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

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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

al., 2002). 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 (Nahahama, 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 *CYP11B1*, *CYP19A1* and *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 (*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 x 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 65ºC 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 42ºC 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 70ºC 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 94ºC for 2 min followed by 20 cycles of 94ºC for 30 sec, the highest annealing temperature (70ºC) for 30 sec, and an extension temperature of 72ºC for 30 sec. The annealing temperature was then decreased 0.5ºC per cycle resulting in a 10ºC span. Final extension was a single cycle of 72ºC 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).

166

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

168 Sea bass gonadal total RNA was isolated from ~ 100 mg of frozen tissue using the FastRNA®
169 Pro Green Kit (Qbiogene Inc., Irvine, CA) and the FastPrep® Instrument (Qbiogene Inc.,
170 Irvine, CA). Purity and concentration of the RNA was verified by spectrophotometry
171 (GeneQuant, Pharmacia Biotech, Cambridge, England). When starting to extract total RNA
172 from sea bass ovaries at distinct gonadal stages we observed great differences in its
173 composition. During previtellogenesis, low molecular weight RNAs were massively
174 accumulated in the sea bass ovary and declined in amount thereafter (data not shown). To
175 avoid an inaccurate quantification of RNA samples and potential interferences of these RNAs
176 with the RT reaction, poly $(A)^+$ enriched RNA, instead of total RNA, was used in the female
177 seasonal expression study. The Oligotex® mRNA Kit (Qiagen GmbH, Germany) was used to
178 isolate poly $(A)^+$ mRNA from ~ 240 µg of ovarian total RNA preparations. The ULTRA
179 Evolution 384™ (Tecan Group Ltd., Männedorf, Switzerland) fluorescence-based microplate
180 reader along with the RediPlate™ 96 RiboGreen® RNA Quantitation Kit (Invitrogen -
181 Molecular Probes, Eugene, OR) were used for poly $(A)^+$ mRNA concentration determination.
182 RT was performed as described above using 1 µg of total RNA treated with DNase I RNase-
183 free (Ambion, Inc., Austin, TX) or 150 ng of poly $(A)^+$ mRNA. The volume of poly $(A)^+$
184 mRNA RTs was then increased to 300 µl. Probes and primers for real-time quantitative RT-
185 PCR assays were designed using the Primer Express software (Applied Biosystems, Inc.,
186 Foster City, CA). All assays were run in triplicate on an iCycler iQ™ (Bio-Rad Laboratories,
187 Inc.), using 96 well optical plates and default settings. For each 25 µl PCR reaction, 1 µl of
188 RT reaction was mixed with the corresponding amount of primers and probe (Table 1) in 1 x
189 ABgene's Absolute™ QPCR Mix (Advanced Biotechnologies Ltd, Epsom, UK). To correct
190 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 genes 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 ln-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

239 displayed a 95.7% identity to the largemouth bass *StAR*. The partial cDNA sequence of sea
240 bass *StAR* is available in the GenBank data base under the accession no. EF409994.
241
242 *Gonadal development and changes in gonadosomatic index (GSI)*
243 Males sampled during the summer (July-September) were immature (stage I) and their testis
244 contained mainly A spermatogonia. The first signs of early spermatogenesis (stage II),
245 characterized by cysts of B spermatogonia and spermatocytes, were seen in animals sampled
246 in October. In November testes reached stage III, with spermatocytes being the dominant
247 germ cell type, although few spermatids and B spermatogonia were also visible. In the
248 following two months, testes were in stage IV. At this stage, spermatocytes and spermatids
249 were the dominant cell type and spermatozoa were observed. In February and March gonads
250 progressed into stage V and testis were filled with spermatozoa. At this point, sperm could be
251 collected by gentle abdominal pressure. Testis from March onwards had no spermatogenic
252 activity and contained residual spermatozoa (stage VI). Females sampled during summer were
253 previtellogenic and their ovaries contained oocytes in primary growth phase or in the early
254 stages of the secondary growth phase. During October and November ovaries were in early
255 vitellogenesis, presenting oocytes recently recruited into the secondary growth phase
256 containing numerous yolk granules in a peripheral position and a clear zona radiata. In
257 December and January, ovaries progressed into late vitellogenesis and post-vitellogenesis,
258 presenting oocytes at the secondary and tertiary granule stages. At this point, some atresic
259 oocytes were already present. The maturation-ovulation stage was first observed in females
260 sampled in January and continued until April although at this point the majority of ovaries
261 were already in atresia. Ovaries constituted mainly by non-spherical shaped, degenerated
262 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

