Seasonal changes in gonadal expression of gonadotropin receptors, steroidogenic acute regulatory protein and steroidogenic enzymes in the European sea bass

Ana Rocha, Silvia Zanuy, Manuel Carrillo*, Ana Gómez

Department of Fish Physiology and Biotechnology, Instituto de Acuicultura de Torre la Sal, Consejo Superior de Investigaciones Científicas (CSIC), Torre la Sal, 12595 Ribera de Cabanes, Castellón, Spain.

*Address for correspondence: Manuel Carrillo

Instituto de Acuicultura de Torre la Sal.

Ribera de Cabanes s/n. 12595 Torre la Sal. Castellón. Spain.
Phone: +34 964 319500. Fax: +34 964 319509

e-mail: carrillo@iats.csic.es
Abstract

The endocrine regulation of gametogenesis, and particularly the roles of gonadotropins, is still poorly understood in teleost fish. This study aimed to investigate transcript levels of both gonadotropin receptors (FSHR and LHR) during an entire reproductive cycle in male and female sea bass (Dicentrarchus labrax). To have a more comprehensive understanding of how different key factors interact to control sea bass gonadal function, changes in the transcript abundance of two important steroidogenic enzymes, P450 11β-hydroxylase (CYP11B1) and P450 aromatase (CYP19A1), and the steroidogenic acute regulatory protein (StAR), were also studied. These expression profiles were analysed in relation to changes in the plasma levels of important reproductive hormones and histological data. Expression of the FSHR was connected with early stages of gonadal development, but also with the spermiation/maturation-ovulation periods. The expression profile of the LHR seen in both sexes supports the involvement of LH in the regulation of the final stages of gamete maturation and spermiation/ovulation. In both sexes StAR expression was strongly correlated with LHR expression. In females high magnitude increments of StAR expression levels were observed during the maturation-ovulation stage. In males, gonadotropin receptors and CYP11B1 mRNA levels were found to be correlated. In females, the expression profiles of FSHR and CYP19A1 and the changes in plasma estradiol (E2) indicate that the follicular production of E2 could be under control of FSH through the regulation of aromatase expression. This study supports the idea that FSH and LH may have different roles in the control of sea bass gonadal function.

Keywords: Follicle-stimulating hormone receptor; luteinizing hormone receptor; gametogenesis; P450 11β-hydroxylase; P450 aromatase; teleost fish.
**Introduction**

In teleost fish, as in mammals, gametogenesis is regulated by the interplay of systemic and intragonadal factors and the importance of each type of regulation varies depending on the developmental stage of the gonad (Patiño and Sullivan, 2002; Schulz and Miura, 2002). The pituitary-derived gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are primary mediators of gonadal steroidogenesis and gametogenesis. They bind and activate specific receptors (FSH receptor (FSHR) and LH receptor (LHR)), present on the surface of gonadal somatic cells, regulating the expression and activity of key steroidogenic enzymes (Themmen and Huhtaniemi, 2000). Although deeply studied in mammals, the precise function of each gonadotropin in teleosts is still largely unknown (Swanson et al., 2003). In the salmonid model, complementary functions of the gonadotropins were suggested by assessment of their transcript and plasma levels. FSH is considered to be involved in the initiation and early stages of gametogenesis, such as vitellogenesis and spermatogenesis, to some extent through the synthesis of estradiol-17β (E2) and 11-ketotestosterone (11-KT), respectively. LH is linked to final maturation and ovulation/spermiation, in part by stimulating the production of maturation inducing hormones (MIHs, the progestins 17α,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS)) (Nagahama, 1994; Swanson et al., 2003).

Synthesis of steroids involves a complex cascade of oxidative enzymes that convert cholesterol into different functional steroids. The cytochrome P450 11β-hydroxylase, encoded by the *CYP11B1* gene is necessary for the final steps of the synthesis of 11-KT (Jiang et al. 1996) whereas cytochrome P450 aromatase (P450arom, encoded by the *CYP19A1* gene), catalyzes the conversion of testosterone (T) to estradiol (E2) (Simpson et al. 1994). The cDNAs encoding these cytochromes have been cloned and characterized in several fish species including the sea bass (*Dicentrarchus labrax* L.) (Socorro et al., 2007; Dalla Valle et
Nevertheless, their expression during gametogenesis of this Perciform has not yet been investigated.

In teleosts, final gamete maturation is initiated by a rapid shift from the synthesis of androgen/estrogen to the synthesis of MIHs (Nakahama, 1994). This steroidogenic shift is typically accompanied by an increase in steroid synthesis. Biosynthesis of steroid hormones has an acute and a chronic hormonal regulation. Whereas chronic, long-term regulation of steroidogenic capacity involves increased transcription/translation of the genes encoding steroidogenic enzymes, the acute regulation of steroidogenesis depends on cholesterol transport into the mitochondria (Miller, 1988; Stocco and Clark, 1996). In mammals, it has been proven that this transport is mediated by the steroidogenic acute regulatory (StAR) protein (Manna and Stocco, 2005). In addition there is evidence of a positive regulation of StAR expression by tropic hormones such as FSH and LH in granulosa cells (Balasubramanian et al., 1997; Sekar et al., 2000) and by LH in Leydig cells (Manna et al., 1999).

