., 2001; González-Martínez et al., 2002a,b; Rodríguez et al., 2000). Furthermore, immunocytochemical studies of the distribution of cells and fibers expressing the different prepro-GnRHs in the brain and pituitary of European sea bass (González-Martínez et al., 2002b) demonstrated that the proximal pars distalis (PPD) and the border of the pars intermedia (PI), where gonadotropes are located, were strongly innervated with sbGnRH associated peptide immunoreactive fibers (sbGAP-*ir*) and receive, as well, a few sGnRH-associated peptide immunoreactive fibers (sGAP-*ir*). In addition, sbGnRH and sGnRH share a common origin near the olfactory system and exhibit overlapping patterns of distribution in the anterior forebrain as observed recently in the Atlantic croaker and medaka (Mohamed et al., 2005; Okubo et al., 2006). These results support the main role of sbGnRH in the stimulation of the secretion of gonadotropins in these species and to some extent suggest the possible participation of sGnRH in the gonadotropic function. The pituitary content of sbGnRH and sGnRH showed sex-related differences and displayed an inverse pattern to that of the brain. Nevertheless, the pituitary content of sbGnRH was about twofold higher than that of sGnRH suggesting a prevailing role in the gonadotropic function. The fact that high levels of sbGnRH in the pituitary appeared just before or at the beginning of sex differentiation, in close association with high sbGnRH brain expression, suggests that the onset of sex differentiation requires a high transcriptional activity of sbGnRH followed by an active synthesis and transport up to the gonadotropes at the distal pituitary. Rodríguez et al. (2000) demonstrated that pituitary sbGnRH peaked around the time of sex differentiation, further supporting the role of sbGnRH in gonadal differentiation in European sea bass.

Recently, Moncaut et al. (2005) have identified five different GnRH-R genes (dlGnRH-R-1A, dlGnRH-R-1B, dlGnRH-R-2A, dlGnRH-R-2B and dlGnRH-R-2C) in the European sea bass. Because the dlGnRH-R-2A is a receptor subtype for the most abundant GnRHs in the forebrain of this species, i.e. sbGnRH and sGnRH (González-Martínez et al., 2002a,b; González-Martínez et al., 2004), its gene expression was examined in the present study. The pattern of pituitary dlGnRH-R-2A mRNA expression in the male-dominant population was similar to the expression of sbGnRH and sGnRH. In the female-dominant population, pituitary dlGnRH-R-2A mRNA levels peaked

coincidently with the second surge of brain sbGnRH and sGnRH gene expression. The significant correlation between sbGnRH and dlGnRH-R-2A gene expression suggests that changes in brain sbGnRH gene expression are reflected in the expression of its receptor at the pituitary level. The dlGnRH-R-2A is highly expressed in European sea bass pituitary gonadotropic cells and is considered as the GnRH-R responsible for the stimulatory actions of sbGnRH and sGnRH on GtH release (González-Martínez et al., 2004). In adult females, the highest expression of dlGnRH-R-2A at the pituitary occurs at the time of maximum ovarian activity during vitellogenesis in November (González-Martínez et al., 2004). In males, sbGnRH pituitary levels were high at the onset of puberty, when the first significant increase of the gonadosomatic index (GSI) and plasma levels of testosterone (T) and 11-ketoT (11-KT) occurred (Rodríguez et al., 2000). In the present study, the strong correlation between gene expression of sbGnRH and dlGnRH-R-2A, in both sexes, is a good indicator of their possible involvement in sex differentiation as well.

In both populations, pituitary changes of GPα, FSHβ and LHβ gene expression displayed similarities to the pattern of variation of dlGnRH-R-2A, with the pattern of expression of FSHβ exhibiting a higher correlation than LHβ. In the pituitary of adult female European sea bass, dlGnRH-R-2A expression activity was mainly detected in LH cells but also in some FSH ones (González-Martínez et al., 2004). Therefore, the similar profiles of synthesis of sbGnRH and sGnRH, and dlGnRH-R-2A and GtHs may indicate the important role of these GnRHs in inducing GtH synthesis and more specifically FSH during the period of sex differentiation in European sea bass. It is important to note that precisely at the onset of male and female sex differentiation, FSHβ transcriptional activity attained its highest values, which were maintained elevated throughout the study period. On the contrary, LHβ mRNA exhibited a progressive and steady increase up to 300 dph, when males attained the tentative gonadal development (TGD) or even spermiation, and females were completely differentiated. Furthermore, the level of expression of FSHβ was higher than that of LHβ (2.6x and 2x in male- and female-dominant populations, respectively). Collectively, these results suggest that mRNA expression of LHβ may not be entirely associated with the onset of sex differentiation, while FSH transcriptional activity seems to be more involved in this process. It has been suggested that the mechanisms regulating FSHβ gene expression in several species of teleosts, including Eu-

ropean sea bass, may be different from that of LHβ, and may depend on the gender and the stage of sexual ontogeny (Gen et al., 2000; Hassin et al., 1995, 1998; Kandel-KWr et al., 2002; Klausen et al., 2001; Kumakura et al., 2004; Mateos et al., 2002; Sohn et al., 2001). Collectively, these results show that the pituitary levels of FSHβ exhibit gender- and reproductive-phase-dependent variation and suggest a prevailing role of FSH in European sea bass sex differentiation, as observed in Cichlasoma dimerus, another perciform species (Pandolfi et al., in press). Unfortunately in the present study it could not be confirmed whether the rise of sbGnRH or sGnRH at the onset of sex differentiation was associated to an increase of pituitary or plasma levels of FSH, since an assay to measure this hormone in European sea bass has not been developed yet. Nevertheless, it is presumed that high levels of FSHβ transcripts in the pituitary during sex differentiation may be indicative also of high FSH content in the pituitary and elevation of its plasma levels, as observed in salmonids (Gómez et al., 1999; Dickey and Swanson, 2000). In fact, in salmonids, it has been suggested that FSH release may be largely constitutive and possibly regulated primarily via control of transcription (Swanson et al., 2003).

The involvement of GnRH and GtHs in gonadal differentiation has already been suggested in other species such as the rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis* spp.), pejerrey (*Odontesthes bonariensis*) and C. dimerus (Feist and Schreck, 1996; Miranda et al., 2001, 2003; Pandolfi et al., 2002, in press; Parhar, 1997). In the European sea bass, the brain machinery responsible for the control of sex differentiation and reproduction is formed well before gonadal development (González-Martínez et al., 2004; Piferrer et al., 2005), and the present results suggest that during sex differentiation sbGnRH regulates the synthesis and release of FSH, which in turn reaches the gonad-stimulating steroidogenesis. A direct stimulation of P450aromA expression by FSH in fish oocytes has been demonstrated (Montserrat et al., 2004), and very recent studies showed that purified European sea bass FSH promotes ovarian synthesis of E2 (Molés et al., in press). Furthermore, the high levels of plasma E2 found at 200 dph in the female-dominant population (Papadaki et al., 2005) suggest that this is indeed the case, as previously observed in tilapia (D'Cotta et al., 2001). Likewise, recent studies on the expression of P450aromB in the brain of European sea bass demonstrated that females exhibited higher mRNA levels than males at the time of gonad sex differentiation (Blázquez and Piferrer, 2004). Finally, it is

interesting to note that in mammals it has been described that the aromatization of T to E2 is the predominant prevailing regulator of FSH secretion (Hayes et al., 2001). Taken together, all this suggests a role of sbGnRH on FSH synthesis and release and their effects on steroidogenesis, both at the brain and the gonad level during sex differentiation in European sea bass.

In the present study, the pituitary content of LH in both populations was correlated with the mRNA levels of LHβ, indicating that transcriptional and synthetic processes are similarly regulated in European sea bass. However, sex differences were observed in the pattern of plasma LH. In the male-dominant population there was a steady increase of plasma LH levels from 150 dph onwards, showing a close relationship with the expression of LHβ and pituitary LH content. On the contrary, the female population exhibited a significant decrease in plasma LH levels at 300 dph, despite the increasing pituitary LH content. This suggests that LH release was arrested through a highly regulated pathway, probably in response to the decrease of sbGnRH input to the pituitary and expression of dlGnRH-R-2A. It has been claimed that LH controls the last stages of the reproductive cycle, while FSH mainly regulates the earlier stages of reproduction such as initiation of meiosis and early spermatogenesis (Gómez et al., 1999; Hassin et al., 2000; Planas and Swanson, 1995; Swanson et al., 1989). In the present study, the observed increase of plasma LH levels from the beginning of sex differentiation toward the end of the studied period, when males were spermiating, confirms this role. Moreover, the profile of LH in the male population correlated well with the presence of precocious males and the progressive increase of plasma 11 KT (Papadaki et al., 2005), which is the regulator of spermatogenesis in teleosts (Amer et al., 2001; Kobayashi et al., 1991; Miura et al., 1991; Schulz and Miura, 2002; Rodríguez et al., 2005). On the contrary, LH plasma levels in the female population did not correlate well with pituitary transcription or synthesis of LH, in agreement with the absence of significant increases, at this time, of plasma E2 and the presence of only primary oocytes in the ovaries (Papadaki et al., 2005).

In conclusion, the present study supports the role of sbGnRH and possibly sGnRH in gonadal differentiation through an enhancement of FSHβ gene expression. A sexual dimorphism of these GnRH forms either at the transcription or synthesis level strengthens the thought that differential neuro-hormonal regulation is required for each sex. Differential endocrine regulation seems to occur at further stages of gonadal development on which

a steady increase of the levels of transcription, synthesis, and release of LH are of paramount importance for maturation and spermiation.

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Capítulo 4:

Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (*Dicentrarchus labrax*)

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Abstract

Follicle-stimulating hormone (FSH) was purified from pituitaries of sea bass (*Dicentrarchus labrax*), and its biochemical and biological properties were studied. Sea bass FSH (sbsFSH) was purified by ethanol extraction-precipitation (40-85%), followed by anion-exchange chromatography on a LKB Ultropac TSK-DEAE column using a linear gradient of ammonium bicarbonate (50-1000 mM) and reverse phase chromatography on a RESOURCE 15RPC column with a linear gradient of acetonitrile (0-50%), using a FPLC system. The molecular mass of the purified sbsFSH, estimated by mass spectrometry, was of 28.5 kDa for the dimer, 12.6 KDa for the glycoprotein α (GPα) and 13.6 KDa for FSHβ subunits. After separation by SDS–PAGE under reducing condition, the intact sbsFSH was dissociated in the respective subunits (GPα and FSHβ). Subunit identity was confirmed by immunological detection and N-terminal amino acid sequencing. Deglycosylation treatment with *N*-glycosidase F, decreased the molecular mass of both subunits. Intact sbsFSH activated the sea bass FSH receptor stably expressed in the cell line HEK 293, in a dose dependent manner. Purified sbsFSH showed gonadotropic activity, by stimulating the release of estradiol-17β (E2) from sea bass ovary and testosterone (T) and 11-ketotestosterone (11KT) from testicular tissue cultured *in vitro*, in a dose and time dependent manner. These results showed that the purified sbsFSH is a heterodimeric hormone, composed of two distinct glycoprotein subunits (GPα and FSHβ), and has biological activity judged by its ability to stimulate its receptor in a specific manner and to promote steroid release from gonadal tissue fragments.

Keywords: European sea bass, perciform, gonadotropin, FSH, steroidogenesis, reproduction

1. Introduction

The gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), produced in the pituitary gland, are essential for the endocrine control of vertebrate reproduction. Together with the also pituitary-derived thyroid stimulating hormone (TSH) and the placental chorionic gonadotropin (CG) that is present only in primate and equine species, they constitute an evolutionarily conserved family of glycoprotein hormones. They are glycosylated heterodimers formed by the non-covalent association of an α-subunit, which is common to all members of the family within a species, with distinct β-subunits that confer hormone specificity (Pierce and Parsons, 1981; Bousfield et al., 1994). Each of the subunits is encoded by single, separate genes (Fiddes and Talmadge, 1984). The dimerization and glycosylation, which occurs in the rough endoplasmic reticulum and Golgi apparatus, are prerequisites for gonadotropins to achieve full biological activity. The FSH and LH are synthesized in the gonadotrope cells of the pituitary (Nozaki et al., 1990; Naito et al., 1993) and transported via the peripheral circulation to the gonads where they regulate steroidogenesis and gametogenesis. Initial biochemical studies of gonadotropins in fish suggested that a single LH-like gonadotropin regulated gametogenesis (Burzawa-Gerard, 1982). In the late 1980´s, the presence and structure of two fish gonadotropins, GTH I and GTH II, was firmly established in the chum salmon (*Oncorhynchus keta*; Suzuki et al., 1988a,b,c,d). Since then, gonadotropins have been isolated and characterized in several fish species (reviewed in Yaron et al., 2003). Moreover, the extended use of molecular biology techniques has resulted in the isolation and characterization of the cDNAs encoding gonadotropin subunits in more than 30 fish species. Structural analyses of their deduced amino acid sequences, together with functional data, undoubtedly 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).

The physiological functions of both GTHs are well established in higher vertebrates, while those in teleosts are still poorly understood. In salmonids FSH is considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH is considered to be responsible for the final maturation processes, such as oocyte maturation, ovulation and

spermiation (Swanson et al., 2003; Yaron and Sivan, 2006). However, the functional duality between FSH and LH has yet to be clarified in perciform fish. During female vitellogenesis in common carp (Van Der Kraak et al., 1992), tuna (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 (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). Unfortunately, purified intact FSH is still not available for most teleost species due to the need of a great number of pituitaries and the reduced content of FSH present in them. Hence, the information on its physiological roles is scarce compared with what is known about LH. The European sea bass is a perciform fish, highly valued in aquaculture. In this species, puberty is attained at 2 years of age in males and at 3 years in females and then reproduction is repeated once a year during winter season. The females present a group-synchronous type of ovarian development, producing 3–4 consecutive spawns during the 1–2 months spawning period (Alvariño et al., 1992; Carrillo et al., 1995, Asturiano et al., 2000). In sea bass, the role of FSH in these processes is not well clarified due mainly to the lack of an assay to measure its levels throughout the reproductive cycle. This paper reports the purification and characterization of native sea bass FSH. Its biochemical properties and biological activities were investigated with the aim to acquire a more accurate knowledge regarding the function of FSH in fish reproduction. The presented results provide the basis for future research on the function of FSH in sea bass.

2. Materials and methods

2.1. Pituitary collection and extraction

Sexually mature male and female sea bass (*Dicentrarchus labrax*) were obtained from a commercial dealer (Culmarex, Aguilas, Spain). Fish were anesthetized and sacrificed by decapitation in accordance with the European Union Animal Care Regulations. Around 1400 pituitary glands (4.1 g wet weight) were collected and immediately frozen in liquid nitrogen and stored at -80°C until use.

Pooled pituitaries were homogenized in 25 ml of 10% ammonium acetate, pH 7.5, containing 1mM phenylmethylsulfonyl fluoride (PMSF) and 5mM EDTA, on ice, using a mechanically driven glass Teflon homogenizer. The homogenate was stirred for 1h at 4°C and centrifuged at 20,000*g* for 30min at 4°C, to eliminate debris. The pellet was re-extracted to recover additional glycoproteins. The supernatant was adjusted to 40% ethanol (v/v) by slow addition of ice-cold ethanol and stirred overnight at 4°C. After centrifugation (20,000*g* for 30min at 4°C) the supernatant was adjusted to 85% ethanol (v/v), and kept at 4°C overnight without stirring. The glycoprotein precipitate was recovered by centrifugation (20,000*g* for 30min at 4°C) and ethanol removed using a vacuum desiccator (10 min).

2.2. Ion-exchange chromatography

The moist pellet (80 mg protein) was solubilized (3h at 4°C) in 20 ml of 50 mM ammonium bicarbonate, pH 8.0, and fractionated by weak anion-exchange chromatography, using a high-performance liquid chromatography (HPLC) system, on an LKB Ultropac TSK-DEAE-5PW HPLC column (21.5 x 150 mm; Pharmacia LKB) equilibrated with the same buffer. Adsorbed proteins were eluted with a linear gradient of 50-1000 mM ammonium bicarbonate, pH 8.0. Fractions of 8 ml were collected at a flow rate of 4 ml/min, and the absorbance read at 280 and 215 nm. Fractions were pooled in eight groups and lyophilized. The molecular mass of the pooled fractions were estimated by mass spectrometry.

2.3. Reversed-phase chromatography

Pooled and lyophilized fractions containing proteins with estimated molecular masses similar to those of known GTHs were reconstituted in 2 ml of 50 mM ammonium acetate buffer, pH 8.0, and further fractionated by reversed-phase chromatography, using a HPLC system (rpHPLC), on a RESOURCE 15RPC 3ml column (6.4 x 100 mm, 15 μm particle size; GE Healthcare Bio-Sciences AB, Sweden). Proteins were eluted with a linear gradient of 0–50% (v/v) acetonitrile in 50 mM ammonium acetate pH 8.0, for 80 min, at a flow rate of 1 ml/min at room temperature and fractions of 1 ml were collected. Peaks of protein were screened for GTHs by SDS–PAGE, Western blot, mass spectrometry and N-terminal amino acid sequencing.

2.4. Electrophoresis

Electrophoresis was performed according to Laemmli (1970), on 4% stacking and 15% separating polyacrylamide gels (0.1% SDS), under reducing (5% 2-Mercaptoethanol (2-ME), 5 min 95°C) and non-reducing conditions. Gels were stained with 0.1% Coomassie brilliant blue R-250 in a 40% methanol/10% acetic acid solution. A prestained SDS-PAGE Standard Broad Range was used as molecular weight marker (Bio-Rad Laboratories, Inc, CA, USA).

2.5. Western blotting

Samples were separated by SDS–PAGE as described above. The gel was equilibrated with a blotting buffer containing 25 mM Tris, 192 mM Glycine and 20% methanol (pH 8.3) and then electrophoretically transferred onto a PVDF membrane (Immobilon P, Millipore Corp.). The membrane was incubated in a blocking buffer (5% dried milk in 10 mM Tris-base, 150 mM NaCl and 0.1% Tween 20 (TBS-T)) overnight at 4°C and then immersed for 90 min at room temperature in a solution containing the specific antisera at a final dilution of 1:3000. The specific antibodies against sea bass GPα (sbsGPα) and LHβ (sbsLHβ) subunits (Mateos et al., 2006) and against a mummichog FSHβ (mFSHβ) synthetic peptide (Shimizu and Yamashita, 2002) were used as specific antisera. A test with sea bass pituitary homogenate was previously performed to determine the anti-mFSHβ specificity for sbsFSHβ and a positive reaction was obtained. The membrane was washed three times with TBS-T solution, and then incubated with 1:2000 goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP, BioRad Laboratories) diluted in the blocking buffer, for 1 h at room temperature. The membrane was washed again with TBS-T solution and the inmunodetection was performed by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc.).

2.6. Mass spectrometry

All MALDI-TOF mass spectra were acquired on a BRUKER Ultraflex spectrometer equipped with a pulsed nitrogen laser (337 nm), in linear, pos-

itive ion mode, using a 25 kV acceleration voltage. Samples were prepared by mixing equal volumes of a saturated solution of the matrix (sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid)) in 0.1% trifluoroacetic acid in water/acetonitrile 2:1, and a protein solution of a concentration on the range of 1-10 μM. From this mixture, 0.5 μL were spotted on the sample slide and allowed to evaporate to dryness. Once evaporated, samples were washed with 1 μL of 0.1% TFA, which was removed after some seconds by aspiration, and then a further 0.5 μL of matrix solution applied. Proteins of known molecular mass were used as standards for mass calibration.

2.7. N-Terminal amino acid sequencing

Samples of the purified proteins, containing about 1 μg of protein, were adsorbed on a PVDF membrane (Millipore) and washed several times with deionized water. The N-terminal sequence was determined by Edman's automated degradation using a Beckman LF3000 protein sequencer equipped with an on-line PTH-amino acid analyzer System Gold (Beckman, Fullerton, CA, USA). Protein homology searches were performed using the BLAST network service (Altschul et al., 1997).

2.8. Heterodimer stability test of intact sbsFSH

To understand the chemical bond of sbsFSH subunits, purified sbsFSH dimers were treated with 0.1% Trifluoroacetic acid (TFA) or 5% 2-Mercaptoethanol (2-ME) for 15 min at 25°C. They were then separated by SDS–PAGE under nonreducing conditions, and the associated states of the respective sbsFSH subunits were analyzed by subsequent Western blotting.

2.9. Glycosidase treatments

Based on supplier recommendations (Roche Diagnostics, Mannheim, Germany), 4 μg of sbsFSH dimer were denatured for 5 min at 94°C in 0.1% SDS and 50 mM 2-Mercaptoethanol. Then, it was incubated with 2.5 Units of peptide-*N*-glycosidase F (PNGase F, Roche Diagnostics) for 2 hour at 37°C in 20 mM sodium phosphate with 0.5% NP-40, pH 7.5, which hydrolyzes all types of *N*-glycans chains. Deglycosylated proteins were subjected to SDS-PAGE followed by Western blotting analysis.

2.10. Activation of FSHR

The biological activity of the purified hormone was analyzed using two cell lines (HEK 293) stably expressing the sea bass FSH or LH receptors (sbsFSHR and sbsLHR) and the firefly luciferase gene under the control of a promoter with cAMP Responsive Elements (CRE) binding sites (Rocha et al., 2007). Receptor activation by FSH was indirectly measured by recording changes in luciferase activity, promoted by a rise of cAMP. HEK 293 cells stably expressing sbsFSHR were plated in 48-well culture plates at $4.5x10^5$ cells/well. For the determination of luciferase activity, cells were incubated with serial dilutions of sbsFSH preparations in Advanced DMEM media (Invitrogen Corp.) containing 100 units/ml Penicillin/Streptomycin, 1% Glutamine and 100 µg/ml Hygromycin. Recombinant sea bass LH, produced using a Baculovirus Expression System, in Sf9 insect cells (Molés, G., Gómez, A., Carrillo, M., Zanuy, S., unpublished), was used as positive control for stimulation of the sbsLHR cell line. After 5 h of incubation cells were lysed in Reporter Lysis Buffer (Promega Corp.) as indicated by the manufacturer. Cell debris were separated by centrifugation for 30s at 15,000*g*, and 20 µl of the supernatant were mixed with 100 µl luciferin reagent (20 mM Tricine KOH, 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 light emitted was measured in a luminometer (Junior, EG&G, Berthold) and expressed as relative light units (RLU).

2.11. *In vitro* bioassay

In vitro tissue cultures of sea bass gonads were performed following the protocol described by Planas et al. (1993) with minor modifications. Briefly, ovaries and testis of adult sea bass sacrificed between July and November were thoroughly chopped on ice with a razor blade until forming a paste. The tissue preparation was washed three times with 10 v/wt of ice-cold Sea Bass Ringer (SBR; according to Sorbera et al., 1999) containing 0.5% bovine serum albumin (BSA, Fraction V, Sigma-Aldrich, Inc.) and 100 units/ml penicillin/streptomycin (Invitrogen Corp.). After each wash, the supernatant was discarded and fresh media was added again to the preparation, the tube was gently mixed by hand and centrifugated for 10 min at 60*g* at

4°C. After the last wash, the preparation was filtered through a 750 μm (for testis) and 150 μm (for ovary) nylon mesh. Pieces of the gonadal preparation were transferred to 24-well plates at about 40 mg per well containing 0.5 ml of SBR. The samples were preincubated in 0.5 ml of SBR for 30 min at 21°C under shaking conditions (100 rpm). After preincubation, the medium was replaced for 0.5 ml of fresh SBR containing different sbsFSH dilutions. Four replicates were used for each treatment and experiments were performed by triplicate. Tissues were then incubated for 20 h under the same conditions as in the preincubation. After incubation, the medium was collected and centrifuged for 15 min at 500*g* at 4°C and the supernatant was stored at -20°C until steroid analysis.

2.12. Steroids measurements

Estradiol (E2) was measured by a conventional competitive enzyme-linked immunosorbent assay (ELISA), developed and validated for the sea bass in our laboratory (Crespo, B., Navas, J.M., Rocha, A., Zanuy, S., Carrillo, M., unpublished). The assay uses specific rabbit antisera against E2 as primary antibody and estradiol acetylcholinesterase conjugate (E-AChE, Cayman Chemical MI, USA) as tracer. The specific rabbit anti-E2 used is the same employed in the E2 RIA described by Prat et al. (1990), where details on cross reactivity of this antibody to other steroids are given. 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). Culture medium 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-E rabbit antiserum (diluted to 1:845,000), E standards (ranging from 80 ng/ml to 0.039 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. Optical density 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 and half-displacement (Bi/B0 = 50%) occurred around 1.90 ng/ml.

The levels of 11-ketotestosterone (11-KT) were determined by enzyme immunoassay (EIA), using an assay developed for the Siberian sturgeon and modified for its use in sea bass (Rodriguez 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. Culture medium testosterone (T) levels were determined by a specific EIA developed by Rodríguez et al. (2000) for sea bass.

3. Results

3.1. Isolation and purification of intact sbsFSH

The pituitary ethanol-extracted glycoproteins were subjected to separation by anion-exchange chromatography (Fig. 1). The collected fractions were analyzed by mass spectrometry and based on molecular weight data

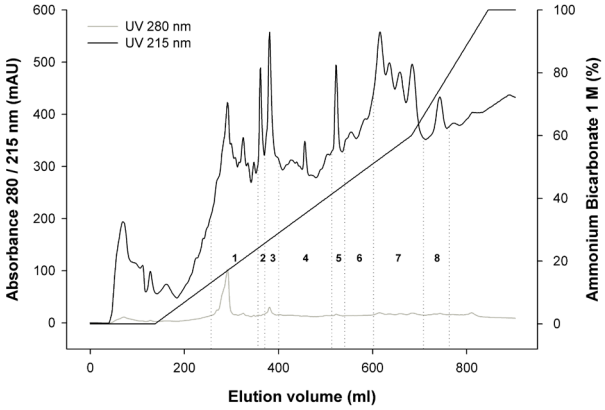

Figure 1. Anion-exchange chromatography on a LKB Ultropac TSK-DEAE column (21.5 x 150 mm) of the ethanol extract from sea bass pituitaries. Adsorbed proteins were eluted with a linear gradient of 50–1000 mM ammonium bicarbonate (line), pH 8.0. Fractions of 8 ml were collected at a flow rate of 4 ml/min, and the absorbance read at 280 and 215 nm. Fractions were pooled in 8 groups as indicated.

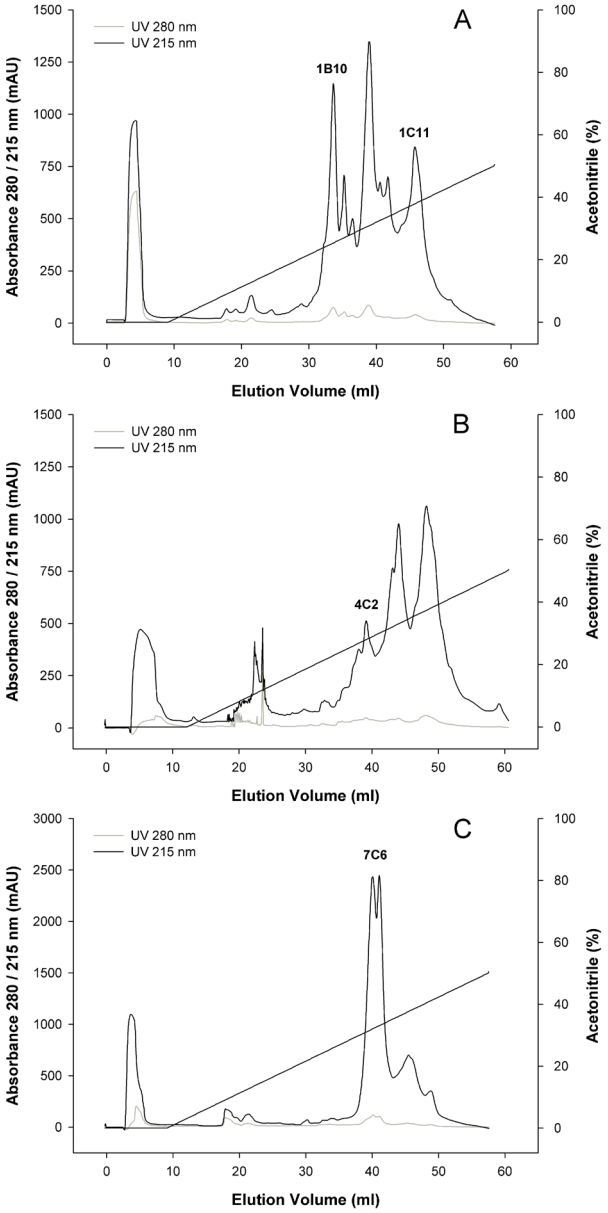

Figure 2. Reverse-phase chromatography on a RESOURCE 15RPC column (6.4 x 100 mm, 15 µm particle size) of pools 1 (A), 4 (B) and 7 (C) from anion-exchange chromatography (Fig. 1), using a HPLC system. Proteins were eluted with a linear gradient of acetonitrile (0–50%) in 50 mM ammonium acetate (line), pH 8.0, and a flow rate of 1 ml/min, 1 ml/tube, at room temperature. After N-terminal amino acid sequencing and Western blotting peaks were identified as sbsFSH (1B10), sbsGPα (1C11), sbsFSHβ (4C2) and sbsLHβ (7C6).

they were pooled in eight groups and lyophilized. The resulting groups were further fractionated by reverse phase chromatography and the eluted fractions were analyzed by Western blotting, mass spectrometry and finally by N-terminal amino acid sequencing, in search of putative intact FSH and its subunits. Results from mass spectrometry and Western blotting revealed that fraction 1B10 (Fig. 2A), contained a highly purified protein reacting with both anti-sbsGPα and anti-mFSHβ but not with anti-sbsLHβ (Fig. 4). The molecular mass of this protein was 28.5 KDa (Fig. 3A) and N-terminal amino acid sequencing revealed that it corresponds to the predicted amino acid sequences of sea bass GPα and FSHβ subunits, respectively (Fig. 5). These results indicated that fraction 1B10 contained highly purified sbsFSH with a yield of 170 μg of protein.

Analysis of three other fractions (1C11, 4C2 and 7C6) showed that they contained different quantities of purified sea bass GPα, FSHβ and LHβ subunits, respectively (Fig. 2). Their identities were also confirmed by Western blot (Fig. 4) and N-terminal amino acid sequencing (Fig. 5) and their molecular masses were 12.6 KDa, 13.6 KDa and 15.3 KDa respectively (Fig. 3).

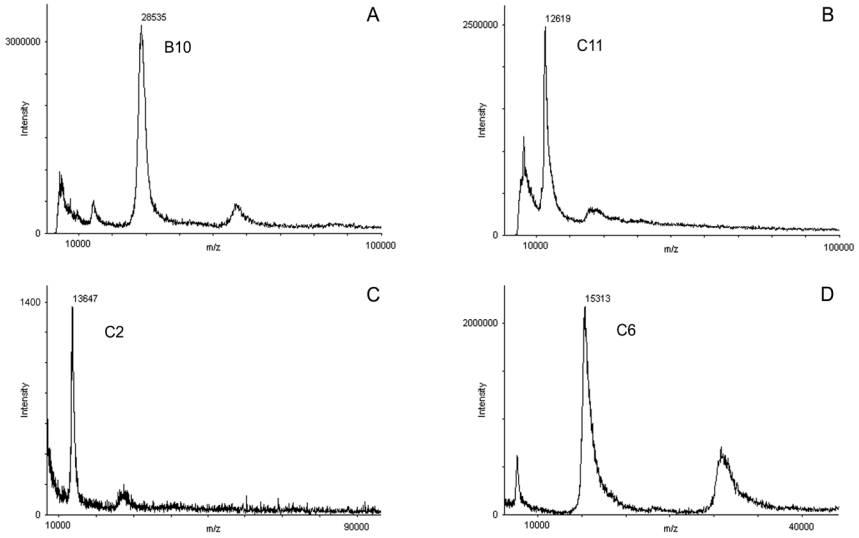

Figure 3. Mass spectrometry of fractions obtained from reverse phase chromatography. (A) sbsFSH (1B10), (B) sbsGPα (1C11), (C) sbsFSHβ (4C2) and (D) sbsLHβ (7C6). MALDITOF mass spectra were acquired on a BRUKER ultraflex spectrometer, with a pulsed nitrogen laser (337 nm), in linear positive ion mode, using a 25 kV acceleration voltage.

3.2. Biochemical and immunological properties of sbsFSH and its subunits

When subjected to SDS-PAGE under non-reducing conditions, the purified sbsFSH (1B10) appeared as a unique band, whereas under reducing conditions two distinct proteins of smaller molecular weight could be visualized (Fig. 4). In the Western blotting analysis, the anti-mFSHβ specifically reacted to the smaller band and the anti-sbsGPα with the bigger band (Fig. 4 B and C). Under non-reducing conditions, the anti-mFSHβ did not react with the sbsFSH dimer due to epitope masking and only when the antigen was reduced the detection was possible (Fig. 4B).