288 the sampling period the expression of these genes changed significantly (P <0.01; P≤0.001) in
289 both sexes. In males, the difference in *18S rRNA* expression between the highest (stage III)
290 and lowest (stage VI) level was lower than threefold (Fig. 3, A). On the other hand, *Ef1-alpha*
291 levels were more than fifteen times higher in stage III than in VI (Fig. 3, B). In females, *18S*
292 *rRNA* levels (measured using total RNA) in late and post-vitellogenesis were approximately
293 twenty four times higher than the levels in previtellogenesis, and they returned to low levels at
294 the end of the reproductive cycle (Fig. 3, C). Although with a lower magnitude, *Ef1-alpha*
295 expression levels also changed during the female study being almost four times higher in the
296 first stage of gonadal development than in maturation/ovulation (Fig. 3, D).
297
298 *Seasonal changes in FSHR, LHR, StAR, CYP11B1 and CYP19A1 expression levels*
299 Changes in gonadal expression of the five genes of interest during a complete reproductive
300 cycle were first examined using non-normalized arbitrary input amounts (Fig. 4). In addition,
301 gene expression was normalized to *18S rRNA* (males) and adjusted *Ef1-alpha* (females). The
302 expression patterns obtained for all genes were similar to those of non-normalized values
303 (data not shown), implying that both methods are feasible. To avoid repeating information,
304 only results from normalized values are described below.
305 **Males** The observed *FSHR* expression profile across the male reproductive cycle was
306 bimodal (Fig. 4, A). Levels gradually increased from the immature to early recrudescence
307 stage followed by a progressive and significant decline during mid and late recrudescence. A
308 second increase in *FSHR* mRNA levels was observed in full spermiating males. The
309 expression patterns of *LHR* and *StAR* genes were very similar (Fig. 4, B and C). A slight and
310 not significant increase was first observed during early recrudescence. Levels decreased
311 during the mid and late recrudescence stages, peaking in full spermiation. Expression then
312 decreased to the lowest levels during the post-spawning stage. The expression of *CYP11B1*

313 remained high during the early stages of gonadal development (Fig. 4, D), decreased during
314 mid recrudescence, and reached significantly low levels at late recrudescence stage. These
315 low levels were maintained until the end of the reproductive cycle.
316 **Females** *FSHR* expression (Fig.4, E) was very low during previtellogenesis With the
317 beginning of vitellogenesis, a slight increase of the expression was observed with values
318 being significantly different form the ones registered in previtellogenesis . During late and
319 post-vitellogenesis, a boost of expression of approximately twenty five times was observed
320 with values peaking during the maturation/ovulation stage until atresia, when they
321 significantly decreased to values similar to the ones in previtellogenesis. *LHR* expression
322 (Fig.4, F) remained low and unchanged during pre- and early vitellogenesis rising during late
323 and post-vitellogenesis and reaching the highest values during the maturation/ovulation stage,
324 which corresponds approximately to an eightfold expression increment. The expression
325 sharply decreased during atresia. As in males, the expression pattern of *StAR* in females (Fig.
326 4, G) was similar to the *LHR* one. Expression remained low during pre- and early
327 vitellogenesis, increased during late and post-vitellogenesis and peaked at
328 maturation/ovulation. In this case, the expression increment was of one hundred and thirty
329 four fold. During atresia, levels were low again. The expression of *CYP19A1* remained low
330 before and during early vitellogenesis (Fig. 4, H). Values were the highest in late and post-
331 vitellogenesis after a sevenfold increase, returning to low levels during the remaining of the
332 cycle (Fig. 4, H).
333
334 *Correlation analysis*
335 Correlation analysis of gene expression in males (Table 2, A) identified significant and
336 positive relationships between changes in *FSHR* expression and transcript levels of *LHR*, and

