

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

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17

**18 Abstract**

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

39

40 *Keywords:* Follicle-stimulating hormone receptor; luteinizing hormone receptor;

41 gametogenesis; P450 11 $\beta$ -hydroxylase; P450 aromatase; teleost fish.

## 42 **Introduction**

43 In teleost fish, as in mammals, gametogenesis is regulated by the interplay of systemic and  
44 intragonadal factors and the importance of each type of regulation varies depending on the  
45 developmental stage of the gonad (Patiño and Sullivan, 2002; Schulz and Miura, 2002). The  
46 pituitary-derived gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone  
47 (LH) are primary mediators of gonadal steroidogenesis and gametogenesis. They bind and  
48 activate specific receptors (FSH receptor (FSHR) and LH receptor (LHR)), present on the  
49 surface of gonadal somatic cells, regulating the expression and activity of key steroidogenic  
50 enzymes (Themmen and Huhtaniemi, 2000). Although deeply studied in mammals, the  
51 precise function of each gonadotropin in teleosts is still largely unknown (Swanson et al.,  
52 2003). In the salmonid model, complementary functions of the gonadotropins were suggested  
53 by assessment of their transcript and plasma levels. FSH is considered to be involved in the  
54 initiation and early stages of gametogenesis, such as vitellogenesis and spermatogenesis, to  
55 some extent through the synthesis of estradiol-17 $\beta$  (E2) and 11-ketotestosterone (11-KT),  
56 respectively. LH is linked to final maturation and ovulation/ spermiation, in part by  
57 stimulating the production of maturation inducing hormones (MIHs, the progestins 17 $\alpha$ ,20 $\beta$ -  
58 dihydroxy-4-pregnen-3-one (17,20 $\beta$ P) and 17 $\alpha$ ,20 $\beta$ ,21-trihydroxy-4-pregnen-3-one (20 $\beta$ S))  
59 (Nagahama, 1994; Swanson et al., 2003).

60 Synthesis of steroids involves a complex cascade of oxidative enzymes that convert  
61 cholesterol into different functional steroids. The cytochrome P450 11 $\beta$ -hydroxylase, encoded  
62 by the *CYP11B1* gene is necessary for the final steps of the synthesis of 11-KT (Jiang *et al.*  
63 1996) whereas cytochrome P450 aromatase (P450arom, encoded by the *CYP19A1* gene),  
64 catalyzes the conversion of testosterone (T) to estradiol (E2) (Simpson *et al.* 1994). The  
65 cDNAs encoding these cytochromes have been cloned and characterized in several fish  
66 species including the sea bass (*Dicentrarchus labrax* L.) (Socorro et al., 2007; Dalla Valle et

67 al., 2002). Nevertheless, their expression during gametogenesis of this Perciform has not yet  
68 been investigated.

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

80 As mentioned above, most of the available information regarding physiological aspects of fish  
81 gonadotropins refers to salmonid species whose germ cells develop in a synchronous fashion.  
82 The fish species selected for this study is the European sea bass that presents a group-  
83 synchronous type of ovarian development (successive clutches of germ cells that will mature  
84 and be spawned are recruited from a population of vitellogenic oocytes), producing 3-4  
85 consecutive spawns during a 1-2 months spawning period that is repeated once a year during  
86 the winter (Asturiano et al., 2000). It is then difficult the extrapolation of salmonid findings to  
87 sea bass (or other fish with a non-synchronous type of gonadal development). Contrary to  
88 what was described for salmonids (reviewed in Swanson et al., 2003), the expression of the  
89 gonadotropin subunits during the reproductive cycle of male sea bass shows overlapping  
90 profiles, suggesting that both hormones could be involved in the control of all stages of  
91 gonadal development (Mateos et al., 2003).

92 Recently, we have described the molecular characterization of sea bass gonadotropin  
93 receptors (Rocha et al., 2007a). In the present study, we aimed to investigate their temporal  
94 expression patterns during an entire reproductive cycle in both male and female sea bass; To  
95 have a more holistic understanding of how different key factors interact to control sea bass  
96 gonadal function, changes in the expression of *CYP11B1*, *CYP19A1* and *StAR* genes were also  
97 evaluated in relation with sex steroid and LH plasma titers as well as gonadal development.

98

## 99 **Materials and Methods**

### 100 *Animals and sample collection*

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

117 spermiating testes and stage VI, post-spawning. The ovarian stages were as follows:  
118 previtellogenesis (prevtg); early vitellogenesis (evtg); late-vitellogenesis and post-  
119 vitellogenesis (lat-postvtg); maturation-ovulation (mat-ovul) and atresia (atre) (Asturiano et  
120 al., 2000). Representative sections showing the different gonadal developmental stages of the  
121 animals used in this work can be found in Rocha et al. (2007b). Gonadosomatic index (GSI)  
122 was determined by the following formula: gonad weight/ body weight x 100.