As mentioned above, most of the available information regarding physiological aspects of fish gonadotropins refers to salmonid species whose germ cells develop in a synchronous fashion. The fish species selected for this study is the European sea bass that presents a group-synchronous type of ovarian development (successive clutches of germ cells that will mature and be spawned are recruited from a population of vitellogenic oocytes), producing 3-4 consecutive spawns during a 1-2 months spawning period that is repeated once a year during the winter (Asturiano et al., 2000). It is then difficult the extrapolation of salmonid findings to sea bass (or other fish with a non-synchronous type of gonadal development). Contrary to what was described for salmonids (reviewed in Swanson et al., 2003), the expression of the gonadotropin subunits during the reproductive cycle of male sea bass shows overlapping profiles, suggesting that both hormones could be involved in the control of all stages of gonadal development (Mateos et al., 2003).
Recently, we have described the molecular characterization of sea bass gonadotropin receptors (Rocha et al., 2007a). In the present study, we aimed to investigate their temporal expression patterns during an entire reproductive cycle in both male and female sea bass; To have a more holistic understanding of how different key factors interact to control sea bass gonadal function, changes in the expression of \textit{CYP11B1}, \textit{CYP19A1} and \textit{StAR} genes were also evaluated in relation with sex steroid and LH plasma titers as well as gonadal development.

**Materials and Methods**

*Animals and sample collection*

Male and female sea bass (\textit{Dicentrarchus labrax}) were obtained from the stock raised at the Instituto de Acuicultura de Torre la Sal (Castellón, Spain, 40ºN) facilities. They were sampled monthly during their first sexual maturation period (puberty), which generally occurs during the second year of life in males and in the third year of life in females. At each sampling point, 5 fish of each sex were anesthetized, weighed, sized and sacrificed in accordance with the Spanish legislation concerning the protection of animals used for experimentation or other scientific purposes. Blood was collected via the caudal vein using heparinized syringes, centrifuged at 2500 x g for 25 min at 4ºC and the obtained plasma was stored at -20ºC until analysis. Gonads were dissected, weighed and one portion was flash frozen in liquid nitrogen and stored at -70ºC. The other portion was fixed by immersion in 4% formaldehyde : 1% glutaraldehyde (McDowell and Trump 1976), embedded in 2-hydroxyethyl methacrylate polymer resin (Technovit 7100, Heraeus Kultzer, Germany), sectioned (3µm) and stained according to Bennett and colleagues (Bennett et al., 1976) for histological analysis. The stages of testicular development were classified by light microscopy, following previously established criteria (Begtashi et al., 2004): stage I, the immature stage; stage II, early recrudescence; stage III, mid recrudescence; stage IV, late recrudescence; stage V, full
spermiating testes and stage VI, post-spawning. The ovarian stages were as follows:

previtellogenesis (prevtg); early vitellogenesis (evtg); late-vitellogenesis and post-vitellogenesis (lat-postvtg); maturation-ovulation (mat-ovul) and atresia (atre) (Asturiano et al., 2000). Representative sections showing the different gonadal developmental stages of the animals used in this work can be found in Rocha et al. (2007b). Gonadosomatic index (GSI) was determined by the following formula: gonad weight/ body weight × 100.

**Hormone analysis**

Plasma E2 was measured by a conventional enzyme immunoassay (EIA), validated for its use on the sea bass in our laboratory (B Crespo, JM Navas, A Rocha, S Zanuy, M Carrillo, unpublished). The assay uses a rabbit antiserum against E2 whose specificity is shown in (Prat et al., 1990). The EIA protocol was similar to that previously developed for testosterone determination (Rodriguez et al., 2000a). Briefly, plasma was extracted with methanol. The organic solvent was evaporated and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA). 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 37ºC. Then, plates were rinsed and colour development was performed by addition of Ellman’s reagent and incubation for 2 h at 20ºC 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 around 0.156 ng/ml (Bi/B0 = 90%).

The plasma levels of 11-KT were determined by an EIA developed for the Siberian sturgeon (Cuisset et al., 1994) and modified for its use in sea bass (Rodriguez et al., 2005). The assay sensitivity of 11-KT was 0.0012 ng/ml. Plasma LH levels were measured by a homologous
competitive ELISA according to (Mateos et al., 2006). The sensitivity of the assay was 0.65 ng/ml.

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

Sea bass total RNA was isolated from head kidney using the TRI Reagent (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer’s instructions. For cDNA synthesis, 4 µg of total RNA were denatured at 65°C for 5 min in the presence of 100 ng of random hexamers and 1 µl of dNTPs (10 mM each dNTP), and then chilled on ice. RT was performed at 42°C for 50 min using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Protection of mRNA from ribonucleases during the cDNA synthesis was assured by using 40 units of RNasin (Promega Corp.). The reaction was stopped by heating at 70°C for 15 min.

In order to obtain a fragment of sea bass *StAR* cDNA, a PCR was performed using 2 µl of cDNA and the degenerate primers star1 (5’-CC(T/A)CCTGCTTC(C/T)TGGC(G/T)GG(A/G)-3’) and star2 (5’-GCATCTTGTGTCAGCAGGC(A/G)TG-3) designed to conserved regions of *StAR* from the largemouth bass (*Micropterus salmoides*, GenBank:DQ166820). Thermal cycling was performed using a touchdown PCR program (Don et al., 1991). The following conditions were used: an initial denaturation step at 94°C for 2 min followed by 20 cycles of 94°C for 30 sec, the highest annealing temperature (70°C) for 30 sec, and an extension temperature of 72°C for 30 sec. The annealing temperature was then decreased 0.5°C per cycle resulting in a 10°C span. Final extension was a single cycle of 72°C for 5 min. The PCR product was cloned into the pGEM-T Easy Vector (Promega Corp.) and sequenced on an automated ABI PRISM 3730 DNA Analyser (Applied Biosystems, Foster City, CA) using the Rhodamine terminator cycle sequencing kit (Perkin-Elmer Inc., Wellesley, Massachusetts).
RNA isolation and reverse transcription for real-time quantitative RT-PCR assays