Deglycosylation treatment of the purified FSH with *N*-glycosidase F decreased the molecular mass of both subunits making them equal in size (Fig. 4A). In addition, both anti-sbsGPα and anti-mFSHβ reacted with bands almost equal in size (Fig. 4 B and C). Considering that the deduced amino acid sequences of the cDNAs encoding the mature sea bass GPα and FSHβ have

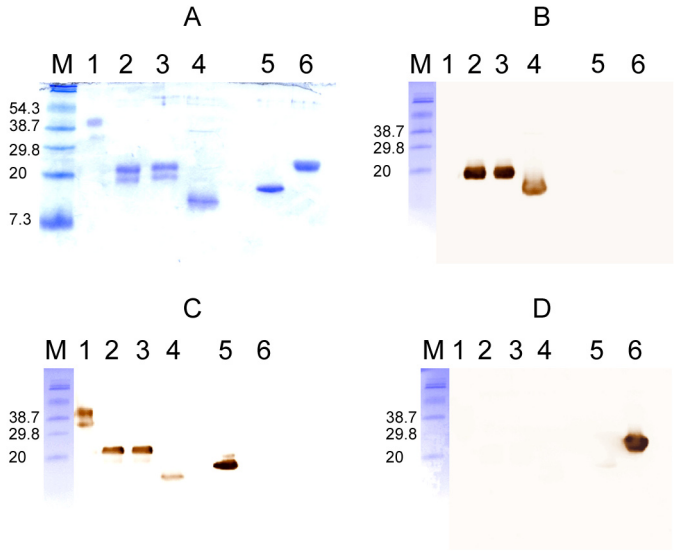

Figure 4. SDS–PAGE and Western blotting of purified sbsFSH (1B10), sbsGPα (1C11) and sbsLHβ (7C6). Electrophoresis was carried out with 3–4 μg of protein sample on 15% SDS–PAGE gels. (A) Proteins stained with Coomassie blue. (B) Western blotting with anti-mFSHβ. (C) Western blotting with anti-sbsGPα. (D) Western blotting with antisbsLHβ. Lane 1: sbsFSH without reduction; Lane 2: sbsFSH treated with 0.1% TFA, 15 min at 25 °C; Lane 3: sbsFSH reduced with 5% 2-ME; Lane 4: Deglycosylation of sbsFSH with 2.5 U of PNGase F and reduced with 5% 2-ME; Lane 5: sbsGPα reduced with 5% 2-ME; Lane 6: sbsLHβ reduced with 5% 2-ME. M: BioRad prestained SDS–PAGE Broad Range molecular weight marker.

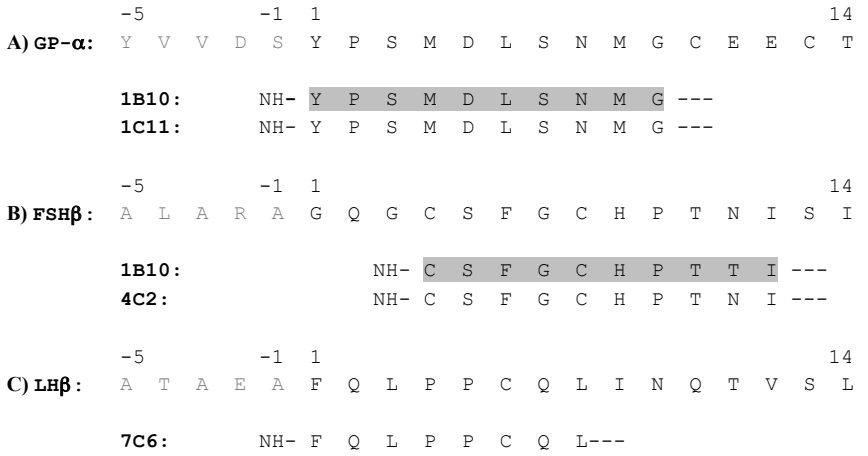

Figure 5. N-terminal amino acid sequence of purified sbsFSH (1B10), sbsGPα (1C11), sbsFSHβ (4C2) and sbsLHβ (7C6). Alignment with those predicted from sea bass gonadotropin cDNA sequences.

almost the same number of residues (94 and 105 amino acids, respectively), these results clearly show that both subunits decreased their molecular mass by deglycosylation treatment and therefore they are *N*-glycosylated.

The stability of the purified sbsFSH heterodimer was tested under acidic and reducing conditions using TFA and 2-ME, respectively. In both cases intact sbsFSH was dissociated into its respective subunits (Fig. 4).

The purified sbsGPα (1C11) and sbsLHβ (7C6) subunits reacted strongly with anti-sbsGPα and anti-sbsLHβ, respectively (Fig.4 C and D). However the purified sbsGPα (1C11) showed a smaller size when compared with the sbsGPα of the dimer (1B10), probably due to differences in the degree of glycosylation or a degradation phenomenon during the process of purification.

3.3. Bioactivities of the purified native sbsFSH

The bioactivity and specificity of the purified sbsFSH was analyzed by determining its capacity to stimulate intracellular cAMP production in HEK 293 cells stably expressing the sbsFSHR or the sbsLHR, as well as its capacity to stimulate steroid production by gonadal tissue fragments cultured *in vitro*. Purified sbsFSH was able to activate the sbsFSHR in a dose depend-

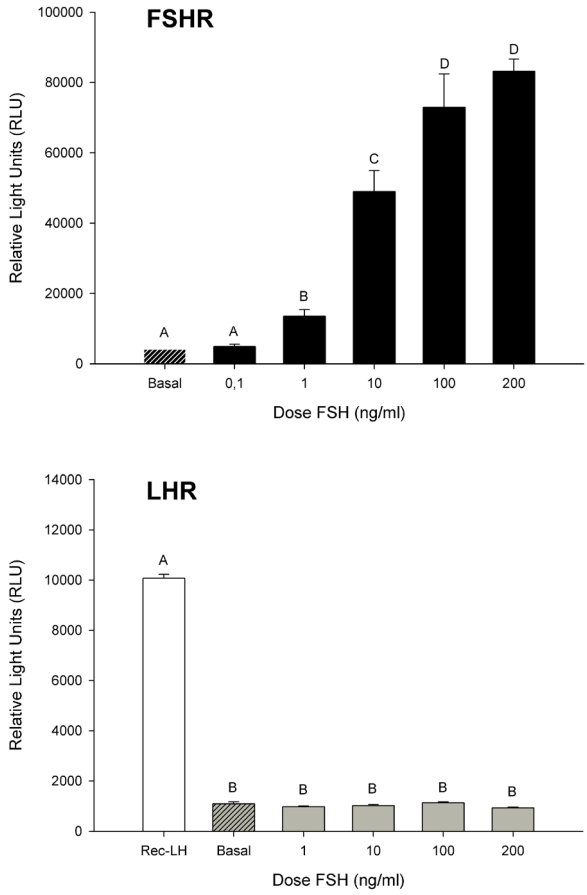

Figure 6. Activation of sea bass FSH and LH receptors with purified sbsFSH. HEK293 cells stably expressing the sbsFSHR or the sbsLHR were treated with increasing concentrations of purified sbsFSH (1B10). Luciferase activity after 5-h treatment of the sbsFSHR or the sbsLHR with different doses of sbsFSH. Recombinant sea bass LH was used as positive control.

ent manner and was unable to stimulate the sbsLHR, exhibiting specificity for its receptor (Fig. 6). Effects of purified sbsFSH on E2 secretion by adult sea bass ovary was analyzed during the previtellogenic (July-August), early vitellogenic (October) and mid vitellogenic (November) stages and showed, a clear dose-dependent effect on E2 secretion from August to November, with a minimal significant (p<0.01) effective concentration of 10 ng/ml in October (Fig. 7B). The stimulatory effects of sbsFSH were also found to be

time-dependent since sbsFSH (100 ng/ml) significantly (p<0.001) stimulated E2 secretion from 2 to 20 hours of incubation (Fig. 7A). In adult sea bass testis, the stimulating effects of sbsFSH were analyzed by measuring T and 11-KT secretion from September to November, which includes from early recrudescence to early spermiogenesis. The analysis showed a dose-dependent tendency on T and 11-KT secretion, although less pronounced than the

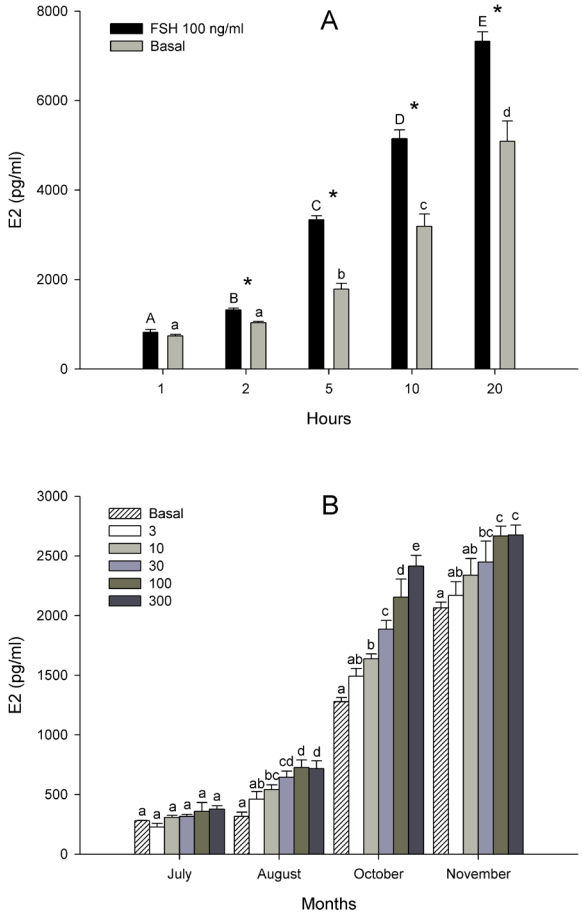

Figure 7. *In vitro* effects of purified sbsFSH (1B10) on E2 production by sea bass ovary. Tissue fragments (40 mg/well) were incubated with 0.5 ml of control medium (basal) or medium containing different concentrations of sbsFSH. Data are expressed as means ± SEM. (A) Temporal E2 production in response to 100 ng/ml of sbsFSH. Different letters indicate significant differences between hours (p < 0.001). Asterisks represent significant differences from the basal (p < 0.001). (B) E2 production after 20 h of incubation with different doses of sbsFSH (3–300 ng/ml). Different letters indicate significant differences between doses (p < 0.05).

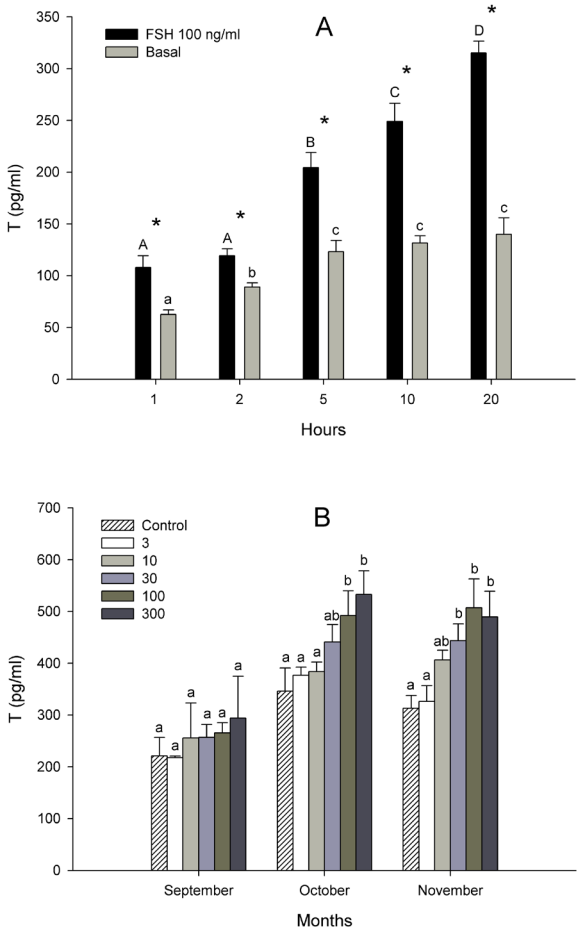

Figure 8. *In vitro* effects of purified sbsFSH (1B10) on T production by sea bass testis. Tissue fragments (40 mg/well) were incubated with 0.5 ml of control medium (basal) or medium containing different concentrations of sbsFSH. Data are expressed as means ± SEM. (A) Temporal T production in response to 100 ng/ml of FSH. Different letters indicate significant differences between hours ($p < 0.03$). Asterisks represent significant differences from the basal ($p < 0.03$). (B) T production after 20 h of incubation with different doses of sbsFSH (3–300 ng/ml). Different letters indicate significant differences between doses ($p < 0.03$).

E2 secretion seen in the ovary. The minimal significant ($p<0.05$) effective concentration of sbsFSH capable of stimulating the *in vitro* production of T and 11-KT, was 30 ng/ml in November (Fig. 8B and 9B). The stimulatory effects of sbsFSH on T and 11-KT secretion were also found to be time-dependent since sbsFSH (100 ng/ml) significantly ($p<0.05$) stimulated the

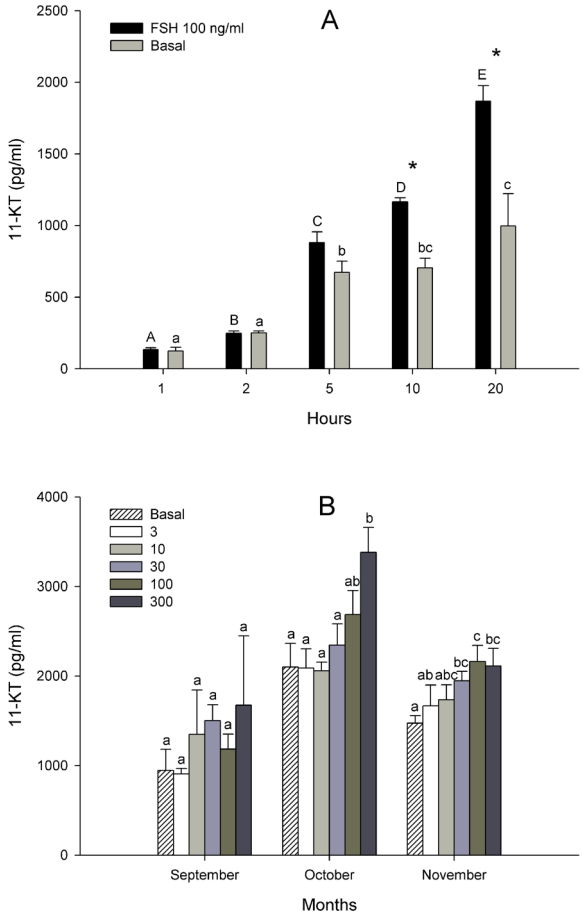

Figure 9. *In vitro* effects of purified sbsFSH (1B10) on 11KT production by sea bass testis. Tissue fragments (40 mg/well) were incubated with 0.5 ml of control medium (basal) or medium containing different concentrations of sbsFSH. Data are expressed as means ± SEM. (A) Temporal 11KT production in response to 100 ng/ml of sbsFSH. Different letters indicate significant differences between hours ($p < 0.05$). Asterisks represent significant differences from the basal ($p < 0.03$). (B) 11KT production after 20 h of incubation with different doses of sbsFSH (3–300 ng/ml). Different letters indicate significant differences between doses ($p < 0.05$).

secretion of T from 1h until 20h of incubation and from 10h until 20h for 11-KT (Fig. 8A and 9A).

4. Discussion

This study describes the purification and characterization of native intact sea bass FSH, free from LH contamination, from the pituitary glands of mature sea bass. Traditionally, the protocols used in the purification of teleost GTH dimers included a combination of gel filtration and ion-exchange chromatography (Suzuki et al., 1988a; Swanson et al., 1991; Van der Kraak et al., 1992; Copeland and Thomas, 1993; Tanaka et al., 1993; Mañanós et al., 1997; Santos et al., 2001; Kamei et al., 2005). Nevertheless, alternative protocols using ion-exchange and reverse phase chromatography under neutral or slightly basic conditions have been described for the purification of FSH and LH from other teleost species including the bonito (Koide et al., 1993), tuna (Okada et al., 1994), Mediterranean yellowtail (García-Hernández et al., 1997) and Atlantic halibut (Weltzien et al., 2003). In this study we used anion-exchange and reverse phase chromatography under slightly basic conditions and it turned out to be particularly effective for the isolation of sbsFSH. However, this protocol might not be suitable for the purification of LH since, as it has been previously described, problems such as low yield or subunit dissociation might occur (Okada et al., 1994). As seen in other species, sbsLH could be considered to be less stable than sbsFSH, since after rpHPLC only small amounts of sbsLH dimer were found while a large amount of its subunits was obtained. It has been described that the amino acid sequence of fish FSHβ subunits is more variable than that of fish LHβ subunits even in regions important for heterodimer formation and receptor interactions, such as the "seatbelt" region. The variation in structure among fish FSHβ subunits in this region may result in species differences in the nature of receptor interactions and possibly stability of the heterodimer (Swanson et al., 2003), which might explain the existence of very stable forms of teleost FSH.

Intact sbsFSH eluted mainly when the linear gradient achieved the 170 mM ammonium bicarbonate on the ion-exchange column, which is comparable to the 200 mM observed for coho salmon (Swanson et al., 1991), bonito (Koide et al., 1993), Mediterranean yellowtail (García-Hernández et al., 1997), and Atlantic halibut (Weltzien et al., 2003). In contrast, FSH from tuna (Okada et al., 1994) eluted with 300 mM ammonium bicarbonate, which indicates that it has a higher negative charge.

On the rpHPLC column under basic conditions, the sbsFSH eluted at 25% acetonitrile, similar to what was observed for Atlantic halibut (Weltzien et al., 2003) and tuna (Okada et al., 1994), while sea bass subunits eluted at 39% (sbsGPα), 28% (sbsFSHβ) and 33% (sbsLHβ) acetonitrile. In other species, as the Mediterranean Yellowtail (García-Hernández et al., 1997) and bonito (Koide et al., 1993), the FSH eluted at minor acetonitrile concentrations (aprox. 15%). In general, the observed differences in the eluting conditions could be due to a different glycosylation degree, which could change the polarity and net charge of the set and hence, the chromatographic mobility of the protein.

The molecular weight estimates for the purified sbsFSH and its subunits were smaller than for other species of teleost. There is a great variability in the molecular weight of those reported so far, finding subunits that range from 15 to 28 KDa and intact FSH from 32 to 50 KDa (Suzuki et al., 1988a; Swanson et al., 1991; Van der Kraak et al., 1992; Koide et al., 1993; Tanaka et al., 1993; Copeland and Thomas, 1993; Okada et al., 1994; García-Hernández et al., 1997; Govoroun et al., 1997; Mañanós et al., 1997; Shimizu and Yamashita, 2002; Weltzien et al., 2003; Kamei et al., 2005). According to the predicted amino acid sequences, the contribution of the polypeptide backbone to the molecular weight is similar among different teleost species, therefore the different molecular weight estimates could be due to differences in the glycosylation degree of the subunits or a consequence of the method of mass determination employed. For example, in the estimation by SDS-PAGE, the lack of SDS binding to the carbohydrate moieties of glycoproteins leads to lower charge-to-mass ratios for SDS-glycoprotein complexes, resulting in slower mobilities in the gel and therefore the over-estimation of molecular weights by SDS-PAGE. However, the mass spectrometry is a technology that makes possible to measure the molecular weight of any protein with a high accuracy.

Additional sureness for the identity and purity of the sbsFSH was confirmed by N-terminal amino acid sequencing of its subunits. The obtained results were compared to the deduced sequences of sea bass subunit cDNAs (Mateos et al., 2003) confirming that the 1B10 fraction contained highly purified hormone composed of sbsGPα and sbsFSHβ subunits. Moreover, the immunobloting also revealed that the mentioned fraction reacted only with anti-sbsGPα and anti-mFSHβ and not with anti-sbsLHβ.

On other hand, the deglycosylation treatment with PNGase F revealed

that both sbsFSH subunits are *N*-glycosylated. This is consistent with previous predictions of N-linked glycosylation sites from deduced amino acid sequences of sbsGPα and sbsFSHβ subunits (Mateos et al., 2003).

Detailed analysis of the isolated sbsFSHβ, both forming the dimer (1B10) and the subunit alone (4C2), showed a N-terminus, which, compared to that of other species, seems to lack the first three residues (Gly-Gln-Gly). Nevertheless, the amino acid sequence deduced from the cDNA sequence (Mateos et al., 2003) is equal in these positions to other teleosts (Fig. 5). It has been reported that N-terminal heterogeneity is normally observed in FSHβ subunits, whereas the N-terminus of the LHβ subunit is usually intact, suggesting that the N-terminus of FSHβ could be more susceptible to proteolytic degradation during the purification process (Okada et al., 1994). In this study only FSHβ missing the first three residues of the N-terminus, as in Atlantic halibut (Weltzien et al., 2003), was identified, making difficult to determine whether this sequence corresponds to the true N-terminal region of the mature sbsFSHβ or it is rather the result of a degradation process during the purification.

In some teleosts such as chum salmon, coho salmon and Mediterranean yellowtail, it is accepted the existence of two types of FSH that are chemically different: one acid-stable type and another acid-unstable type (Suzuki et al., 1988b; Swanson et al., 1991; García-Hernández et al., 1997). This unusual stability to acid treatment could suggest that its subunits are linked by disulfide bonds (Suzuki et al., 1988b). In this study, sbsFSH did not remain stable when subjected to a 0.1% TFA treatment, which is consistent with a non-covalent type of association between sbsGPα and sbsFSHβ subunits.

During the purification process, two types of sbsGPα with a different molecular weight were identified. The sbsGPα of the sbsFSH dimer (1B10) has a larger size than the isolated sbsGPα subunit (1C11). Different forms of GPα have also been found in other species of teleosts such as the Mediterranean yellowtail (García-Hernández et al., 1997), the Japanese eel (Kamei et al., 2005), the chum salmon (Suzuki et al., 1988b) and the coho salmon (Swanson et al., 1991). In some cases, they even showed different amino acid composition (Suzuki et al., 1988b; Swanson et al., 1991). In general, these differences in size could be due to a degradation process during the purification or to different carbohydrate composition. SbsGPα is predicted to have two *N*-glycosylation sites (Mateos et al., 2003). Addition of sugar chains to only one or to both potential sites of glycosylation would originate proteins

with different molecular weights, which would explain the observed SDS-PAGE results. Furthermore, glycosylation of mammalian GPα subunits is known to be required for heterodimer stability (Boime and Ben-Menahem, 1999). This could explain why the larger sbsGPα (1B10), a molecule potentially more glycosylated, was found in the sbsFSH dimer while the smaller form (1C11) was found free.

The biological activity and specificity of the purified sbsFSH were studied by evaluating its ability to stimulate sea bass gonadotropin receptors. 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 is evidence indicating that the specificity of the piscine gonadotropin receptors is less obvious (Bogerd et al., 2005).
In this study, we demonstrate that the purified sbsFSH is a biologically active protein since it was able to activate the sbsFSHR in a dose dependent manner. In addition, we provide evidence for specific interaction between this hormone and its cognate receptor since the purified sbsFSH was unable to activate the sbsLHR even when high doses were used (200 ng/ml). These results are in accordance with previous results regarding sea bass gonadotropin receptors ligand binding specificity (Rocha et al., 2007).

The steroidogenic activity of the purified sbsFSH was also evaluated by using an *in vitro* culture system for gonadal tissue. Purified sbsFSH significantly stimulated the release of T and 11-KT from adult sea bass testes and E2 from ovary fragments in a dose and time dependent manner. This steroid stimulation was greater in testis from males in early recrudescence (October) and in females in early and mid vitellogenic stages (October and November). T and 11-KT are the major androgens produced in male fish (Borg, 1994) and are known to be essential for spermatogenesis and spermiation (Fostier et al., 1983; Schulz and Miura, 2002). Besides, in Japanese eel it has been recently suggested that 11-KT, regulated by FSH, is the key endocrine factor for the initiation of spermatogonial proliferation (Ohta et al., 2007). Also, several reports have demonstrated that the entire process of vitellogenesis is regulated by E2 (Nagahama, 1994) and it has been described the stimulatory effect of FSH on the activity and expression of P-450 aromatase in vitellogenic trout follicles, suggesting that FSH plays a fundamental role in the regulation of E2 production in salmonid ovary (Montserrat et al., 2004). In the sea bass, plasma levels of 11-KT and T increase during the

spermatogenesis period (October-January) (Prat et al., 1990; Rodriguez et al., 2004) and plasma levels of E2 increase in parallel with the vitellogenin and the oocyte growth (Asturiano et al., 2000; 2002). The results obtained in this study are consistent with the expression profile seen for sbsFSHR in testis and ovary of adult sea bass, since high levels of sbsFSHR mRNA were observed in October in males and a significant increase in the expression of this gene was appreciated in October-November in females. In both sexes, the highest levels of sbsFSHR expression appear before those of sbsLHR (Rocha, A., Zanuy, S., Carrillo, M., Gómez, A., unpublished). All together, these data support the idea that in sea bass, as in salmonids, the FSH could be acting in the early stages of gametogenesis by promoting the synthesis of sexual steroids and consequently inducing spermatogonial proliferation in males and vitellogenesis in females.

In conclusion, highly purified and biologically active intact FSH, and their free GPα and FSHβ subunits were isolated from sea bass pituitary glands. The availability of purified sbsFSH will enable to study and understand the mechanisms involved in the gametogenesis process of this species.

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Capítulo 5:

Receptor Specificity and Functional Comparison of Recombinant Sea Bass (*Dicentrarchus labrax*) Gonadotropins (Fsh and Lh) Produced in Different Host Systems

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Abstract

Different yields, biopotency, and in vivo pharmacokinetics are obtained for recombinant sea bass gonadoltropins depending on the production system and DNA construct, but they show specific activation of their corresponding receptors. Gonadotropins (GTHs) are glycoprotein hormones that play a major role in the regulation of gonadal functions. Recently, we succeeded in isolating the native sea bass Fsh from sea bass pituitaries, but to ensure the availability of bioactive GTHs and no cross-contamination with other related glycoproteins, recombinant sea bass GTHs were produced using two expression systems—insect and mammalian cells—and different constructs that yielded tethered or noncovalently bound dimers. Their production levels, binding specificity to their homologous cognate receptors, and bioactivity were investigated and compared. Both expression systems were successful in the generation of bioactive recombinant GTHs, but insect Sf9 cells yielded higher amounts of recombinant proteins than mammalian Chinese Hamster Ovary (CHO) stable clones. All recombinant GTHs activated their cognate receptors without cross-ligand binding and were able to stimulate sea bass gonadal steroidogenesis in vitro, although with different biopotencies. To assess their use for in vivo applications, their half-life in sea bass plasma was evaluated. Sf9-GTHs had a lower in vivo stability compared with CHO-GTHs due to their rapid clearance from the blood circulation. Cell-dependent glycosylation could be contributing to the final in vivo stability and biopotency of these recombinant glycoproteins. In conclusion, both insect and mammalian expression systems produced bioactive sea bass recombinant gonadotropins, although with particular features useful for different proposes (e.g., antibody production or in vivo studies, respectively).

Keywords: fish, follicle-stimulating hormone, FSH/FSH receptor, gametogenesis, in vivo half-life, LH/LH receptor, luteinizing hormone, perciform, protein expression, single-chain GTHs, steroidogenesis

1. Introduction

The gonadotropins (GTHs)—follicle-stimulating hormone (FSH) and luteinizing hormone (LH)—produced in the pituitary gland are essential in the endocrine control of vertebrate reproduction. Together with the pituitary-derived thyroid-stimulating hormone (TSH) and the placental chorionic gonadotropin (CG), which is present only in primate and equine species, they constitute an evolutionarily conserved family of glycoprotein hormones. They are glycosylated heterodimers formed by the noncovalent association of an α subunit (Cga), which is common to all members of the family within a species, with distinct β subunits (Fshb and Lhb) that confer hormone specificity [1, 2]. Each subunit is encoded by a single, separate gene [3]. The dimerization and glycosylation are prerequisites for GTHs to achieve full biological activity.

Since late 1980s, GTHs have been isolated and characterized in several fish species [4]. As in higher vertebrates, GTHs regulate gametogenesis and steroidogenesis. Nevertheless, the functional duality between Fsh and Lh has not yet been well clarified in perciform fish, mainly because of the lack of appropriate tools for their study. In salmonids, from which most of the information regarding GTHs comes, Fsh is considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas Lh is considered to be responsible for the final maturation processes, such as oocyte maturation, ovulation, and spermiation [5, 6].

Access to bioactive and pure fractions of homologous GTHs is essential for studies aiming to reveal their differential functions in each fish species. Traditionally, pure, intact fish GTHs have been obtained by purification of the native hormones from pituitary glands. This is a highly demanding process with regard to time, cost, and the substantial amount of pituitary glands required; in addition, it has not always been successful [7, 8]. The availability of recombinant GTHs is an attractive alternative to native hormones that can facilitate physiological studies and be used in the development of biotechnological applications. They can be continually produced, assuring their availability and no cross-contamination with other related glycoproteins. The isolation and characterization of cDNAs encoding GTH subunits in a wide range of fish species provide the possibility to produce species-specific recombinant GTHs, although this has only been accomplished in

a handful of fish species in the last decade. Fish recombinant GTHs have been produced by means of various expression platforms, mainly eukaryotic systems that permit appropriate posttranslational modifications, such as glycosylation [7–13]. Production was accomplished by coexpression of both subunit genes (*cga* and *fshb*/*lhb*) or by fusion of their cDNAs, giving rise to a single protein known as single-chain GTH. All different approaches have generated bioactive recombinant GTHs, but they show different strengths and weaknesses, mainly concerning high-yield production and stability.

As a second step toward elucidating the gonadotropic regulation of gametogenesis in fish, full-length cDNAs encoding the receptors for Fsh (Fshr) and Lh (Lhr) were isolated from the gonads of several fish species [14]. As in other vertebrates, they are G protein-coupled receptors. Ligand binding activates the Gs/adenylate cyclase pathway, and the increased cAMP levels initiate a signaling cascade that leads to steroid synthesis [15, 16]. Despite their overall structural conservation, in vitro binding and functional studies revealed promiscuous ligand recognition of fish GTH receptors, in contrast to the strict ligand selectivity described in mammals. Depending on the species and origin of the GTHs (homologous/heterologous), a promiscuous activation of the Fshr or the Lhr was reported in studies using mammalian cell lines expressing recombinant fish GTH receptors [8, 10, 17–22]. These observations may complicate the interpretation of their specific roles.

The European sea bass is a perciform fish whose reproduction period is once a year during the winter season. Females present a group-synchronous type of ovarian development, producing three to four consecutive spawns during the 1- to 2-mo spawning period [23–25]. In this fish, the roles of Fsh in the reproductive processes are not clarified. Recently, the purification of sea bass Fsh from pituitary glands [26] has allowed the study of some aspects of steroidogenesis in this species. However, the limited quantities of hormone obtained, together with the unavailability of native Lh dimer for functional comparative studies motivated the development of recombinant GTHs.

In the available reports on production of recombinant fish GTHs, a specific expression system and DNA construct have been chosen in each case. In the present study, we intended to produce and characterize recombinant Fsh and Lh for sea bass, but also to compare expression systems and expression constructs. We chose two expression systems representing distant eukaryotes: insect (invertebrate) and mammalian cells, and DNA constructs

leading to the production of both separate and tethered subunits. Finally, we assessed and compared their function regarding receptor activation specificity and biopotency, gonadal steroidogenic ability, and in vivo stability, using homologous targets and receptors. This functional characterization provides new data on sea bass GTH action in GTH-receptor interactions, and sets a fundamental basis for the use of recombinant GTHs for future applications.

2. Materials and methods

2.1. Production of recombinant Fsh and Lh in insect cells (Sf9)

Construction of cga, fshb and lhb subunit transfer vectors

Complementary DNA fragments containing the entire open reading frames (ORFs) of sea bass *cga* (416 bp), *fshb* (480 bp), and *lhb* (487 bp; GenBank accession numbers: AF269157, AF543314, and AF543315) were obtained by PCR using specific primers (Supplemental Table S1, Nos. 1 to 6; available online at www. biolreprod.org) and the proofreading *Pfu*Turbo

Table S1. PCR primers used in this study.

Gene	*N°*[a]	*Direction*[a]	*Primer sequence*[b,c,d]	*Restriction sites*
cga	**1**	F:	5′-GG**GGATCC**CTTTCTCTCAACATGGTAAC-3′	*Bam* HI
	2	R:	5′-GG**AAGCTT**GAATGGTCTCCAGTTCCCAT-3′	*Hind* III
fshb	**3**	F:	5′-GG**GAATTC**GTTTAGAGATTGACAGAAGAAGC-3′	*Eco* RI
	4	R:	5′-AT**GCGGCCGC**TTAATGATGATGATGATGATGAA AGGACAGACAGCTGGGTAT-3′	*Not* I
lhb	**5**	F:	5′-GG**GGATCC**CCAGAGAGGATGATGGCTGT-3′	*Bam* HI
	6	R:	5′-GG**AAGCTT**ATGTGCTTCATGCTGCTATT-3′	*Hind* III
fshb-CTP	**7** (B1)	F	5′-CCG**GGATCC**GTTTAGAGATTGACAGAAGAA-3′	*Bam* HI
	8 (B2)	R	5′-*TGAGGAAGAGGA*AAAAGGACAGACAGCTGGG-3′	
	9 (B3)	F	5′-TGTCTGTCCTTT*TCCTCTTCCTCAAAGGCC*-3′	
lhb-CTP	**10** (B1)	F	5'-CCGG**GGATCC**CCAGAGAGGATGATGGCTGTG-3'	*Bam* HI
	11 (B2)	R	5'-*TGAGGAAGAGGA*GTAGTAGAAAGGTATGTC-3'	
	12 (B3)	F	5'-CCTTTCTACTAC*TCCTCTTCCTCAAAGGCC*-3'	
cga-CTP	**13** (A1)	R	5'-CATGCTGGGGTA*TTGTGGGAGGATCGG*-3'	
	14 (A2)	F	5'-*ATCCTCCCACAA*TACCCCAGCATGGACTTA-3'	
	15 (A3)	R	5'-CG**GAATTC**CATGAAAGTGCAACATCTG-3'	*Eco* RI

[a] F: Forward, R: Reverse; [b] Bold: Restriction enzyme recognition sites; [c] Underline: sequence for six histidine residues; [d] Italics: CTP.

DNA polymerase (Stratagene). Restriction sites were added at the 5' end of the forward and reverse primers. These fragments were directionally cloned into the donor plasmid pFastBac1 between *Bam*HI/*Hin*dIII (pFastBac1-Cga and pFastBac1-Lhb) or *Eco*RI/*Not*I (pFastBac1-Fshb) restriction sites. All of the constructs were sequenced to confirm their identity.