123

#### 124 *Hormone analysis*

125 Plasma E2 was measured by a conventional enzyme immunoassay (EIA), validated for its use  
126 on the sea bass in our laboratory (B Crespo, JM Navas, A Rocha, S Zanuy, M Carrillo,  
127 unpublished). The assay uses a rabbit antiserum against E2 whose specificity is shown in  
128 (Prat et al., 1990). The EIA protocol was similar to that previously developed for testosterone  
129 determination (Rodriguez et al., 2000a). Briefly, plasma was extracted with methanol. The  
130 organic solvent was evaporated and the dry extract was reconstituted in assay buffer (EIA  
131 buffer, Cayman Chemical MI, USA). Each component, E2-acetylcholinesterase tracer, anti-  
132 E2 rabbit antiserum and E2 standards (Sigma-Aldrich, Inc) or samples, were added to 96-well  
133 microtiter plates coated with mouse anti-rabbit IgG monoclonal antibodies (Clone RG-16,  
134 Sigma-Aldrich, Inc) and incubated overnight at 37°C. Then, plates were rinsed and colour  
135 development was performed by addition of Ellman's reagent and incubation for 2 h at 20°C in  
136 the dark. Optical density was read at 405 nm using a microplate reader (Bio-Rad microplate  
137 reader model 3550). The sensitivity of the assay was around 0.156 ng/ml (Bi/B0 = 90%).  
138 The plasma levels of 11-KT were determined by an EIA developed for the Siberian sturgeon  
139 (Cuisset et al., 1994) and modified for its use in sea bass (Rodriguez et al., 2005). The assay  
140 sensitivity of 11-KT was 0.0012 ng/ml. Plasma LH levels were measured by a homologous

141 competitive ELISA according to (Mateos et al., 2006). The sensitivity of the assay was 0.65  
142 ng/ml.

143

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

145 Sea bass total RNA was isolated from head kidney using the TRI Reagent (Molecular  
146 Research Center, Inc. Cincinnati, OH) according to the manufacturer's instructions. For  
147 cDNA synthesis, 4 µg of total RNA were denatured at 65°C for 5 min in the presence of 100  
148 ng of random hexamers and 1 µl of dNTPs (10 mM each dNTP), and then chilled on ice. RT  
149 was performed at 42°C for 50 min using Superscript II reverse transcriptase (Invitrogen Corp.,  
150 Carlsbad, CA). Protection of mRNA from ribonucleases during the cDNA synthesis was  
151 assured by using 40 units of RNasin (Promega Corp.). The reaction was stopped by heating at  
152 70°C for 15 min.

153 In order to obtain a fragment of sea bass *StAR* cDNA, a PCR was performed using 2 µl of  
154 cDNA and the degenerate primers star1 (5'-  
155 CC(T/A)CCTGCTTC(C/T)TGGC(G/T)GG(A/G)-3') and star2 (5'-  
156 GCATCTTGTGTCAGCAGGC(A/G)TG-3) designed to conserved regions of *StAR* from the  
157 largemouth bass (*Micropterus salmoides*, GenBank:DQ166820). Thermal cycling was  
158 performed using a touchdown PCR program (Don et al., 1991). The following conditions  
159 were used: an initial denaturation step at 94°C for 2 min followed by 20 cycles of 94°C for 30  
160 sec, the highest annealing temperature (70°C) for 30 sec, and an extension temperature of  
161 72°C for 30 sec. The annealing temperature was then decreased 0.5°C per cycle resulting in a  
162 10°C span. Final extension was a single cycle of 72°C for 5 min. The PCR product was cloned  
163 into the pGEM-T Easy Vector (Promega Corp.) and sequenced on an automated ABI PRISM  
164 3730 DNA Analyser (Applied Biosystems, Foster City, CA) using the Rhodamine terminator  
165 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<sup>®</sup>169 Pro Green Kit (Qbiogene Inc., Irvine, CA) and the FastPrep<sup>®</sup> 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)<sup>+</sup> enriched RNA, instead of total RNA, was used in the female177 seasonal expression study. The Oligotex<sup>®</sup> mRNA Kit (Qiagen GmbH, Germany) was used to178 isolate poly (A)<sup>+</sup> mRNA from ~ 240 µg of ovarian total RNA preparations. The ULTRA179 Evolution 384<sup>™</sup> (Tecan Group Ltd., Männedorf, Switzerland) fluorescence-based microplate180 reader along with the RediPlate<sup>™</sup> 96 RiboGreen<sup>®</sup> RNA Quantitation Kit (Invitrogen -181 Molecular Probes, Eugene, OR) were used for poly (A)<sup>+</sup> 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)<sup>+</sup> mRNA. The volume of poly (A)<sup>+</sup>