Sea bass gonadal total RNA was isolated from ∼ 100 mg of frozen tissue using the FastRNA® Pro Green Kit (Qbiogene Inc., Irvine, CA) and the FastPrep® Instrument (Qbiogene Inc., Irvine, CA). Purity and concentration of the RNA was verified by spectrophotometry (GeneQuant, Pharmacia Biotech, Cambridge, England). When starting to extract total RNA from sea bass ovaries at distinct gonadal stages we observed great differences in its composition. During previtellogenesis, low molecular weight RNAs were massively accumulated in the sea bass ovary and declined in amount thereafter (data not shown). To avoid an inaccurate quantification of RNA samples and potential interferences of these RNAs with the RT reaction, poly (A)^+ enriched RNA, instead of total RNA, was used in the female seasonal expression study. The Oligotex® mRNA Kit (Qiagen GmbH, Germany) was used to isolate poly (A)^+ mRNA from ∼ 240 µg of ovarian total RNA preparations. The ULTRA Evolution 384™ (Tecan Group Ltd., Männedorf, Switzerland) fluorescence-based microplate reader along with the RediPlate™ 96 RiboGreen® RNA Quantitation Kit (Invitrogen - Molecular Probes, Eugene, OR) were used for poly (A)^+ mRNA concentration determination. RT was performed as described above using 1 µg of total RNA treated with DNase I RNase-free (Ambion, Inc., Austin, TX) or 150 ng of poly (A)^+ mRNA. The volume of poly (A)^+ mRNA RTs was then increased to 300 µl. Probes and primers for real-time quantitative RT-PCR assays were designed using the Primer Express software (Applied Biosystems, Inc., Foster City, CA). All assays were run in triplicate on an iCycler iQ™ (Bio-Rad Laboratories, Inc.), using 96 well optical plates and default settings. For each 25 µl PCR reaction, 1 µl of RT reaction was mixed with the corresponding amount of primers and probe (Table 1) in 1 x ABgene’s Absolute™ QPCR Mix (Advanced Biotechnologies Ltd, Epsom, UK). To correct for variability in amplification efficiency between different cDNAs, standard curves were
prepared for the sea bass target genes (FSHR, LHR, StAR, CYP11B1 and CYP19A1) and the
sea bass endogenous reference genes (18S rRNA and elongation factor 1-alpha (Ef1-alpha)).
Ten-fold serial dilutions of known concentrations of the plasmids containing each of the
genesis were used. Data were capture and analyzed by the iCycler iQ™ software (version
3.0.6070). Correlation coefficients of the standard curves ranged from 0.99 to 1.00. PCR
efficiencies are shown in Table 1. For each experimental sample, the amount of target and
endogenous reference was determined from the appropriate standard curve.
The expression of the genes of interest was analysed using two separate methods: (a) Raw
arbitrary input amount (non-normalized) and (b) Input amount normalized against a control
gene. The 18S rRNA and Ef1-alpha endogenous genes were tested for their ability to be used
as control genes. They were chosen based on previous studies performed in gonads of other
fish species (e.g., (Kumar et al., 2000; Bobe et al., 2004; Kusakabe et al., 2006) and because
sea bass 28S rRNA and β-actin have already been proved no to be suitable (Halm et al., 2008).
Male data normalization was done by dividing the input amount by the 18S rRNA amount.
Concerning females, the input amount was normalized against adjusted Ef1-alpha values.
This method involves the standardization of expression of the reference gene in each sample
of each month to a randomly chosen “control” group and it has been used in the
characterization of the expression levels of several genes at different stages of ovarian
follicular development in zebrafish (Danio rerio) (Ings and Van Der Kraak, 2006). This is
done by using the following formula according to Billiau et al. (2001): individual value within
a group/(mean value within a group/mean value of control group), where the previtellogenic
stage was chosen as the control group. Data are presented as relative mRNA levels. In males,
the mean of samples in stage VI was set as 1, while in females the mean of samples from
previtellogenesis was the chosen value to be set as 1.
Further information regarding the adopted strategy for real-time PCR data normalization is available on Supplementary Methods online.

**Data representation and statistical analysis**

The data are presented as the mean plus/minus the standard error of the mean (SEM). Gene expression levels of *StAR*, LH and 11-KT in males and *FSHR* in females were analyzed by one-way ANOVA followed by the Holm-Sidak test. Before the analysis, values were In-transformed to meet normality and homoscedasticity requirements. Percentage data (GSI) were arcsine transformed before being used for analysis. Since the remaining data did not meet the criteria for parametric statistics, the Kruskal-Wallis nonparametric test was used to compare differences between groups. If differences were found (*P*<0.05), the Dunn's method or Tukey test (GSI) were used for multiple comparison tests. The strength of the association between pairs of parameters (gene expression levels and plasma hormone levels) was evaluated by calculating the correlation coefficient, *r*, using the Spearman rank order correlation nonparametric test. The significance level was adjusted by Bonferroni correction to reduce type I error. Probability level alpha at 0.05. This was calculated by dividing the alpha level set at 0.05 by the number of comparisons (0.05/6) which means that only *p*<0.008 were considered significant. All the analyses were conducted using the statistical software SigmaStat version 3.0 (SYSTAT Software Inc., Richmond, CA).

**Results**

*Cloning of a partial cDNA of sea bass StAR*

A partial 290 bp cDNA for sea bass *StAR* was amplified to allow the design of specific primers and a probe for real-time quantitative RT-PCR assays. The obtained sequence
displayed a 95.7% identity to the largemouth bass *StAR*. The partial cDNA sequence of sea bass *StAR* is available in the GenBank database under the accession no. EF409994.