Generation of recombinant baculovirus

Recombinant baculovirusexpressing sea bass *cga*, *fshb*, and *lhb* were generated independently using the Bac-to-Bac Baculovirus Expression System (Invitrogen). Briefly, competent *Escherichia coli* DH10Bac was transformed with the recombinant donor plasmids pFastBac1-Cga, pFastBac1-Fshb, or pFastBac1-Lhb for transposition into the bacmid. Recombinant bacmids were then used to transfect insect cells, *Spodoptera frugiperda* (Sf9), with Cellfectin Reagent (Invitrogen) according to the manufacturer's protocol. Sf9 cells were grown in monolayer at 28°C in Sf-900 II SFM medium (Invitrogen) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). After 72 h, recombinant baculovirus was harvested from the medium. Viral stocks were amplified by infection of Sf9 cells at a multiplicity of infection (MOI) of 0.1 for 48 h at 28°C to achieve final viral titers of $2x10^7$ to $5x10^7$ plaque-forming units (pfu) per milliliter as assessed by viral plaque assay.

Production of recombinant Fsh and Lh in Sf9 cells

Monolayer cultures of Sf9 cells were grown in 75-cm^2 flasks to 80%–85% confluence (approximately $12x10^6$ cells). Optimal results for the expression of GTH subunits were obtained when Sf9 cells were infected at an MOI of 3 pfu/ml during 4–5 days. Cells and medium were then harvested by centrifugation (110 x *g*, 10 min) and stored at −80°C until used. The infected cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0; 100 mM KCl; 1% Nonidet P-40; 1 mM PMSF; and 1 μg/ml aprotinin) and their cell membrane disrupted by brief periods of sonication on ice using a cell disrupter (Vibracell; Sonics & Materials, Inc.). Supernatants containing soluble proteins were recovered after removal of cell debris by centrifugation at 35000 x *g* for 30 min at 4°C.

For production of sea bass Fsh and Lh dimers, Sf9 cells were coinfected with a 1:1 ratio of baculovirus Cga:β subunit (Fshb or Lhb, respectively) at an MOI of 3 pfu/ml. Twenty-four hours after infection, fresh culture medium was added, and cells were incubated at 28°C for 4 days. Finally, the medium

was harvested and the presence of recombinant dimeric GTHs assessed.

2.2. Production of recombinant Fsh and Lh in Chinese Hamster Ovary cells

Construction of expression plasmids

A cDNA fragment containing the entire ORF of sea bass *fshb* and a C-terminal 6xHis tag was obtained by PCR using the specific primers 3 and 4 (Supplemental Table S1) and directionally subcloned into the *Eco*RI/*Not*I-digested pcDNA3 plasmid (Invitrogen) to generate pcDNA3-Fshb. The pCMVtk-Cga plasmid contains the full-length *cga* cDNA under the control of the CMVtk promoter, followed by an SV40 polyadenylation signal. All of the constructs were sequenced to confirm sequence identity.

Single-chain Fsh (scFsh) and Lh (scLh) were constructed by in-frame fusion of the sea bass *cga* and *β* subunit cDNAs of each hormone using the carboxy-terminal peptide (CTP; 28 amino acids) from the human chorionic gonadotropin (hCG) β subunit as a linker. Sequential overlapping PCR [27, 28] reactions were performed using primers 7 to 15 (Supplemental Table S1; see Fig. 1 for a diagram) and the high-fidelity *Pfu*Turbo DNA polymerase

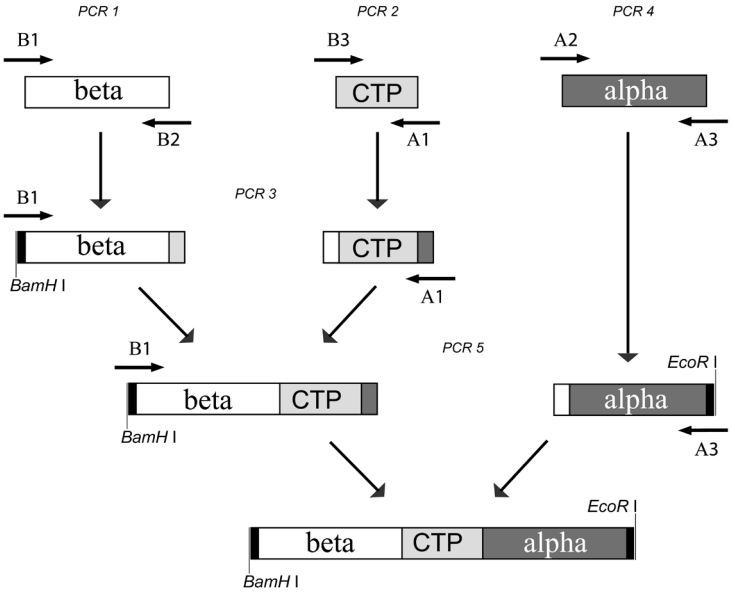

Figure 1. Diagram of construction of single-chain GTHs using overlapping PCR.

(Stratagene). Expression plasmids containing the *hCGB* (kindly provided by Dr. Irving Boime, Washington University), and the cDNAs for sea bass *fshb*, *lhb*, and *cga* were used as templates. In the instance of *cga*, the sequence coding for the signal peptide was removed. The PCR-generated fusion fragments for Fsh and Lh were completely sequenced to ensure sequence identity. Each of these tethered cDNAs (*scfsh* and *sclh*) was inserted into the expression vector pcDNA3 (Invitrogen) as *Bam*HI/*Eco*RI fragments, and the resulting expression plasmids were named pCMV-scFsh and pCMV-scLh, respectively.

Generation of Chinese Hamster Ovary stable clones and production of recombinant GTHs

Chinese Hamster Ovary (CHO) cells were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal bovine serum (FBS), at 37°C with 5% CO_2. Cells were transfected in 24-well plates using Lipofectamine Reagent (Invitrogen) according to the manufacturer's protocol. Equal amounts of pCMVtk-Cga and pcDNA3-Fshb were cotransfected to express the Fsh dimer, and pCMV-scFsh or pCMV-scLh was transfected to express single-chain Fsh or Lh, respectively. To obtain the stable clones, 48 h after transfection cells were replated in 96-well plates and selected in medium containing 500 μg/ml G418 (Geneticin; Invitrogen). The isolated stable clones (CHO-Fsh, CHO-scFsh, and CHO-scLh) were expanded, and the cells and conditioned medium were screened for the presence of Fsh and Lh by Northern blotting, Western blotting, and receptor activation.

For each construct, one selected clone expressing the hormone was grown according to the protocol described by Schatz et al. [29] in 75-cm^2 cell culture flasks until 80%–90% confluence in selective medium. The FBS-containing medium was then replaced by Advanced DMEM (Invitrogen) serum-free medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine. The cells were further incubated for 9 days at 25°C. Then, the culture supernatants were harvested and stored at -80°C.

2.3. Recombinant hormone concentration

The harvested media (Sf9-Fsh, Sf9-Lh, Sf9-Fshb, Sf9-Lhb, CHO-Fsh, CHO-scFsh, CHO-scLh, and CHO control) were concentrated by ultrafil-

tration using Centricon Plus-20 Biomax 5 (subunits and dimers) and Ultracel PL 30 (single chains) centrifugal filter devices (Amicon; Millipore Corp.). In the different experiments, the concentration of recombinant GTHs used is expressed as a dilution factor of the culture medium (Table 1).

2.4. SDS-PAGE and Western blot analysis

Recombinant proteins were electrophoresed through 15% SDS-PAGE gels under reducing conditions (5% 2-mercaptoethanol [2-ME]). The separated proteins were transferred to PVDF membranes (Immobilon P; Millipore Corp.), blocked overnight with 5% skimmed milk at 48C, incubated with the specific antisera (dilutions 1:2000 and 1:3500) for 90 min at room temperature, washed, and further incubated with 1:2000 goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (GAR-HRP; Bio-Rad Laboratories) for 60 min at room temperature. The immunodetection was performed by chemiluminescence (Western Blotting Luminol Reagent; Santa Cruz Biotechnology Inc.). Antibodies against sea bass (sbs) Cga and Lhb subunits (1:2000) [30], and a mummichog (*Fundulus heteroclitus*) Fshb (mFshb) antibody (1:3500) [31] already tested for sea bass [26] were used as antisera. In all cases, the molecular weights were estimated by SDS-PAGE.

2.5. Glycosidase treatments

Concentrated media (3–10 µl) containing sea bass GTH dimers were denatured at 94°C for 5 min in 0.1% SDS and 50 mM 2-ME. Then, media were incubated with 2.5 units of peptide-*N*-glycosidase F (PNGase F; Roche Di-

Table 1. Amount of recombinant sea bass gonadotropins after concentration by ultrafiltration.

Hormone	Fold concentration[a]	Concentration (1x)	Quantification method
Sf9-Fsh	40x	24.8 - 4.12 µg/ml	I. dot-blot / Bioassay FSH-R
Sf9-Lh	50x	2.8 µg/ml	ELISA LH
Sf9-Lhb	25x	2.2 µg/ml	ELISA LH
CHO-Fsh	294x	0.650 - 0.011 µg/ml	I. dot-blot / Bioassay FSH-R
CHO-scFsh	160x	0.405 - 0.1 µg/ml	I. dot-blot / Bioassay FSH-R
CHO-scLh	195x	0.03 µg/ml	ELISA LH

[a] Number of folds that the culture medium was concentrated.

agnostics) at 37°C for 16 h in 20 mM sodium phosphate with 0.5% Nonidet P-40, pH 7.5. Deglycosylated proteins were subjected to SDS-PAGE followed by Western blot analysis.

2.6. Activation of sea bass Fsh and Lh receptors

The biological activity of the recombinant hormones were analyzed using Human Embryonic Kidney (HEK) 293 cell lines stably expressing the sea bass Fsh or Lh receptor (sbsFshr and sbsLhr) and the firefly luciferase gene under the control of a promoter with cAMP responsive element (CRE)-binding sites (pCRE-LUC) [20]. Receptor activation by recombinant Fsh or Lh was indirectly measured by recording changes in luciferase activity, promoted by a rise of cAMP. Cells from the HEK 293 clones were seeded in 48-well plates and incubated with serial dilutions of recombinant sea bass Fsh or Lh preparations in Advanced DMEM media (Invitrogen). After 5 h of incubation, cells were lysed in Reporter Lysis Buffer (Promega Corp.) as indicated by the manufacturer. Cell debris was separated by centrifugation for 30 sec at 15000 x *g*, and 20 µl of the supernatant was mixed with 100 µl of luciferin reagent (20 mM Tricine KOH, pH 7.8; 0.1 mM ethylenediaminetetraacetic acid; 8 mM $MgCl_2$; 33.3 mM dithiothreitol; 270 µM coenzyme A; 530 µM ATP; and 400 µM luciferin). Light emission was measured in a luminometer (Junior; EG&G Berthold) and expressed as relative light units.

HEK 293 cells expressing the sbsFshr were also used to calculate the effective ligand concentrations (native and recombinant Fsh) inducing a half-maximal stimulation (EC_{50}).

Recombinant human FSH (rhFSH; AFP8468A; biopotency 8000 IU/mg) was obtained from the National Hormone & Peptide Program, Harbor-UCLA Medical Center. Lyophilized hCG from urine of pregnant women was purchased at a local pharmacy (hCG-lepori 2500 IU; Farma-Lepori).

2.7. *In vitro* sex steroid production and follicle maturation

In vitro tissue culture of sea bass gonads was performed following the protocol described by Molés et al. [26]. Briefly, ovaries (in early to mid vitellogenesis) and testis (in late spermatogenesis) of adult sea bass killed between October and November were thoroughly chopped on ice with a razor blade until forming a paste. The tissue preparation was washed three times

with 10 vol/wt of ice-cold Sea Bass Ringer (SBR; according to Sorbera et al. [32]) containing 0.5% bovine serum albumin (Fraction V; Sigma-Aldrich Inc.) and 100 U/ml penicillin/ streptomycin (Invitrogen Corp.). After each wash, the tissue was centrifuged at 60 x *g* for 10 min at 4°C, and the supernatant was discarded. In the last wash, the preparation was filtered through a 750-μm(for testis) or 150-μm(for ovary) nylon mesh. Pieces of the gonadal preparation were transferred to 24-well plates (40 mg per well) and pre-incubated shaking (100 rpm) in SBR (0.5 ml per well) for 30 min at 18°C. Then, the medium was replaced with fresh SBR (0.5 ml per well) containing different dilutions of recombinant sea bass Fsh or Lh. Tissues were further incubated for 20 h at 18°C and 100 rpm. Finally, the medium was collected, centrifuged at 500 x *g* for 15 min at 4°C, and the supernatant stored at -20°C until steroid analysis. Each treatment was repeated four times, and the experiments were performed in triplicate.

Follicles from adult sea bass killed in January were cultured according to Sorbera et al. [32]. Only vitellogenic and postvitellogenic intact follicles with opaque cytoplasm and no indication of yolk clarification or lipid droplet coalescence were chosen for incubation. Oocytes were manually isolated from the ovarian tissue on ice, placed in SBR, and allowed to stabilize at 18°C for 30 min prior to treatments. For scFsh stimulation, follicles were placed in 48-well plates (45–70 follicles per well) containing different doses of scFsh in 250 μl of SBR. After 48 h of incubation at 18°C, the medium was collected and stored at -20°C until estradiol (E2) analysis. Each dose was tested in triplicate, and the experiment was repeated three times. For scLh treatment, 30 postvitellogenic follicles per dose were individually incubated in 96-well plates in 100 μl of SBR for 48 h. The experiment was repeated twice. Maturation, which included both mature/preovulatory and ovulated oocytes, was confirmed by visual assessment of volume increase, lipid droplet coalescence, yolk clarification, and germinal vesicle migration and breakdown [32].

2.8. Hormone analysis

Estradiol was measured by a conventional enzyme immunoassay (EIA), which was validated for sea bass in our laboratory. The protocol was similar to that previously developed for testosterone determination [33]. Briefly, culture medium was extracted with methanol, which was further evapo-

rated. The dry extract was reconstituted in assay buffer (EIA buffer; Cayman Chemical). Each component, E2-acetylcholinesterase tracer, anti-E2 rabbit antiserum, and E2 standards (Sigma-Aldrich Inc.), or samples were added to 96-well microtiter plates coated with mouse anti-rabbit IgG monoclonal antibodies (Clone RG-16; Sigma-Aldrich Inc.) and incubated overnight at 37°C. Then, plates were rinsed, and color development was performed by addition of Ellman reagent and incubation for 2 h at 20°C in the dark. Optical density was read at 405 nm using a microplate reader (Bio-Rad microplate reader model 3550). The sensitivity of the assay was 0.156 ng/ml (Bi/B0 = 90%) with a slope of 0.956. The interassay coefficient of variation (n = 12) was 9.38%.

11-Ketotestosterone (11-KT) was determined by an EIA developed for the Siberian sturgeon [34] and modified for use in sea bass [35]. The assay sensitivity was 0.0012 ng/ml (Bi/B0 = 90%).

Levels of recombinant sea bass Lh in culture medium or plasma samples were measured by a homologous competitive ELISA according to Mateos et al. [30]. The sensitivity of the assay was 0.65 ng/ml (Bi/B0 = 80%).

The amount of recombinant sea bass Fsh produced by Sf9 and CHO cells was determined by an immunodot blot assay with the mFshb antisera already described. Briefly, the proteins present in the culture media were denatured (5% 2-ME, 4 min at 95°C) and immobilized in a PVDF membrane using a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories). The membrane was processed as in the Western blot assay (anti-mFshb, 1:3500; GAR-HRP, 1:2000). The immunodetection was performed by chemiluminescence (Luminol Reagent) using the image system Versadoc (Bio-Rad Laboratories). The intensity of the dots was quantified with the image analysis software Quantity One v 4.4 (Bio-Rad Laboratories). Known quantities of purified native sea bass Fsh [26] were used as standard curve.

Levels of bioactive sea bass Fsh in plasma samples were estimated by receptor activation as described above using the sbsFshr/pCRE-LUC HEK 293 stable clone [26] and 96-well plates. Plasma samples were diluted 1:25 and measured in triplicate. After 6 h of incubation, luciferase activity was quantified directly on the plates using the Steady-Glo Luciferase Assay System (Promega Corp.) and the ULTRA Evolution (Tecan) detection platform. Intraassay and interassay coefficients of variation were 6.9% and 9.5%, respectively. The sensitivity of the bioassay was 0.104 ng/ml.

By using both determination methods, the in vitro biological:immuno-

logical ratio (B:I) could be calculated (i.e., Fsh concentration from the in vitro bioassay relative to that yielded by the immunodot blot).

2.9. In vivo half-life evaluation

Juvenile sea bass (51–94 g body mass) were used for pharmacokinetic analysis of recombinant sea bass GTHs (7–10 fish per group). A single dose of Sf9-Fsh (200 ng per fish), Sf9-Lh (500 ng per fish), scLh (52 ng per fish), or scFsh (200 ng per fish) diluted in PBS was administered by intramuscular injection. Noninjected animals or animals injected with Sf9-Fshb, Sf9-Lhb, or wild-type CHO conditioned concentrated medium served as controls. Blood samples (0.2 ml) were collected at different times (0, 0.5, 1, 3, 6, 12, 24, 48, and 72 h) after injection. Plasma was obtained by centrifugation (3000 x *g* for 30 min at 4°C) and stored at -20°C until hormone measurements. Clearance rate was calculated as the relationship clearance rate = dose/area under the concentration-time curve. The area under the concentration-time curve was calculated using NCSS software.

Animals used for in vivo studies or as a source of gonadal tissue were treated in accordance with the Spanish legislation concerning the protection of animals used for experimentation or other scientific purposes.

2.10. Statistical analysis

Data are presented as mean ± SEM. The significance of the differences between group means of hormone levels or receptor activation (luciferase activity) was determined by one-way ANOVA followed by the Holm-Sidak method using SigmaStat 3.5 (Systat Software Inc.). Student *t*-test was performed to determine significant differences between treatments.

3. Results

3.1. Production of recombinant sea bass GTHs and biochemical properties

Dimeric or single-chain recombinant sea bass GTHs have been produced and efficiently secreted in two systems, insect (Sf9) and mammalian (CHO) cells, as detailed in *Materials and Methods*. Culture media from Sf9 infections and CHO selected clones were concentrated by ultrafiltration (Table

1) and analyzed for recombinant proteins. To identify and characterize the biochemical properties of the secreted hormones, a Western blot analysis was performed. After SDS-PAGE and immunoblotting of the medium containing the sea bass Sf9-Fsh or Sf9-Lh, two proteins of different molecular mass could be visualized. In the case of recombinant Sf9-Fsh, the anti-mFshb and anti-sea bass Cga reacted with proteins of approximately 12 kDa and 15.6 kDa, respectively. Both recombinant subunits were slightly smaller than the native forms purified from sea bass pituitary (Fig. 2, A and B). For recombinant Sf9-Lh, the anti-sea bass Lhb and anti-sea bass Cga reacted with proteins of approximately 22 kDa and 15.6 kDa, respectively (Fig. 2, E and F). As expected, because of the larger number of amino acids, the recombinant Sf9-Lhb showed a larger size than the Sf9-Fshb. When the β subunits were individually expressed in Sf9 cells, only Lhb was secreted and detected in the culture media (Fig. 2E), whereas Fshb remained in the infected cells, because a strong band was detected in the cell lysates but not in the culture medium (Fig. 2A).

Concerning the production of the Fsh dimer in CHO cells, anti-mFshb and anti-sea bass Cga reacted with two proteins of a similar size (approximately 27 kDa and approximately 24 kDa, respectively; Fig. 2, I and J). Both subunits were bigger than the Sf9-Fsh and native sea bass Fsh subunits. The Western blot analysis of culture medium from CHO cells producing single-chain Fsh or Lh revealed proteins reacting with the anti-mFshb (Fig. 2K) and anti-sea bass Lhb (Fig. 2L) with sizes of roughly 38 and 48 kDa, respectively, which would be in the range expected for the linked subunits.

The size differences found in the recombinant subunits depending on the expression system are most likely due to the degree and type of glycosylation. A deglycosylation treatment with *N*-glycosidase F was performed to confirm this aspect. The enzymatic treatment, which hydrolyzes all types of *N*-glycan chains, resulted in a decrease in size of both subunits of the recombinant Fsh and Lh dimers (Fig. 2, C, D, and J, and G and H, respectively). The observed reduction in size clearly shows that in both expression systems, the recombinant subunits were *N*-glycosylated, and thus the differences in molecular mass between Sf9-Fsh and CHO-Fsh were due to a greater glycosylation degree in the CHO cells.

The levels of recombinant Sf9-Lh and Sf9-Lhb secreted to the culture media by coinfected and infected Sf9 cells were 2.8 μg/ml and 2.2 μg/ml, respectively, whereas the production of CHO-scLh was 0.03 μg/ml, as quan-

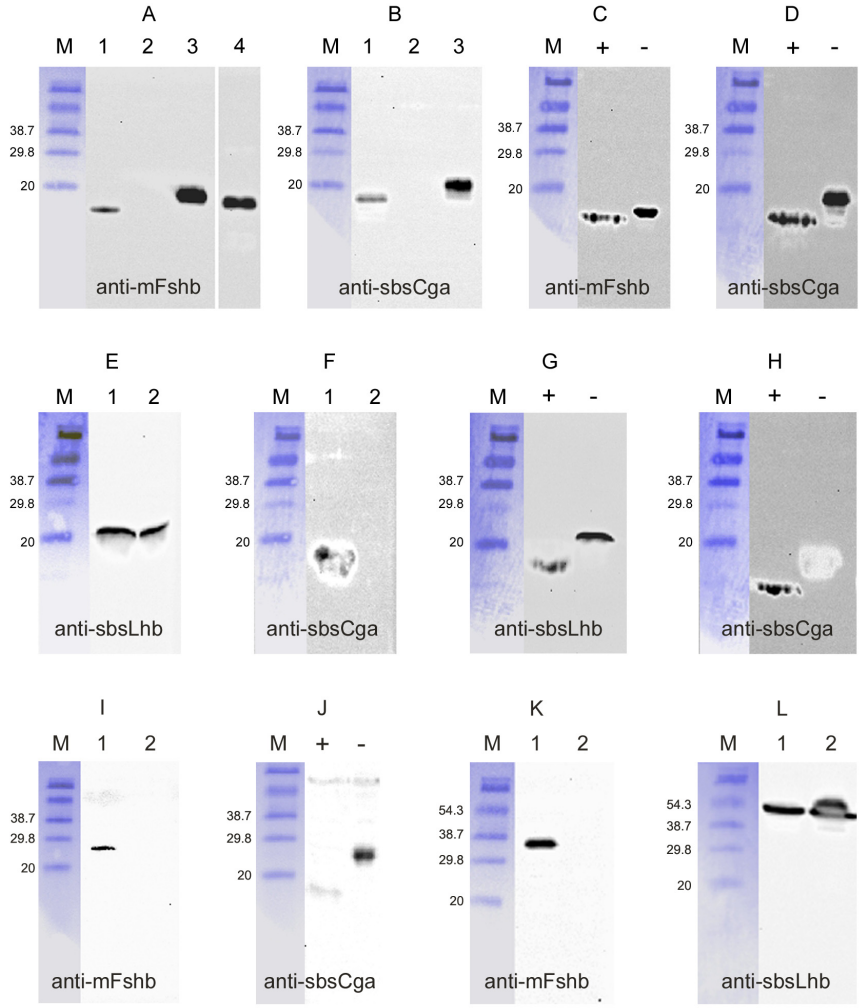

Figure 2. Western blot analysis of recombinant GTHs. Proteins in culture medium (CM) from cells producing different recombinant forms were electrophoresed and incubated with specific antibodies. Analysis of Sf9-Fsh (**A-D**). **A** and **B**) Lane 1: CM from Sf9 cells producing Fsh dimer; lane 2: CM from Sf9 cells producing Fshb subunit; lane 3: purified native sea bass Fsh; lane 4: cell lysates from Sf9 producing Fshb subunit. **C** and **D**) Lane +: Sf9-Fsh deglycosylated; Lane -: Sf9-Fsh nondeglycosylated. Analysis of Sf9-Lh (**E-H**). **E** and **F**) Lane 1: CM from Sf9 cells producing Lh dimer; lane 2: CM from Sf9 cells producing Lhb subunit. **G** and **H**) Lane + : Sf9-LH deglycosylated; lane - : Sf9-Lh nondeglycosylated. Analysis of CHO-Fsh, CHO-scFsh and CHO-scLh (**I-L**). **I**) Lane 1: CM from CHO cells producing Fsh dimer; Lane 2: CM from control CHO cells. **J**) Lane + : CHO-Fsh deglycosylated; lane - : CHO-Fsh nondeglycosylated. **K**) Lane 1: CM from CHO cells producing scFsh; lane 2: CM from control CHO cells. **L**) Lanes 1 and 2: CM from CHO cells producing scLh. M: Bio Rad prestained SDS-PAGE broad range molecular weight marker.

tified by ELISA. On the other hand, the production level of recombinant sea bass Fshs was estimated by using two different methods: an immunodot blot assay using anti-mFshb antisera and an in vitro bioassay using a recombinant sea bass Fshr. This approach allowed us to assess both quantity and bioactivity for each preparation. For both methods, serial dilutions of purified native sea bass Fsh were used as standard. According to the immunodot blot, the level of Sf9-Fsh production was 24.8 μg/ml, whereas the levels of CHO-Fsh and CHO-scFsh were 0.650 μg/ml and 0.405 μg/ml, respectively. According to the activation of sbsFshr, when compared with the bioactivities of native sea bass Fsh, the Sf9-Fsh production was 4.12 μg/ml, whereas CHO-Fsh and CHOscFsh production was 0.011 μg/ml and 0.1 μg/ml, respectively (Table 1).

3.2. Bioactivity and specific receptor (Fshr and Lhr) activation

The bioactivity and specificity of the recombinant sea bass Fsh and Lh were analyzed by determination of their capacity to stimulate intracellular cAMP production in HEK 293 cells stably expressing sea bass Fshr or Lhr. Recombinant dimers (Sf9-Fsh, CHO-Fsh, and Sf9-Lh) and single-chain GTHs (CHO-scFsh and CHO-scLh) activated their corresponding receptors in a dose-dependent manner. Additionally, the β subunits obtained from infections with baculovirus Fshb or Lhb were unable to stimulate their cognate receptors (Fig. 3, A and B), demonstrating that only the dimers are bioactive. Dilutions of culture medium from the different expression systems (Sf9 and CHO cells) were used to stimulate the respective receptors. The minimal concentration of culture medium able to induce cAMP production significantly was 1:1000 for Sf9-Fsh and Sf9-Lh (Fig. 3, A and B), 1:10 for CHO-Fsh and CHO-scLh (Fig. 3, C and D), and 1:100 for CHO-scFsh (Fig. 3C). In all cases, the maximal induction obtained was 22- to 30-fold the basal value. To evaluate ligand receptor specificity for the sea bass GTHs, all of the recombinant hormones were used to activate both sea bass GTH receptors, sbsFshr and sbsLhr. None of the GTHs showed cross-receptor binding at any of the concentrations tested because they were only able to stimulate their cognate receptors (Fig. 3, A–D).

To expand our knowledge of ligand recognition by the sea bass GTH receptors, heterologous human GTHs (hFSH and hCG) were tested and compared with the homologous hormones. Interestingly, hFSH was able to

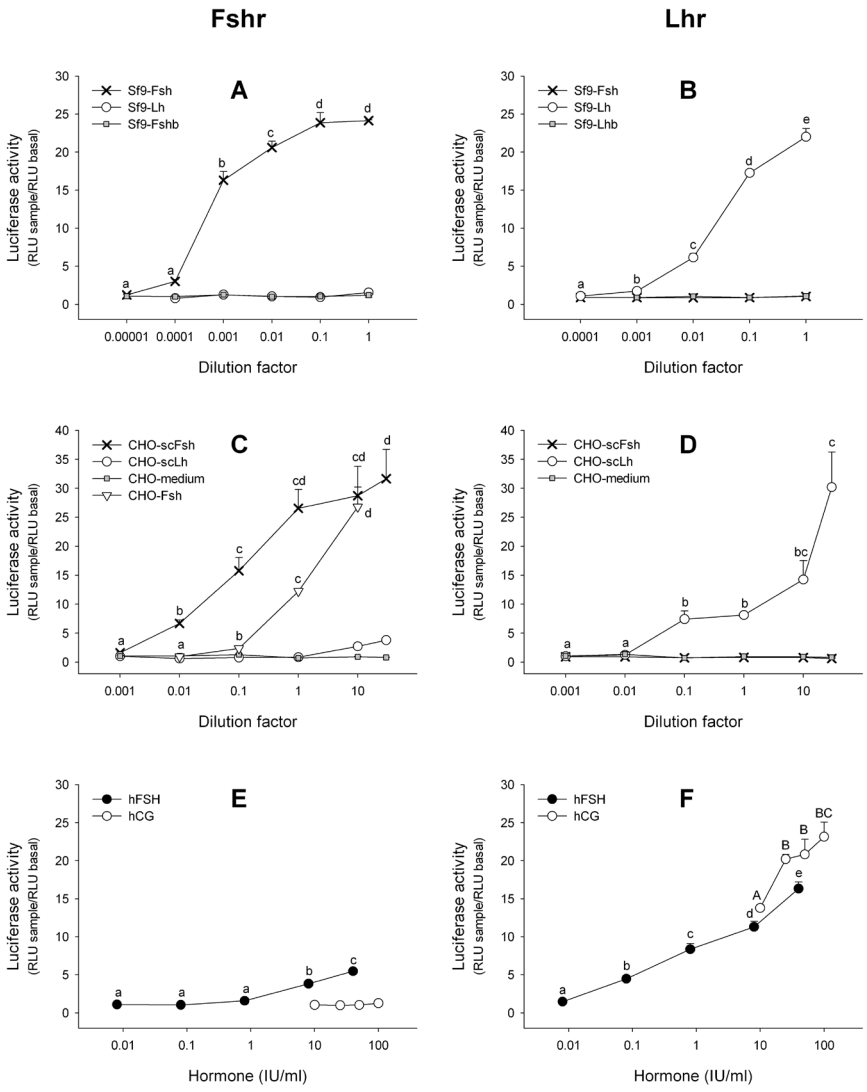

Figure 3. Activation of sea bass Fshr (**A**, **C** and **E**) and Lhr (**B**, **D** and **F**) by sea bass and human GTHs. HEK 293 cells expressing the GTH receptors were incubated with increasing doses of GTHs. The concentration is expressed as dilution factor of the culture medium for sea bass recombinant GTHs (**A**-**D**) or international units per milliliter for human GTHs (**E** and **F**). Sf9-Fshb, Sf9-Lhb or wild-type CHO conditioned concentrated medium served as control. Each point represents mean ± SEM of three determinations. Each experiment was repeated at least three times. RLU, relative light units. Different letters indicate significant differences between doses.

stimulate the sbsLhr in a dosedependent manner (Fig. 3F) but not the sbsFshr, although a slight stimulation was observed when high doses (8–40 IU/ml) were used (Fig. 3E). On the other hand, hCG activated the sbsLhr (Fig. 3F) but not the sbsFshr (Fig. 3E).

To compare the biopotency of Fsh dimer proteins produced by each expression system (Sf9 and CHO cells) with that of the native sea bass Fsh, which is the only available GTH purified from sea bass pituitary glands, we calculated the EC_{50} to induce cAMP-dependent luciferase expression of

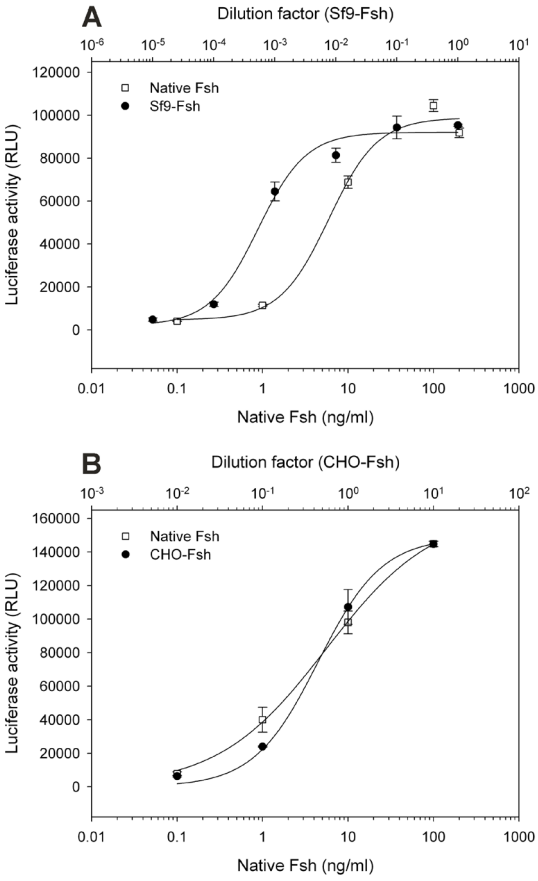

Figure 4. Parallelism of dose-response curves and comparative bioactivity of recombinant Fsh dimers with native sea bass Fsh. HEK 293 cells expressing the sea bass Fshr were incubated with increasing doses of native sea bass Fsh, Sf9-Fsh (**A**) and CHO-Fsh (**B**). Each point represents mean ± SEM of three determinations. RLU, relative light units.

Table 2. Bioactivities of the recombinant Fshs.