337 *CYP11B1*. Changes in *LHR* expression were significantly positively correlated with changes
338 in *StAR* and *CYP11B1* expression. Other correlations were not significant.
339 In females (Table 2, B), changes in *FSHR* expression were significantly correlated to *LHR*,
340 *StAR* and *CYP19A1* transcript levels. Changes in *LHR* expression were significantly positively
341 correlated to those of *StAR*. Significant positive correlations were found between E2 plasma
342 levels and the expression of all the analysed genes except for the *LHR*. Plasma LH levels and
343 changes in the titers of E2 were also found to increase together.
344

345 ## Discussion

346 In this study, we investigated the seasonal expression of the sea bass gonadotropin receptor
347 genes during the first gonadal maturation in males and females, and searched for relationships
348 between their expression profiles and those of *StAR*, *CYP11B1*, and *CYP19A1*, and plasma
349 profiles of essential reproductive hormones.
350 In male sea bass, both gonadotropin receptors show parallel expression patterns during the
351 reproductive cycle, with highest expression levels observed during spermiation. In male
352 yellowtail (*Seriola quinqueradiata*) were expression profiles of gonadotropin receptors were
353 studied by Northern blot, *FSHR* mRNA levels showed an increase during early
354 spermatogenesis, but opposite to sea bass, transcript levels decreased at spermiation (Rahman
355 et al., 2003). However, for both species the expression of their *FSHR*s agrees with the one of
356 their *FSHβ* genes, which code for the specific subunit of FSH. In yellowtail, *FSHβ* expression
357 decreased in spermiating males, while in sea bass expression of *FSHβ* increased with the
358 progression of gonadal growth, reaching a maximum at the initiation of the spermiation
359 period, and remaining high during all this period (Mateos et al., 2003). Increased expression
360 of the *FSHR* during spermiation has also been recently described in the rainbow trout
361 (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

462 reflects the average of the existing follicles, including that of growing oocytes that would still
463 express *FSHR*. This idea is supported by a previous *in situ* hybridization study on post-
464 vitellogenic sea bass ovary, which showed a strong expression of *FSHR* only in the follicular
465 cells of previtellogenic and vitellogenic oocytes (Rocha et al., 2007a).
466 The profile of E2 plasma levels observed in this study (Fig. 2, C) is in agreement with
467 previous works on sea bass (Prat et al., 1990; Mañanós et al., 1997; Asturiano et al., 2000),
468 with a single annual peak at late vitellogenesis (December) and constant high levels during
469 the maturation/ovulation period. The maintenance of constant high E2 levels during the entire
470 maturation/ovulation stage has been attributed to a prolongation of the vitellogenic process, as
471 vitellogenic oocytes are also present during this stage (Mañanós et al., 1997).
472 In sea bass ovaries cultured *in vitro* FSH stimulates the production of E2 (Moles et al., 2008)
473 and in salmonid fish it was established that FSH influences ovarian P450arom expression and
474 activity (Montserrat et al., 2004). The positive relationship among sea bass *FSHR* and
475 *CYP19A1* mRNA levels and E2 plasma profile (Table 2) could indicate that the ovarian
476 production of E2 in sea bass, as in salmonid fish, would be under the stimulatory effect of
477 FSH by upregulation of P450arom expression. In mammalian ovaries, FSH, estrogens and
478 growth factors induce the expression of the *LHR* in granulosa cells of preovulatory follicles
479 (Dufau, 1998). It is interesting to note that in the sea bass ovary, the expression levels of the
480 *LHR* remained basal until *FSHR* expression and E2 plasma levels were high (Fig. 2 and 5),
481 indicating that a similar induction mechanism could occur during late vitellogenesis and post-
482 vitellogenesis in this fish.
483 In this study, a significant elevation of *StAR* expression was observed at the end of
484 vitellogenesis coinciding with an increase in plasma E2 levels. The highest expression values
485 were observed at the maturation-ovulation stage (Fig. 4, G), when LH plasma levels were
486 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.

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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.