**Gonadal development and changes in gonadosomatic index (GSI)**

Males sampled during the summer (July-September) were immature (stage I) and their testis contained mainly A spermatogonia. The first signs of early spermatogenesis (stage II), characterized by cysts of B spermatogonia and spermatocytes, were seen in animals sampled in October. In November testes reached stage III, with spermatocytes being the dominant germ cell type, although few spermatids and B spermatogonia were also visible. In the following two months, testes were in stage IV. At this stage, spermatocytes and spermatids were the dominant cell type and spermatozoa were observed. In February and March gonads progressed into stage V and testis were filled with spermatozoa. At this point, sperm could be collected by gentle abdominal pressure. Testis from March onwards had no spermatogenic activity and contained residual spermatozoa (stage VI). Females sampled during summer were previtellogenic and their ovaries contained oocytes in primary growth phase or in the early stages of the secondary growth phase. During October and November ovaries were in early vitellogenesis, presenting oocytes recently recruited into the secondary growth phase containing numerous yolk granules in a peripheral position and a clear zona radiata. In December and January, ovaries progressed into late vitellogenesis and post-vitellogenesis, presenting oocytes at the secondary and tertiary granule stages. At this point, some atresic oocytes were already present. The maturation-ovulation stage was first observed in females sampled in January and continued until April although at this point the majority of ovaries were already in atresia. Ovaries constituted mainly by non-spherical shaped, degenerated vitellogenic/post-vitellogenic oocytes which are reabsorbed were seen until June.
Data collected on the GSI of the fish used in this study are shown in Fig. 1. In both male and female, the GSI values were low during the summer and early fall (July-October). In males, the GSI (Fig. 1, A) started to increase in November to reach high levels in December remaining high during spermatogenesis (II, III, IV) and full spermiation (V) stages. In females, the GSI (Fig. 1, B) rapidly increased from November on until it peaked in February, during the maturation-ovulation stage. A progressive decrease of the GSI was then observed in both sexes from March onwards until low values were reached again.

**Seasonal changes in hormone plasma levels**

In order to correlate all the variables used in this study, different hormones were measured in the plasma of these specific animals, as extrapolation of previous data might be inaccurate. Plasma 11-KT levels in males started to increase in stage III and peaked in stage IV. These high levels significantly dropped in full spermiating testis remaining low during post-spawning (Fig. 2, A). Plasma LH levels showed a significant elevation in stage IV that was maintained until the end of the cycle (Fig. 2, B).

In females, E2 levels gradually increased during early vitellogenesis. They peaked during late and post-vitellogenesis and then decreased during the maturation/ovulation stage although to levels not statistically different from the previous stage (Fig. 2, C). During pre- and early vitellogenesis female plasma LH values remained low. Levels started to increase during late and post-vitellogenesis and peaked during maturation/ovulation. These levels remained high during atresia (Fig. 2, D).

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

The seasonal changes in the expression of the reference genes, 18S rRNA and Ef1-alpha, during gonadal development in both sea bass male and female are presented in Fig. 3. During
the sampling period the expression of these genes changed significantly ($P < 0.01; P \leq 0.001$) in both sexes. In males, the difference in $18S$ rRNA expression between the highest (stage III) and lowest (stage VI) level was lower than threefold (Fig. 3, A). On the other hand, $Efl$-alpha levels were more than fifteen times higher in stage III than in VI (Fig. 3, B). In females, $18S$ rRNA levels (measured using total RNA) in late and post-vitellogenesis were approximately twenty four times higher than the levels in previtellogenesis, and they returned to low levels at the end of the reproductive cycle (Fig. 3, C). Although with a lower magnitude, $Efl$-alpha expression levels also changed during the female study being almost four times higher in the first stage of gonadal development than in maturation/ovulation (Fig. 3, D).

Seasonal changes in FSHR, LHR, StAR, CYP11B1 and CYP19A1 expression levels

Changes in gonadal expression of the five genes of interest during a complete reproductive cycle were first examined using non-normalized arbitrary input amounts (Fig. 4). In addition, gene expression was normalized to $18S$ rRNA (males) and adjusted $Efl$-alpha (females). The expression patterns obtained for all genes were similar to those of non-normalized values (data not shown), implying that both methods are feasible. To avoid repeating information, only results from normalized values are described below.

**Males** The observed FSHR expression profile across the male reproductive cycle was bimodal (Fig. 4, A). Levels gradually increased from the immature to early recrudescence stage followed by a progressive and significant decline during mid and late recrudescence. A second increase in FSHR mRNA levels was observed in full spermiating males. The expression patterns of LHR and StAR genes were very similar (Fig. 4, B and C). A slight and not significant increase was first observed during early recrudescence. Levels decreased during the mid and late recrudescence stages, peaking in full spermiation. Expression then decreased to the lowest levels during the post-spawning stage. The expression of CYP11B1...
remained high during the early stages of gonadal development (Fig. 4, D), decreased during mid recrudescence, and reached significantly low levels at late recrudescence stage. These low levels were maintained until the end of the reproductive cycle.

**Females**  
*FSHR* expression (Fig. 4, E) was very low during previtellogenesis. With the beginning of vitellogenesis, a slight increase of the expression was observed with values being significantly different from the ones registered in previtellogenesis. During late and post-vitellogenesis, a boost of expression of approximately twenty-five times was observed with values peaking during the maturation/ovulation stage until atresia, when they significantly decreased to values similar to the ones in previtellogenesis. *LHR* expression (Fig. 4, F) remained low and unchanged during pre- and early vitellogenesis rising during late and post-vitellogenesis and reaching the highest values during the maturation/ovulation stage, which corresponds approximately to an eightfold expression increment. The expression sharply decreased during atresia. As in males, the expression pattern of *StAR* in females (Fig. 4, G) was similar to the *LHR* one. Expression remained low during pre- and early vitellogenesis, increased during late and post-vitellogenesis and peaked at maturation/ovulation. In this case, the expression increment was of one hundred and thirty-four fold. During atresia, levels were low again. The expression of *CYP19A1* remained low before and during early vitellogenesis (Fig. 4, H). Values were the highest in late and post-vitellogenesis after a sevenfold increase, returning to low levels during the remaining of the cycle (Fig. 4, H).

**Correlation analysis**

Correlation analysis of gene expression in males (Table 2, A) identified significant and positive relationships between changes in *FSHR* expression and transcript levels of *LHR*, and
CYP11B1. Changes in LHR expression were significantly positively correlated with changes in StAR and CYP11B1 expression. Other correlations were not significant.