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<sup>™</sup> (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<sup>™</sup> QPCR Mix (Advanced Biotechnologies Ltd, Epsom, UK). To correct

190 for variability in amplification efficiency between different cDNAs, standard curves were



191 prepared for the sea bass target genes (*FSHR*, *LHR*, *StAR*, *CYP11B1* and *CYP19A1*) and the  
192 sea bass endogenous reference genes (*18S rRNA* and elongation factor 1-alpha (*Ef1-alpha*)).  
193 Ten-fold serial dilutions of known concentrations of the plasmids containing each of the  
194 genes were used. Data were capture and analyzed by the iCycler iQ™ software (version  
195 3.0.6070). Correlation coefficients of the standard curves ranged from 0.99 to 1.00. PCR  
196 efficiencies are shown in Table 1. For each experimental sample, the amount of target and  
197 endogenous reference was determined from the appropriate standard curve.  
198 The expression of the genes of interest was analysed using two separate methods: (a) Raw  
199 arbitrary input amount (non-normalized) and (b) Input amount normalized against a control  
200 gene. The *18S rRNA* and *Ef1-alpha* endogenous genes were tested for their ability to be used  
201 as control genes. They were chosen based on previous studies performed in gonads of other  
202 fish species (*e.g.*, (Kumar et al., 2000; Bobe et al., 2004; Kusakabe et al., 2006) and because  
203 sea bass *28S rRNA* and *β-actin* have already been proved no to be suitable (Halm et al., 2008).  
204 Male data normalization was done by dividing the input amount by the *18S rRNA* amount.  
205 Concerning females, the input amount was normalized against adjusted *Ef1-alpha* values.  
206 This method involves the standardization of expression of the reference gene in each sample  
207 of each month to a randomly chosen “control” group and it has been used in the  
208 characterization of the expression levels of several genes at different stages of ovarian  
209 follicular development in zebrafish (*Danio rerio*) (Ings and Van Der Kraak, 2006). This is  
210 done by using the following formula according to Billiau et al. (2001): individual value within  
211 a group/(mean value within a group/mean value of control group), where the previtellogenic  
212 stage was chosen as the control group. Data are presented as relative mRNA levels. In males,  
213 the mean of samples in stage VI was set as 1, while in females the mean of samples from  
214 previtellogenesis was the chosen value to be set as 1.

215 Further information regarding the adopted strategy for real-time PCR data normalization is  
216 available on Supplementary Methods online

217

#### 218 *Data representation and statistical analysis*

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

234

## 235 **Results**

### 236 *Cloning of a partial cDNA of sea bass StAR*

237 A partial 290 bp cDNA for sea bass *StAR* was amplified to allow the design of specific  
238 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.

263 Data collected on the GSI of the fish used in this study are shown in Fig. 1. In both male and  
264 female, the GSI values were low during the summer and early fall (July-October). In males,  
265 the GSI (Fig. 1, A) started to increase in November to reach high levels in December  
266 remaining high during spermatogenesis (II, III, IV) and full spermiation (V) stages. In  
267 females, the GSI (Fig. 1, B) rapidly increased from November on until it peaked in February,  
268 during the maturation-ovulation stage. A progressive decrease of the GSI was then observed  
269 in both sexes from March onwards until low values were reached again.

270

#### 271 *Seasonal changes in hormone plasma levels*

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

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

284

#### 285 *Seasonal changes in 18S rRNA and Efl-alpha expression levels*

286 The seasonal changes in the expression of the reference genes, *18S rRNA* and *Efl-alpha*,  
287 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 ( $P < 0.01$ ;  $P \leq 0.001$ ) 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, *Efl-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, *Efl-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 *Efl-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 from 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 *FSHRs* agrees with the one of  
356 their *FSH $\beta$*  genes, which code for the specific subunit of FSH. In yellowtail, *FSH $\beta$*  expression  
357 decreased in spermiating males, while in sea bass expression of *FSH $\beta$*  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).

362 In fish, as in mammals, *FSHR* is expressed in Sertoli cells (Miwa et al., 1994; Petersen and  
363 Söder, 2006), although it has been recently demonstrated that Leydig cells also express the  
364 *FSHR* in Japanese eel (*Anguilla japonica*) and African catfish (*Clarias gariepinus*) (Ohta et  
365 al., 2007; Garcia-Lopez et al., 2009). In maturing and adult testis from African catfish and  
366 Nile tilapia (*Oreochromis niloticus*), Sertoli cell proliferation occurs primarily during  
367 spermatogonial proliferation and ceases in postmeiotic cysts. At the beginning of  
368 spermiogenesis, due to the expansion of cyst volume and the stabilization of Sertoli cell  
369 number per cyst, there is a dilution of Sertoli cells. However, during the spermiogenic process  
370 there is a striking reduction of cyst volume in Nile tilapia testis (Schulz et al., 2005).