Hormone	EC_{50}	B:I ratio
Sf9-Fsh	23 ng/ml	0.16
CHO-Fsh	260 ng/ml	0.017
CHO-scFsh	14 ng/ml	0.25
Pituitary Fsh	6 ng/ml	

each recombinant sea bass Fsh and that obtained with different doses of native sea bass Fsh. The Sf9-Fsh was approximately 11-fold more potent (EC_{50} = 23 ng/ml) than the CHO-Fsh (EC_{50} = 260 ng/ml), and both were approximately 4- and 43-fold less potent than purified pituitary Fsh (EC_{50} = 6 ng/ml), respectively (Fig. 4, A and B). However, when the CHO-scFsh was analyzed (EC_{50} = 14 ng/ml), the EC_{50} was in the same range as that of the Sf9-Fsh and was 18-fold more potent than the CHO-Fsh. Moreover, with the measurement of both Fsh quantity (immunodot blot) and bioactivity (bioassay), the B:I could be estimated. The Sf9-Fsh and CHO-scFsh exhibited similar B:I ratios (0.16 and 0.25, respectively), whereas the B:I ratio estimated for CHO-Fsh (0.017) was about 10-fold lower (Table 2). This indicated a higher bioactivity for the baculovirus derived and the single-chain Fshs and supports the EC_{50} results mentioned above.

3.3. *In vitro* bioactivity in gonad culture

To determine the activity of sea bass recombinant GTHs on the target tissue, their ability to induce steroidogenesis and/or maturation in sea bass ovary and testis was tested. Preliminary in vitro experiments using several doses of sea bass recombinant GTHs (data not shown) showed a clear dosedependent effect on E2 production by ovarian tissue. In further experiments, only two doses of all recombinant dimers (Sf9-Fsh, CHO-Fsh, and Sf9-Lh) were used. The stimulatory effects of recombinant sea bass Fsh (Sf9 and CHO) and Lh (Sf9) dimers were analyzed in adult sea bass ovary during the early and mid vitellogenic stages (October to November), and all of them showed a dose-dependent effect on E2 secretion (Fig. 5A). We observed that equivalent concentrations of Sf9-Fsh and Sf9-Lh in the range of 250 ng/ml had a similar effect in ovary E2 production. In testis of adult male sea

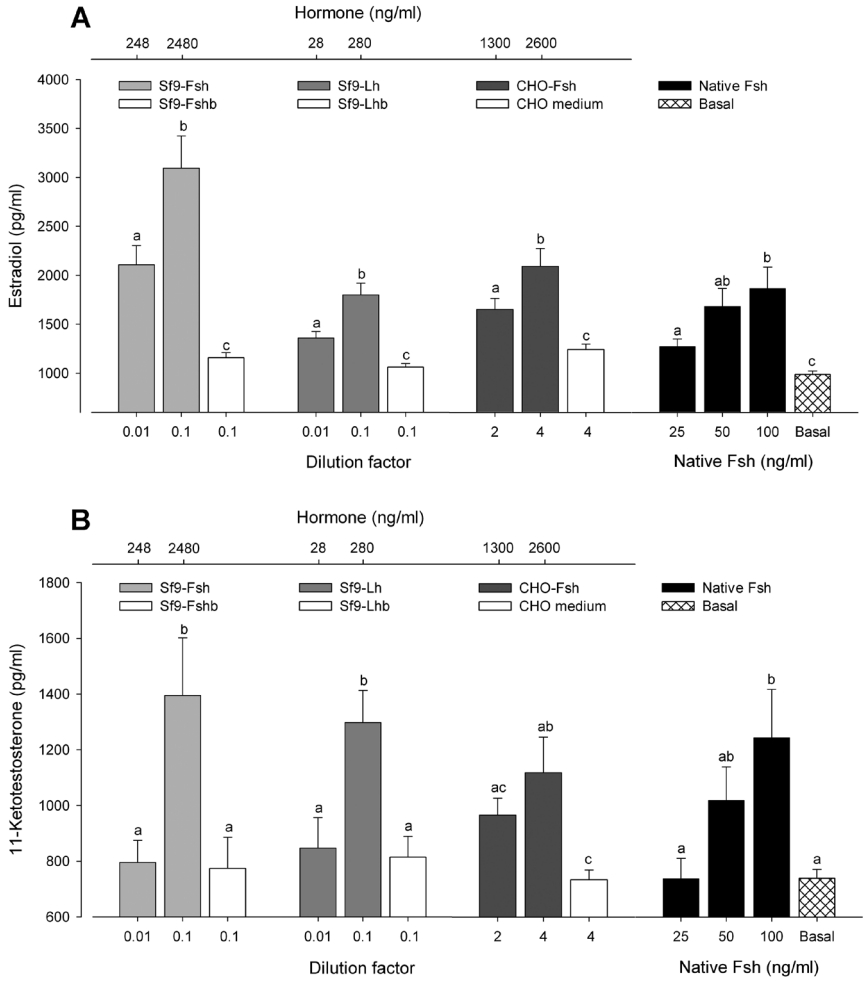

Figure 5. In vitro effects of recombinant GTH dimers on E2 (**A**) and 11-KT (**B**) production by sea bass ovary and testis, respectively. Tissue fragments were incubated with two doses of each recombinant GTH. In parallel, the effect of purified native Fsh in the same tissue is shown. The upper axis shows the concentrations used calculated by immunoassay (Lh ELISA and Fsh immunodot blot). Each bar represents mean ± SEM of four determinations. Each experiment was repeated three times. Different letters indicate significant differences between doses.

bass, the 11-KT secretion during late spermatogenesis (October to November) was evaluated. The analysis showed that 280 ng/ml Sf9-Lh was able to stimulate 11-KT production in testis, whereas a concentration of Sf9-Fsh 10 times higher (2.480 ng/ml) was needed to obtain the same effect (Fig.

5B). When comparing the steroid production between Sf9-Fsh and CHO-Fsh treatments, the results were consistent with those calculated for the EC_{50}, and equivalent immunologically detected concentrations in the range of 2500 ng/ml had different potency. The ability of single-chain proteins to induce E2 production and maturation in vitelogenic and postvitellogenic oocytes was also analyzed. The scFsh was effective to induce E2 production in a dose-dependent manner (Fig. 6A), and scLh was able to increase the percentage of maturing oocytes compared with CHO medium (Fig. 6B).

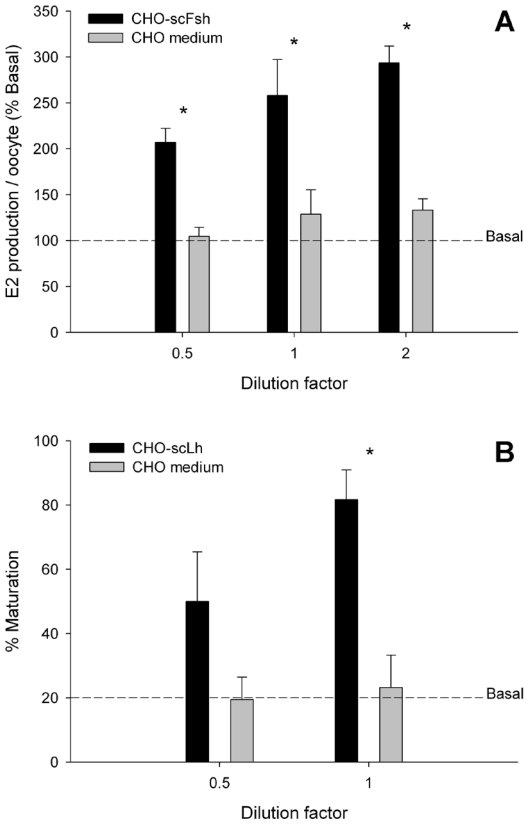

Figure 6. In vitro effects of recombinant single-chain Fsh and Lh on E2 production (**A**) and the percentage of maturation induction (**B**), respectively, by sea bass oocytes. Estrogen production is expressed by oocyte and percentage with respect to basal levels. Each bar represents mean ± SEM of 3 (**A**) and 30 (**B**) determinations, and each experiment was repeated three and two times respectively. Asterisks represent significant differences from the control (CHO medium).

3.4. *In vivo* half-life evaluation

Those GTH preparations showing the highest in vitro biopotency were used to evaluate their in vivo stability, allowing the comparison of recombinant hormones produced in two systems, Sf9 and CHO, which generate different glycosylation patterns. Single intramuscular injections with recombinant Fsh (Sf9-Fsh vs. CHO-scFsh) or Lh (Sf9-Lh vs. CHO-scLh) were performed, plasma was collected at designated intervals (0, 6, 12, 24, 48, and 72 h) after injection, and GTH levels were analyzed by bioassay (Fsh) or ELISA (Lh). Plasma concentration-time curves for Sf9-Lh and CHO-scLh are illustrated in Figure 7A. The analysis showed that Sf9-Lh is rapidly cleared from circulation, with a clearance rate of 3.16 ml/h. Six hours after Sf9-Lh injection, Lh levels were slightly greater (2.1-fold) than before the injection (t0), whereas Lh levels of CHO-scLh injected animals were approximately 11-fold higher than at t0 and were maintained significantly high until 48 h (Fig. 7A). Accordingly, the clearance rate of CHO-scLh was 40 times lower than that of Sf9-Lh (0.079 ml/h vs. 3.16 ml/h). Because very low levels of the Lh produced by the insect cells could be detected already at 6 h after injection, another injection of 1000 ng of Sf9-Lh was performed to further determine whether Sf9-Lh entered circulation or was degraded before. Blood samples were collected at 0, 0.5, 1, 3, and 6 h after injection. High levels of Lh were already bioavailable at 0.5 h, declining rapidly during the next 6 h (Fig. 7A, inset). The calculation of the clearance rate rendered a value of 6.5 ml/h, which is in the range but further rises the one calculated before.

Regarding Fsh, the injection of either recombinant hormone, Sf9-Fsh or CHO-scFsh, led to an increase in Fsh plasma levels at 6 h after injection and decreased gradually in the next hours. CHO-scFsh showed a more prolonged life in circulation than Sf9-Fsh, as high levels of CHO-scFsh could be detected until 72 h after injection, whereas Sf9-Fsh was cleared just in 24 h (Fig. 7, B and C). Both the mammalian- and the insect-derived Fshs were more stable than the corresponding recombinant Lhs (Fig. 7C), and could be detected longer in circulation.

4. Discussion

In the present study, bioactive recombinant sea bass Fsh and Lh proteins were successfully produced using two different expression systems, insect

(Sf9) and mammalian (CHO) cells.

In both systems, dimer and single-chain sea bass Fsh and Lh were efficiently expressed and secreted, although the production levels obtained in the baculovirus system were considerably higher than those obtained through stable transfection of CHO cells. The levels of recombinant Sf9-Lh produced were comparable to those reported by other authors in the production of fish and mammalian GTHs using this system [36–38]. Regarding the production levels of Sf9-Fsh, CHO-Fsh, and CHO-scFsh, the combined use of an immunodot blot and an in vitro bioassay specific for sea bass Fsh allowed the evaluation of the relative bioactivity of concrete amounts of recombinant protein produced. These assays permitted us to assess protein production levels and to compare the bioactivities of the different recombinant dimer Fsh forms. The data reported here clearly showed a higher production of Sf9-Fsh than that of CHO-Fsh. When we analyzed the potency and B:I ratio, Sf9-Fsh was more bioactive than CHO-Fsh but oddly less than CHO-scFsh. These different bioactivities of the recombinant GTHs could be due to a different degree and type of glycosylation according to the expression system used [39–41]. Insect cells produce glycoproteins containing high mannose-type oligosaccharides. In contrast, mammalian cells are able to express glycoproteins with complex sialylated oligosaccharides [42]. Functionally, GTH isoforms containing more sialylated oligosaccharides are less biopotent, as has been shown for rat FSH [41] and LH [40]. This information supports our findings that Sf9-Fsh is more bioactive than CHO-Fsh. In the particular case of CHO-scFsh, the higher B:I ratio might be influenced by other aspects (e.g., being a fusion protein instead of a dimer, which avoids subunit dissociation, or the presence of the CTP that has been shown to increase extracellular stability) [43, 44].

When β subunits were individually expressed in the baculovirus system, only Sf9-Lhb was readily detectable in the culture medium, whereas Sf9-Fshb was only found in the cell lysate. Similar situations have been reported for other recombinant GTH subunits. In a mammalian expression system, recombinant human FSHb and LHb subunits were inefficiently secreted and slowly degraded intracellularly, whereas CGa and hCGb were rapidly secreted [45–47]. In insect cells, recombinant bovine CGa was found in culture medium at 10-fold higher levels than FSHb [48], and recombinant catfish Cga was the only of the three subunits to be successfully produced in abundance as a single subunit [49]. The reason for this behavior is not

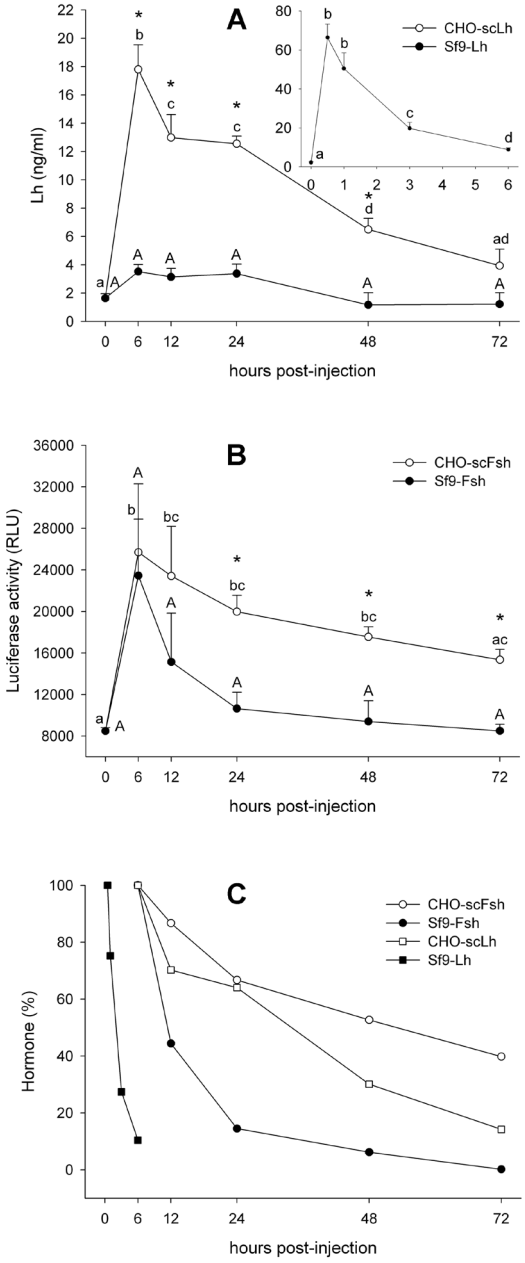

Figure 7. In vivo half-life of recombinant GTHs. Juvenile sea bass were injected with Sf9 and CHO recombinant GTHs. **A**) Plasma levels of Sf9-Lh and CHO-scLh at different times after injection. The inset graphic shows plasma levels of Sf9-Lh during the 0-6 h postinjection period. Lh was determined by specific ELISA. **B**) Plasma levels of Sf9-Fsh and CHO-scFsh at different times after injection. Fsh was determined by bioassay using sbsFshr. **C**) Clearance rate of injected hormones is presented as percentage of plasma levels at 6 h (100%). For Sf9-Lh, plasma levels at 0.5h were taken as 100%. Each point represents mean ± SEM of 5-10 fish. RLU, relative light units. Different letters indicate significant differences between hours. Asterisks represent significant differences between treatments.

totally clear, but it is known that proteins that fail to reach their native conformation in the endoplasmic reticulum, orphan subunits, and some heterologously expressed proteins can be selectively retained and later returned to the cytosol, where they are degraded [50, 51]. Nevertheless, coexpression of both GTH subunits leads to heterodimer assembly and efficient secretion [47–49].

The functionality of the recombinant Fshs and Lhs was initially evaluated through activation of their homologous receptors (Fshr and Lhr). All sea bass recombinant GTHs activated their cognate receptors in a dose-dependent manner, showing no cross-activity on a wide range of concentrations. Besides, neither the presence of the CTP included in the single chains nor the His-tag of the Fsh dimers influenced hormone receptor binding specificity. Selective ligand-receptor interactions, which are well established in mammals, have been also described for rainbow trout by using species-specific gonadotropins [22], and amago salmon receptors by using the ligands from a closely related species [21]. Opposite to the sea bass and rainbow trout situation, in most of the fish species analyzed, promiscuous Fshr-Lh interactions have been observed in addition to the cognate ligand-receptor pairs [38], although the structural basis or physiological significance of this promiscuity is still unclear. When human GTHs were used to activate the sea bass receptors, sbsLhr was activated by hCG and hFSH with nearly the same potency, whereas the sbsFshr scarcely responded to hFSH. This behavior of the human GTHs on the sea bass receptors is the same as that found for the bovine GTHs [20]. The same promiscuous behavior was observed in zebrafish, amago salmon, and African catfish Lhrs [18, 19, 52] when using mammalian GTHs. The promiscuous response of the sbsLhr to mammalian FSHs (hFSH and bFSH) but not to sea bass Fsh could have its basis in the higher similarity in sequence and structure of mammalian FSHs to sea bass Lh than to sea bass Fsh, as we previously proposed [20]. On the other hand, the very weak response of sbsFshr to hFSH, together with the fact that many residues described as important for hFSH/hFSHR binding [53] are lacking in sea bass [20], or that specificity determinants in fish β subunits are not related to charge differences as in mammals [54], would recommend caution when using mammalian models to explain fish Fsh-receptor interactions.

In this study, all recombinant sea bass GTHs were able to induce steroid production in cultured ovarian and testis fragments. Sf9-Fsh and Sf9-Lh had a similar effect in stimulating E2 production in the ovary, whereas

in testis, Sf9-Lh was more potent in stimulating 11-KT production. Other studies using recombinant fish GTHs also showed that both Fsh and Lh were able to stimulate in vitro steroid synthesis, albeit with different potencies. In red seabream, as in sea bass, Lh was also more effective than Fsh in stimulating 11-KT release [55]. In coho salmon, Lh was progressively more potent than Fsh in stimulating 11-KT production as spermatogenesis progressed through stage IV [56]. However, in zebrafish and channel catfish, recombinant Fsh was more effective in stimulating androgen release than Lh [49, 57]; and in African catfish testis, the amount of Fsh needed to obtain a significant steroid release was lower than that of Lh [58]. These different responses could be based on the developmental stage of the gonadal tissue, and therefore in the presence of the corresponding GTH receptors. In zebrafish it has been suggested that the higher levels of *fshr* expression could explain the higher potency of Fsh [57]. In our study, the cultured testes were in late spermatogenesis, and there is no difference in GTH receptor expression at that gonadal stage [59]. Although in teleosts both GTHs are steroidogenic, further research is needed to elucidate the differential role of each hormone in each spermatogenic stage.

Before undertaking any in vivo functional experiment, it is essential to gain knowledge on the in vivo pharmacokinetics of the recombinant GTHs. Other studies in fish had suggested that insect-produced GTHs could be rapidly cleared from circulation [13, 60], but this has not been demonstrated or quantified. In this report, CHO-scGTHs showed a higher stability in plasma than Sf9-GTHs, which could be based in their different glycosylation because the content in terminal sialic acid residues can be a determinant for the rate at which glycoproteins are cleared from circulation [40, 41, 61]. The fusion of the two subunits and the presence of *O*-linked oligosaccharides in the CTP may additionally contribute to prolonging the circulating half-life of the scGTHs [43, 44, 62]. The reason for the highest stability of Fsh compared with Lh in both systems is unknown. A relationship between the stability and the physiological role of the hormones could be hypothesized. Maintained high levels of Fsh in the bloodstream would be necessary for long-term stimulation of gonadal growth, whereas short-term increases in plasma Lh would have a more specific effect in a certain phase of the reproductive cycle and should be cleared more quickly.

In conclusion, although heterologous GTHs may bind to fish GTH receptors, their physiological effects could not reproduce exactly those of

homologous hormones. Thus, the production of homologous recombinant GTHs is a fundamental tool for studies on the physiology of GTHs and the development of biotechnological applications. In this study we have produced potent recombinant sea bass FSHs and LHs that mimic the natural hormones and can be used in future studies of this species. The baculovirus system is more efficient than mammalian expression systems in terms of large amounts of production, necessary to develop different detection assays (production of antibodies and standards). Because of its high potency, it is also attractive for in vitro structure-function studies of recombinant GTH analogs. However, the data obtained in vivo show a rapid clearance of the insect-produced proteins in contrast with those produced by mammalian cells. Thus, recombinant CHO-GTHs, in particular single-chain GTHs in combination with CTP, seem to be better candidates for in vivo experiments because of their increased half-life.

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Capítulo 6:

Determination of Fsh Quantity and Bioactivity During Sex Differentiation and Oogenesis in European Sea Bass

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Abstract

Follicle-stimulating hormone (FSH) is a glycoprotein hormone that plays a key role in the regulation of gonadal functions in vertebrates. The present study reports the monitoring of pituitary and plasma Fsh levels during sex differentiation and oogenesis in European sea bass (*Dicentrarchus labrax*) using a homologous immunoassay and an in vitro bioassay. Both assays were used complementarily for the first time in a fish species. High levels of Fsh bioactivity in plasma were found during the initial phases of sexual differentiation. Plasma and pituitary Fsh (quantity and bioactivity) levels and Biological to Immunological (B:I) ratios were higher in females than in males, suggesting sexual dimorphism in the synthesis and potency of Fsh. In females, the B:I ratios in adult were lower than during sex differentiation indicating that Fsh would be less biopotent in the adult stage. Plasma Fsh bioactivity levels increased during vitellogenesis pointing to that Fsh would be involved in the regulation of the mid phases of oogenesis, whereas luteinizing hormone (Lh) would be responsible for the final events.

Keywords: Gonadotropin, FSH receptor, biopotency, dot-blot immunoassay, hormone levels, Perciform fish

1. Introduction

The gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are heterodimeric glycoproteins synthesized and secreted by the pituitary gland. The GTHs are formed by the non-covalent association of a common alpha subunit (CGA) with distinct beta subunits (FSHB and LHB) that confer hormone specificity [1, 2]. Both GTHs are essential in the endocrine control of vertebrate reproduction, by regulating gonadal steroidogenesis and gametogenesis through the specific interactions with their respective receptor, LH receptor (LHR) and FSH receptor (FSHR). In most teleost orders, the functional duality between Fsh and Lh in critical moments of the reproductive life cycle, such as sex differentiation, puberty or gametogenesis has not yet been clarified adequately, despite the recognized importance of the two GTHs in reproduction. This is caused primarily by the lack of appropriate tools for measuring fish GTHs, especially assays for determination of Fsh levels.

Among teleosts, homologous immunoassays for Fsh have been developed only in three fish species with synchronous ovarian development, the chum salmon (*Oncorhynchus keta*) [3] coho salmon (*Oncorhynchus kisutch*) [4, 5] and rainbow trout (*Oncorhynchus mykiss*) [6, 7], and one with an asynchronous ovarian development, the tilapia (*Oreochromis niloticus*) [8]. In other fish species, such quantitative tools have been restricted to Lh, a fact that hampered studies on the functional duality of Fsh and Lh in fish. Results obtained so far indicate that in salmonids Fsh regulates the early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas Lh is responsible for the late phases, such as oocyte maturation, ovulation and spermiation [9]. Nevertheless, in tilapia, the only non-salmonid species studied so far, a concomitant increase of Fsh and Lh levels was observed during the vitellogenic phase, suggesting that in this perciform species Lh may play a role not only during oocyte maturation, but also at vitellogenesis [8].

A few studies have also focused on the role of GTHs during sex differentiation. The endocrine control of sex differentiation involves a communication between the brain, pituitary and gonad through the production of GTHs and steroids [10]. The GTHs have been shown to play a critical role in sex differentiation both in gonochoristic and hermaphroditic fishes [11-13], but the underlying mechanisms behind these observations remain largely

unknown. A variation in the chronological appearance of pituitary Fsh and Lh cells during sex differentiation has been reported in several teleost species [12, 14, 15, 16, 17]. Furthermore, sexually dimorphic expression of *fshb* was found in the hermaphrodite teleost honeycomb grouper (*Epinephelus merra*), suggesting that Fsh may trigger sex reversal in this species [11].

The European sea bass (*Dicentrarchus labrax*) is a perciform fish used in both applied and basic studies, due to its high commercial value and the large number of molecular and physiological tools available for the study of its reproductive process. This marine fish has a group-synchronous type of ovarian development [18], and the role of Fsh during sex differentiation and the reproductive cycle are still not well clarified. Studies performed in the last few years have allowed the elucidation of some physiological and biochemical aspects of Fsh actions in this species. For example, expression analysis of the genes coding for the GTH subunits [19] and the enzyme cytochrome P450 aromatase (Cyp19a1, previously known as P450aromA) [20] during sex differentiation suggested a role of Fsh during this period. Studies with European sea bass Fsh demonstrated a high specificity in the activation of its cognate receptor in a dose-dependent manner and its ability to stimulate the production of sex steroids by in vitro culture of gonads [21, 22]. However, while in females both GTHs were equally potent in stimulating 17β-estradiol (E2) secretion, in males Lh seemed to be more potent than Fsh in stimulating 11-ketotestosterone (11-KT) production. Analysis of seasonal expression of European sea bass GTH receptor genes (*fshr* and *lhr*) in sexually mature females revealed that the maximum *fshr* expression occurred before (late- and post-vitellogenesis) the maximum *lhr* expression (oocyte maturation and ovulation). A positive relationship was observed between ovarian levels of *fshr* mRNA, *cyp19a1* mRNA and those of plasma E2, and *lhr* mRNA and Lh plasma levels [23]. However, in order to clarify how Fsh modulates gonadal functions, knowledge about the kinetics of the levels of this hormone in the pituitary and plasma is imperative, but this information is still lacking for the European sea bass, as well as for most fish species.

Different types of assays have been developed for the measurement of FSH in various animals. These methods can be grouped in assays that determine a response of a biological system to stimulation with FSH (*e.g.* bioassays, both in vivo and in vitro) and assays that estimate high affinity binding to molecules which exhibit specific properties of molecular recognition (*e.g.* immunoassays). In contrast to the former, these latter assays are intended to

measure the number of molecules or their mass. However, bioassays are an ideal means to determine the functional aspects of GTHs. Since the pituitary glicoproteins are highly heterogeneous in terms of carbohydrate composition, the bioactivity of the different isoforms present in the pituitary and blood may vary greatly and does not always match their immunoreactivity [24, 25]. Since the early 1990s a new generation of bioassays based on cell lines expressing the FSHR gene has emerged as a tool to measure serum FSH in mammalian species [26-30].

Based on the above information, the objective of the present study was to develop methods to measure both bioactivity and quantity of Fsh in this important European aquaculture and fish reproduction research species, and to generate new information on its role in the process of sex differentiation and control of the reproductive cycle of European sea bass.

2. Materials and methods

2.1. Experimental animals and sample collection

Two sibling populations of European sea bass, one of very small fish (predominantly males) and another of very large fish (predominantly females) were generated by four sequential size gradings during the period between 66 and 223 days post hatching (dph) [31]. At each grading the fish were split approximately in a 50:50 ratio into large and small populations. After the first grading, the large fish from the large population and the small fish from the small population were kept. Since the eventual very small and very large populations consisted of 70% males and 96% females, respectively, these populations in the present study were referred to as males and females. Samples were collected from both populations every 50 days from 150 to 300 dph and 6 pituitaries and 10 plasma samples per sampling point were analyzed. The body weight and total length (mean±SD) at 150 dph were 6.6±0.24 g and 85.1±0.96 mm in males; 9.3±0.24 g and 96.3±0.76 mm in females. At 300 dph males weighted 66.7± 0.93 g and were 180.3±0.72 mm while females had 130.9±1.75 g and a total length of 218.8±0.75 mm.

Sexually mature females maintained in Torre la Sal, Spain (40°NL), were sampled monthly during their first reproductive period, which generally occurs during the third year of life. For each pituitary and blood collection, 5 fish were anesthetized and sacrificed in accordance with the Spanish

legislation concerning the protection of animals used for experimentation or other scientific purposes. Blood was collected by caudal puncture with heparinized syringes and centrifuged (3000*g* for 30 min at 4°C). The upper plasma layer was separated and stored at -20°C until use. Pituitary glands were collected, immediately frozen in liquid nitrogen and stored at -80°C. For analysis, individual pituitaries were homogenized mechanically in TBS-T (10 mM Tris-base, 150 mM NaCl and 0.05% Tween-20) using sterile syringes. The extract was centrifuged (3000*g* for 15 min at 4°C) to eliminate debris and stored at -80°C until assayed. The stages of ovarian development were classified by light microscopy as in Rocha et al. [23], following previously established criteria [32]: previtellogenesis (prevtg), early vitellogenesis (evtg), late-vitellogenesis and post-vitellogenesis (lvtg-pvtg), maturation and ovulation (mat-ovul), and atresia (atre).

2.2. Biological activity determinations for European sea bass Fsh

In order to determine bioactive Fsh an in vitro bioassay was developed. The biological activity of Fsh in pituitary and plasma samples was analyzed using a human embryonic kidney (HEK) 293 cell line stably expressing the European sea bass Fsh receptor (sbsFshr) and the firefly luciferase reporter gene under the control of a promoter with cAMP Responsive Elements (CRE) binding sites (pCRE-LUC) [33]. The HEK 293 cells were grown at 37°C in DMEM medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Invitrogen) in a humidified incubator supplied with 5% CO_2. Receptor activation by FSH was indirectly measured by recording changes in luciferase activity, promoted by a rise of cAMP. Cells from the sbsFshr/pCRE-LUC HEK293 stable clone were seeded in 96 well cell culture plates (Corning) at a density of 4.5 x 10^5 cells $well^{-1}$ in 300 µl of growth medium. After 16-20 h, medium was removed and cells were incubated with 100 µl of FSH standards or sample preparations per well (1/25 plasma; 1/2000-12000 pituitary extract) in OPTIMEM or Advanced-DMEM media (Invitrogen) supplemented with 1% glutamine, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. After 6 h incubation, luciferase activity was directly quantified on the plates using the Steady-Glo Luciferase Assay System (Promega) and the ULTRA Evolution (TECAN) detection platform. All samples were measured in triplicate and the light emitted expressed as relative lights units (RLU). Known quantities

of recombinant European sea bass Fsh dimer produced in Chinese Hamster Ovary cells (CHO-scFsh) or insect cells (Sf9-Fsh) [22] were used as standard curve. After logarithmic transformation, the standard curve was linearized and a concentration could be extrapolated for each sample analyzed.

2.3. Plasma Lh and Fsh extraction by lectin

Extraction of plasma Fsh and Lh was performed by affinity chromatography with Concanavalin A (Con A) Sepharose 4B (GE Healthcare). Briefly, a column with 1 ml of Con A Sheparose was equilibrated with 20 mM Tris buffer containing 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4, and loaded with a pool of plasma samples (6.84 mg protein). The flow-through plasma, consisting of plasma free of Lh and Fsh, was stored at -20°C until use. Con A-bound GTHs were eluted with 10 mM methyl α-D-glucopyranoside and 300 mM methyl α-D-mannopyranoside.

2.4. Production and purification of recombinant European sea bass Fshb

To generate specific polyclonal antibodies, recombinant Fshb was produced using the Bac-to-Bac Baculovirus Expression System as described previously by Molés et al. [22]. Briefly, a cDNA fragment containing the open reading frame of European sea bass *fshb* and a C-terminal 6xHis tag were obtained by PCR using specific primers. The cDNA was directionally cloned into donor plasmid pFastBac1 and used to generate recombinant baculovirus by transposition. The baculovirus-Fshb was used to infect insect cells derived from the fall armyworm *Spodoptera frugiperda* (Sf9). The Sf9 cells were grown at 28ºC in Sf-900 II SFM medium (Invitrogen), containing 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen). Monolayer cultures of Sf9 cells were grown to 80-85% of confluence (roughly $12x10^6$ cells) and later infected at a multiplicity of infection (MOI) of 3 pfu ml^{-1} during 4-5 days. Cells and medium were then harvested by centrifugation (110*g*, 10 min) and stored at -80ºC until used. The infected cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 1% Nonidet P-40, 1 mM PMSF and 1 μg ml^{-1} Aprotinin) and their cell membrane disrupted by brief periods of sonication on ice using a cell disrupter (Vibracell; Sonics & Materials, Inc.). Supernatants containing soluble proteins were recovered after removal of cell debris by centrifugation at 35000*g* for 30 min at 4ºC.

Recombinant Fshb from supernatants of Sf9 cell lysates was purified by immobilized metal affinity chromatography (IMAC Ni^{2+}) using His GraviTrap prepacked columns (GE Healthcare) according to manufacturer instructions. Briefly, the pH of the supernatant was adjusted to 7-8 with diluted acetic acid and the prepacked columns were equilibrated with PBS (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 20 mM imidazole. After loading the supernatants, three washes with PBS (20 mM, 70 mM and 100 mM Imidazole) were performed. Finally the bound Fshb was eluted with PBS containing 500 mM Imidazole. The purity of fractions obtained was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blot.

A second purification step was performed consisting of a protein extraction from the SDS-PAGE gel by passive elution. Proteins obtained by the IMAC purification were separated by a SDS-PAGE (11%) under reducing conditions (5% 2-mercaptoethanol). The gel was stained with a negative and reversible protein stain for polyacrylamide gels (E-Zinc Stain; PIERCE) and the band corresponding to Fshb was excised. Individual slices were placed in microcentrifuge tubes and protein was extracted from the gel by passive elution in 0.1-0.2 ml $band^{-1}$ of buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5) following a method adapted from the PIERCE technical resource (TR0051.0). The tubes were incubated overnight at 4°C followed by 2h incubation at 25°C in rotary shaker (300 rpm). Finally, a centrifugation at 10,000*g* for 10 minutes at 4°C was performed and the supernatants stored at -80°C. Protein purity was confirmed by SDS-PAGE (15%) and staining with Coomassie brilliant blue.