In females (Table 2, B), changes in FSHR expression were significantly correlated to LHR, StAR and CYP19A1 transcript levels. Changes in LHR expression were significantly positively correlated to those of StAR. Significant positive correlations were found between E2 plasma levels and the expression of all the analysed genes except for the LHR. Plasma LH levels and changes in the titers of E2 were also found to increase together.

Discussion

In this study, we investigated the seasonal expression of the sea bass gonadotropin receptor genes during the first gonadal maturation in males and females, and searched for relationships between their expression profiles and those of StAR, CYP11B1, and CYP19A1, and plasma profiles of essential reproductive hormones.

In male sea bass, both gonadotropin receptors show parallel expression patterns during the reproductive cycle, with highest expression levels observed during spermiation. In male yellowtail (Seriola quinqueradiata) were expression profiles of gonadotropin receptors were studied by Northern blot, FSHR mRNA levels showed an increase during early spermatogenesis, but opposite to sea bass, transcript levels decreased at spermiation (Rahman et al., 2003). However, for both species the expression of their FSHRs agrees with the one of their FSHβ genes, which code for the specific subunit of FSH. In yellowtail, FSHβ expression decreased in spermatiating males, while in sea bass expression of FSHβ increased with the progression of gonadal growth, reaching a maximum at the initiation of the spermiation period, and remaining high during all this period (Mateos et al., 2003). Increased expression of the FSHR during spermiation has also been recently described in the rainbow trout (Sambroni et al., 2007).
In fish, as in mammals, FSHR is expressed in Sertoli cells (Miwa et al., 1994; Petersen and Söder, 2006), although it has been recently demonstrated that Leydig cells also express the FSHR in Japanese eel (Anguilla japonica) and African catfish (Clarias gariepinus) (Ohta et al., 2007; Garcia-Lopez et al., 2009). In maturating and adult testis from African catfish and Nile tilapia (Oreochromis niloticus), Sertoli cell proliferation occurs primarily during spermatogonial proliferation and ceases in postmeiotic cysts. At the beginning of spermiogenesis, due to the expansion of cyst volume and the stabilization of Sertoli cell number per cyst, there is a dilution of Sertoli cells. However, during the spermiogenic process there is a striking reduction of cyst volume in Nile tilapia testis (Schulz et al., 2005). Assuming an analogous behaviour for sea bass Sertoli cell proliferation during testicular development, the progressive increase in FSHR expression observed in stages I and II (Fig. 4, A) could be related with a proliferation of Sertoli cells, and the decrease of expression in stages III and IV could be the result of a dilution of somatic cells with respect to germ cells, rather than a reduction in FSHR transcripts. During spermiation this dilution effect is no longer observed, resulting in a second increase in the expression levels. Nevertheless, the decline in FSHR expression during mid recrudescence could also be the result of a transient transcription downregulation to prevent Sertoli cell overstimulation by FSH (Themmen et al., 1991). Then, the observed enhancement of expression during sea bass spermiation could be due to an upregulation of FSHR expression, and/or connected with a new proliferation of Sertoli cells needed for the maintenance of spermatogenesis in several clutches of gametes present in the testis, since spermiation is associated with the degeneration of at least some of the Sertoli cells (Billard, 1986; Prisco et al., 2003).

The LHR expression profile in sea bass testis (Fig. 4, B) is consistent with data from maturating rainbow trout and yellowtail males, showing maximum receptor mRNA levels during spermiation (Rahman et al., 2003; Kusakabe et al., 2006; Sambroni et al., 2007).
However, in yellowtail and rainbow trout, according to Kusakabe et al. (2006), receptor expression steadily increases during testicular maturation, while in sea bass and rainbow trout, according to Sambroni et al. (2007), LHR mRNA levels were maintained almost constant until the end of the recrudescence stage. Analysis of LH levels in sea bass plasma (Fig. 2, B) showed an increase of this hormone during spermatogenesis reaching the highest levels in spermiation, which is in agreement with the expression profiles of sea bass LHβ (Mateos et al., 2003) and LHR (Fig. 4, B). These results support the already suggested role of LH in the regulation of the final stages of fish gamete maturation and spermiation (reviewed in Swanson et al., 2003).

11-KT is considered to play an important role in stimulating spermatogenesis in several fish species (Schulz and Miura, 2002) including sea bass (Rodriguez et al., 2000b). The profile of 11-KT obtained in this study (Fig. 2, A) is in accordance with previous results obtained by us in sea bass (Rodriguez et al., 2000b), with levels increasing during mid recrudescence, and dropping once spermiation begins. In fish, very little information is available on the specific roles of FSH and LH in regulating androgen production by the testis. In coho salmon, FSH and LH were equipotent in stimulating the production of T, 11-KT and the MIH 17,20βP by testicular tissue in late stages of spermatogenesis, nevertheless the steroidogenic effects of LH increased as spermatogenesis progressed (Planas and Swanson, 1995). In red seabream, both FSH and LH stimulated the production of 11-KT in sliced testis of animals in the spawning season (Kagawa et al., 1998) and in sexually immature cultivated Japanese eel FSH induces spermatogenesis via stimulation of 11-KT production (Ohta et al., 2007). Recent studies using in vitro culture of sea bass testis have shown that purified native FSH stimulates 11-KT secretion in a dose and time dependent manner (Moles et al., 2008). In this study, we did not find a correlation between 11-KT profile in plasma and sea bass FSHR or CYP11B1 expression; however, the expression profiles of both FSHR and CYP11B1 genes were highly
and positively correlated (Table 2), suggesting, all together, that FSHR signalling could be involved in CYP11B1 expression in sea bass. CYP11B1 expression was high during early gonadal growth, declining in late recrudescence, when 11-KT plasma levels were highest. This delayed profile of plasma 11-KT with respect to enzyme expression has also been observed in rainbow trout males (Kusakabe et al. 2006), and could be the result of a mismatch between expression and activity of the steroidogenic enzyme P450β11. Changes in CYP11B1 transcripts were also found to positively vary together with LHR expression. Thus, further studies will be needed to understand the action of each gonadotropin in the synthesis of sex steroids and the specific role of all of them in the spermatogenic process of sea bass.