371 Assuming an analogous behaviour for sea bass Sertoli cell proliferation during testicular  
372 development, the progressive increase in *FSHR* expression observed in stages I and II (Fig. 4,  
373 A) could be related with a proliferation of Sertoli cells, and the decrease of expression in  
374 stages III and IV could be the result of a dilution of somatic cells with respect to germ cells,  
375 rather than a reduction in *FSHR* transcripts. During spermiation this dilution effect is no  
376 longer observed, resulting in a second increase in the expression levels. Nevertheless, the  
377 decline in *FSHR* expression during mid recrudescence could also be the result of a transient  
378 transcription downregulation to prevent Sertoli cell overstimulation by FSH (Themmen et al.,  
379 1991). Then, the observed enhancement of expression during sea bass spermiation could be  
380 due to an upregulation of *FSHR* expression, and/or connected with a new proliferation of  
381 Sertoli cells needed for the maintenance of spermatogenesis in several clutches of gametes  
382 present in the testis, since spermiation is associated with the degeneration of at least some of  
383 the Sertoli cells (Billard, 1986; Prisco et al., 2003).

384 The *LHR* expression profile in sea bass testis (Fig. 4, B) is consistent with data from  
385 maturing rainbow trout and yellowtail males, showing maximum receptor mRNA levels  
386 during spermiation (Rahman et al., 2003; Kusakabe et al., 2006; Sambroni et al., 2007).



387 However, in yellowtail and rainbow trout, according to Kusakabe et al. (2006), receptor  
388 expression steadily increases during testicular maturation, while in sea bass and rainbow trout,  
389 according to Sambroni et al.(2007), *LHR* mRNA levels were maintained almost constant until  
390 the end of the recrudescence stage. Analysis of LH levels in sea bass plasma (Fig. 2, B)  
391 showed an increase of this hormone during spermatogenesis reaching the highest levels in  
392 spermiation, which is in agreement with the expression profiles of sea bass *LH $\beta$*  (Mateos et  
393 al., 2003) and *LHR* (Fig. 4, B). These results support the already suggested role of LH in the  
394 regulation of the final stages of fish gamete maturation and spermiation (reviewed in Swanson  
395 et al., 2003).

396 11-KT is considered to play an important role in stimulating spermatogenesis in several fish  
397 species (Schulz and Miura, 2002) including sea bass (Rodriguez et al., 2000b). The profile of  
398 11-KT obtained in this study (Fig. 2, A) is in accordance with previous results obtained by us  
399 in sea bass (Rodriguez et al., 2000b), with levels increasing during mid recrudescence, and  
400 dropping once spermiation begins. In fish, very little information is available on the specific  
401 roles of FSH and LH in regulating androgen production by the testis. In coho salmon, FSH  
402 and LH were equipotent in stimulating the production of T, 11-KT and the MIH 17,20 $\beta$ P by  
403 testicular tissue in late stages of spermatogenesis, nevertheless the steroidogenic effects of LH  
404 increased as spermatogenesis progressed (Planas and Swanson, 1995). In red seabream, both  
405 FSH and LH stimulated the production of 11-KT in sliced testis of animals in the spawning  
406 season (Kagawa et al., 1998) and in sexually immature cultivated Japanese eel FSH induces  
407 spermatogenesis via stimulation of 11-KT production (Ohta et al., 2007). Recent studies using  
408 *in vitro* culture of sea bass testis have shown that purified native FSH stimulates 11-KT  
409 secretion in a dose and time dependent manner (Moles et al., 2008). In this study, we did not  
410 find a correlation between 11-KT profile in plasma and sea bass *FSHR* or *CYP11B1*  
411 expression; however, the expression profiles of both *FSHR* and *CYP11B1* genes were highly