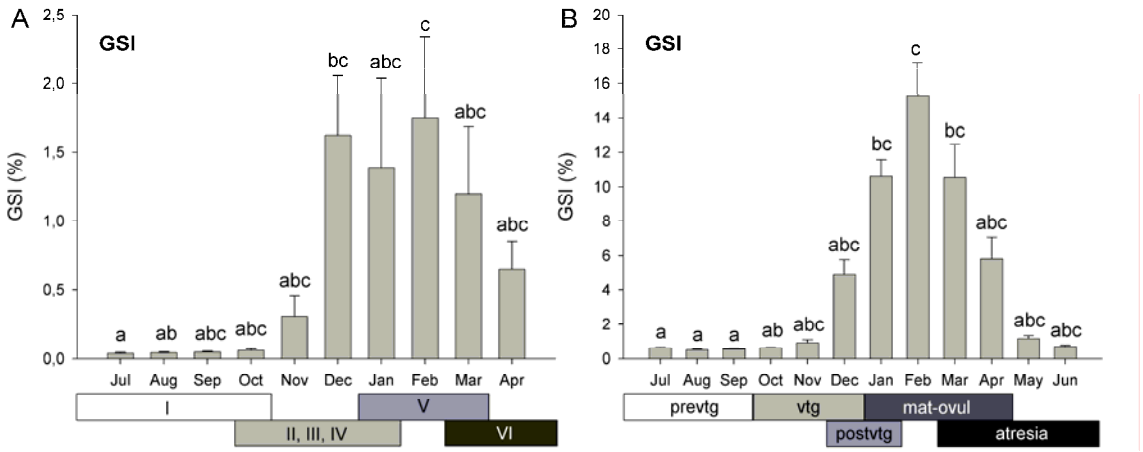
Figure 3 - Changes in the amount of *18S rRNA* and *Ef1-alpha* mRNAs in testes (A, B) and ovaries (C, D) of sea bass sampled during their first sexual maturation. Data, shown as the mean ± SEM, are represented by stages of gonadal development as determined by histology (see Materials and Methods). One-way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

728 to compensate for changes in expression across stages, and expressed as a proportion of the
729 mean value in the prevtg stage. Statistically significant differences are indicated with different
730 letters above the bars.

731 Figure 1

732



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735 Figure 2

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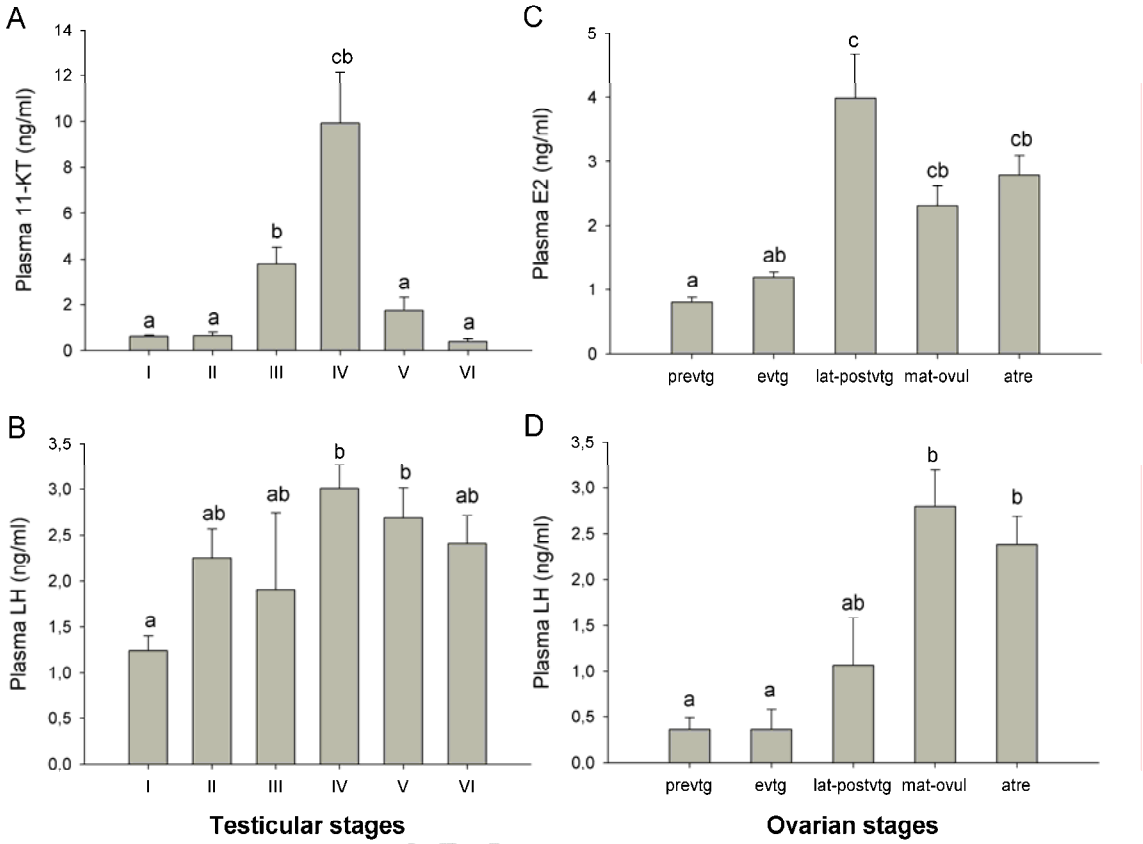