Interestingly, the quantification of StAR transcripts in sea bass testis (Fig. 4, C) revealed a profile identical to the one observed for the LHR (Fig. 4, B), what was supported by a significant positive correlation between both gene mRNA levels (Table 2). These results are in line with the ones reported in rainbow trout males (Kusakabe et al., 2006). The acute, steroidogenic effect of LH in mammalian Leydig cells is based on an increased availability of cholesterol for the mitochondrial P450scc. This is achieved via induction of Star (Stocco et al., 2005). Our results indicate that a similar regulation may occur in the sea bass testis.

Like in males, in sea bass females both gonadotropin receptors follow a similar expression pattern (Fig. 4, E and F). Expression of these genes is strongly positively correlated (Table 2), although the expression levels of FSHR are remarkably higher than those of LHR (Fig. 4).

Before yolk incorporation, during primary growth (previtellogenesis), both receptors are expressed at extremely low levels in sea bass ovary. In early vitellogenesis (October), the expression level of FSHR slightly increased while LHR mRNA levels remained unchanged. Recent work in channel catfish (Ictalurus punctatus) and zebrafish (Danio rerio) has suggested that an enhancement in ovarian FSHR expression occurs at the beginning of vitellogenesis and this upregulation continues through vitellogenesis (Kumar and Trant, 2004;
Kwok et al., 2005). In coho salmon, FSHR expression remained unchanged during previtellogenesis and enlargement of expression was only observed with the appearance of lipid droplets in the oocyte (Campbell et al., 2006). Contrary to what was believed for long time, recent studies have proved that vitellogenins enter the ovarian follicle at the same time as cortical alveoli and lipid globules appear (Le Menn et al., 2007). Taken together, these data suggest that the initial increase of FSHR expression is connected with the start of yolk proteins uptake, at least in the above mention species. Increases in sea bass ovarian LHR mRNA levels were only observed when postvitellogenesis began (December). At that stage, FSHR mRNA levels were already at their maximum. During the maturation-ovulation period, expression levels of both receptors remained elevated, returning to their basal levels only after spawning.

Studies on female salmonids, which have a synchronous type of oocyte development, suggest that secondary oocyte growth is regulated primarily by FSH, whereas LH plays a major role in regulating final oocyte maturation. Nonetheless, the observed expression pattern of FSHR in sea bass (Fig. 4, E) involves this receptor (and FSH) also in processes occurring after secondary oocyte growth. Various studies performed on rainbow trout ovary have shown that increased FSHR expression is related with high maturational competence (Bobe et al., 2004), oocyte maturation and ovulation (Sambroni et al., 2007). Regarding sea bass, we consider that the observed high expression level of FSHR during maturation could be connected with oocyte growth and is explained by the reproductive strategy of this species. Sea bass ovary exhibits a group-synchronous type of development, and so, during the maturation-ovulation stage at least two populations of oocytes can be distinguished at the same time; a fairly synchronous population of larger oocytes (defined as a clutch) and a more heterogeneous population of smaller oocytes from which the clutch is recruited (Mayer et al., 1990; Asturiano et al., 2000). Therefore, the expression of any gene measured at the ovary level
reflects the average of the existing follicles, including that of growing oocytes that would still express FSHR. This idea is supported by a previous in situ hybridization study on post-vitellogenic sea bass ovary, which showed a strong expression of FSHR only in the follicular cells of previtellogenic and vitellogenic oocytes (Rocha et al., 2007a).

The profile of E2 plasma levels observed in this study (Fig. 2, C) is in agreement with previous works on sea bass (Prat et al., 1990; Mañanós et al., 1997; Asturiano et al., 2000), with a single annual peak at late vitellogenesis (December) and constant high levels during the maturation/ovulation period. The maintenance of constant high E2 levels during the entire maturation/ovulation stage has been attributed to a prolongation of the vitellogenic process, as vitellogenic oocytes are also present during this stage (Mañanós et al., 1997).

In sea bass ovaries cultured in vitro FSH stimulates the production of E2 (Moles et al., 2008) and in salmonid fish it was established that FSH influences ovarian P450arom expression and activity (Montserrat et al., 2004). The positive relationship among sea bass FSHR and CYP19A1 mRNA levels and E2 plasma profile (Table 2) could indicate that the ovarian production of E2 in sea bass, as in salmonid fish, would be under the stimulatory effect of FSH by upregulation of P450arom expression. In mammalian ovaries, FSH, estrogens and growth factors induce the expression of the LHR in granulosa cells of preovulatory follicles (Dufau, 1998). It is interesting to note that in the sea bass ovary, the expression levels of the LHR remained basal until FSHR expression and E2 plasma levels were high (Fig. 2 and 5), indicating that a similar induction mechanism could occur during late vitellogenesis and post-vitellogenesis in this fish.

In this study, a significant elevation of StAR expression was observed at the end of vitellogenesis coinciding with an increase in plasma E2 levels. The highest expression values were observed at the maturation-ovulation stage (Fig. 4, G), when LH plasma levels were high. In a study performed on individual plasma samples of sea bass, successive elevations of
plasma E2 levels were observed prior to peaks of the progestins 17,20βP and 20βS (the sea bass MIHs), which resulted in the maturation/ovulation of different clutches of oocytes (Asturiano et al., 2002). The shift from estrogen to MIHs synthesis needs both the partial reworking of the steroidogenic pathway and the rapid delivery of cholesterol substrate, which requires the StAR protein. Thus, the expression profile of sea bass StAR obtained in this study is consistent with an important involvement of the coded protein in the synthesis of sea bass MIHs.