412 and positively correlated (Table 2), suggesting, all together, that FSHR signalling could be  
413 involved in *CYP11B1* expression in sea bass. *CYP11B1* expression was high during early  
414 gonadal growth, declining in late recrudescence, when 11-KT plasma levels were highest.  
415 This delayed profile of plasma 11-KT with respect to enzyme expression has also been  
416 observed in rainbow trout males (Kusakabe et al. 2006), and could be the result of a mismatch  
417 between expression and activity of the steroidogenic enzyme P450 $\beta$ 11. Changes in *CYP11B1*  
418 transcripts were also found to positively vary together with *LHR* expression. Thus, further  
419 studies will be needed to understand the action of each gonadotropin in the synthesis of sex  
420 steroids and the specific role of all of them in the spermatogenic process of sea bass.  
421 Interestingly, the quantification of *StAR* transcripts in sea bass testis (Fig. 4, C) revealed a  
422 profile identical to the one observed for the *LHR* (Fig. 4, B), what was supported by a  
423 significant positive correlation between both gene mRNA levels (Table 2). These results are  
424 in line with the ones reported in rainbow trout males (Kusakabe et al., 2006). The acute,  
425 steroidogenic effect of LH in mammalian Leydig cells is based on an increased availability of  
426 cholesterol for the mitochondrial P450 $scc$ . This is achieved via induction of *StAR* (Stocco et  
427 al., 2005). Our results indicate that a similar regulation may occur in the sea bass testis.  
428 Like in males, in sea bass females both gonadotropin receptors follow a similar expression  
429 pattern (Fig. 4, E and F). Expression of these genes is strongly positively correlated (Table 2),  
430 although the expression levels of *FSHR* are remarkably higher than those of *LHR* (Fig. 4).  
431 Before yolk incorporation, during primary growth (previtellogenesis), both receptors are  
432 expressed at extremely low levels in sea bass ovary. In early vitellogenesis (October), the  
433 expression level of *FSHR* slightly increased while *LHR* mRNA levels remained unchanged.  
434 Recent work in channel catfish (*Ictalurus punctatus*) and zebrafish (*Danio rerio*) has  
435 suggested that an enhancement in ovarian *FSHR* expression occurs at the beginning of  
436 vitellogenesis and this upregulation continues through vitellogenesis (Kumar and Trant, 2004;

437 Kwok et al., 2005). In coho salmon, *FSHR* expression remained unchanged during  
438 previtellogenesis and enlargement of expression was only observed with the appearance of  
439 lipid droplets in the oocyte (Campbell et al., 2006). Contrary to what was believed for long  
440 time, recent studies have proved that vitellogenins enter the ovarian follicle at the same time  
441 as cortical alveoli and lipid globules appear (Le Menn et al., 2007). Taken together, these data  
442 suggest that the initial increase of *FSHR* expression is connected with the start of yolk  
443 proteins uptake, at least in the above mention species. Increases in sea bass ovarian *LHR*  
444 mRNA levels were only observed when postvitellogenesis began (December). At that stage,  
445 *FSHR* mRNA levels were already at their maximum. During the maturation-ovulation period,  
446 expression levels of both receptors remained elevated, returning to their basal levels only after  
447 spawning.

448 Studies on female salmonids, which have a synchronous type of oocyte development, suggest  
449 that secondary oocyte growth is regulated primarily by FSH, whereas LH plays a major role  
450 in regulating final oocyte maturation. Nonetheless, the observed expression pattern of *FSHR*  
451 in sea bass (Fig. 4, E) involves this receptor (and FSH) also in processes occurring after  
452 secondary oocyte growth. Various studies performed on rainbow trout ovary have shown that  
453 increased *FSHR* expression is related with high maturational competence (Bobe et al., 2004),  
454 oocyte maturation and ovulation (Sambroni et al., 2007). Regarding sea bass, we consider that  
455 the observed high expression level of *FSHR* during maturation could be connected with  
456 oocyte growth and is explained by the reproductive strategy of this species. Sea bass ovary  
457 exhibits a group-synchronous type of development, and so, during the maturation-ovulation  
458 stage at least two populations of oocytes can be distinguished at the same time; a fairly  
459 synchronous population of larger oocytes (defined as a clutch) and a more heterogeneous  
460 population of smaller oocytes from which the clutch is recruited (Mayer et al., 1990;  
461 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

487 plasma E2 levels were observed prior to peaks of the progestins 17,20 $\beta$ P and 20 $\beta$ S (the sea  
488 bass MIHs), which resulted in the maturation/ovulation of different clutches of oocytes  
489 (Asturiano et al., 2002). The shift from estrogen to MIHs synthesis needs both the partial  
490 reworking of the steroidogenic pathway and the rapid delivery of cholesterol substrate, which  
491 requires the StAR protein. Thus, the expression profile of sea bass *StAR* obtained in this study  
492 is consistent with an important involvement of the coded protein in the synthesis of sea bass  
493 MIHs.

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

506 **Acknowledgements**

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

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695 **Figure Legends**

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

701

702 **Figure 2** - Changes in plasma levels of 11-KT (A), LH (B and D) and E2 (C) in male and  
 703 female sea bass during their first sexual maturation. Data, shown as the mean  $\pm$  SEM, are  
 704 represented by stages of gonadal development as determined by histology (see Materials and  
 705 Methods). Males: stage I ( $n = 19$ ), immature; stage II ( $n = 6$ ), early recrudescence; stage III ( $n$   
 706  $= 3$ ), mid recrudescence; stage IV ( $n = 6$ ), late recrudescence; stage V ( $n = 10$ ), full  
 707 spermiating testes and stage VI ( $n = 4$ ), post-spawning. Females: prevtg ( $n = 15$ ),  
 708 previtellogenesis; evtg ( $n = 10$ ), early vitellogenesis; lat-postvtg ( $n = 7$ ), late-post-  
 709 vitellogenesis; mat-ovul ( $n = 14$ ), maturation-ovulation and atre ( $n = 13$ ), atresia. Different  
 710 significance levels are indicated with different letters above the bars.