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738 Figure 3

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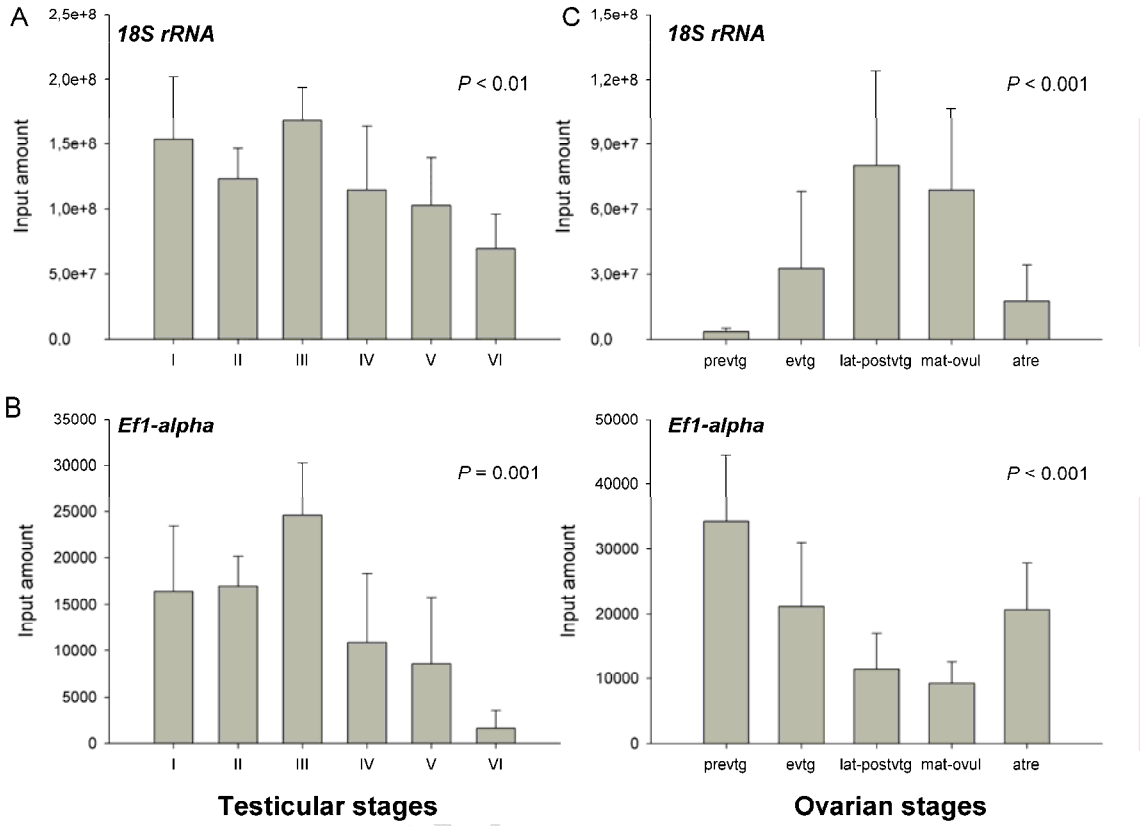