In summary, the present study describes for the first time in a multiple group-synchronous spawner teleost, the sea bass, the expression profiles of gonadotropin receptors during the first gonadal recrudescence in males and females. These expression profiles support the involvement in gonadal growth and final stages of maturation/ovulation of FSHR and LHR respectively. In addition, the elevated expression of FSHR in spermiation/ovulation could be due to the group-synchronous nature of sea bass gonadal development, which could require maintaining FSHR expression in some clutches of developing gametes. All together, the relation among these profiles, gonadal development, transcript abundance of genes involved in steroidogenesis and plasma levels of important reproductive hormones intends to draw a first picture on the role of gonadotropins in sea bass gonadal function, and their relation to sex steroids. Further in vitro and in vivo studies will be needed to understand how gonadotropins, sex steroids and other gonadal factors interact to regulate sea bass reproduction.
Acknowledgements

We thank Conrado Marin and Joaquin Salvador for assistance with fish maintenance and sampling, and Javier Beltran and Jose Monfort for assistance with immunoassays and histology, respectively. This work was financed by Spanish Ministry of Science and Technology funding; grant AGL 2001-1257 to MC and grant AGL2005-00796, and a Generalitat Valenciana grant (Group 04/80) to AG. The European Social Fund and Portuguese Operational Programme “Science and Innovation 2010” funds under Portuguese National Science Foundation (FCT) POCI-2010 SFRH/BD/6901/2001 covered a fellowship received by AR.

References


Mañanós, E.L., Zanuy, S., Carrillo, M., 1997. Photoperiodic manipulations of the reproductive cycle of sea bass (Dicentrarchus labrax) and their effects on gonadal development, and plasma 17\beta-estradiol and vitellogenin levels. Fish Physiol. Biochem. 16, 211-222.


Figure Legends

Figure 1 - Changes in the gonadosomatic index (GSI) in male (A) and female (B) sea bass, during the sampling period. Values represent the mean ± SEM ($n = 5$ fish/month). The stages of gonadal development as determined by histology (see Materials and Methods) are represented by horizontal bars below each graph. Different significance levels are indicated with different letters above the bars.

Figure 2 - Changes in plasma levels of 11-KT (A), LH (B and D) and E2 (C) in male and female sea bass during their first sexual maturation. Data, shown as the mean ± SEM, are represented by stages of gonadal development as determined by histology (see Materials and Methods). Males: stage I ($n = 19$), immature; stage II ($n = 6$), early recrudescence; stage III ($n = 3$), mid recrudescence; stage IV ($n = 6$), late recrudescence; stage V ($n = 10$), full spermiating testes and stage VI ($n = 4$), post-spawning. Females: prevtg ($n = 15$), previtellogenesis; evtg ($n = 10$), early vitellogenesis; lat-postvtg ($n = 7$), late-post-vitellogenesis; mat-ovul ($n = 14$), maturation-ovulation and atre ($n = 13$), atresia. Different significance levels are indicated with different letters above the bars.

Figure 3 - Changes in the amount of $18S$ rRNA and Ef1-alpha mRNAs in testes (A, B) and ovaries (C, D) of sea bass sampled during their first sexual maturation. Data, shown as the mean ± SEM, are represented by stages of gonadal development as determined by histology (see Materials and Methods). One-way ANOVA was performed. The $P$ value is indicated in each graphic.

Figure 4 - Relative changes in expression of FSHR, LHR, StAR, CYP11B1 and CYP19A1 in male (A, B, C, D) and female (E, F, G, H) sea bass, sampled during their first sexual maturation. Values, shown as the mean ± SEM, are represented by stages of gonadal development as determined by histology. Males: stage I ($n = 19$), immature; stage II ($n = 6$), early recrudescence; stage III ($n = 3$), mid recrudescence; stage IV ($n = 6$), late recrudescence; stage V ($n = 10$), full spermiating testes and stage VI ($n = 4$), post-spawning. Females: prevtg ($n = 15$), previtellogenesis; evtg ($n = 10$), early vitellogenesis; lat-postvtg ($n = 7$), late-post-vitellogenesis; mat-ovul ($n = 14$), maturation-ovulation and atre ($n = 13$), atresia. Male expression values are normalized to $18S$ rRNA and expressed as a proportion of the mean value in stage VI. Female expression values are normalized to Ef1-alpha, which was adjusted.
to compensate for changes in expression across stages, and expressed as a proportion of the mean value in the prevtg stage. Statistically significant differences are indicated with different letters above the bars.
Figure 1

Testicular stages

Ovarian stages
Figure 2

Testicular stages

Ovarian stages

Plasma 1-XT (ng/ml)

Plasma E2 (ng/ml)

Plasma LH (ng/ml)

Plasma LH (ng/ml)
Figure 3

(A) 18S rRNA expression across different testicular stages. Bars represent mean ± SD. P < 0.01.

(B) E. aiptasia expression across different testicular stages. Bars represent mean ± SD. P = 0.001.

(C) 18S rRNA expression across different ovarian stages. Bars represent mean ± SD. P < 0.001.