711

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

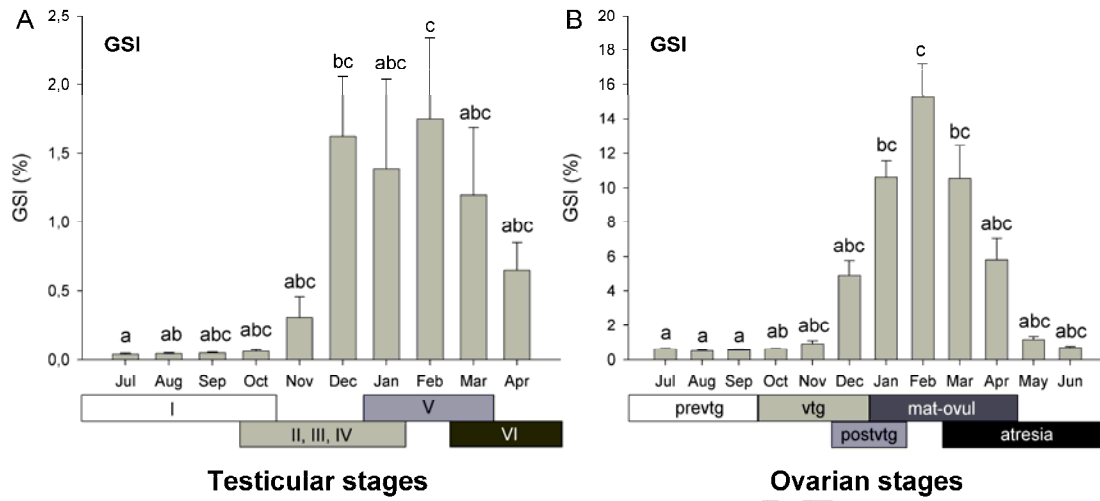
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718 **Figure 4** - Relative changes in expression of *FSHR*, *LHR*, *StAR*, *CYP11B1* and *CYP19A1* in  
 719 male (A, B, C, D) and female (E, F, G, H) sea bass, sampled during their first sexual  
 720 maturation. Values, shown as the mean  $\pm$  SEM, are represented by stages of gonadal  
 721 development as determined by histology. Males: stage I ( $n = 19$ ), immature; stage II ( $n = 6$ ),  
 722 early recrudescence; stage III ( $n = 3$ ), mid recrudescence; stage IV ( $n = 6$ ), late recrudescence;  
 723 stage V ( $n = 10$ ), full spermiating testes and stage VI ( $n = 4$ ), post-spawning. Females: prevtg  
 724 ( $n = 15$ ), previtellogenesis; evtg ( $n = 10$ ), early vitellogenesis; lat-postvtg ( $n = 7$ ), late-post-  
 725 vitellogenesis; mat-ovul ( $n = 14$ ), maturation-ovulation and atre ( $n = 13$ ), atresia. Male  
 726 expression values are normalized to *18S rRNA* and expressed as a proportion of the mean  
 727 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



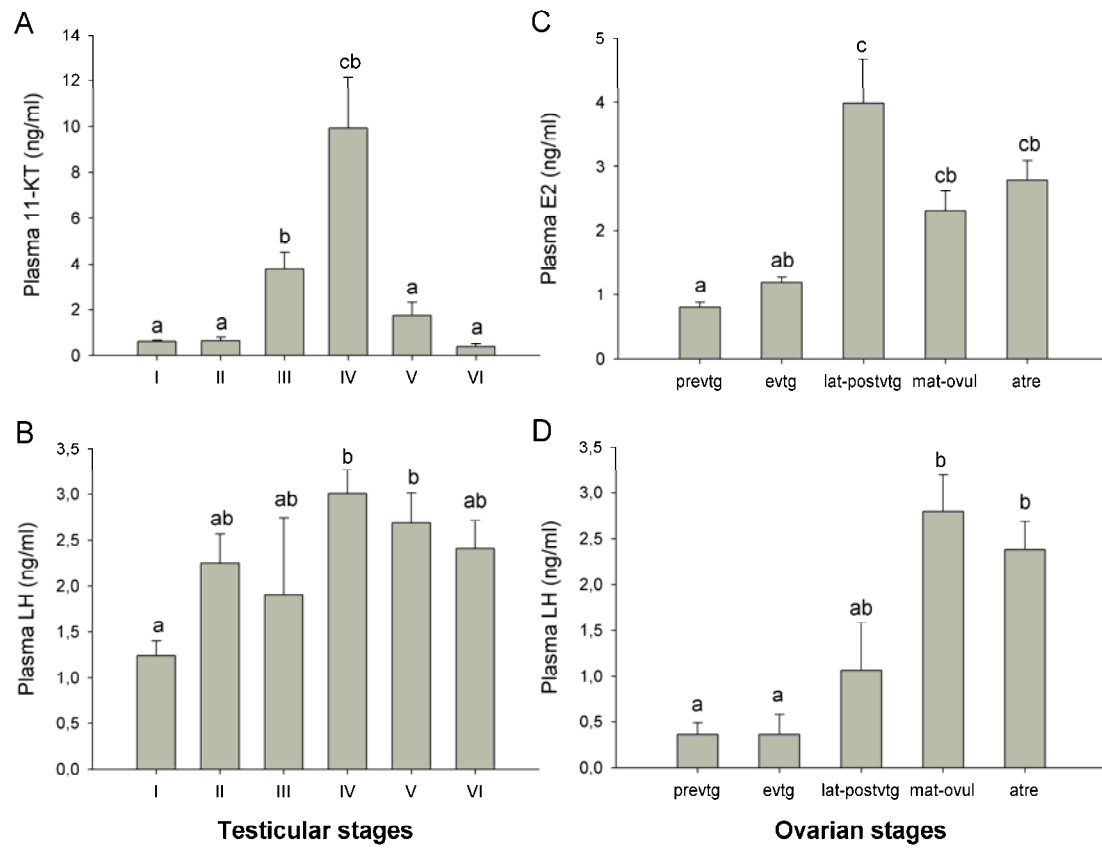
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735 Figure 2