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741 Figure 4

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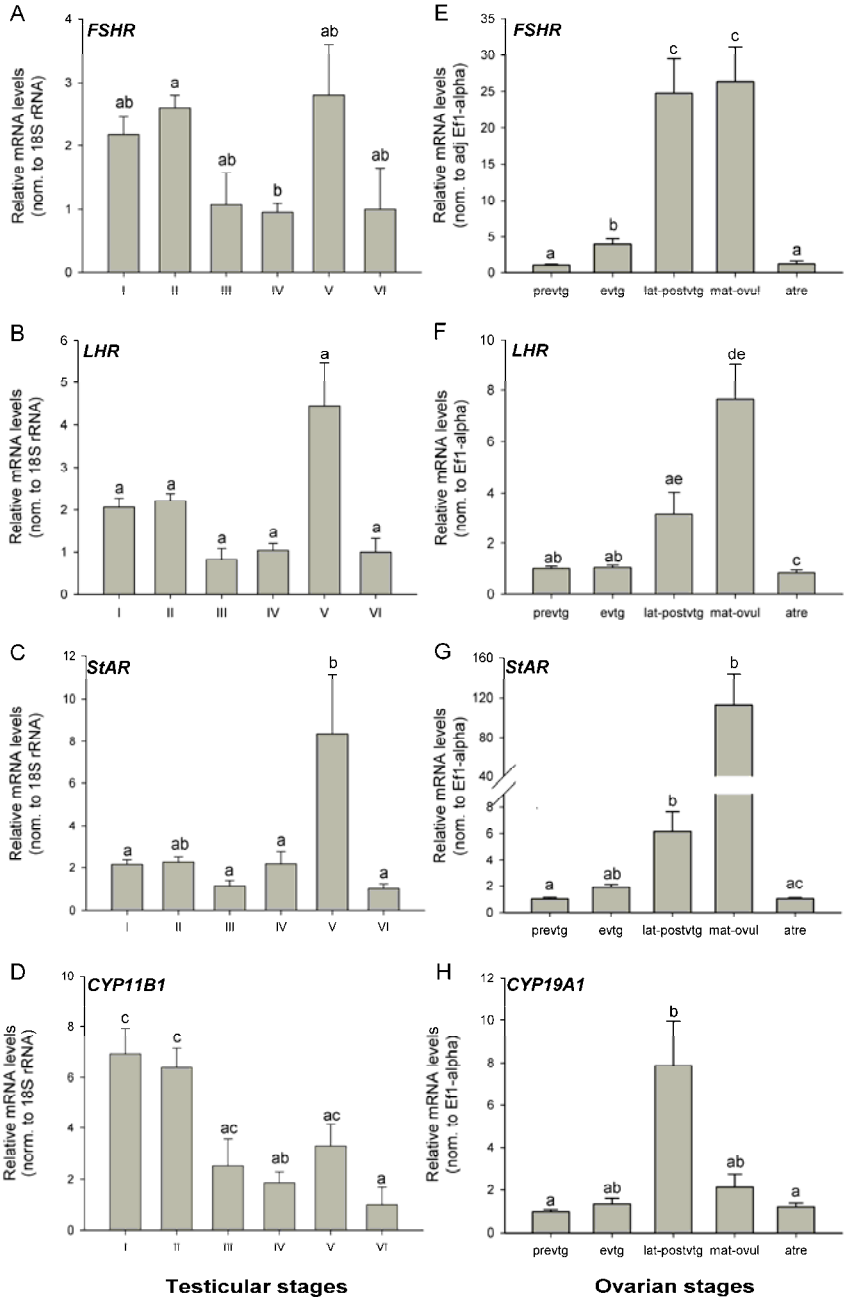

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Table 1 Primers and TaqMan™ fluorogenic probes[a] used in this study