Testicular stages

Ovarian stages
Figure 4

A  FSHR

B  LHR

C  STAR

D  CYP11B1

E  FSHR

F  LHR

G  STAR

H  CYP19A1

Testicular stages

Ovarian stages
Table 1 Primers and TaqMan™ fluorogenic probes used in this study

<table>
<thead>
<tr>
<th>Primer or Probe</th>
<th>Sequence (5’→3’)</th>
<th>nM</th>
<th>Amplicon size; PCR efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSHR (AY642113)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fshr 1074 fw</td>
<td>CCGCCCCCAATCTGAAG</td>
<td>50</td>
<td>63 bp; 0.89</td>
</tr>
<tr>
<td>fshr 1136 rv</td>
<td>GGTTGGCCTCTGAGATTT</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>fshr 1092 pr</td>
<td>[6-FAM]AGGTTCTCTCCTGAGCTT[TAMRA]</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>LHR (AY642114)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lhr 1231 fw</td>
<td>ACTTCTGTAGACCGAGCAAA</td>
<td>900</td>
<td>67 bp; 0.92</td>
</tr>
<tr>
<td>lhr 1297 rv</td>
<td>TCCTCACAGGGATTGGAAGCA</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>lhr 1253 pr</td>
<td>[6-FAM]TTTGGTTTGCACACTTGAGCA[TAMRA]</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>STAR (EF409994)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>star 142 fw</td>
<td>GGCTGGATCCCGAAGCAAA</td>
<td>900</td>
<td>72 bp; 0.99</td>
</tr>
<tr>
<td>star 213 rv</td>
<td>CCTGAGTGTTGGCAAGAT</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>star 162 pr</td>
<td>[6-FAM]CTAAACAAAGTGCTCTCAGACACGG[TAMRA]</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>CYP19A1 (AJ311177)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyp19 1328 fw</td>
<td>TCCTCGCCGCTACTTCCA</td>
<td>300</td>
<td>65 bp; 0.98</td>
</tr>
<tr>
<td>cyp19 1392 rv</td>
<td>TGGCGATGTGACCCAGAT</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>cyp19 1348 pr</td>
<td>[6-FAM]CATTCGTTACGGCCCTCGA[TAMRA]</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CYP11B1 (AF449173)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyp11 351 fw</td>
<td>CCTGTTGCTCCGCTGTGCTTG</td>
<td>300</td>
<td>66 bp; 1.02</td>
</tr>
<tr>
<td>cyp11 416 rv</td>
<td>CTGAGATGTAGCCATCGGCA</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>cyp11 373 pr</td>
<td>[6-FAM]CTCTGTTGGACGACCCCTCGA[TAMRA]</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S fw</td>
<td>GCATGCCGGAGTCTCGTT</td>
<td>900</td>
<td>71 bp; 0.92</td>
</tr>
<tr>
<td>18S rv</td>
<td>TGCATGCCGGATCTTGTTG</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>18S pr</td>
<td>[6-FAM]TTATCGGAATTAACCAGAC[TAMRA]</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>EF1-α (AJ866727)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EF1-α 156 fw</td>
<td>GGAGTGACGAGCTCTCGTT</td>
<td>50</td>
<td>69 bp; 0.99</td>
</tr>
<tr>
<td>EF1-α 224 rv</td>
<td>GCGGGCCTGGCTGTAAG</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>EF1-α 179 pr</td>
<td>[6-FAM]AGTCAAAAGATGGACTCCACTGAGC[TAMRA]</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Forward (fw) and reverse (rv) primers were obtained from Invitrogen Corp. (Carlsbad, CA). Fluorogenic probes (pr) were purchased from Operon Biotechnologies GmbH (Cologne, Germany).
<sup>b</sup> GenBank accession nos. for sea bass genes.
<sup>c</sup> Amount of primer or probe in the PCR reaction.
<sup>d</sup> Values represent the average numbers of two, three or five assays.
Table 2 Correlation analysis among changes in relative expression levels of *FSHR*, *LHR, StAR, CYP11B1* and *CYP19A1* and plasma hormones in individual sea bass males (n=48) (A) and females (n=59) (B).

<table>
<thead>
<tr>
<th></th>
<th>LHR</th>
<th>StAR</th>
<th>CYP11B1</th>
<th>11-KT</th>
<th>LH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>FSHR</em></td>
<td><em>P=0.000</em></td>
<td><em>r=0.54</em></td>
<td><em>P=0.000</em></td>
<td><em>P=0.21</em></td>
<td><em>P=0.32</em></td>
</tr>
<tr>
<td><em>LHR</em></td>
<td><em>P=0.000</em></td>
<td><em>r=0.40</em></td>
<td><em>P=0.000</em></td>
<td><em>P=0.03</em></td>
<td><em>P=0.68</em></td>
</tr>
<tr>
<td><em>StAR</em></td>
<td><em>P=0.050</em></td>
<td><em>r=0.67</em></td>
<td><em>P=0.067</em></td>
<td><em>P=0.09</em></td>
<td><em>P=0.025</em></td>
</tr>
<tr>
<td><em>CYP11B1</em></td>
<td><em>P=0.000</em></td>
<td><em>r=0.56</em></td>
<td><em>P=0.017</em></td>
<td><em>P&lt;0.001</em></td>
<td><em>P&lt;0.001</em></td>
</tr>
<tr>
<td>11-KT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>FSHR</em></td>
<td><em>P=0.000</em></td>
<td><em>r=0.80</em></td>
<td><em>P=0.000</em></td>
<td><em>P=4.4 x 10^{-3}</em></td>
<td><em>P=0.52</em></td>
</tr>
<tr>
<td><em>LHR</em></td>
<td><em>P=0.000</em></td>
<td><em>r=0.82</em></td>
<td><em>P=0.000</em></td>
<td><em>P=0.37</em></td>
<td><em>r=0.09</em></td>
</tr>
<tr>
<td><em>StAR</em></td>
<td><em>P=0.110</em></td>
<td><em>r=0.72</em></td>
<td><em>P=0.110</em></td>
<td><em>P=0.24</em></td>
<td><em>P=0.25</em></td>
</tr>
<tr>
<td><em>CYP19A1</em></td>
<td><em>P=0.000</em></td>
<td><em>r=0.43</em></td>
<td><em>P=3.4 x 10^{-3}</em></td>
<td><em>P=0.30</em></td>
<td><em>r=0.14</em></td>
</tr>
<tr>
<td>E2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Probability value.

*b* Correlation coefficient value.

*c* Values sowed in bold were considered statistically significant after applying Bonferroni correction.