2.5. Western blot analysis

The proteins separated by SDS-PAGE were transferred to PVDF membranes (Immobilon P, Millipore). The membranes were blocked overnight with 5% skimmed milk at 4°C, incubated for 90 min at room temperature with anti-6xHis (1:6000) (mAb/HRP Conjugate; Clontech) or anti-mummichog Fshb (anti-mFshb) (1:3500) already validated for European sea bass Fshb [21], washed and incubated with 1:2000 goat anti-rabbit IgG horseradish peroxidase conjugate (GAR-HRP, Bio-Rad), for 60 min at room temperature. The immunodetection was performed by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc.) using the

Versadoc image system (Bio-Rad).

2.6. European sea bass Fshb antibody production

Polyclonal antibodies against the purified recombinant Fshb were produced by a commercial company (Agrisera, Sweden). Two rabbits were immunized with 60 μg of Fshb in Freund's complete adjuvant by subcutaneous injection. Four subsequent immunizations were carried out with 25 μg of antigen in Freund's incomplete adjuvant at 3-week intervals. Rabbits were bled 2 weeks before immunizations (pre-immune serum) and 2 weeks after immunizations III, IV and V, in order to perform the corresponding titration test. Final bleeding was done at 2 weeks after the fifth immunization.

2.7. Immunological determination of European sea bass Fsh

A dot-blot immunoassay was developed for immunological determination of Fsh. The sample preparations (1/50-250 pituitary extracts) were previously denatured (5% 2-mercaptoethanol, 4 min 95ºC) and immobilized on a PVDF membrane (Immobilon P, Millipore) using a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was blocked overnight with 5% skimmed milk in TBS-T at 4ºC, incubated with the anti-European sea bass Fshb produced (anti-sbsFshb) (1/2000) for 90 min at room temperature, washed and incubated with goat anti-rabbit IgG (1/1000) horseradish peroxidase conjugate (GAR-HRP; Bio-Rad) for 60 min at room temperature. The immunodetection was performed by chemiluminescence (Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Inc.) using the Versadoc image system (Bio-Rad). All samples were measured in duplicate. Immunoreactivity signal (Intensity $(mm^2)^{-1}$) was calculated for each dot and compared to those obtained from serial dilutions of standard using the image analysis software Quantity One v 4.4 (Bio-Rad). Known quantities of purified native European sea bass Fsh [21] were used as standard curve. After logarithmic transformation, the standard curve was linearized and a concentration could be extrapolated for each sample analyzed.

2.8. Pituitary European sea bass Lh measurements

Levels of Lh in pituitary were measured by homologous competitive en-

zyme-linked immunosorbent assay (ELISA) according to Mateos et al. [34]. The sensitivity of the assay was 0.65 ng ml^{-1} (Bi/Bo 80%) and intra- and inter-assay coefficients of variation were 11.7% and 11%, respectively.

2.9. Immunohistochemistry

In order to locate the gonadotrope cells in the pituitary an immunohistochemistry was performed. Two-year old sexually mature male and three year old mature female European sea bass were anesthetized in 0.1% (2)-phenoxyethanol (Merck) and then transcardially perfused with a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4). The brains were carefully extracted with the pituitary attached and postfixed overnight at 4ºC, dehydrated and embedded in paraffin. Before immunostaining, 6 µm transversal sections were deparaffinized and washed in TBS-T (0.1% Triton X-100) for 10 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 in TBS-T for 30 min. For epitope unmasking, samples were warmed up at 90-95ºC in 0.01M Sodium Citrate buffer (pH 6) for 10 min and left to temper at room temperature for 15 min. Sections were saturated with 3% goat serum (Sigma-Aldrich) in TBS-T for 45 min in order to reduce non-specific reactions. They were then incubated with rabbit anti-sbsFshb (1/500) or anti-European sea bass Lhb (anti-sbsLhb) (1/1000) [34] overnight at room temperature. Sections were rinsed three times with TBS-T for 10 min and were incubated with GAR-HRP (1/200) for 100 min. Finally, sections were rinsed with TBS-T (2x10 min) and 0.05 M Tris-HCl (pH 7.6) (1x10 min) and peroxidase activity was visualized by treatment with 0.025% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.01% (v/v) H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.6) for 5-15 min. The sections were then rinsed, counterstained with hematoxylin (25%), dehydrated in ethanol, cleared in xylene, and cover slipped with DPX mounting medium (Panreac).
The immunostained sections were observed under a light microscope (Nikon Elipse) and were photographed digitally. The specificity of the immunoreaction was confirmed by incubating the sections with pre-immune rabbit serum instead of the specific anti-sbsFshb (data not shown).

2.10. Data representation and statistical analysis

Due to the different concentrations obtained according to the assay used,

the Fsh levels have been expressed as relative values for a better understanding and comparison of the results. The 200 dph males exhibiting the first histological signs of gonadal differentiation [31] were chosen as the reference group to study the Fsh profile during the sex differentiation period. To study the Fsh profile of females during oogenesis the samples were grouped according to the ovarian stage of development (see section 2.1) and early vitellogenic females were chosen as the reference group. The Fsh levels of these reference groups were set as 1. Statistical significance of the differences between group means of hormone levels or receptor activation (luciferase activity) was determined by one-way Analysis of Variance (ANOVA) followed by Holm-Sidak method using SigmaStat 3.5 (Systat Software Inc.). When the test of equal variance failed, ANOVA on Ranks (Kruskal-Wallis) was performed followed by all pair-wise multiple comparison procedures (Dunn´s method). The correlation coefficents (R) between the two assays were calculated using the Pearson product moment test. Data are presented as mean ± SEM.

After conversion of the results to relative levels, the pituitary Fsh biological to immunological (B:I) ratio was calculated by dividing the pituitary values obtained from the bioassay by those from the immunoassay.

3. Results

3.1. Development and validation of the European sea bass Fsh bioassay

Previous studies have shown that the sbsFshr/pCRE-LUC HEK293 stable clone expressed the luciferase gene in a dose dependent manner when stimulated with European sea bass Fsh [21, 22]. Since stimulation of luciferase activity declines with time, the luminescent signal was monitored over time after treatment with Fsh. Incubation of sbsFshr/pCRE-LUC HEK293 cells with a single dose of recombinant CHO-scFsh for 3, 6, 9, 15, 20 or 24 h showed maximum stimulation between 6 and 9 h (data not shown). A 6 h incubation time was used for all further studies.

The Fsh specificity of the sbsFshr cell line was examined previously by the addition of recombinant European sea bass Lh and other heterologous gonadotropins [22]. Dilutions of recombinant Fshs (CHO-scFsh and Sf9-Fsh), pituitary extracts and plasma samples were tested in the luciferase assay system. All of them stimulated the sbsFshr in a dose-dependent manner,

similar to native Fsh standard curve (Fig. 1A). To detect potential nonspecific plasma effects in the rise of intracellular cAMP, receptor activation with Con A-treated plasma was analyzed and no activation was detected (Fig. 1B).

The sensitivity of the bioassay, defined as the lowest concentration of Fsh able to stimulate a luciferase activity higher than the mean plus 2 times the standard deviation of the zero concentration of Fsh (basal), was 0.104 ng

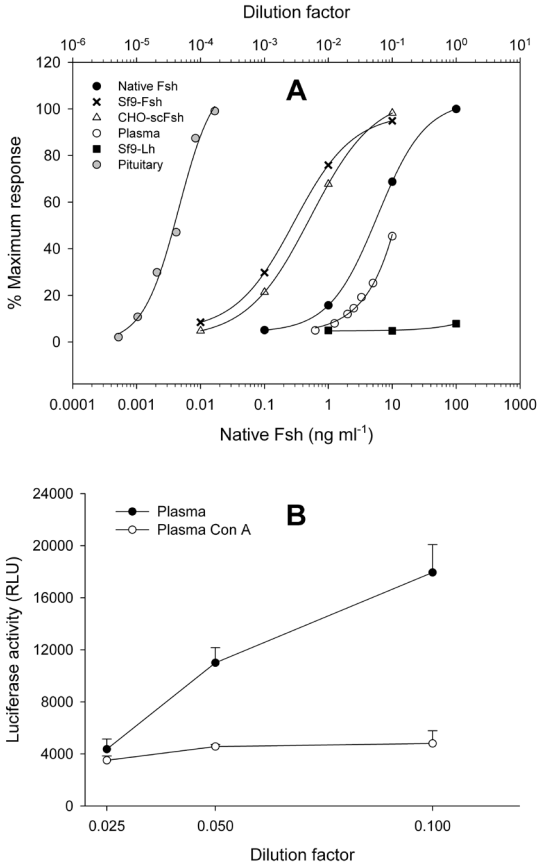

Figure 1. Development of European sea bass Fsh bioassay. A) Dose-response curves. SbsFshr/pCRE-LUC HEK293 cells were incubated with standard curve of purified native Fsh and serial dilutions of plasma samples, pituitary extract and recombinant European sea bass Fshs (Sf9-Fsh and CHO-scFsh) and Lh (Sf9-Lh). Data points represent the mean of three independent determinations. B) Effect of GTHs-free plasma in luciferase stimulation. SbsFshr/pCRE-LUC HEK293 cells were incubated with GTHs plasma extracted and non-extracted with lectin Con A. Samples were measured in triplicate (Mean± SEM) and the light emitted expressed as relative lights units (RLU).

ml^{-1}. The intra-assay coefficient of variation (CV), calculated by measuring replicates of the same sample (n=14) within the assay, was estimated at 9.3%. The inter-assay CV, calculated by measuring replicates of same sample in different assays (n=10), was 10.9%.

3.2. Development and validation of European sea bass Fsh dot-blot immunoassay

In order to generate antibodies against European sea bass Fshb and develop a specific immunoassay, recombinant Fshb was produced using the baculovirus expression system. In this system, recombinant Fshb remained inside the infected cells instead of being secreted [22]. Therefore, recombinant Fshb was purified from cell lysates by affinity chromatography (IMAC Ni^{2+}). Analysis by SDS-PAGE and western blot with anti-6xHis (data not shown) and anti-mFshb confirmed the identity and abundance of Fshb in the obtained fractions (Fig. 2A and 2B). However, the Coomassie blue stain showed the presence of high molecular weight proteins in addition to Fshb (Fig. 2A) and, thus, another step of purification was necessary to obtain a satisfactory degree of purity. Due to its excellent ability to resolve individual components of protein mixtures, SDS-PAGE was used as an active step in the purification process. Finally, after protein extraction by passive elution, a high degree of antigen purity was achieved (Fig. 2C).

Polyclonal antibodies against European sea bass Fshb were obtained by rabbit immunizations using this purified protein. The antibodies reacted

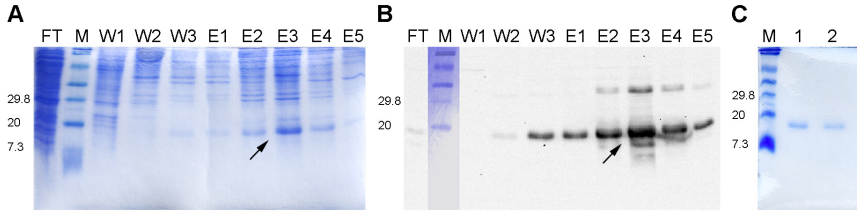

Figure 2. SDS-PAGE and western blot analysis of purified recombinant Fshb. A) Proteins stained with Coomassie blue and B) western blot with anti-mFshb, after affinity chromatography (IMAC Ni^{2+}). FT: Flow-through (10 µl $lane^{-1}$); W1-W3: wash with PBS containing 20 mM, 70 mM and 100 mM Imidazole, respectively (15 µl $lane^{-1}$); E1-E4: Elution of Fshb (arrows) with PBS containing 500 mM Imidazole (15 µl $lane^{-1}$). C) Two fractions of Fshb (≤0.5 µg $lane^{-1}$) stained with Comassie blue after protein extraction from SDS-PAGE by passive elution. M: Bio-Rad prestained Broad Range molecular weight marker.

strongly and specifically with Fshb under reducing conditions, however, a very weak reaction was obtained under non-reducing conditions (Fig. 3A and 3B). Therefore, a dot-blot immunoassay was developed for Fsh determination in denatured samples. Validation of the assay was performed only for pituitary samples because the plasma Fsh concentrations were below or near the limit of detection. The sensitivity of the immunoassay, defined as the lowest dose of Fsh able to increase a dot density higher than the mean plus 2 times the standard deviation of zero dose of Fsh (basal), was of 162.8 ng ml^{-1}. Dose-response curves of pituitary extracts and recombinant Fsh (Sf9-Fsh) were similar to the standard curve of native purified Fsh. No cross-reactivity was detected with serial dilutions of recombinant Lh (Fig. 4). The intra-assay CV, calculated by measuring replicates of the same sample within the assay (n=10), was estimated at 9.8%. The inter-assay CV, calculated by measuring replicates of same sample in different assays (n=8), was 11.5%.

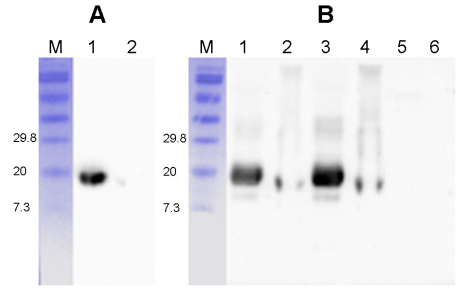

Figure 3. Analysis of polyclonal antibodies for European sea bass Fshb by Western blot. A) Test of the antiserum after the third immunization (1/4000). Lanes 1-2: 300 ng of purified native Fsh under reduction and native conditions, respectively. B) Test of the antiserum after final bleeding (1/2000). Lanes 1 to 4: 760 ng (1-2) and 1520 ng (3-4) of pituitary extract under reducing (1, 3) and non-reducing (2, 4) conditions. Lanes 5-6: 1000 and 2000 ng of European sea bass Lh under reducing conditions. M: Bio-Rad prestained Broad Range molecular weight marker.

3.3. Determination of Fsh levels during sex differentiation and the reproductive cycle

The assays developed in this study were used to analyse the profiles of Fsh in pituitary extracts and plasma samples from male and female sibling populations during sex differentiation, specifically from 150 dph to 300 dph. The quantity and bioactivity profiles of pituitary Fsh were very similar,

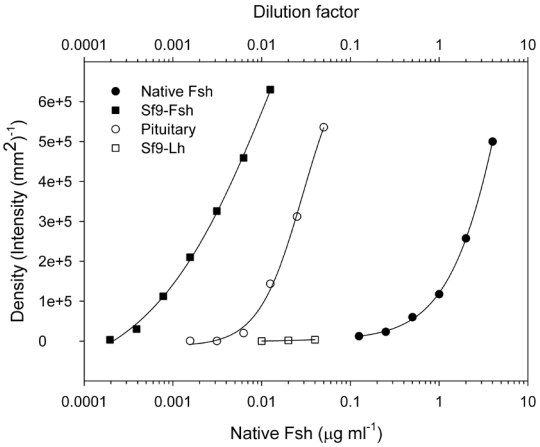

Figure 4. Dose-response curves for the European sea bass Fsh immunoassay. Standard curve of purified native Fsh, serial dilutions of pituitary extract and recombinant European sea bass GTHs produced in Sf9 insect cells (Sf9-Fsh and Sf9-Lh) were immobilized on a membrane and incubated with the anti-sbsFshb antibody. The 1/25 (0.04) dilution of Sf9-Lh corresponds with a concentration of 5.6 µg ml^{-1}. Data points represent the mean of two independent determinations.

showing high coefficient of correlation both in males and females (Fig. 5A), nevertheless the absolute concentrations calculated by immunoassay were always higher than by bioassay. Relative values, respect to males at 200 dph, were used to plot the profiles of pituitary and plasma Fsh along this period. At 200 dph, the mean content of pituitary Fsh in males, measured by immunoassay, was 6.04 µg whereas the bioactivity was equal to 0.87 µg of standard. At this sampling point, the plasma Fsh bioactivity was equal to 216 ng ml^{-1} of standard. Relative levels showed that the bioactivity increased much more than the quantity respect to reference group, *e.g.* while the bioactivity increased ~16-fold, the amount of Fsh increased only ~ 6-fold in females at 300 dph (Fig. 5B). At 150 dph, pituitary Fsh levels were very low in both sexes and increased significantly beginning 200 - 250 dph (Fig. 5B). During the period studied, the Fsh levels tended to be higher in females than in males, regardless of the assay used. Moreover, the B:I ratio was higher in females than males (Table 1). The profile of plasma Fsh bioactivity was completely opposite to those of pituitary and in both sexes Fsh levels were higher at 150 dph than at 300 dph (Fig. 5C). As observed in the pituitary, there was a tendency for plasma Fsh levels to be higher in females than males.

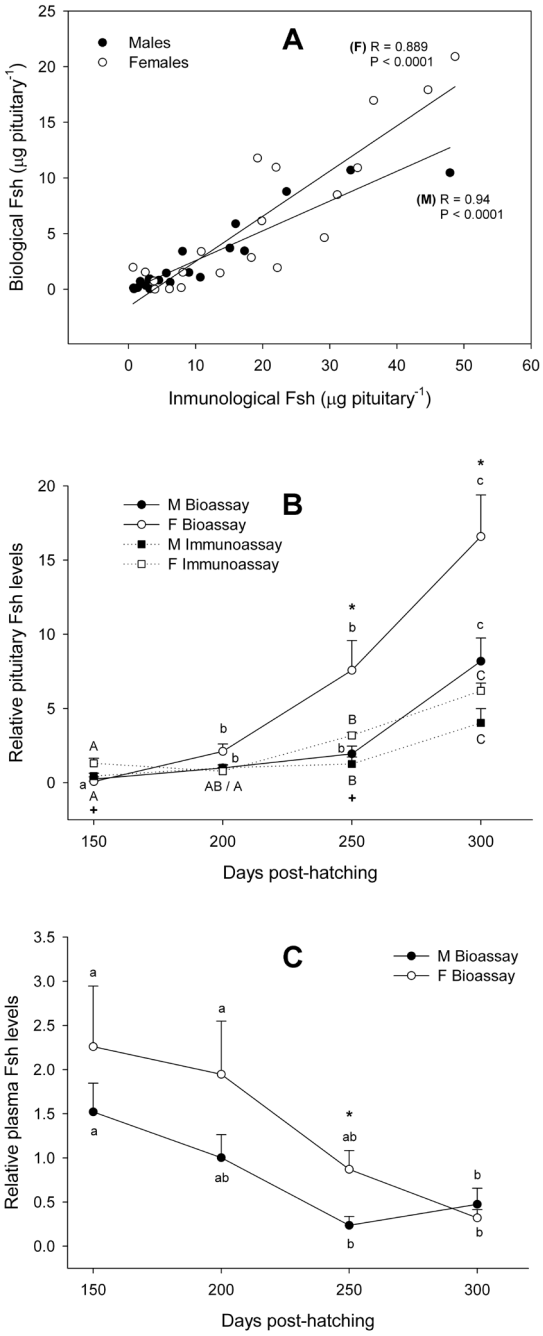

Figure 5. Profiles of Fsh in European sea bass males (M) and females (F) during sex differentiation. A) Correlation between pituitary Fsh values as determined by bio- and immunoassay. B) Pituitary Fsh quantity and bioactivity levels. C) Plasma Fsh bioactivity levels. Data (mean ± SEM of 6-10 fish) are expressed as a proportion of the mean FSH values in males at 200 dph that was set as 1(*Pituitary*: Bioassay = 0,87 µg /pituitary, Immunoassay = 6.04 µg/pituitary; *Plasma*: Bioassay = 216 ng ml^{-1}). Different letters indicate significant differences over time for female or male populations (capital letters for immunoassay and lowercase letters for bioassay). Asterisks (bioassay) and plus (immunoassay) indicate significant differences between the two populations at equivalent sampling points. Note: Statistics at 200 dph in immunoassay is AB in males and A in females.

Similar to the study during sex differentiation, a high coefficient of correlation was obtained in the Fsh determinations of the quantity and bioactivity during the reproductive cycle of adult females (Fig. 6A). As above, the absolute concentrations calculated by the immunoassay were always higher than bioassay, and relative levels respect to early vitellogenic females were used to plot the Fsh profiles. In this stage (evtg), the mean content of pituitary Fsh, measured by immunoassay, was 71.01 µg whereas the bioactivity was equal to 12.14 µg of standard. The plasma Fsh bioactivity was equal to 4.03 ng ml^{-1}

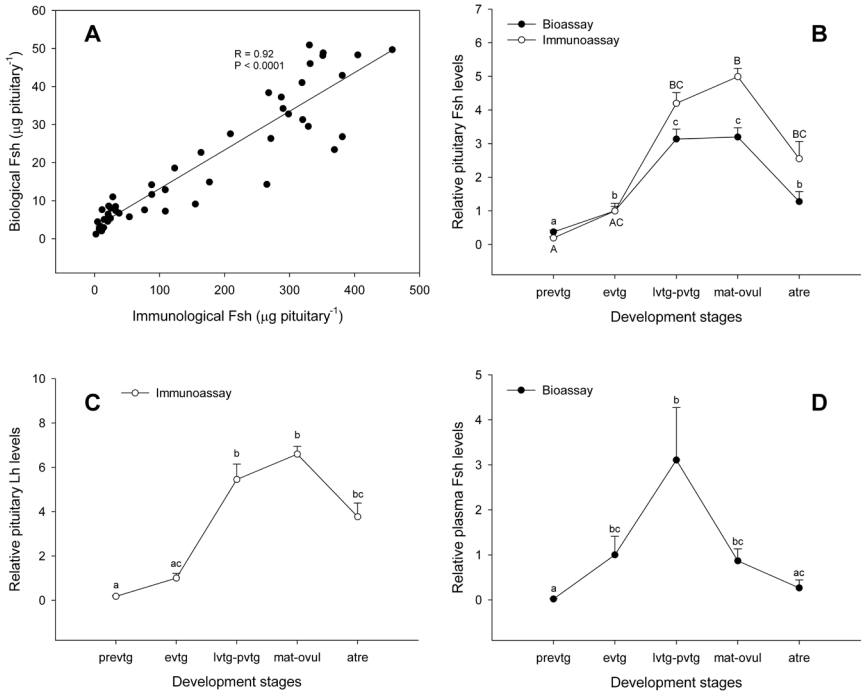

Figure 6. Annual profile of Fsh in adult female Europea sea bass during the first reproductive period. A) Correlation of pituitary Fsh values as determined by bio- and immunoassay. B) Pituitary Fsh quantity and bioactivity levels. C) Pituitary Lh quantity levels. D) Plasma Fsh bioactivity levels. Values were classified according to the stage of gonadal development as determined by histology. Previtellogenesis (prevtg), early vitellogenesis (evtg), late- and post-vitellogenesis (lvtg-pvtg), maturation-ovulation (mat-ovul), and atresia (atre). Data (mean ± SEM of 7-14 fish) are expressed as a proportion of the mean Fsh value of females in the early vitellogenesis stage that was set as 1 (*Pituitary*: Bioassay= 12.14 µg/pituitary, Immunoassay= 71.01 µg/pituitary; *Plasma*: Bioassay=4.03 ng ml^{-1}). Different letters indicate significant differences between developmental stages (capital letters for immunoassay and lowercase letters for bioassay).

Table 1. Biological to Immunological (B:I) ratio in pituitary samples.

	Sex differentiation (dph)				Reproductive cycle (stage)				
	150	**200**	**250**	**300**	**prevtg**	**evtg**	**lvtg-pvtg**	**mat-ovul**	**atre**
Males	0.54	1	1.55	2.03					
Females	0.06	2.74	2.38	2.68	1.93	1	0.75	0.64	0.50

of standard. The quantity and bioactivity relative levels of pituitary Fsh in the pituitary increased during early vitellogenesis, peaked in late- and post-vitellogenesis until maturation-ovulation, and finally in the atresia stage the levels tended to decline (Fig 6B). When the Fsh B:I ratio was calculated, this decreased throughout the cycle (Table 1). Concurrently, pituitary Lh levels had an identical profile to that of Fsh (Fig. 6C). In the plasma, the trend was that Fsh bioactivity levels increased during early vitellogenesis, peaked in late-vitellogenesis and post-vitellogenesis and decreased in maturation-ovulation stage (Fig 6D).

3.4. Immunohistochemistry

The antibodies against European sea bass Fshb or Lhb were used to differentially stain the pituitary gonadotropin producing cells of sexually mature male. Two different GTH cell populations expressing Fsh and Lh were observed (Fig. 7). The Fsh cells were distributed throughout the whole *proximal pars distalis* (PPD), isolated or arranged in small groups (Fig. 7A). Conversely, Lh formed strands or compact groups (Fig. 7B) distributed mostly in the ventral part of the PPD and surrounding the *pars intermedia* (PI) and were more numerous than Fsh cells. The same results were observed in a pituitary of a sexually mature female (data not shown).

4. Discussion

The present study reports, for the first time in a fish with group-synchronous ovarian development, the monitoring of the Fsh levels during critical moments of the reproductive life cycle using a newly developed bioassay and immunoassay. The immunological determinations of Fsh, based on antigen-antibody reaction, do not necessarily reflect the biological signal

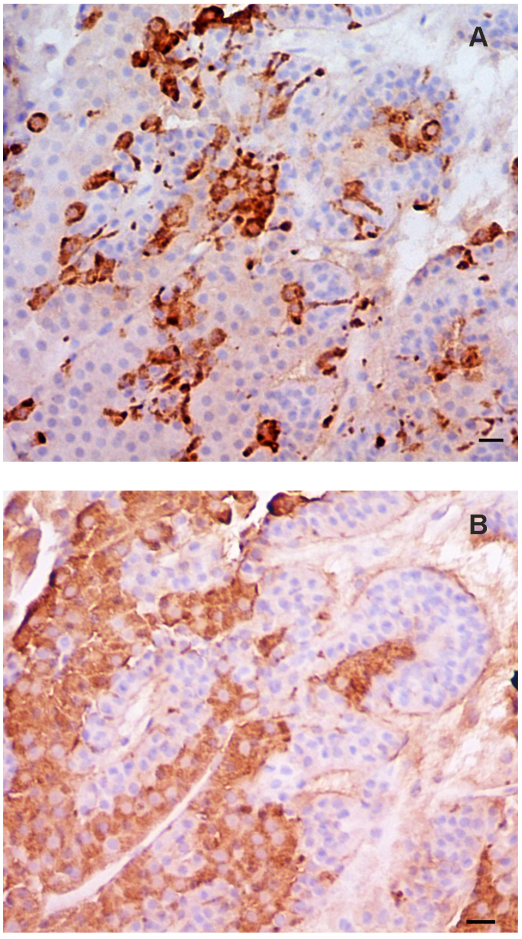

Figure 7. Two adjacent sections of a male European sea bass pituitary (*proximal pars distalis*) immunostained with anti-sbsFshb (A) and anti-sbsLhb (B). Bar = 10 μm.

perceived by its cognate receptor [24]. Consequently, an in vitro bioassay for Fsh constitutes an ideal approach to determine functional aspects of this GTH. The bioassay developed here, using co-transfection of *sbsFshr* cDNA and a luciferase reporter gene in HEK 293 cells, exhibited dose-dependent receptor activation and was able to measure the bioactivity of European sea bass Fsh with a high degree of sensitivity and specificity. In fish, receptor transactivation systems have been used transiently transfected or as stable lines to investigate functional specificity of the receptor/hormone interactions using recombinant or native fish GTHs [9]. However, our homologous Fsh bioassay is the first one developed and validated in fish to measure Fsh bioactivity in pituitary and plasma samples. The bioassay showed optimal

characteristics of precision, specificity and sensitivity, in concordance with those reported for other FSH bioassays in mammals [35].

Traditionally, the immunoassays developed to determine GTH levels in fish have been radioimmunoassays (RIA) or ELISA based on native GTHs subunits purified from fish pituitaries and their specific antibodies. Native Fsh purification is a highly demanding process as regards time, cost and substantial amount of pituitary glands required that often limits the production of specific antibodies [9]. In the present study, recombinant Fshb was produced using a baculovirus expression system. The production yield was high, but Fshb was undetectable in the culture medium, since most of the recombinant subunit was found in the cell lysate. The recombinant Fshb was isolated after two-step purification from cell lysates and used to produce polyclonal antibodies, which exhibited a strong reaction under reducing conditions, but a very weak reaction under native conditions. There is no clear explanation for this observation, but we could hypothesize that denaturation of the antigen during the purification process led to a different conformation from the native subunit that could have been crucial in the generation of antibodies that recognize mainly internal epitopes of the subunit. This explanation could account for the observed differences in reactivity under reducing versus native conditions. Despite this, a specific dot-blot immunoassay able to measure European sea bass Fsh in pituitary was developed. This assay did not show cross-reactivity with European sea bass Lh and showed a low intra- and inter-assays CVs, comparable to those reported for other fish Fsh immunoassays [3, 6, 8].

Studies in mammals have shown a relative abundance of intra-pituitary and circulating isoforms of GTHs that differ from each other not only in their carbohydrate composition, but also in their ability to remain in the blood stream and their biopotency, provoking a range of biological responses in vitro and in vivo [25]. Also, it is known that in experimental animals as well as in humans a close relationship exists between the presence of a particular isoform pattern in the pituitary and in circulation, and the functional state of the gonad [25, 27, 36]. Based on the assumption that immunoreactivity is a measure of the quantity of Fsh and that bioactivity provides information relative to the ability to elicit a biological response, the B:I ratio should provide an assessment of the average quality of individual Fsh molecules in a biological sample at a given time. Therefore, the two methods are different but complementary, since their combination allows the evaluation

of the relative bioactivity of defined amounts of Fsh according to the biological stage of the animal.

The pituitary Fsh profiles of European male and female sea bass during sex differentiation and during the female reproductive cycle obtained with the immunoassay were similar to those obtained with the bioassay. However, the calculated concentrations (absolute values) were always higher with the immunoassay (Fig. 5A and 6A). One possible explanation could be related with the biopotency of the Fsh variant used as standard, since, in the bioassay, a more potent standard than the samples would result in an extrapolation of lower levels of concentration, moreover must be taken into account that the immunoassay recognizes Fshb and it does not distinguish between dimers (alpha-beta) or monomers (beta), whereas in the bioassay only the dimers (alpha-beta) are bioactive and, therefore, measured. The first histological signs of gonadal differentiation were observed around 150 dph in females and 200 dph in males, which coincided with the time that sex differentiation of the ovary and testes, respectively, was well underway [31]. At 250 dph, gonads were completely differentiated in females and males. In both sexes, plasma Fsh bioactivity levels were high at 150-200 dph and progressively decreased by 300 dph. In the pituitary, the profiles of Fsh quantity and bioactivity were exactly the opposite, increasing from 150-200 to 300 dph. These results indicate that high levels of Fsh could be required at initial stages of sexual differentiation. Thus, the data obtained suggest a high Fsh release in plasma at early stages followed by a later accumulation in pituitary. In this regard, previous studies performed on the same animals and during the same period showed that the maximum levels of *fshb* and *cga* pituitary expression were achieved at 200 dph, keeping high until 300 dph, and they were higher than *lhb*. Moreover, plasma levels of Lh were low at early stages, with an elevation only observed at 250 dph when gonads were completely differentiated in both sexes [19]. On the other hand, the immunohistochemical analysis in this study revealed that, as in other teleost species [16, 37,38, 39, 40], Fsh and Lh in European sea bass are produced in different cell populations of the adenohypophysis. Moreover, in rainbow trout (*Oncorhynchus mykiss*), platyfish (*Xiphophorus maculatus*) and acará (*Cichlasoma dimerus*) it has been reported that the Fsh cells appeared prior to the onset of sexual differentiation, while Lh cells were observed several days after this process started or upon completion [12, 14, 15, 17]. Taken together, these data support a key role for Fsh during sex differentiation.

Additionally, both plasma and pituitary European sea bass Fsh levels were higher in females than in males, and the pituitary B:I ratio during this period was also higher in females, suggesting a possible sexual dimorphism in the synthesis and potency of Fsh during sex differentiation of European sea bass. Expression analysis of cytochrome P450 aromatase genes in ovary (*cyp19a1*) and brain (*cyp19a2*) in the same fish populations and period [20, 41] showed that females exhibited higher expression levels than males, suggesting that the high levels of Fsh seen in this study could be involved in the control of their expression. The enzyme Cyp19a1 catalyzes the conversion of Testosterone (T) into E2 and previous studies have demonstrated a direct stimulation of *cyp19a1* expression by Fsh in fish oocytes [42].

Regarding the annual cycle of sexually mature females, the Fsh quantity and bioactivity levels in the pituitary were significantly higher during late- and post-vitellogenesis and maturation-ovulation, with a tendency to decrease at the stage of atresia at the end of the reproductive season. In the plasma, Fsh bioactivity levels peaked at late- and post-vitellogenesis and declined gradually thereafter. This Fsh profile is concordant with those found in synchronous species. In coho salmon, Swanson [43] reported that plasma Fsh concentration increased during vitellogenesis, with the highest levels occurring during mid- to late-vitellogenesis, after which plasma Fsh decreased as ovulation approached. In rainbow trout, plasma Fsh levels increased significantly at the onset of vitellogenesis [44], being maintained during active vitellogenesis and decreasing prior to maturation [45]. On the other hand, when the pituitary content of European sea bass Lh was analyzed in the same samples of the female cycle, the quantity profile turned out to be identical to that of Fsh. Previously, in the same animals, the Lh and E2 plasma levels and gene expression of *fshr*, *lhr* and *cyp19a1* were analyzed [23]. Correlation between *lhr* receptor expression and its ligand levels was observed, Lh plasma levels and *lhr* gene expression peaked at maturation-ovulation, whereas *fshr* gene expression exhibited very high levels at late- and post-vitellogenesis coincident with the highest plasma levels of Fsh bioactivity (Rocha et al. [23] and this study, respectively). At maturation-ovulation *fshr* gene expression remained elevated and plasma Fsh bioactivity levels decreased although not significantly. The high levels of *fshr* expression in the ovary [23] and the Fsh content in pituitary and bioactivity in plasma (this study) during maturation could be explained by the reproductive strategy of this species. European 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 for maturation [32, 46]. Therefore, during the maturation stage, Fsh would be needed by clutches of oocytes that are still in vitellogenesis. The data presented here indicate similar synthesis and accumulation of Fsh and Lh in the pituitary gland, but different release to the bloodstream. Moreover, the maximum of plasma FSH bioactivity observed here coincided with the maximum expression of *cyp19a1* and E2 plasma levels reported earlier [23]. Estradiol is known to stimulate the hepatic synthesis of vitellogenin, which is then incorporated progressively into the growing oocytes during the period of gametogenesis [47]. Several studies have demonstrated that Fsh stimulates in vitro production of E2 in ovarian explants or isolated vitellogenic follicles [21, 42, 48, 49] and promotes the incorporation of vitellogenin into the oocytes [50, 51]. Therefore, according to the obtained results it appears that in the European sea bass, Fsh regulates the vitellogenesis by stimulation of the ovarian production of E2 via activation of Cyp19a1, whereas Lh would be responsible for the final events, such as oocyte maturation and ovulation.