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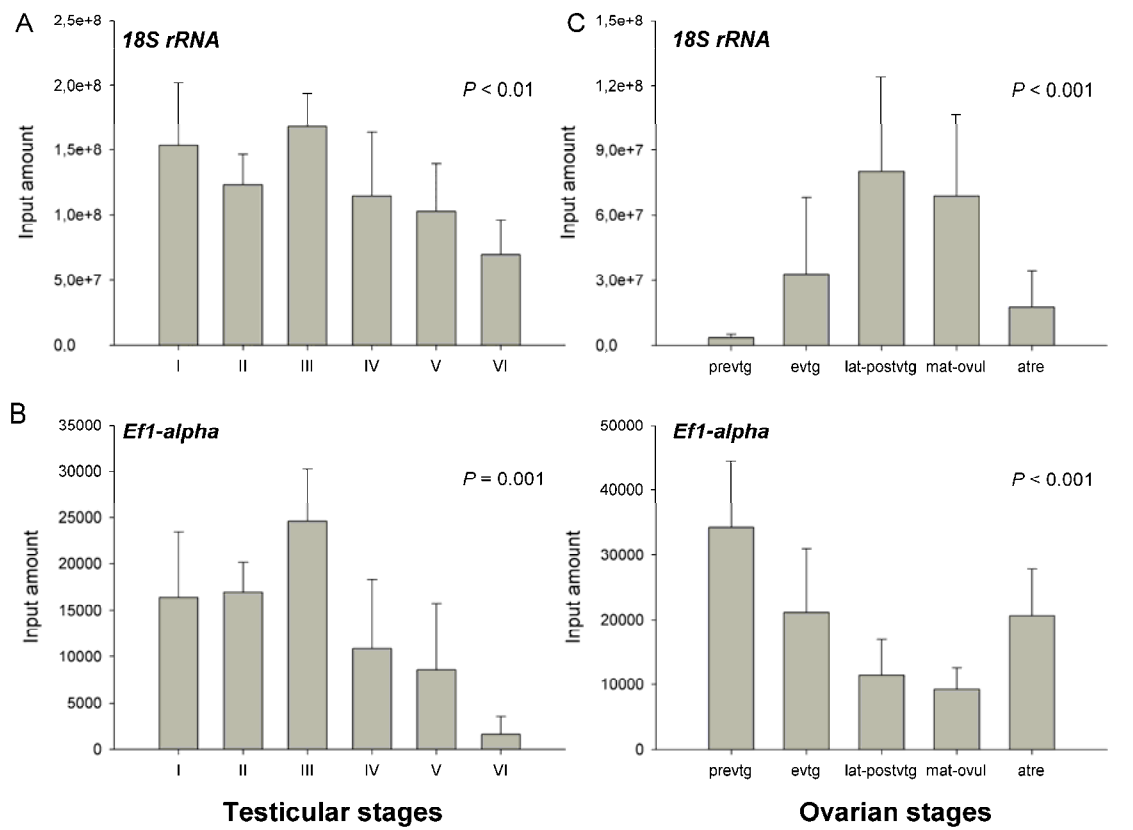