Primer or Probe	Sequence (5'→3')	nM[c]	Amplicon size; PCR efficiency[d]
FSHR (AY642113)[b]			
fshr 1074 fw	CCGCCCCCAATCTGAAG	50	63 bp; 0.89
fshr 1136 rv	GGTTGGCCTGGTGCAGTTT	900	
fshr 1092 pr	[6~FAM]AGCTTCCTCCTCTGGAGCTCTTC[TAMRA]	75	
LHR (AY642114)			
lhr 1231 fw	ACTTCTGTCAGACCCGACCAA	900	67 bp; 0.92
lhr 1297 rv	TCCTCACAGGGATTGAAAGCA	900	
lhr 1253 pr	[6~FAM]TTTGGTTTGCACACCTGAAGCA[TAMRA]	125	
StAR (EF409994)			
star 142 fw	GGCTGGATCCCGAAGACAA	900	72 bp; 0.99
star 213 rv	CCTGAGGTGGTTGGCAAAGT	900	
star 162 pr	[6~FAM]CATAAACAAAGTGCTCTCTCAGACGCAGGTG[TAMRA]	75	
CYP19A1 (AJ311177)			
cyp19 1328 fw	TCCTCGCCGCTACTTCCA	300	65 bp; 0.98
cyp19 1392 rv	TGGCGATGTGCTTACCAACA	300	
cyp19 1348pr	[6~FAM]CATTCGGTTCAGGCCCTCGCG[TAMRA]	100	
CYP11B1 (AF449173)			
cyp11 351 fw	CCTGTTGCTCCGTGTTCGT	300	66 bp; 1.02
cyp11 416 rv	CTGAAGATGTGATCCCATGCA	900	
cyp11 373 pr	[6~FAM]CCTCTGTGGACCAAGCACGCCA[TAMRA]	100	
18S rRNA			
18S fw	GCATGCCGGAGTCTCGTT	900	71 bp; 0.92
18S rv	TGCATGGCCGTTCTTAGTTG	900	
18S pr	[6~FAM]TTATCGGAATTAACCAGAC[TAMRA]	200	
Ef1-α (AJ866727)			
Ef1-α 156 fw	GGAGTGAAGCAGCTCATCGTT	50	69 bp; 0.99
Ef1-α 224 rv	GCGGGCCTGGCTGTAAG	300	
Ef1-α 179 pr	[6~FAM]AGTCAACAAGATGGACTCCACTGAGCCC[TAMRA]	200	

[a] Forward (fw) and reverse (rv) primers were obtained from Invitrogen Corp. (Carlsbad, CA). Fluorogenic probes (pr) were purchased from Operon Biotechnologies GmbH (Cologne, Germany)

[b] GenBank accession nos. for sea bass genes.

[c] Amount of primer or probe in the PCR reaction.

[d] 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**).

A	*LHR*	*StAR*	*CYP11B1*	11-KT	LH
FSHR	[a c] ***P*=0.000** [b] **r=0.54**	*P*=0.005 r=0.40	***P*=0.000** **r=0.74**	*P*=0.21 r=-0.19	*P*=0.32 r=-0.15
LHR		***P*=0.000** **r=0.67**	***P*=0.000** **r=0.56**	*P*=0.03 r=-0.31	*P*=0.68 r=-0.06
StAR			*P*=0.050 r=0.29	*P*=0.75 r=0.05	*P*=0.09 r=0.25
CYP11B1				*P*=0.17 r=-0.20	*P*<0.01 r=-0.37
11-KT					*P*=0.13 r= 0.22

B	*LHR*	*StAR*	*CYP19A1*	E2	LH
FSHR	***P*=0.000** **r=0.80**	***P*=0.000** **r=0.82**	**P=0,000** **r=0.43**	***P*=4.4 x 10^{-3}** **r=0.37**	*P*=0.52 r=0.09
LHR		***P*=0.000** **r=0.72**	*P*=0.110 r=0.21	*P*=0.06 r=0.24	*P*=0.25 r=0.15
StAR			***P*=0.000** **r=0.43**	***P*=3.4 x10^{-3}** **r=0.38**	*P*=0.30 r=0.14
CYP19A1				***P*=2.4 x 10^{-3}** **r=0.39**	*P*=0.94 r=-0.009
E2					***P*=0.000** **r=0.43**

[a] Probability value.
[b] Correlation coefficient value.
[c] Values sowed in bold were considered statistically significant after applying Bonferroni correction.