In the pituitary of adult female European sea bass, the B:I ratio decreased as ovarian recrudescence progressed whereas the opposite occurred during sex differentiation. This observation was true for both ratios calculated from absolute values and relative values, keeping proportions between the two experiments. This could suggest the presence of Fsh isoforms in the pituitary, with different biopotencies associated to the reproductive stage of the animal. Studies in mammals have demonstrated that changes in the content of sialic acid affect the bioactivity of FSH isoforms. In humans, less acidic FSH isoforms exhibit higher B:I ratios than more acidic counterparts [52]. In rats it has also been suggested that more sialylated FSH isoforms are less biopotent whereas less acidic isoforms exhibit higher biopotency but shorter half-life in circulation [36]. Besides, it seems that intrapituitary FSH molecular microheterogenity may change depending on the age, sexual development or/and the steroidogenic milieu [36, 53]. Our results seem to indicate that during sex differentiation Fsh isoforms with higher potency are needed, whereas less biopotent but possibly long-lived Fsh could be needed as ovarian growth progresses in adult females.

In conclusion, two homologous and specific assays have been developed to measure European sea bass Fsh in plasma and pituitary. The assays have provided, for the first time in a fish species, quantitative and qualitative in-

formation on Fsh in critical moments of the reproductive life cycle. The obtained results reinforce the hypothesis that Fsh has an important role during sex differentiation of European sea bass. In addition, a sexual dimorphism in the synthesis and potency of Fsh is suggested. On the other hand, in adult females, a Fsh less biopotent form than that found during sex differentiation would regulate secondary oocyte growth (vitellogenesis), whereas Lh would be responsible for the final reproductive events, such as oocyte maturation and ovulation.

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Capítulo 7:

Development of homologous enzyme-linked immunosorbent assay for European sea bass FSH. Reproductive cycle plasma levels in both sexes and in yearling precocious and non-precocious males

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General and Comparative Endocrinology (en revisión)

Abstract

Since the late 1980s, gonadotropins have been isolated and characterized in several fish species, but specific immunoassays for the follicle-stimulating hormone (FSH) have only been developed for a few. The present study reports the development and use of a specific and homologous competitive ELISA for measuring FSH in European sea bass (*Dicentrarchus labrax*) using a recombinant FSH and its specific antiserum. Recombinant European sea bass FSHβ and FSH dimer were produced in the methylotrophic yeast *Pichia pastoris* and a baculovirus expression system, respectively. Specific polyclonal antibodies, generated by rabbit immunization against recombinant FSHβ, were used at a final dilution of 1:8000. Recombinant FSH dimer was used to generate a standard curve and for coating of microplates (166 ng/ml). The sensitivity of the assay was 0.50 ng/ml [B_0-2SD], and the intra- and inter-assay coefficients of variation were 2.12% (n=10) and 5.44% (n=16) (B_i/B_0 ~ 45%), respectively. A high degree of parallelism was observed between the standard curve and serially diluted plasma and pituitary samples of European sea bass.

The ELISA developed was used to study the plasma FSH profiles of mature males and females during the reproductive cycle, and those of immature juvenile males under different light regimes. The analysis showed that FSH increased significantly during the intermediate stages of spermatogenesis and during vitellogenesis. Analyses in immature juvenile males showed that the continuous light photoperiod significantly reduced plasma FSH levels, and consequently, testicular growth. In conclusion, the immunoassay developed has proven to be sensitive, specific and accurate for measuring European sea bass FSH, and it represents a valuable tool for future studies on the reproductive endocrinology of this species.

Keywords: FSH, recombinant, yeast, ELISA, reproductive cycle, Perciform

1. Introduction

The gonadotropins (GTHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are key hormones in the control of reproduction in vertebrates. These heterodimeric glycoproteins are synthesized and secreted by the pituitary gland. GTHs are formed by the non-covalent association of a common α-subunit with distinct β-subunits that confer hormone specificity [5, 27]. Each subunit is encoded by a single, separate gene [11], while dimerization and glycosylation are prerequisites for GTHs to achieve full biological activity.

As in higher vertebrates, fish GTHs regulate gametogenesis and steroidogenesis. In salmonids, from which most of the information on seasonal GTHs profiles comes, FSH is believed to regulate the early stages of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH is generally accepted to be the hormone responsible for the final maturation processes, such as oocyte maturation, ovulation and spermiation [15]. However, the functional duality between FSH and LH at critical moments in the reproductive process in other fish species has not yet been fully clarified, mainly due to a lack of appropriate research tools like assays to measure GTH levels. Traditionally, the immunoassays developed to determine GTH levels in fish have been radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) based on native GTHs purified from fish pituitaries, as well as their specific antibodies. The purification of native GTHs is a highly demanding process in terms of time, cost and the large number of pituitary glands required [15]. Since the late 1980s, GTHs have been isolated and characterized in several fish species, but homologous immunoassays for FSH have only been developed for three fish species with synchronous ovarian development: chum salmon (*Oncorhynchus keta*) [37], coho salmon (*Oncorhynchus kisutch*) [40, 41] and rainbow trout (*Oncorhynchus mykiss*) [12]. For other fish species, quantitative tools like these have only been available for LH, a fact that has hampered studies. Over the last decade, the isolation and characterization of cDNAs encoding GTH subunits in a wide range of fish species has made it possible to produce species-specific recombinant GTHs. The production of recombinant GTHs represents a good alternative to native hormones, as they can be continuously produced, ensuring their availability and preventing cross-contamination with other related glyco-

proteins [15]. However, over the last few years, homologous immunoassays for FSH and LH using recombinant gonadotropins have been successfully developed for only one perciform species with asynchronous ovarian development: the tilapia (*Oreochromis niloticus*) [1].

The European sea bass (*Dicentrarchus labrax*) is a marine perciform fish with a group-synchronous ovarian type development [9], and there is information available on the endocrine control of its reproductive function, particularly regarding the role of LH in adults [24, 25, 30] and juveniles [31, 32]. Nevertheless, there is a lack of information on the role of FSH in either adults or juveniles. The European sea bass is a very important species for intensive aquaculture, and under these conditions, a large number of males anticipate puberty just after sexual differentiation [33]. In light of this, precocity becomes one of the most significant problems to be solved, since these fish experience growth depletion and vulnerability to diseases [43]. Several works have demonstrated the effectiveness of photoperiod in preventing early puberty in male European sea bass [6, 31]. Nevertheless, further studies are needed to understand the underlying mechanisms of this inhibition, in particular with regard to the role of FSH.

Recently, we have produced recombinant European sea bass FSHβ subunit in a baculovirus expression system and developed a dot-blot immunoassay capable of measuring FSH in pituitary samples [22]. However, this assay could not be validated for plasma, because plasma FSH levels were often below the assay detection limit.

The objectives of this study were threefold: (1) the production of a new batch of recombinant European sea bass FSHβ in the methylotrophic yeast *Pichia pastoris* and the generation of its specific antibodies; (2) the development of a more sensitive and versatile immunoassay for European sea bass FSH, capable of determining FSH levels in both plasma and pituitary samples; and (3) to study the plasma FSH profiles in adult males and females during the reproductive cycle, and in juvenile males under different light regimes.

2. Materials and methods

2.1. Experimental fish and sample collection

Male and female European sea bass from the stock raised at the facili-

ties of the Torre de la Sal Aquaculture Institute (40° NL) were used to study seasonal profiles during the reproductive cycle. Fish were sampled monthly throughout the entire period of their first sexual maturation. At each sampling point, five fish of each sex were anesthetized, weighed, measured and sacrificed. Blood was collected via a caudal vein puncture using heparinized syringes, centrifuged at 2500*g* for 25 min at 4°C and plasma stored at -20°C until the time of analysis. Pituitaries were extracted, immediately frozen in liquid nitrogen and stored at -80°C. For analysis, pituitaries were mechanically homogenized in TBS-T (10 mM Tris-base, 150 mM NaCl and 0.05% Tween-20) using sterile syringes. The extract was centrifuged (3000*g* for 15 min at 4°C) to eliminate debris and stored at -80°C until assayed. Gonads were dissected, fixed, sectioned (3 μm) and stained according to the method described by Bennett et al. [4] for histological analysis. The stages of testicular development were classified by means of light microscopy, following previously established criteria [6]: stage I, immature testes; stage II-IV, testicular growth (early, mid and late recrudescence); stage V, fully spermiating testes and stage VI, post-spawning. The ovarian stages were classified as described by Rocha et al. [30], following previously established criteria [3]: previtellogenesis (prevtg); early vitellogenesis (evtg); late-vitellogenesis and post-vitellogenesis (lvtg-pvtg); maturation–ovulation (mat–ovul) and atresia (atre).

Juvenile male European sea bass (seven months of age) obtained from Aquanord (Gravelines, France) were used to study the effect of a continuous light regime, which is known to impair precocious puberty in males [6], in terms of plasma FSH levels. Immature juvenile males were subjected to either simulated natural photoperiod (NP) or continuous light (LL) (24h light/day) treatments for ten months (June-March). In October, December, January and March, 13 fish from each group were anesthetized, weighed, measured and sacrificed. Blood was collected as described above.

All fish were sacrificed in accordance with Spanish legislation concerning the protection of animals used for experimentation and other scientific purposes.

2.2. Construction of FSHβ expression plasmid

Complementary DNA fragments containing the entire open reading frame (ORF) of mature European sea bass FSHβ (480 bp) (GenBank ac-

cession number: AF543314) and a C-terminal 6xHis tag were obtained by PCR, using specific primers (Forward: 5'GG GAATTC GGG CAG GGC TGC AGC TTC3'; Reverse: 5'AT GCGGCCGC TTA ATG ATG ATG ATG ATG ATG AAA GGA CAG ACA GCT GGG TAT3') and the proofreading *PfuTurbo* DNA polymerase (Stratagene). Restriction sites were added at the 5' end of the forward and reverse primers. The fragment was directionally cloned into the donor plasmid pPIC9K (Invitrogen) between *Eco*RI / *Not*I restriction sites. The pPIC9K plasmid contains the yeast AOX1 promoter followed by the α-Factor signal sequence, which directs the recombinant protein to the secretory pathway. The pPIC9K-FSHβ construct was sequenced to confirm its identity.

2.3. Recombinant FSHβ production in yeast

The pPIC9K-FSHβ construct was linearized with *Bgl*II and used to transform methylotrophic yeast *Pichia pastoris*, strain GS115 (Invitrogen), by electroporation, using an ECM 830 Electroporation system (BTX). The host strain GS115 has a mutation in the histidinol dehydrogenase gene (*his4*) that prevents it from synthesizing histidine. The pPIC9K expression plasmid complements *his4* in the host, so 85 transformants were selected for their ability to grow on histidine-deficient MD medium (1.5% agar, 2% dextrose, 1.34% yeast nitrogen base, $4x10^{-5}$% biotin). The pPIC9K plasmid contains the bacterial kanamycin gene that confers resistance to the antibiotic G418 (Geneticin; Invitrogen). Due to the fact that multiple plasmid integration events may increase the levels of expressed recombinant protein, the transformants were further screened for high resistance to G418 on YPD medium (2% agar, 1% yeast extract, 2% peptone, 2% dextrose and G418 at various concentrations (0.5-2 mg/ml)). Screening of FSHβ production was performed by Western blot analysis. The clones with the highest production levels were cultured to examine the time-course of FSHβ synthesis. Initially they were grown in BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 1% glycerol, $4x10^{-5}$ % biotin and 100 mM potassium phosphate, pH 6) under shaking for 21 h at 29°C. The cells were then harvested by centrifugation at 2000*g* for 5 min at room temperature (RT) and afterwards re-suspended and cultured in 1/4 volume of BMMY medium (BMGY with 0.5% methanol instead of 1% glycerol). Incubation continued for another 144 h at 29°C, and methanol was added at a concentration of

0.5% every 24 h (induction phase). Samples of the culture supernatant were collected at different times (0, 24, 48, 72, 96, and 144 h) by centrifugation at 15000*g* for 3 min at RT and analyzed by FSH dot-blot immunoassay. As a negative control, GS115 cells were transformed using an expression plasmid without the sea bass FSHβ cDNA and treated in the same manner.

2.4. FSH dot-blot immunoassay analysis

The production level of FSHβ during the time course was measured by homologous FSH dot-blot immunoassay according to Molés et al. [22]. This assay has a sensitivity of 162 ng/ml, with intra- and inter-assay coefficients of variation of 9.8% and 11.5%, respectively.

2.5. Recombinant FSHβ purification

The European sea bass FSHβ was produced and harvested at 72 h after induction by methanol. The supernatant (1L) of a culture of GS115-FSHβ clone 6 was concentrated (5-6 ml) by ultrafiltration using Centricon Plus-70 Biomax 5 centrifugal filter devices (Amicon, Millipore) and later purified by immobilized metal affinity chromatography (IMAC Ni^{2+}) using His GraviTrap prepacked columns (GE Healthcare), according to the manufacturer instructions. Briefly, the pH of the supernatant was adjusted to 7-8 with diluted acetic acid and the prepacked columns were equilibrated with PBS (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 20 mM imidazole. After loading the concentrated supernatant, two washings with PBS (20 mM and 70 mM imidazole) were performed. Finally, the bound FSHβ was eluted with PBS containing 250 mM imidazole. The purity of the fractions obtained was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

2.6. SDS-PAGE and Western blot analysis

Proteins were electrophoresed through 15%-SDS-PAGE gels under reducing conditions (5% 2-mercaptoethanol (2-ME)). The separated proteins were stained with Coomassie blue or transferred to PVDF membranes (Immobilon P, Millipore). The membranes were blocked overnight with 5% skim milk at 4°C, incubated with 1:3000 antibodies against European sea bass

FSHβ (AbFSHβ-1) [22] for 90 min at room temperature, washed, and further incubated with 1:2000 goat anti-rabbit IgG-horseradish peroxidase conjugate (GAR-HRP, Bio-Rad) for 60 min at room temperature. The immunodetection was performed by chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz Biotechnology, Inc.).

2.7. European sea bass FSHβ antibody production

Polyclonal antibodies against the purified recombinant FSHβ (AbFSHβ-2) were produced by a commercial company (Agrisera, Sweden). Two rabbits were immunized with 150 μg of FSHβ in Freund's complete adjuvant by subcutaneous injection. Four subsequent immunizations (II-V) were carried out with 30 μg of antigen in Freund's incomplete adjuvant at 3-week intervals. Rabbits were bled 2 weeks before immunizations (pre-immune serum) and 2 weeks after immunizations III, IV and V, in order to perform the corresponding titration test. The final bleeding was performed 2 weeks after the fifth immunization.

2.8. European sea bass FSH ELISA

A competitive ELISA for sea bass FSH determination was developed using the specific antiserum generated (AbFSHβ-2) and recombinant FSH dimer produced in a baculovirus expression system [21]. The protocol was based on that previously described for ELISAs of striped bass LH [17] and sea bass LH [18], and was adapted as follows:

1) **Coating**. Polystyrene ELISA 96-well microplates (Maxisorp, Nunc) were coated with 50 μl/well of recombinant European sea bass FSH dimer solution (166.5 ng/ml, diluted in sodium carbonate buffer 0.05 M, pH 9.6) overnight at 4°C. Three wells were coated with the same concentration of bovine serum albumin (BSA, Sigma) to determine the non-specific binding. After coating, the wells were washed (3 x 1 min) with PBST (sodium phosphate buffer 0.01 M, pH 7.2, containing 0.9% NaCl and 0.05% Tween-20). The plate wash was repeated after every step of the assay.

2) **Blocking**. To reduce background, wells were blocked with 100 μl/well of PBST buffer containing 2% BSA for 30 min at 37°C.

3) **Incubation with primary antibodies**. Before distribution into the wells, standard and unknown samples (final dilution 1:4 for plasma and 1:4000 for pituitary) were first preincubated with AbFSHβ-2 (final dilution 1/8000) in microcentrifuge tubes (overnight at 4°C). The standard curve ranged from 0.32 to 162.5 ng/ml of sea bass FSH. All standards, samples and the antiserum solution were diluted in PBST buffer containing 2% normal goat serum (NGS, Sigma). After preincubation, samples and standards were dispensed in duplicate (50 µl/well) into the coated wells and incubated for 48 h at 4°C without shaking. The non-specific binding wells and five FSH coated wells (maximum binding, B_0) received only AbFSHβ-2 solution.

4) **Incubation with secondary antibodies**. The antigen-antibody complexes formed were detected by incubation with 50 µl/well of GAR-HRP, diluted 1:1000 in PBST-2% NGS buffer for 1 h at 37°C.

5) **Color development**. The presence of enzyme complexes was detected by the addition of 100 µl/well of TMB reagent (Bio-Rad). The reaction was carried out in complete darkness at RT for 25 min, and was stopped with 100 µl/well of 1 N sulphuric acid. Absorbances were read after 5 min at 450 nm, using an automatic microplate reader (Bio-Rad).

2.9. Data representation and Statistical analysis

To study the FSH profiles of males and females during the reproductive cycle, the samples were grouped according to gonadal stage of development (see Section 2.1). Data are presented as mean ± SEM. The significance of the differences between group means of hormone levels was determined by one-way analysis of variance (ANOVA) followed by post hoc testing using the Holm-Sidak method, performed with SigmaStat 3.5 software (Systat Software Inc.). When the test of equal variance failed, an ANOVA on Ranks (Kruskal-Wallis) was performed, followed by all pairwise multiple comparison procedures (Dunn's method).

For ELISA data calculations, sigmoid curves were linearized using the logit transformation (logit $(B_i/B_0) = \ln (B_i - NSB/B_0 - B_i)$), where B_i represents the binding of each point, B_0 is the maximum binding and NSB the non-specific binding.

3. Results

3.1. Production of Recombinant European sea bass FSHβ

The pPIC9K-FSHβ construct was used to transform GS115 cells by electroporation. Transformants were selected for their ability to grow on histidine-deficient MD medium and according to their resistance to high doses of G418. Thirteen colonies with His^+ Mut^+ phenotype and resistance to 2 mg/ml G418 were selected and used for screening FSHβ production by Western blot analysis. A candidate protein reacted intensely with the AbFSHβ-1 antibody [22] in all the colonies analyzed (Fig. 1). Yeast transformed with the vector alone served as a negative control and yielded no band (Fig. 1). The clones that produced the highest amount of recombinant FSHβ were selected and examined for time-course production and secretion of FSHβ. Samples of culture supernatant were collected at different times (0, 24, 48, 72, 96, and 144 h) and subjected to dot-blot immunoassay analysis, which revealed that the yields of FSHβ increased up to 48 h after induction with methanol and remained constant between 72 and 144 h (Fig. 2). The clone with highest level of production was cultured to large scale for 72 h. After the purification process, the collected fractions were analyzed by SDS-PAGE and Western blot. The Coomassie blue stain showed a production with a high yield and good degree of purity. A single band was intensely stained, although proteins of lower molecular weight were also observed (Fig. 3A). A

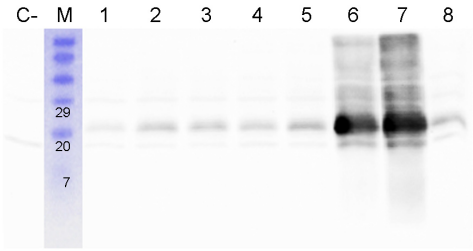

Figure 1. Screening of yeast FSHβ production. Analysis by Western blot with AbFSHβ-1. Lanes 1-8: Cell lysates (15 µl) from different GS115 colonies transformed with pPIC9K-FSHβ. C-: Negative control; Cell lysate (15 µl) from a colony containing pPIC9K. M: Bio-Rad prestained Broad Range molecular weight marker.

Western blot analysis confirmed that the purified protein and the low molecular weight products were FSHβ (Fig. 3B), indicating that some protein degradation may have taken place. The molecular weight detected for FSHβ produced in yeast turned out to be slightly higher than that of the FSHβ previously produced in the baculovirus system (Fig. 3A). The purified FSHβ was used to generate specific polyclonal antibodies through rabbit immunizations. The antiserum produced was tested in Western blot, and specifically immunoreacted with pituitary extract and recombinant European sea bass FSHβ forms produced in yeast and the baculovirus system, but did not recognize recombinant European sea bass LH (Fig. 4), indicating the FSHβ specificity of the antibodies.

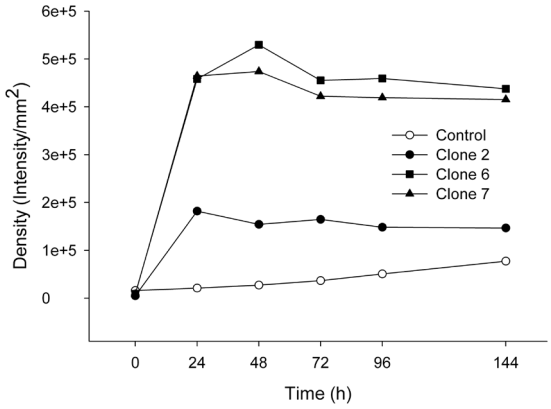

Figure 2. Time-course of yeast FSHβ production. Analysis by FSH dot-blot immunoassay of three GS115 clones expressing FSHβ and one wild type control. Each point is the mean of two replicates (250 µl supernatant/sampling point).

3.2. Development and validation of an ELISA for European sea bass FSH

A homologous competitive ELISA was developed for FSH determination in pituitary and plasma samples, using recombinant FSH dimer for coating and the standard curve, and the specific antiserum generated (AbFSHβ-2) for immunodetection. A series of tests were performed to optimize the ELISA protocol by studying the behavior of the standard curve under different temperatures, incubation times and coating concentrations.

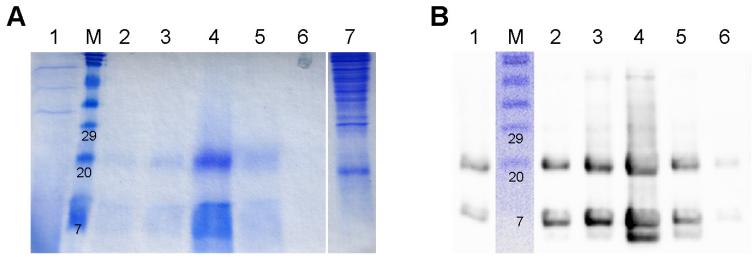

Figure 3. SDS-PAGE and Western blot analysis of purified recombinant European sea bass FSHβ. A) Staining with Coomassie blue and B) Western blot with AbFSHβ-1 after affinity chromatography (IMAC Ni^{2+}) of recombinant FSHβ produced in yeast (GS115). Lane 1: Flow-through (10 µl); Lane 2: Wash with PBS containing 70 mM Imidazole (20 µl); Lanes 3 to 6: Elution with PBS containing 250 mM Imidazole (20 µl); Lane 7: Elution with PBS containing 500 mM Imidazole of FSHβ produced in baculovirus system (20 µl). M: Bio-Rad prestained Broad Range molecular weight marker.

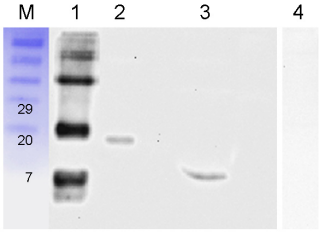

Figure 4. Analysis by Western blot of the polyclonal antibodies against recombinant European sea bass FSHβ produced in yeast (AbFSHβ-2; 1:15000). Lane 1: FSHβ produced in yeast (GS115) (100 ng), Lane 2: Pituitary extract (300 ng), Lane 3: FSHβ of FSH dimer produced in baculovirus system (320 ng). Lane 4: LH dimer produced in baculovirus system (420 ng). M: Bio-Rad prestained Broad Range molecular weight marker.

Under optimized conditions, described in Materials and methods, the sensitivity of the assay, defined as the lowest dose of FSH capable of reducing the optical density more than the mean plus 2 standard deviations of the zero dose of FSH [B_0 – 2SD], was 0.50 ng/ml (B_i/B_0 > 93,9%). The half maximum displacement occurred at 11.32 ng/ml (B_i/B_0 = 50%).

The precision of the assay was tested by calculating the intra- and inter-assay coefficients of variation (CV). The intra-assay CV, tested by measuring replicates of the same sample in a single assay plate, was 4.7, 2.1 or 1.4%, according to the calculations at 25, 48 or 74% of binding, respectively (n = 10).

The inter-assay CV, calculated by measuring the same sample in different assays was 8.6, 5.4 or 3%, according to the calculations at 23, 44 or 83% of binding, respectively (n=16).

The specificity of the assay was tested by cross-reaction of AbFSHβ-2 with recombinant European sea bass LH produced in a baculovirus expression system [21], which showed no immunoreaction even at concentrations as high as 1000 ng/ml (Fig 5A).

Finally, the assay was validated for European sea bass plasma and pituitary samples by testing the parallelism with the standard curve. Displacement curves obtained with serial dilutions of plasma and pituitary extracts produced sigmoid curves similar to the FSH standard curve (Fig. 5A). In order to test the possibility of using the European sea bass FSH ELISA for FSH measurements in other fish species, displacement curves of serial dilutions of pituitary extracts and plasma from other fish species were compared with the FSH standard curve. A scarce or non-linear parallelism with the FSH standard curve was observed for thicklip grey mullet (*Chelon labrosus*), sea bream (*Sparus aurata*) and Senegalese sole (*Solea senegalensis*), which shows the high degree of specificity of AbFSHβ-2 for European sea bass FSH (Fig. 5B).

3.3. Determination of FSH levels

FSH levels during the European sea bass reproductive cycle

Plasma profiles of FSH were examined throughout the first reproductive cycle of both sexes, which occurred during the second and third year of life in males and females, respectively. In males, FSH levels increased significantly during active spermatogenesis (stages II-IV) and fell to baseline levels just before full spermiation (stage V) (Fig. 6A). In females, the FSH levels peaked at vitellogenesis and post-vitellogenesis, while the minimum values were detected at maturation-ovulation (Fig. 6B). In both sexes, the baseline levels of FSH were higher than 15 ng/ml.

FSH levels of juvenile European sea bass under different light regimes

Plasma FSH levels of juvenile European sea bass exposed to simulated natural photoperiod (control group) or continuous light were examined from October to March. The analysis showed that continuous light significantly reduced plasma FSH levels as compared to the control group. Under

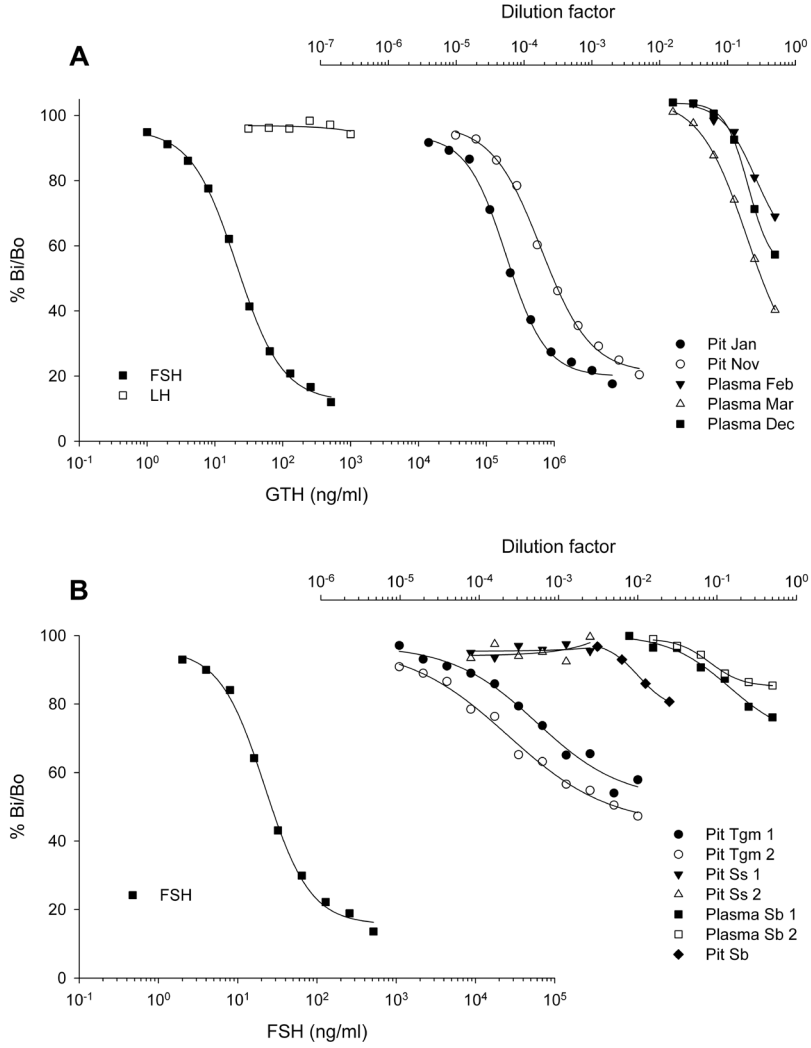

Figure 5. Validation of European sea bass FSH ELISA. A) Parallelism between FSH standard curve and displacement curves obtained with serial dilutions of plasma, pituitary extracts (Pit) and recombinant European sea bass LH. B) Parallelism between standard curve and displacement curves obtained with serial dilutions of plasma and pituitary extracts from different fish (1 and 2). Thicklip grey mullet (Tgm), sea bream (Sb) and Senegalese sole (Ss). Each point is the mean of two replicates.

natural photoperiod, plasma FSH levels increased steadily until reaching their maximum level in March, while FSH levels of fish subjected to continuous light, remained unchanged (Fig. 7). The percentage of precocious juvenile males in the control group was about 65%, on the contrary, under a regime of continuous light, the number of premature males was much lower, about 10% (data not shown).

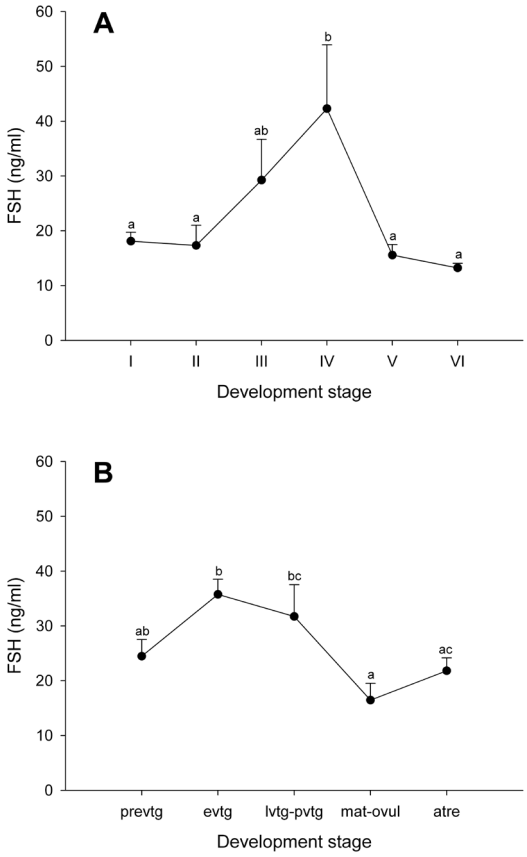

Figure 6. Seasonal profiles of plasma FSH in male and female European sea bass during the reproductive cycle. Values were classified according to stage of gonadal development as determined by histology. A) Males: immature (I); early recrudescence (II); mid recrudescence (III); late recrudescence (IV); fully spermiating (V) and post-spawning (VI). B) Females: previtellogenesis (prevtg); early vitellogenesis (evtg); late- and post-vitellogenesis (lvtg-pvtg); maturation-ovulation (mat-ovul) and atresia (atre). Points represent mean ± SEM values (n= 5-19, depending on the stage). Different letters indicate significant differences between developmental stages (P=0.05).

4. Discussion

This study describes the development and use of a specific and homologous ELISA for European sea bass FSH, using recombinant FSH and its specific antiserum. In teleosts, homologous immunoassays for FSH have only been developed for three salmonid species and one perciform species [1,

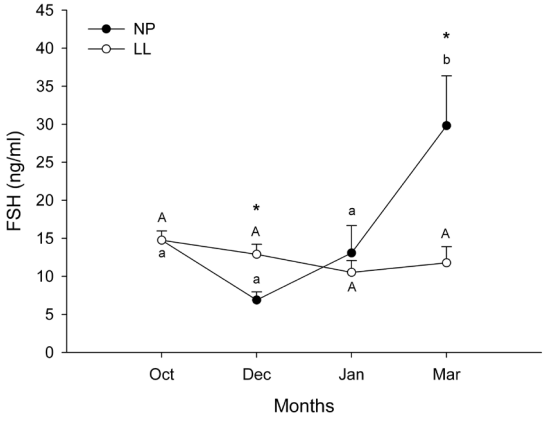

Figure 7. Plasma FSH levels in immature males of European sea bass exposed to simulated natural photoperiod (NP) or continuous light (LL) regimes for ten months (between June and March). Points represent mean ± SEM (n= 5-9, depending on the sampling point). Different letters indicate significant differences between sampling points (P=0.05) (lowercase letters for NP and capital letters for LL). Asterisks indicate significant differences between the two treatments at equivalent sampling points (P<0.05).

12, 37, 40]. Traditionally, the immunoassays developed to determine GTHs levels in fish have been based on native GTHs purified from fish pituitaries and their specific antibodies. Since purification of native FSH is a very demanding process, the production of recombinant forms is a good alternative that yields large quantities of protein, ensuring its availability and no cross-contamination with other related glycoproteins. In the present study, we have developed a sensitive and versatile immunoassay for FSH, an ELISA able to measure FSH in plasma and pituitary samples. For this purpose, we produced recombinant European sea bass FSHβ in the methylotrophic yeast *Pichia pastoris*, which was used to generate specific antibodies. Prior to this, we had produced recombinant European sea bass FSH dimer and

FSHβ subunit using a baculovirus expression system [21, 22], as well as its specific antibodies (AbFSHβ-1). However, AbFSHβ-1 reacted very weakly with FSH under native conditions; in spite of which we were still able to develop a dot-blot immunoassay capable of measuring pituitary FSH under denaturalized conditions. Nevertheless, to develop a more sensitive and versatile ELISA-like immunoassay, it was necessary to produce new antibodies that could recognize native forms of FSH. Accordingly, a new batch of recombinant FSHβ was produced in a yeast system. The main advantage of this system, compared to the baculovirus system previously used [22], is that the FSHβ was efficiently secreted and easily purified from the culture medium, although the production yield in yeast was somewhat lower than in the baculovirus system. In addition, yeast culture does not require costly sophisticated growing media and is fairly easy to establish and scale up.

The size of recombinant FSHβ generated in yeast was slightly higher than that previously produced in the baculovirus system. This fact might be due to different degrees of glycosylation, since *Pichia pastoris* have mostly a high-mannose-type N-linked glycosylation [13] while insect cells, used in the baculovirus system, assemble insect-specific paucimannose-type N-glycans [14].

To develop the FSH specific ELISA, recombinant FSH dimer [21] was used as coating and to generate the standard curve. The ELISA developed for European sea bass FSH showed optimal characteristics in terms of precision, specificity and sensitivity, similar to those reported for other fish GTH immunoassays. The precision was high, ensured by low intra- and inter-assay coefficients of variation, which were both below 5% and 9%, respectively. These variations are lower or similar to those reported for FSH immunoassays in other fish species, which are in the range of 4-8% and 10-12% for intra- and inter-assay CVs, respectively [1, 12, 37]. The specificity was demonstrated by the absence of AbFSHβ-2 immunoreaction with European sea bass LH; no cross-reactivity was detected in either the Western blot or ELISA. Finally, the sensitivity of the European sea bass FSH ELISA (0.50 ng / ml) was much higher than that of the FSH dot-blot immunoassay previously developed (162 ng/ml) [22], and high enough to measure plasma FSH levels. Moreover, the sensitivity was comparable to those reported for other fish GTH immunoassays, which ranged from 0.2 to 2.34 ng/ml for RIAs [12, 29, 35, 37, 40, 42] and 0.24 pg/ml to 0.65 ng/ml for ELISAs[1, 17, 18, 34].

The European sea bass FSH ELISA was validated for plasma and pituitary samples by testing the parallelism with the standard curve. The results indicated that native sea bass FSH, both in blood and the pituitary, was parallel to the recombinant FSH dimer used in the standard curve, and therefore was immunologically similar. We also tested the usefulness of the developed European sea bass FSH ELISA to measure FSH in the pituitary and plasma of other fish species. Non-linear parallelism was observed between the FSH standard curve and the pituitaries of Senegalese sole, and scarce parallelism with thicklip grey mullet and sea bream pituitaries. It can therefore be determined that AbFSHβ-2 has a high degree of specificity for European sea bass FSH, and is therefore not suitable to measure FSH levels in either the pituitary or the plasma of these species.

The ELISA developed was used to analyze, for the first time, immunological plasma FSH levels in a marine perciform with multiple-batch group-synchronous ovarian development, the European sea bass. In adult males, the plasma levels of FSH increase during testicular growth (II-IV stages) and decline just before full spermiogenesis-spermiation (V stage), suggesting that in European sea bass, FSH plays an important role in the regulation of early-mid phases of spermatogenesis, but not in spermiogenesis and spermiation. The plasma levels of 11-Ketotestosterone (11-KT) and LH have been previously analyzed in the same animals [30]. The 11-KT profile coincided with that of FSH in the present study, with levels gradually increasing during testicular growth, and dropping off once full spermiation begins. In eels, it has been demonstrated that recombinant FSH induces complete spermatogenesis by stimulating the production of spermatogenesis-inducing steroids, such as 11-KT [26]. Similarly, in European sea bass, we have shown a stimulatory effect of native FSH in the production of 11-KT in testicular tissue cultured *in vitro* [20]. 11-KT is the major androgen of teleost species and is considered to play an important role in the spermatogenesis of several fish species, mainly in the initiation of spermatogonial proliferation toward meiosis [36]. On the other hand, plasma LH levels increased gradually, although not significantly, as spermatogenesis progressed, with a significant elevation in late recrudescence (stage IV) that was maintained in full spermiation [30], although with concentrations consistently lower than those of FSH. These results are comparable to those observed in male rainbow trout, where maximum levels of plasma FSH appear during mid- to late testicular growth, and maximum plasma LH levels during spermiation [29].

In female European sea bass, the maximum plasma FSH levels were detected during vitellogenesis and post-vitellogenesis, whereas a significant decrease was observed in maturation-ovulation. Nevertheless, the pituitary FSH profile presented an opposite trend, showing low FSH levels in previtellogenesis and early vitellogenesis, high in late- and post-vitellogenesis and maturation-ovulation and again low in atresia [22]. These results indicate an active synthesis and secretion of FSH in the course of vitellogenesis, whereas during maturation-ovulation FSH secretion seems to decrease, accumulating in the pituitary. On the other hand, the plasma FSH bioactivity profile, measured in the same samples by *in vitro* bioassay [22], showed a tendency to achieve maximums of bioactivity in late- and post-vitellogenesis although plasma FSH concentration is maintained. Interestingly, the lowest plasma FSH levels obtained in the present study were found in maturation-ovulation coinciding with a FSH bioactivity moderately high [22]. This could be indicating an increase of the FSH potency in these stages. In fact, several studies have consistently demonstrated the occurrence of FSH isoforms with different potencies throughout the human menstrual cycle [44]. Moreover, in our case, estradiol (E2) and LH plasma levels have been previously analyzed in these same animals [30]. The maximum plasma E2 levels were observed during late- and post-vitellogenesis, coinciding with the maximums of plasma FSH levels calculated here and the maximum of plasma FSH bioactivity [22]. In females, E2 is known to stimulate the hepatic synthesis of vitellogenin, which is then progressively incorporated into the growing oocytes during oogenesis [16]. Moreover, several studies have shown that FSH stimulates the *in vitro* production of E2 in ovarian explants or isolated vitellogenic follicles [20, 21, 23, 28, 38]. LH plasma levels peaked at maturation-ovulation, the point when FSH reached minimum values, although at levels higher than those for LH (Rocha et al., [30] and this study, respectively). These profiles for female European sea bass GTHs are concordant with those found in salmonids. In coho salmon, Swanson [39] reported that the plasma FSH concentration increased during vitellogenesis, with the highest levels occurring during mid- to late-vitellogenesis, after which they decreased as ovulation approached. In rainbow trout, plasma FSH levels significantly increased at the onset of vitellogenesis [29], were maintained during vitellogenesis and then decreased prior to maturation [7]. On the contrary, in rainbow trout, plasma LH levels peaked at maturation-ovulation [7, 29]. Previous analysis of European sea bass pituitaries

revealed similar FSH and LH contents (and profiles) during the reproductive cycle of females [22]; nevertheless, the data obtained in plasma suggest a different control of release to the bloodstream (Rocha et al., [30] and this study). Additionaly, pharmacokinetic studies of GTHs in European sea bass [21] and in mammals [8] have shown that FSH has a longer half-life in blood than LH, which indicates greater clearance rates for LH. This fact would be in agreement with the lower plasma levels of LH, compared to FSH, that have been observed in males and females. Thus, our results suggest that in the case of European sea bass, high levels of FSH in the bloodstream would be necessary for long-term stimulation of gonadal growth, whereas short-term increases in plasma LH levels might have a more specific effect during certain phases of the reproductive cycle, and therefore are likely to be cleared more quickly.

The plasma FSH levels in the group of juvenile males under a simulated natural photoperiod (control group) increased gradually until reaching a maximum value in March. On the contrary, under a regime of continuous light, the levels of plasma FSH were maintained constantly low throughout the experimental period, which probably led to a lower percentage of precocious juvenile males. The FSH content in the pituitary gland showed the same profiles (M. Carrillo, M., Felip, A., Molés, G., Yilmaz, O., Zanuy, S., unpublished data), indicating that the inhibitory effect of continuous light on FSH was at the level of synthesis, not release. These results are in agreement with previous studies in juvenile male European sea bass and adult Atlantic Cod (*Gadus morhua*), where continuous light produced a significant reduction of gonadotropin subunit gene expression, 11-KT plasma levels and testicular growth [2, 10, 31]. All these results suggest that continuous light reduces precocity by impairing the synthesis of FSH. This results in a decrease of 11-KT levels that very likely reduces spermatogonial proliferation towards meiosis, and consequently, testicular growth.

In conclusion, the production of recombinant FSHβ in yeast has allowed us to generate specific antibodies and develop a homologous ELISA capable of measuring FSH in both plasma and pituitary samples from European sea bass. The results of the analysis performed suggest that FSH plays an important role during active spermatogenesis and vitellogenesis, whereas LH would seem to be involved in the final reproductive events, such as spermiation, oocyte maturation and ovulation. In juvenile males, the continuous light regime significantly reduced FSH synthesis, which is probably the

cause of the reduction of precocious puberty rates. Finally, this assay represents a valuable tool for future studies on the reproductive endocrinology of this species.

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Capítulo 8. DISCUSIÓN GENERAL

Discusión general

Esta sección pretende ofrecer una visión general de los resultados discutiendo los aspectos más relevantes de los trabajos presentados. Para ello, la discusión se ha desglosado en tres apartados en relación a (1) la caracterización de la FSH y producción de GTHs recombinantes, (2) desarrollo de inmunoensayos para la determinación de niveles de FSH y (3) estudio de los perfiles de GnRHs y GTHs durante la diferenciación sexual y ciclo reproductor.

1. Caracterización de la FSH de lubina y producción de GTHs recombinantes

En la presente Tesis Doctoral se ha aislado y caracterizado el dímero FSH y las tres subunidades gonadotropas (GPα, FSHβ y LHβ) a partir de hipófisis de lubina. Además, mediante el uso de técnicas de expresión de proteínas recombinantes se han producido diferentes formas de FSH y LH, y se han estudiado algunas de sus propiedades bioquímicas y funcionales.

La purificación de GTHs se realizó a partir de 1400 hipófisis mediante extracción alcohólica de glicoproteínas, seguida de técnicas cromatográficas de intercambio iónico y fase reversa bajo condiciones ligeramente básicas. La metodología empleada fue idónea para aislar FSH, pero no LH, pues se obtuvo una gran cantidad de sus subunidades (GPα y LHβ) pero escaso dímero. Al igual que en otras especies de peces (Okada et al., 1994; Suzuki et al., 1988b; Weltzien et al., 2003), parece que en la lubina la LH es menos estable que la FSH, ya que durante el proceso de purificación tuvo lugar una considerable disociación de sus subunidades. En peces, los análisis de las secuencias de aminoácidos de las subunidades FSHβ muestran que estas son más variables que las de LHβ en regiones importantes para la formación del heterodímero e interacciones con el receptor. Esta variación estructural de las FSHβ podría dar lugar diferencias inter-especificas en la naturaleza de

las interacciones con el receptor y posiblemente en la estabilidad del heterodímero (Swanson et al., 2003).

La identidad y el grado de pureza de la FSH y de las subunidades gonadotropas purificadas se confirmó por secuenciación N-terminal de aminoácidos y por Western blot. Los resultados de la secuenciación se compararon con las secuencias de aminoácidos deducidas a partir de los ADNc (Mateos et al., 2003), confirmando que la fracción 1B10 contenía una proteína altamente pura, compuesta por las subunidades GPα y FSHβ, mientras que las fracciones 1C11, 4C2 y 7C6 correspondían a las subunidades libres GPα, FSHβ y LHβ, respectivamente (ver Fig. 5, pag. 107). Los análisis por Western blot, usando anticuerpos disponibles contra la GPα y LHβ de lubina (Mateos et al., 2006) y un péptido sintético de la FSHβ del fúndulo (Fundulus heteroclitus) cedido por el Dr. Akio Shimizu (Shimizu y Yamashita, 2002), ratificaron que se trataba de las subunidades gonadotropas de lubina (ver Fig. 4, pag. 106). Además, la secuenciación N-terminal mostró que, cuando se comparaba la secuencia de la FSHβ purificada (1B10 y 4C2) con la secuencia de otras especies o con la deducida a partir del propio ADNc, le faltaban los tres primeros aminoácidos (G-Q-G). Esta heterogeneidad N-terminal también se ha observado en otras especies como el atun (*Thunnus obesus*) o el fletán (*Hippoglossus hippoglossus*) y sugiere que esta porción de FSHβ podría ser más susceptible a degradación proteolítica durante el proceso de purificación que la de LHβ (Okada et al., 1994; Weltzien et al., 2003). En este estudio solo se identificó una forma de FSHβ a la que le faltaban los tres primeros residuos del extremo N-terminal, lo que hace difícil saber si esta secuencia corresponde a la verdadera región N-terminal de la FSH madura o si es más bien resultado de un proceso de degradación durante la purificación.

Los pesos moleculares de la FSH (28.5 KDa) y de las tres subunidades gonadotropas (GPα: 12.6 KDa, FSHβ: 13.6 KDa, LHβ: 15.3 KDa), estimados por espectrometría de masas, son más pequeños que los calculados en otras especies de teleósteos. De todas formas, cabe decir que existe una gran variabilidad en los cálculos de los pesos moleculares de las GTHs de peces descritas hasta el momento, encontrando subunidades GPα, FSHβ y LHβ en el rango de 15 a 27 KDa y dímeros FSHs desde 32 a 50 KDa (ver Tabla 2, pag. 37). Según algunos autores (Hearn y Gomme, 2000) los oligosacáridos de las

GTHs pueden contribuir entre un 20 y 40 % del peso total de la molécula. Para confirmar la presencia de oligosacáridos en la FSH purificada en este estudio, se realizó un tratamiento con una *N*-glicosidasa (PNGase F) (ver Fig. 4, pag. 106). Este redujo considerablemente el tamaño de las subunidades GPα y FSHβ, lo que indicaba que ambas estaban *N*-glicosiladas y corroboraba las predicciones de sitios potenciales de *N*-glicosilación realizadas a partir de los ADNc (Mateos et al., 2003). En general, según las secuencias de aminoácidos, la contribución del esqueleto polipeptídico al peso molecular de las GTHs es similar entre las diferentes especies de teleósteos. Por ello, la variabilidad observada entre los pesos moleculares podría atribuirse a diferencias en la porción glicosídica de las subunidades o a diferencias asociadas al método de medida.

La especificidad y actividad biológica de la FSH purificada se evaluó por su capacidad de estimular los receptores homólogos (FSHR y LHR) expresados en una línea celular heteróloga (HEK 293) (ver Fig. 6, pag. 108). En mamíferos, las barreras de especificidad entre cada pareja receptor-gonadotrofina están bien definidas y son tales que bajo condiciones fisiológicas no hay señalización cruzada (Braun et al., 1991; Moyle et al., 1994). Por el contrario, en peces hay evidencias que indican que la especificidad entre las GTHs y sus receptores es menos obvia (Bogerd et al., 2005; Levavi-Sivan et al., 2010). En este estudio, se demostró que la FSH purificada era biológicamente activa y altamente especifica ya que activó FSHR de manera dosis dependiente pero no activó LHR incluso a dosis elevadas (200 ng/ml). Además de la activación de receptores, se estudió la actividad esteroidogénica de la FSH en cultivos *in vitro* de tejido gonadal y se demostró que estimulaba significativamente la producción de T y 11-KT en testículos y de E2 en ovarios de lubinas adultas de manera dosis y tiempo dependiente (ver Fig. 7-9, pag. 109-111). Esta estimulación fue mayor en machos en recrudescencia testicular (octubre) y en hembras vitelogénicas (octubre y noviembre). La T y 11-KT son los principales andrógenos producidos por los peces machos (Borg, 1994) y son esenciales para que tenga lugar la espermatogénesis (Fostier et al., 1983; Schulz y Miura, 2002). Además se ha demostrado que la 11-KT, aparentemente regulada por la FSH, es un factor endocrino clave del inicio de la proliferación espermatogonial hacia la meiosis (Schulz et al., 2010). Por otro lado, numerosos trabajos han demostrado que la vitelogénesis está regulada por el E2 (Nagahama, 1994) y se ha descrito que en folículos vite-

logénicos de trucha (*Salmo trutta*), la FSH estimula la expresión y actividad de la enzima P-450 aromatasa (CYP19A1) que cataliza la conversión de T a E2, jugando un papel fundamental en la regulación de la producción de E2 en ovarios de salmónidos (Montserrat et al., 2004). En la lubina, los niveles plasmáticos de 11-KT y T incrementan durante el periodo de espermatogénesis (octubre-enero) (Prat et al., 1990; Rocha et al., 2009; Rodriguez et al., 2004) y los de E2 aumentan en paralelo con los de vitelogenina y el crecimiento folicular (Asturiano et al., 2000; Asturiano et al., 2002). En conjunto todos estos datos apoyan la idea de que en la lubina, la FSH estimula la proliferación espermatogonial en machos y la vitelogénesis en hembras mediante la síntesis de esteroides sexuales.

La purificación de la FSH de lubina ha permitido estudiar algunos aspectos bioquímicos y fisiológicos de esta hormona. Sin embargo, para el desarrollo de nuevas investigaciones en el área de endocrinología reproductiva de peces, y en particular de la lubina, es esencial disponer de ambas GTHs de forma continuada. La purificación de GTHs nativas es un proceso muy exigente en términos de tiempo y dinero, para el cual se requiere una gran cantidad de hipófisis que a menudo limita su disponibilidad. Por ello, la producción de formas recombinantes de GTHs representa una excelente alternativa a las hormonas nativas ya que pueden ser producidas continuamente, asegurando su disponibilidad y evitando contaminaciones cruzadas con otras glicoproteínas hipofisarias.

En el transcurso de la presente Tesis Doctoral, se han producido diferentes formas recombinantes de FSH y LH de lubina usando dos sistemas de expresión, células de insecto (Sf9) y células de mamífero (CHO). En el primer caso se infectaron células Sf9 con baculovirus modificados que contenían los genes de las subunidades gonadotropas de lubina. Tras la infección, los virus se apoderaron de la maquinaria celular y expresaron sus genes de forma abundante, entre ellos los de las subunidades gonadotropas. En el segundo caso, la expresión de las GTHs se realizo mediante transfecciones estables de células CHO. Después de transfectar con plásmidos portadores de los genes de las subunidades gonadotropas, se seleccionaron aquellos clones de células CHO que habían incorporado el casete de expresión en el genoma de la célula y producían mayores niveles de GTHs.

Estos dos sistemas han servido para expresar y secretar eficientemen-

te formas diméricas (subunidades unidas por enlaces no covalentes: Sf9-FSH, Sf9-LH y CHO-FSH) y de cadena única (fusión de ambas subunidades: CHO-scFSH y CHO-scLH). Los niveles de producción fueron comparables a los descritos por otros autores en la producción de GTHs de peces (Levavi-Sivan et al., 2010), y considerablemente mas altos con el sistema baculovirus usando células Sf9 que con transfecciones estables de células CHO. Cuando se analizó la biopotencia de las formas recombinantes de FSH (EC_{50} y ratios de determinación Biológica : Inmunológica, (B:I)), se vio que Sf9-FSH era más bioactiva que CHO-FSH pero menos que CHO-scFSH. Análisis funcionales realizados en mamíferos han demostrado que las GTHs presentan bioactividades diferentes en función del tipo de glicosilación presente en cada isoforma (Ambao et al., 2009; Olivares et al., 2009; Zambrano et al., 1996). Concretamente, se ha demostrado que isoformas de FSH y LH con menos contenido en acido siálico exhiben mayores ratios B:I y son más potentes que sus homologas más acidas. Por tanto, las diferencias en bioactividad observadas con las FSHs recombinantes de lubina podrían estar relacionadas con el tipo y grado de glicosilación que reciben según el sistema de expresión empleado. En este sentido, hay que decir que las células de insecto carecen de la maquinaria enzimática necesaria para añadir oligosacáridos terminales de galactosa y/o acido siálico y producen un tipo glicosilación muy sencilla del tipo "paucimanosa", constituida principalmente por una estructura básica de residuos de manosa. Por el contrario, las células de mamíferos son capaces de añadir oligosacáridos complejos sializados (Kost et al., 2005). Todos estos datos apoyan nuestros resultados, ya que la Sf9-FSH, no sializada, fue más bioactiva que la CHO-FSH. En el caso concreto de la forma de cadena única, CHO-scFSH, su mayor potencia podría estar influenciada o relacionada con otros aspectos como el hecho de ser una proteína de fusión o por la presencia del péptido carboxi-terminal (CTP) de la CG humana, el cual se sabe que incrementa la estabilidad extracelular (Ben-Menahem and Boime, 1996; Fares et al., 1992).

Paralelamente a la producción de díferos recombinantes también se expresaron de forma individual las subunidades FSHβ y LHβ en células Sf9. De las dos subunidades solo Sf9-LHβ se secretó eficientemente al medio de cultivo, mientras que Sf9-FSHβ quedaba retenida en el interior de las células infectadas (ver Fig. 2, pag. 138). Situaciones similares se han descrito en la producción de otras subunidades gonadotropas recombinantes. Por ejem-

plo, al contrario que GPα y hCGβ, las subunidades FSHβ y LHβ humanas, expresadas en células de mamífero (C-127 y CHO), fueron secretadas deficientemente y degradadas lentamente en el interior de las células (Corless et al., 1987a; Corless et al., 1987b; Keene et al., 1989). En células de insecto (Sf21), la GPα bovina se encontró en el medio de cultivo a niveles 10 veces superiores a los de la FSHβ (van de Wiel et al., 1998) y la GPα de pez gato (*Clarias gariepinus*) fue la única de las tres subunidades gonadotropas producida en abundancia como subunidad libre en células de *Drosophila* (S2) (Zmora et al., 2007). La razón de este comportamiento no está clara, pero se sabe que proteínas que no alcanzan su conformación nativa en el retículo endoplasmático rugoso, subunidades huérfanas y algunas proteínas expresadas de forma heteróloga pueden ser retenidas selectivamente y más tarde devueltas al citosol donde son degradadas (Helenius y Aebi, 2001; Kleizen y Braakman, 2004). Sin embargo, generalmente, la coexpresión de ambas subunidades (α y β) lleva a un ensamblaje correcto y a una secreción eficiente (Keene et al., 1989; van de Wiel et al., 1998; Zmora et al., 2007).

Al igual que se hizo con la FSH nativa, también se evaluó la bioactividad y especificidad de las GTHs recombinantes por su capacidad para activar los receptores homólogos (FSHR y LHR) y por su actividad esteroidogénica en cultivos *in vitro* de gónadas de lubina. Todas las GTHs recombinantes producidas activaron específicamente a sus respectivos receptores de manera dosis dependiente, sin mostrar actividad cruzada en un amplio rango de concentraciones (ver Fig. 3, pag. 140). Además, ni la presencia del CTP, incluido en las recombinantes de cadena única, ni la cola de histidinas de los dímeros FSH, influyeron en dicha especificidad de unión al receptor. Como se comento anteriormente, al contrario de lo que ocurre en mamíferos, donde las interacciones ligando-receptor son muy específicas, en la mayoría de especies de peces analizadas se han observado interacciones promiscuas, principalmente con FSHR-LH (Levavi-Sivan et al., 2010). Las bases estructurales y/o significado fisiológico de esta promiscuidad todavía no están claros. Para ampliar nuestro conocimiento acerca de la especificidad de ligandos por parte de los receptores de lubina, se estudió su capacidad de unión a GTHs heterólogas humanas (hFSH y hCG). Curiosamente, el LHR se activó con hCG y hFSH casi con igual potencia, mientras que el FSHR apenas respondió a la hFSH (ver Fig. 3, pag. 140). Este comportamiento de los receptores de lubina con las GTHs humanas fue similar al observado an-

teriormente por Rocha et al. (2007) con las GTHs bovinas. Esta promiscuidad con las GTHs de mamíferos también se ha observado para los LHRs de pez cebra (*Danio rerio*), salmón amago (*Oncorhynchus rhodurus*) y pez gato (*Clarias gariepinus*) (Kwok et al., 2005; Oba et al., 1999a; Vischer y Bogerd, 2003). De acuerdo con lo sugerido por Rocha et al. (2007), en el caso de la lubina, la respuesta promiscua del LHR a las FSHs de mamíferos (hFSH y bFSH) pero no a la FSH de lubina, podría deberse a una mayor similitud entre la estructura tridimensional de FSHs de mamíferos y la LH de lubina que con la propia FSH. Todos estos datos, junto con la débil respuesta de FSHR a la hFSH y al hecho de que en la lubina están ausentes muchos residuos considerados importantes para la unión hFSH/hFSHR de humanos (Fan y Hendrickson, 2005; Rocha et al., 2007), recomiendan cierta precaución a la hora de usar modelos de mamíferos para explicar las interacciones FSH-receptor de peces.

La actividad biológica de las GTHs recombinantes también se analizó por su capacidad esteroidogénica en cultivos *in vitro* de gónadas de lubina. En este caso, todas las GTHs recombinantes fueron capaces de inducir la producción de esteroides en ovario y testículo (ver Fig. 5 y 6, pag. 143-144). Sf9-FSH y Sf9-LH tuvieron un efecto similar en la estimulación de la producción de E2 en el ovario, mientras que en el testículo Sf9-LH fue más potente en estimular la producción de 11-KT. Otros estudios *in vitro* con GTHs nativas y recombinantes de otras especies de peces también han demostrado su capacidad esteroidogénica aunque con diferentes potencias. En la dorada japonesa (*Pagrus major*), como en la lubina, la LH fue más efectiva que la FSH en estimular la liberación de 11-KT (Kagawa et al., 1998b). En salmón coho (*Oncorhynchus kisutch*), la potencia de la LH para estimular la producción de 11-KT supero a la de FSH a medida que avanzaba la espermatogénesis (Planas y Swanson, 1995). Sin embargo, en pez cebra (*Danio rerio*) y en azul o bagre del canal (*Ictalurus punctatus*), la FSH recombinante fue más efectiva que la LH en estimular la liberación de andrógenos (García-López et al., 2010; Zmora et al., 2007). Además, en testículos de pez gato, la cantidad de FSH necesaria para obtener una liberación significativa de esteroides fue menor que la de LH (García-López et al., 2009). Estas diferencias en la respuesta esteroidogénica podrían estar más relacionadas con el estado de desarrollo gonadal y por lo tanto con la presencia del receptor correspondiente (FSHR y/o LHR), que con la propia capacidad esteroidogénica de cada una de ellas. En este sentido, en el pez cebra se ha sugerido que la mayor potencia

observada de la FSH podría deberse a mayores niveles de expresión de FSHR que de LHR (García-López et al., 2010). En nuestro estudio, los cultivos de testículo estaban en espermatogénesis tardía y en este estado gonadal no se han detectado diferencias en la expresión de los receptores de lubina (Rocha et al., 2009). Así, aunque se ha demostrado que en los teleósteos ambas GTHs son esteroidogénicas, serán necesarios más estudios para dilucidar totalmente el papel de cada hormona en cada etapa de la espermatogénesis.

Algunos estudios en peces sugieren que las GTHs recombinantes producidas en células de insecto podrían ser eliminadas rápidamente de la circulación sanguínea (Kazeto et al., 2008; Kobayashi et al., 2010a), aunque no ha sido demostrado ni cuantificado. Por ello, en nuestro caso, antes de realizar experimentos *in vivo* decidimos estudiar la estabilidad en sangre y conocer la farmacocinética de las GTHs recombinantes producidas. Los análisis mostraron que las CHO-scGTHs tenían mayor estabilidad en plasma que las Sf9-GTHs (ver Fig. 7, pag. 147). Una vez más, la presencia o ausencia de ciertos oligosacáridos en su molécula podría estar detrás de dicha estabilidad. Numerosos estudios con GTHs de mamíferos han demostrado que el contenido de ciertos oligosacáridos, mas concretamente de acido siálico, a parte de influir en la bioactividad, pueden ser determinantes en la velocidad con que las glicoproteínas se eliminan de la circulación sanguínea. Así, por ejemplo, a mayor contenido de acido siálico menor potencia pero mayor vida media (Ambao et al., 2009; Olivares et al., 2009; Ulloa-Aguirre et al., 2001). Como se mencionó anteriormente, al contrario que a las células CHO, a las células Sf9 les falta la maquinaria enzimática necesaria para añadir estos oligosacáridos. Además, en el caso de las CHO-scGTHs, la fusión de las dos subunidades y la presencia de oligosacáridos unidos mediante enlaces *O*-glicosídicos al CTP, podrían contribuir a prolongar su vida media en circulación (Ben-Menahem y Boime, 1996; Fares et al., 1992; Klein et al., 2003). En ambos sistemas de expresión (CHO y Sf9), las FSHs producidas presentaron mayor estabilidad que las LHs. Dicha particularidad, junto con otros datos que veremos mas adelante (apartado 3 de esta discusión), nos lleva a pensar en una posible relación entre la estabilidad y el papel fisiológico de estas hormonas.

En general, aunque los resultados obtenidos aquí y en otras especies indican que GTHs heterólogas pueden unirse a los receptores de peces, puede

que sus efectos fisiológicos no reproduzcan exactamente los de las hormonas homologas. Por lo tanto, debido a las dificultades para aislar hormonas nativas, la producción de GTHs recombinantes es una excelente alternativa para disponer de estas hormonas de forma continuada y facilitar la realización de estudios relacionados con la fisiología de las mismas. En este estudio, hemos producido FSHs y LHs de lubina similares a las hormonas nativas que pueden ser usadas en estudios futuros con esta especie. Los dos sistemas de expresión empleados fueron eficaces en la producción de GTHs recombinantes, sin embargo cada sistema ofreció unas ventajas diferentes que hay que tener en cuenta según los objetivos que se pretendan conseguir. En términos de producción, el sistema baculovirus con células Sf9 fue más eficiente que el sistema de células CHO. El primero resulto particularmente útil para producir grandes cantidades de GTHs y sus subunidades, necesarias, por ejemplo, para el desarrollo de diferentes ensayos de detección y determinación (producción de anticuerpos y estándares). Sin embargo, los datos obtenidos *in vivo* mostraron que las GTHs producidas en células de insecto tenían eliminación plasmática más rapida que las producidas en células de mamífero. Así, debido a su mayor estabilidad en sangre, las recombinantes CHO-GTHs y en particular las de cadena única parecen ser mejores candidatas para realizar futuros estudios *in vivo*.

Los trabajos realizados en esta sección han proporcionado las bases y herramientas necesarias para el desarrollo de nuevas metodologías importantes en el estudio de la reproducción de la lubina.

2. Desarrollo de ensayos para la determinación de niveles de FSH

En la presente Tesis Doctoral se han desarrollado diferentes ensayos homólogos (Bioensayo, Dot-blot y ELISA) para determinar niveles de FSH en hipófisis y plasma, imprescindibles para la consecución de los objetivos planteados en la misma.

Básicamente, los métodos para cuantificar moléculas biológicamente activas como las GTHs se agrupan en dos categorías, aquellos que estiman una cantidad o numero de moléculas mediante la unión especifica de anticuerpos (inmunoensayos) y aquellos que determinan una respuesta biológica (bioensayos). Las estimaciones inmunológicas de una hormona, suelen ser altamente precisas, sin embargo no reflejan necesariamente la señal perci-

bida por su correspondiente receptor y por consiguiente su efecto biológico (Christin-Maitre y Bouchard, 1996). Por ello, los bioensayos *in vitro* constituyen una excelente aproximación para determinar aspectos funcionales de las GTHs. En esta Tesis Doctoral se han desarrollado inmunoensayos específicos y un bioensayo que han permitido evaluar conjuntamente tanto cantidad, como bioactividad de la FSH en momentos críticos del ciclo reproductor de la lubina.

Hasta la fecha solo se han logrado desarrollar inmunoensayos homólogos de FSH en tres especies de teleósteos con desarrollo ovárico síncrono, salmón chum (*Oncorhynchus keta*), salmón coho (*Oncorhynchus kisutch*) y trucha arcoiris (*Oncorhynchus mykiss*), y una con desarrollo asíncrono, tilapia (*Oreochromis niloticus*) (Aizen et al., 2007; Govoroun et al., 1998; Suzuki et al., 1988a; Swanson et al., 1989; Swanson et al., 1991). Tradicionalmente los inmunoensayos desarrollados para estudiar los niveles de GTHs de peces han sido radioinmunoensayos (RIAs) o ensayos de inmunoabsorción ligados a enzimas (ELISAs), ambos basados en GTHs nativas y sus respectivos anticuerpos específicos. En nuestro caso usamos FSH y FSHβ recombinantes producidas en diferentes sistemas de expresión. Inicialmente, mediante el sistema de expresión baculovirus, se produjo gran cantidad de FSHβ que se utilizó para generar anticuerpos policlonales específicos. Estos mostraron una fuerte reacción específica bajo condiciones reductoras pero muy débil en condiciones nativas, lo cual condicionó el tipo de inmunoensayo a desarrollar. No hemos encontrado una razón clara que explique la baja afinidad de estos anticuerpos por la hormona en condiciones nativas. No obstante, todo parece indicar que la desnaturalización de la FSHβ durante el segundo paso de purificación pudo haber sido crucial en la generación de unos anticuerpos que reconocían principalmente epítopos internos de la subunidad. Esta circunstancia explicaría las diferencias observadas en la reactividad del antígeno bajo condiciones reductoras o nativas. Debido a la débil reacción de los anticuerpos en condiciones nativas no fue posible desarrollar un ELISA, en su lugar se puso apunto un inmunoensayo tipo "Dot-blot" capaz de usar muestras desnaturalizadas. Este ensayo no presento reacción cruzada con la LH de lubina y mostró bajos CV intra- e interensayo, comparables a los descritos para otros inmunoensayos de FSH en peces (Aizen et al., 2007; Govoroun et al., 1998; Suzuki et al., 1988a). La sensibilidad del ensayo (162.8 ng/ml) fue adecuada para el análisis de niveles hipofisarios de FSH, sin em-

bargo no fue suficiente para determinar niveles en plasma, ya que estos estuvieron siempre por debajo del limite de detección.

La necesidad de disponer de un inmunoensayo capaz de medir FSH en plasma, impulsó la producción de un nuevo lote de FSHβ recombinante para generar nuevos anticuerpos más adecuados. En esta ocasión se uso un sistema de expresión de proteínas recombinantes en levadura (*Pichia pastoris*). Este sistema ha demostrado ser muy eficaz en la producción y secreción de subunidades recombinantes libres (Levavi-Sivan et al., 2010) y se caracteriza por su bajo coste y fácil manejo. Los niveles de producción de FSHβ fueron ligeramente inferiores a los obtenidos previamente en el sistema baculovirus usando células Sf9. Sin embargo, al contrario de este último, la FSHβ producida en levadura se secretó eficientemente al medio de cultivo y pudo ser fácilmente purificada, conservando su conformación nativa. El tamaño de la FSHβ producida fue levemente superior al obtenido con el sistema baculovirus (ver Fig. 3, pag. 199). No obstante, debido a que la secuencia utilizada es la misma, estas diferencias se pueden atribuir al grado de glicosilación recibido, ya que se sabe que *Pichia pastoris* realiza una glicosilación del tipo "alto contenido en manosa" (Grinna y Tschopp, 1989) mientras que las células de insecto del tipo "paucimanosa" (Kost et al., 2005). De nuevo, la FSHβ producida se usó para generar anticuerpos policlonales y esta vez reconocieron específicamente la FSHβ, tanto en su forma desnaturalizada, como en su forma nativa. Los nuevos anticuerpos generados fueron aptos para desarrollar un inmunoensayo tipo ELISA que mostró características óptimas de precisión, especificidad y sensibilidad, comparables a las descritas para otros inmunoensayos de GTHs de peces (ver Tabla 5, pag. 54). Además, la alta precisión (CV intra- e inter-ensayo < 5% y 9%, respectivamente), la ausencia de reacción cruzada con LH y su mayor sensibilidad (0.50 ng/ml), permitió validar el ELISA tanto para muestras de hipófisis como de plasma. Por otro lado, el ELISA fue altamente específico para la FSH de lubina ya que se observó un paralelismo escaso o nulo con diluciones seriadas de muestras de otras especies (ver Fig. 5, pag. 201).

Por último, se desarrolló un bioensayo *in vitro* usando un clon estable de la línea celular HEK 293 que expresa el FSHR de lubina y el gen de la luciferasa (Rocha et al., 2007). Este ensayo mostró una activación del FSHR dosis-dependiente y fue capaz de medir niveles de bioactividad de FSH con un alto grado de sensibilidad (0.104 ng/ml) y especificidad, en concordancia

con otros bioensayos de FSH descritos en mamíferos (Christin-Maitre et al., 2000). Sin embargo, hay que mencionar que las concentraciones calculadas por los inmunoensayos siempre fueron mayores que con el bioensayo. Una posible explicación a este hecho podría estar relacionada con la potencia de la FSH usada como estándar en el bioensayo, ya que en el caso de un estándar mas potente que las muestras daría lugar a niveles más bajos al extrapolar una concentración. Por otro lado, hay que tener en cuenta que los inmunoensayos no distinguen entre dímeros (FSH) y monómeros (FSHβ), mientras que en el bioensayo solo los dímeros (FSH) son bioactivos y por lo tanto cuantificados. En peces, los sistemas de transactivación de los receptores de GTHs se han usado frecuentemente para investigar la especificidad de las interacciones hormona/receptor (Levavi-Sivan et al., 2010). Sin embargo, nuestro bioensayo para FSH es el primero desarrollado y validado en peces que permite medir niveles de actividad FSH en plasma e hipófisis.

En conjunto, los diferentes métodos desarrollados han permitido determinar, por primera vez en la lubina, niveles plasmáticos e hipofisarios de FSH en diversos experimentos y han sentado las bases metodológicas precisas para abordar estudios posteriores.

3. Estudio de perfiles de GnRHs y GTHs durante la diferenciación sexual y ciclo reproductor

La disponibilidad de los ensayos descritos en el apartado anterior permitió analizar los niveles de FSH en hipófisis y en plasma durante la diferenciación sexual, la prepubertad y el ciclo reproductor de la lubina. Además, durante el proceso de diferenciación sexual, se estudiaron los perfiles de expresión génica de las GnRHs, GnRHR y GTHs así como los correspondientes niveles hormonales.

Algunos estudios en mamíferos han demostrado que existe una relativa abundancia de isoformas de GTHs que difieren en su composición glicosídica y cuya presencia esta estrechamente relacionada con el estado funcional de la gónada (Ambao et al., 2009; Christin-Maitre et al., 1996; Ulloa-Aguirre et al., 2003). Estas isoformas muestran diferencias en su estabilidad y biopotencia, provocando un rango de respuestas biológicas tanto *in vitro* como *in vivo* (Ulloa-Aguirre et al., 2003). Partiendo del supuesto de que la

inmunoreactividad es una medida de la cantidad de FSH y que bioactividad proporciona información relativa a la capacidad de provocar una respuesta biológica, la relación de las determinaciones Biológicas/Inmunológicas (B:I) debería proporcionar una evaluación de la calidad media de las moléculas individuales de FSH en una muestra biológica, en un momento dado. Así, el desarrollo y uso combinado de un bioensayo y un inmunoensayo para FSH en el transcurso de la presente Tesis Doctoral, ha permitido evaluar la actividad biológica de cantidades definidas de FSH de acuerdo al estado biológico del animal.

Para estudiar el control endocrino de la diferenciación sexual se crearon dos poblaciones de machos y hembras de lubina mediante selección de tamaños, en base a la existencia de una fuerte relación entre el crecimiento y el fenotipo sexual (Blázquez et al., 1999; Saillant et al., 2003). Los primeros signos histológicos de diferenciación gonadal se observaron alrededor de los 150 días después de la eclosión (dph) en hembras y 200 dph en machos, mientras que a los 250 dph las gónadas estaban completamente diferenciadas en ambos sexos (Papadaki et al., 2005). Los análisis de expresión génica mostraron que en ambas poblaciones había un pico de expresión cerebral de sbGnRH (GnRH-1) y sGnRH (GnRH-3) que coincidía con el inicio de la diferenciación sexual (ver Fig. 1, pag. 76). En el mismo periodo, se detectaron niveles máximos de expresión de FSHβ, que se mantuvieron altos hasta el final del proceso y fueron claramente superiores a los de LHβ (ver Fig. 3, pag. 79). Además, en ambos sexos se observó una fuerte correlación entre la expresión cerebral de sbGnRH y el receptor hipofisario dlGnRH-R-2A (actualmente denominado dlGnRHRII-1a), y de este último con la expresión hipofisaria de FSHβ y LHβ, sugiriendo su participación en la diferenciación sexual. Al mismo tiempo, los análisis neurohormonales mostraron un contenido alto de GnRHs en la hipófisis antes o al inicio de la diferenciación sexual, siendo el de sbGnRH mayor que el de sGnRH, y en ambos casos mayor en hembras (ver Fig. 2, pag. 77). Estos resultados concuerdan con el hecho de que sbGnRH es considerada la principal forma hipofisiotrófica implicada en la secreción de GTHs en la lubina (González-Martínez et al., 2002a; González-Martínez et al., 2002b). En cuanto a las GTHs, los análisis de FSH mostraron que había una fuerte bioactividad de FSH en plasma al inicio de la diferenciación y una acumulación progresiva de FSH en hipófisis (ver Fig. 5, pag. 173). Sin embargo, como ya insinuaba la actividad transcripcional

de LHβ, los niveles plasmáticos de LH fueron muy bajos durante de la diferenciación sexual, mostrando únicamente una elevación discreta a los 250 dph, cuando las gónadas ya estaban completamente diferenciadas (ver Fig. 4, pag. 81). Todos estos resultados refuerzan la hipótesis de que la FSH tiene un papel importante durante la diferenciación sexual y que su secreción esta regulada principalmente por sbGnRH y en menor medida por sGnRH a través de la activación del dlGnRH-R-2A. Por otra parte, cabe señalar que los análisis inmunohistoquímicos realizados en hipófisis de lubina adultas (ver Fig. 7, pag. 176) revelaron que, como en otras especies de teleósteos (Calman et al., 2001; García-Ayala et al., 1998; Kagawa et al., 1998a; Miranda et al., 2001; Nozaki et al., 1990), la FSH y LH se sintetizan en diferentes poblaciones de células de la adenohipófisis. En este sentido, en estudios previos con trucha arcoiris (*Oncorhynchus mykiss*), platy (*Xiphophorus maculatus*) y acará (*Cichlasoma dimerus*) se ha descrito que las células gonadotropas de FSH aparecen antes del inicio de la diferenciación sexual, mientras que las de LH surgen varios días después de que haya empezado el proceso o justo al finalizar (Pandolfi et al., 2006; Feist y Schreck, 1996; Magliulo-Cepriano et al., 1994; Saga et al., 1993). En conjunto, todos estos datos apoyan una posible acción de la FSH durante la diferenciación gonadal de peces teleósteos. Además cabe destacar que en la lubina los niveles plasmáticos e hipofisarios de FSH y los ratios B:I calculados en hipófisis, fueron más altos en hembras que en machos, lo que sugiere un dimorfismo sexual en la síntesis y potencia de la FSH durante la diferenciación sexual. Este hecho esta además apoyado por los mayores niveles hipofisarios de sbGnRH y sGnRH detectados en hembras. Al mismo tiempo, en estudios paralelos con las mismas poblaciones y en el mismo periodo (Blázquez y Piferrer, 2004; Piferrer et al., 2005), se observó que en las hembras los niveles de expresión de la enzima citocromo P450 aromatasa en gónada (CYP19A1) y en cerebro (CYP19A2) eran mayores que en los machos, sugiriendo que los altos niveles de FSH vistos en este estudio podrían estar involucrados en el control de su expresión.

Transcurrido el periodo de diferenciación sexual, las gónadas diferenciadas (ovarios o testículos) permanecen inmaduras (1 o 2 años) hasta la llegada de la pubertad. Muy a menudo bajo condiciones de cultivo intensivo aparecen machos precoces que adelantan la primera maduración sexual. La pubertad prematura en la lubina es un gran problema que causa importantes perdidas al sector acuícola ya que los peces precoces experimentan un re-

ducción del crecimiento y son más vulnerables a enfermedades (Taranger et al., 2010). Con el fin de evitar el inicio prematuro de la pubertad, en nuestro grupo se han venido realizando en los últimos años numerosas experiencias (Begtashi et al., 2004; Felip et al., 2008; Rodríguez et al., 2005; Rodríguez et al., 2001) que han demostrado la eficacia de determinados tratamientos de luz para reducir el porcentaje de machos precoces. Sin embargo, se necesitan más estudios para comprender los mecanismos subyacentes de esta inhibición, en particular en lo que respecta a las GTHs. En el transcurso de la presente Tesis Doctoral se analizaron los perfiles de FSH en machos de lubina prepúberes sometidos a diferentes condiciones de fotoperiodo (durante 10 meses) para averiguar como afecta la luz en la reducción de la precocidad (ver Fig. 7, pag. 203). En machos juveniles inmaduros sometidos a fotoperiodo natural, los niveles plasmáticos de FSH aumentaron gradualmente, alcanzando máximos en Marzo, al final del experimento. En ese momento el porcentaje de machos espermiantes (precoces) fue del 65%. Sin embargo, el tratamiento de luz continua (24h/día) mantuvo los niveles de FSH invariables a lo largo del periodo experimental, mostrando una reducción significativa respecto el grupo control, tanto de los niveles de FSH en Marzo, como del número de machos precoces que no supero el 10%. Además, el análisis del contenido de FSH en hipófisis (datos no mostrados) presento los mismos perfiles, indicando que el efecto inhibitorio de la luz continua sobre la FSH era a nivel de síntesis y no de liberación. Estos resultados apoyan estudios previos con machos juveniles de lubina donde se vio que la luz continua producía una reducción significativa de la expresión génica de las subunidades gonadotropas, de los niveles plasmáticos de 11-KT y del crecimiento testicular (Felip et al., 2008; Rodríguez et al., 2005). Por otro lado, análisis histológicos de testículos de peces tratados con luz continua han mostrado que existe una fuerte reducción de la proliferación espermatogonial (Carrillo, M., Felip, A., Molés, G., Yilmaz, O., Zanuy, S., en consideración). Puesto que, la 11-KT es un andrógeno clave en la iniciación de la proliferación espermatogonial (Schulz et al., 2010) y dado el efecto estimulador de la FSH de lubina en la producción de 11-KT en cultivos *in vitro* de fragmentos de testículo (Molés et al., 2008, Molés et al., 2011a o presente Tesis Doctoral), todo parece indicar que el tratamiento de luz continua reduce la precocidad influyendo negativamente sobre la síntesis de FSH, lo que causa una disminución de los niveles de 11-KT y como consecuencia una reducción de la proliferación espermatogonial y del crecimiento testicular.

Por último, se estudió el papel de las GTHs durante el la gametogénesis. Para ello se analizaron los perfiles de FSH en machos y hembras adultas a lo largo de un ciclo reproductor. Anteriormente Rocha et al. (2009) habían estudiado los perfiles plasmáticos de LH y esteroides sexuales (11-KT y E2) en los mismos animales.

En machos, los niveles de FSH en plasma incrementaron considerablemente durante la recrudescencia testicular (estados II a IV) y disminuyeron justo antes de la espermiación (estado V) (ver Fig. 6, pag. 202). El perfil de FSH en plasma fue idéntico al observado anteriormente para la 11-KT (Rocha et al., 2009), resaltando la importancia de la FSH en la regulación de las fases tempranas-medias de la espermatogénesis pero no en espermiogénesis y espermiación. Por el contrario, el perfil de LH mostró un ligero incremento durante el crecimiento testicular, con niveles muy inferiores a los de FSH, que se mantuvieron hasta la espermiación (Rocha et al., 2009). En machos de trucha arcoiris se observo algo parecido, en estos, los niveles plasmáticos de FSH fueron más altos que los de LH durante todas las fases de la espermatogénesis, con máximos niveles de FSH detectados durante la fase de crecimiento testicular medio y tardío (Prat et al., 1996). Por el contrario, los niveles de LH en plasma, en la misma especie, no se detectaron durante la mayor parte del proceso (< 0,3 ng/ml) y únicamente aumentaron durante la espermiación (Prat et al., 1996). Por tanto, al igual que en salmónidos, se puede afirmar que en machos de lubina la FSH tiene un papel claro en la iniciación y mantenimiento de la espermatogénesis, mediado por la 11-KT, mientras que la LH estaría más implicada en las fases de espermiogénesis y espermiación.

En hembras, los perfiles hipofisarios de FSH y de LH fueron idénticos (ver Fig. 6, pag. 174). Los niveles de ambas hormonas fueron significativamente altos durante la vitelogénesis tardía, post-vitelogénesis y maduración-ovulación, con una tendencia a disminuir en el estado de atresia, al final de la temporada reproductiva. En plasma, se observaron niveles máximos de FSH durante todo el proceso de vitelogénesis y post-vitelogénesis y mínimos significativos en maduración-ovulación (ver Fig. 6, pag. 202). Estos resultados indican una activa síntesis y secreción de FSH en vitelogénesis, y una posterior acumulación en hipófisis durante la maduración-ovulación. Al mismo tiempo, los niveles máximos de bioactividad de FSH en plasma se observaron en vitelogénesis tardía y post-vitelogénesis, disminuyendo después de forma gradual hasta el estado de atresia (ver Fig. 6, pag. 174). Por el contra-

rio, los niveles máximos de LH en plasma se observaron en maduración-ovulación (Rocha et al., 2009), aunque su concentración fue siempre muy inferior a la de FSH durante todo el proceso de oogénesis. Los resultados obtenidos sugieren una síntesis y acumulación semejante de FSH y LH en la hipófisis, pero un control distinto de la secreción al torrente sanguíneo. Los perfiles plasmáticos de GTHs obtenidos en hembras de lubina son similares a los observados en algunas especies con desarrollo ovárico síncrono, como el salmón coho, donde Swanson (1991) describió que la concentración de FSH en plasma aumentaba durante la vitelogénesis, con máximos niveles durante la fase media-tardía de la misma, y disminuía a medida que la ovulación se aproximaba. De forma similar en trucha arcoiris, se describió un incremento significativo de los niveles plasmáticos de FSH en el inicio de la vitelogénesis (Prat et al., 1996), manteniéndose elevados durante la misma y disminuyendo antes de la maduración (Breton et al., 1998). Por el contrario, los niveles de LH alcanzaron máximos en maduración-ovulación (Breton et al., 1998; Prat et al., 1996). Por otro lado, en hembras de lubina se ha observado niveles máximos de expresión de la enzima P450 aromatasa (CYP19A1) y de E2 plasmático (Rocha et al., 2009) durante la vitelogénesis tardía y la post-vitelogénesis, los cuales coincidieron con los máximos plasmáticos de FSH detectados en la presente estudio. Como se comento anteriormente, la enzima CYP19A1 cataliza la conversión de T a E2 y se ha demostrado que la FSH estimula su expresión en folículos vitelogénicos de trucha (Montserrat et al., 2004). Además, varios estudios, incluida esta Tesis Doctoral, han demostrado que el tratamiento con FSH de explantes ováricos o folículos vitelogénicos aislados, aumenta la producción *in vitro* de E2 (Molés et al., 2008; Molés et al., 2011a; Montserrat et al., 2004; Planas et al., 2000; Suzuki et al., 1988c). En hembras, el E2 estimula la síntesis hepática de vitelogenina, la cual es incorporada progresivamente por los oocitos en crecimiento (Mañanós et al., 1994; Nagahama, 1994). Por lo tanto, de acuerdo con los resultados obtenidos y con la bibliografía existente, se desprende que en la lubina, al igual que en otras especies de teleósteos analizadas, la FSH regula la vitelogénesis mediante la producción ovárica de E2 a través de la activación de la enzima CYP19A1, mientras que la LH sería responsable de los eventos finales de la oogénesis, como son la maduración de los oocitos y la ovulación.

Por otra parte, los ratios B:I de FSH calculados en hipófisis y plasma de hembras adultas durante la gametogénesis fueron menores que los calcula-

dos en hipófisis durante la diferenciación sexual. En plasma se observo un claro incremento del ratio B:I durante la vitelogénesis tardía y post-vitelogénesis, mientras que en hipófisis disminuyo progresivamente a medida que la oogénesis avanzaba (Tabla 1). Este hecho podría indicar la presencia de isoformas de FSH con diferentes potencias asociadas a el estado reproductivo del animal. En mamíferos se ha sugerido que la microheterogeneidad molecular de FSH en hipófisis y plasma puede cambiar dependiendo de la edad, desarrollo sexual y/o el medio esteroidogénico (Ambao et al., 2009; Rulli et al., 1999; Zariñan et al., 2001). Por ejemplo, en roedores y humanos se ha observado un aumento de formas de FSH menos sializadas (más potentes) durante la fase preovulatoria (Ulloa-Aguirre et al., 1999). Teniendo en cuenta todo esto, nuestros resultados sugieren la presencia de diferentes isoformas de FSH según el estado desarrollo del animal y se podría hipotetizar que durante la diferenciación sexual, serian necesarias isoformas de FSH más potentes pero probablemente menos duraderas en plasma que las presentes durante la oogénesis.

Tabla 1. Ratios de determinación Biológica e Inmunologica (B:I).

		Diferenciación sexual (dph)				Ciclo reproductor (estado)				
		150	200	250	300	Prevtg	evtg	lvtg-pvtg	mad-ovul	atre
Hipófisis	Machos	0,08	0,14	0,22	0,29					
	Hembras	0,01	0,39	0,34	0,39	0,33	0,17	0,13	0,11	0,09
Plasma	Hembras					0,001	0,04	0,12	0,07	0,02

Nota: Para poder comparar mejor los ratios obtenidos en diferentes experimentos, estos se han calculado a partir de las concentraciones obtenidas en ng/ml.

Por último, cabe señalar que en general los niveles de LH en plasma fueron siempre inferiores a los de FSH, cuyos valores basales estuvieron alrededor de 15 ng/ml, tanto en machos como en hembras. Hecho que podría estar relacionado con la diferente estabilidad de ambas GTHs, ya que los análisis farmacocinéticos realizados en la presente Tesis Doctoral (Molés et al 2011a) y en otros estudios con mamíferos (Campbell, 2005), muestran una mayor estabilidad en sangre para FSH que para LH y consecuentemente, un mayor ratio de eliminación plasmática para LH. Además, durante el proceso de purificación de GTHs nativas de lubina quedó patente que la LH se disocia más fácilmente que la FSH. Todos estos datos sugieren que en la lubina

podrían ser necesarios altos niveles de FSH en plasma para la estimulación a largo plazo del crecimiento gonadal, mientras que elevaciones puntuales de los niveles de LH podrían tener un efecto más específico en ciertas fases del ciclo reproductor que requerirían de una rápida eliminación.

En resumen, los resultados obtenidos muestran que la FSH tiene un papel clave durante la diferenciación sexual de la lubina, y sugieren un dimorfismo sexual en la síntesis y potencia de la misma. En machos juveniles, los tratamientos de luz continua reducen significativamente los niveles de FSH y, probablemente como consecuencia, la maduración sexual precoz. Por ultimo, los análisis en lubinas adultas muestran que la FSH tiene un papel importante durante la recrudescencia testicular y el crecimiento secundario del oocito (vitelogénesis), mientras que la LH seria responsable de eventos finales como espermiogénesis-espermiación en machos y maduración-ovulación en hembras. Para terminar, cabe decir que los ensayos desarrollados han permitido obtener una visión más amplia y exacta de la función de la FSH en la lubina, contribuyendo al esclarecimiento de la dualidad funcional de las GTHs. Ademas, dichos ensayos representan una valiosa herramienta para futuros estudios en la endocrinología reproductiva de esta especie.

Capítulo 9. CONCLUSIONES

Conclusiones

Primera: Tanto la FSH nativa de lubina, como las GTHs recombinantes producidas en este estudio, activan a sus respectivos receptores de manera específica y dosis dependiente, sin mostrar actividad cruzada en un amplio rango de concentraciones. Además, son capaces de inducir la síntesis de esteroides sexuales en cultivos *in vitro* de ovario y de testículo. En el caso de la FSH, esta estimulación es mayor en machos en recrudescencia testicular y en hembras vitelogénicas.

Segunda: El sistema de expresión baculovirus, en células Sf9, es más eficiente en términos de producción que las transfecciones estables de células CHO y produce formas diméricas de FSH menos glicosiladas pero más bioactivas. Por el contrario, las células CHO producen formas recombinantes con glicosilaciones complejas que les confiere mayor estabilidad en sangre. A su vez, dentro de cada sistema, la estabilidad en sangre de las FSHs es mayor que la de las LHs.

Tercera: Los ensayos desarrollados para medir FSH han permitido determinar, por primera vez en lubinas de ambos sexos, los niveles plasmáticos e hipofisarios de esta hormona en diversos momentos de su proceso reproductor y bajo diferentes condiciones experimentales.

Cuarta: Los perfiles hormonales obtenidos durante la diferenciación sexual sugieren que la FSH tiene un papel importante en este proceso y que sbGnRH, y en menor medida sGnRH, regula su síntesis y secreción a través de la activación del dlGnRH-R-2A (actualmente denominado dlGnRHRII-1a). Además, los diferentes niveles de FSH y los ratios B:I en machos y hembras sugiere un dimorfismo sexual en la síntesis y potencia de la FSH.

Quinta: Los análisis inmunohistoquímicos en hipófisis de lubinas adultas muestran que, como en otras especies de teleósteos, la FSH y la LH se sintetizan en diferentes poblaciones de células de la adenohipófisis.

Sexta: El tratamiento de machos juveniles de lubina con luz continua reduce los niveles de FSH en plasma. Esto previene la pubertad precoz de los mismos, probablemente como consecuencia de una disminución de la proliferación espermatogonial y del crecimiento testicular.

Séptima: Los perfiles de GTHs en lubinas adultas sugieren una síntesis y acumulación similar de FSH y LH en hipófisis, pero un control distinto de su secreción al torrente sanguíneo. Además, dichos perfiles indican que la FSH tiene un papel clave en la iniciación y mantenimiento de la espermatogénesis y en la vitelogénesis, mientras que la LH parece estar involucrada en las fases de espermiogénesis, espermiación, maduración de los oocitos y ovulación.

Octava: La mayor cantidad y estabilidad de la FSH en sangre durante el ciclo reproductor sugiere que esta podría ser necesaria para una estimulación a largo plazo del crecimiento gonadal, mientras que en el caso de la LH solo se requerirían elevaciones puntuales para un efecto más específico en las fases finales de la gametogénesis.

Novena: Los diferentes ratios B:I de FSH calculados en hipófisis y plasma sugieren la presencia de isoformas con diferentes potencias según el estado desarrollo del animal. Siendo en hipófisis, aparentemente más potentes durante la diferenciación sexual que durante la oogénesis, y dentro de esta última, en plasma, más potente durante la vitelogénesis tardía y postvitelogénesis.

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RESUMENES

Resumen en castellano

La reproducción es un proceso biológico de gran interés en acuicultura debido a que su control es un factor limitante para la producción de peces en cautividad. El éxito reproductivo de una especie en régimen de cultivo depende de un profundo conocimiento de los procesos básicos que regulan la puesta en funcionamiento del ciclo reproductor y su alternancia. En peces, como en otros vertebrados, la reproducción esta regulada por una cascada hormonal que ocurre a lo largo de una red neuro-endocrina compuesta por el cerebro, la hipófisis y las gónadas (eje CHG). Entre las principales hormonas implicadas destacan las hormonas liberadoras de gonadotrofinas (GnRHs), las gonadotrofinas (GTHs) y los esteroides sexuales. En este marco, las GTHs - hormona folículo estimulante (FSH) y hormona luteinizante (LH) - tienen un papel central y el conocimiento de su estructura y modo de acción es esencial para comprender y controlar el ciclo reproductor. Los estudios realizados en los últimos años han permitido dilucidar algunos aspectos bioquímicos y fisiológicos de estas hormonas en peces. Sin embargo, la dualidad funcional de las GTHs no esta totalmente clara en muchas especies de teleósteos debido a la falta de herramientas apropiadas para su estudio, principalmente de ensayos para determinar sus niveles en momentos críticos del ciclo reproductor.

Para abordar este problema en la lubina (*Dicentrarchus labrax*), un Perciforme marino de gran interés en acuicultura, se ha purificado la FSH nativa y se han producido diferentes GTHs recombinantes. Esto ha permitido caracterizarlas bioquímica y funcionalmente y desarrollar ensayos específicos (biológicos e inmunológicos) para medir FSH, los cuales representan una valiosa herramienta para el estudio de la endocrinología reproductiva de esta especie. Además, se han puesto a punto herramientas moleculares para estudiar los perfiles de expresión génica de las GnRHs, GnRHR y GTHs. Todas estas herramientas, junto con otras disponibles en nuestro laboratorio han permitido investigar la función de las GTHs durante la diferenciación sexual y la gametogénesis de la lubina.

Los resultados obtenidos muestran que ambas GTHs activan específicamente sus respectivos receptores, sin mostrar actividad cruzada en un amplio rango de concentraciones, y son capaces de inducir la síntesis de esteroides sexuales en cultivos *in vitro*. Los perfiles hormonales y de ex-

presión génica obtenidos durante la diferenciación sexual sugieren un papel clave para la FSH, cuya síntesis y potencia presenta cierto dimorfismo sexual y parece estar regulada principalmente por sbGnRH. Además, los análisis inmunohistoquímicos en hipófisis muestran que la FSH y la LH se sintetizan en células de la adenohipófisis diferentes. Por ultimo, los perfiles hormonales obtenidos en lubinas prepúberes y adultas sugieren que la FSH esta implicada en la iniciación y mantenimiento de la espermatogénesis y la vitelogénesis. Por el contrario, la LH parece estar involucrada en las fases finales de la gametogénesis como la espermiogénesis, espermiación, maduración y ovulación.

Resum en valencià

La reproducció és un procés biològic de gran interès en aqüicultura ja que, el seu control és un factor limitant per a la producció de peixos en captivitat. L'èxit reproductiu d'una espècie en règim de cultiu depèn d'un profund coneixement dels processos bàsics que regulen la posada en funcionament del cicle reproductor i la seva alternança. En peixos, com en altres vertebrats, la reproducció està regulada per una cascada hormonal que passa al llarg d'una xarxa neuro-endocrina composta pel cervell, la hipòfisi i les gònades (eix CHG). Entre les principals hormones implicades destaquen les hormones alliberadores de gonadotrofines (GnRHs), les gonadotrofines (GTHs) i els esteroides sexuals. En aquest marc, les GTHs - hormona fol·licle estimulant (FSH) i hormona luteïnitzant (LH) - tenen un paper central i el coneixement de la seva estructura i manera d'acció és essencial per a comprendre i controlar el cicle reproductor. Els estudis realitzats en els últims anys han permès dilucidar alguns aspectes bioquímics i fisiològics d'aquestes hormones en peixos. No obstant això, la dualitat funcional de les GTHs no està totalment clara en moltes espècies de teleostis a causa de la falta d'eines apropiades per al seu estudi, principalment d'assaigs per determinar els seus nivells en moments crítics del cicle reproductor.

Per abordar aquest problema en el llobarro (*Dicentrarchus labrax*), un Perciforme marí de gran interès en aqüicultura, s'ha purificat la FSH nativa i s'han produït diferents GTHs recombinants. Això ha permès caracteritzar bioquímica i funcionalment i desenvolupar assaigs específics (biològics i immunològics) per mesurar FSH, els quals representen una valuosa eina per al'estudi de l'endocrinologia reproductiva d'aquesta espècie. A més, s'han posat a punt eines moleculars per estudiar els perfils d'expressió gènica de les GnRHs, GnRHR i GTHs. Totes aquestes eines, juntament amb altres disponibles al nostre laboratori han permès investigar la funció de les GTHs durant la diferenciació sexual i la gametogènesi del llobarro.

Els resultats obtinguts mostren que les dues GTHs activen específicament els seus respectius receptors, sense mostrar activitat creuada en un ampli rang de concentracions, i són capaços d'induir la síntesi d'esteroides sexuals en cultius *in vitro*. Els perfils hormonals i d'expressió gènica obtinguts durant la diferenciació sexual suggereixen un paper clau per a la FSH, la síntesi i potència de la qual presenta cert dimorfisme sexual i sembla

estar regulada principalment per sbGnRH. A més, els anàlisis immunohistoquímics en hipòfisi mostren que la FSH i la LH es sintetitzen en cèl·lules de la adenohipòfisi diferents. Finalment, els perfils hormonals obtinguts en llobarros prepúbers i adults suggereixen que la FSH esta implicada en la iniciació i manteniment de l'espermatogènesi i la vitelogénesis. Per contra, la LH sembla estar involucrada en les fases finals de la gametogènesi com l'espermiogènesi, espermiació, maduració i ovulació.

English summary

Reproduction is a biological process of great interest for aquaculture since its control is a limiting factor for production of fish in captivity. The reproductive success of a species under confinement depends on a deep understanding of the basic processes that govern the initiation of puberty and maintenance of reproductive cyclicity. In fish, as in other vertebrates, reproduction is regulated by a hormonal cascade that occurs over a neuroendocrine network composed of the brain, the pituitary and the gonads (BPG axis). The main hormones involved include the gonadotropin-releasing hormone (GnRHs), gonadotropins (GTHs) and sex steroids. In this context, GTHs - follicle stimulating hormone (FSH) and luteinizing hormone (LH) - have a central role. Knowledge of its structure and mode of action is essential to understand and control the reproductive cycle. Studies in recent years have allowed elucidating biochemical and physiological aspects of these hormones in fish. However, the functional duality of GTHs is not entirely clear in many teleost species due to the lack of appropriate tools for their study, primarily assays to determine their levels at critical times of the reproductive cycle.

To address this problem in the sea bass (*Dicentrarchus labrax*), a marine Perciform of great interest for aquaculture, native FSH has been purified and different recombinant GTHs have been produced. This has allowed to characterize, biochemically and functionally, the sea bass GTHs and to develop specific assays (biological and immunological) to measure FSH, which represents a valuable tool for studying the reproductive endocrinology of this species. In addition, molecular tools have also been developed to study gene expression profiles of GnRHs, GnRHR and GTHs. These tools, along with others already available in our laboratory, have allowed investigating the role of GTHs during sexual differentiation and gametogenesis in the sea bass.

The results show that both GTHs specifically activate their respective receptors, showing no cross-reactivity in a wide range of concentrations, and that are able to induce the synthesis of sex steroids *in vitro*. During sexual differentiation, hormonal profiles and gene expression levels suggest a key role for FSH. Furthermore, the synthesis and biopotency of this hormone presents a sexual dimorphism and appears to be regulated primarily by

sbGnRH. In addition, immunohistochemical analysis indicates that in the pituitary, FSH and LH are synthesized by different adenohypophysis cells. Finally, hormonal profiles obtained from prepubertal and adult sea bass suggest that FSH is involved in the initiation and maintenance of spermatogenesis and vitellogenesis. On the contrary, the LH appears to be involved in the final stages of gametogenesis such as spermiogenesis, spermiation, maturation and ovulation.