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738 Figure 3

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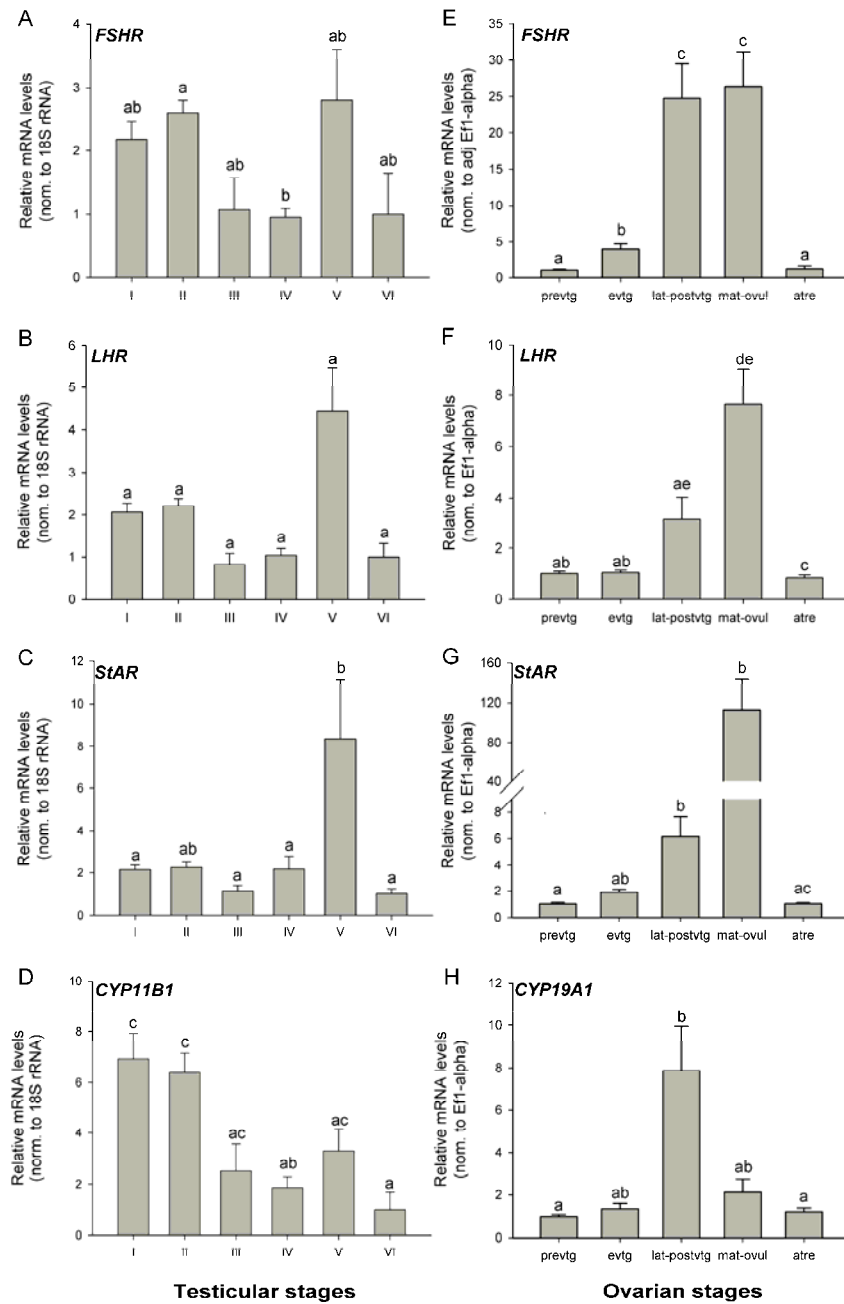


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741 Figure 4

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744



**Table 1** Primers and TaqMan™ fluorogenic probes<sup>a</sup> used in this study

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

<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).

A	<i>LHR</i>	<i>StAR</i>	<i>CYP11B1</i>	11-KT	LH
<i>FSHR</i>	<sup>a,c</sup> <b><i>P</i>=0.000</b> <sup>b</sup> <b><i>r</i>=0.54</b>	<i>P</i> =0.005 <i>r</i> =0.40	<b><i>P</i>=0.000</b> <b><i>r</i>=0.74</b>	<i>P</i> =0.21 <i>r</i> =-0.19	<i>P</i> =0.32 <i>r</i> =-0.15
<i>LHR</i>		<b><i>P</i>=0.000</b> <b><i>r</i>=0.67</b>	<b><i>P</i>=0.000</b> <b><i>r</i>=0.56</b>	<i>P</i> =0.03 <i>r</i> =-0.31	<i>P</i> =0.68 <i>r</i> =-0.06
<i>StAR</i>			<i>P</i> =0.050 <i>r</i> =0.29	<i>P</i> =0.75 <i>r</i> =0.05	<i>P</i> =0.09 <i>r</i> =0.25
<i>CYP11B1</i>				<i>P</i> =0.17 <i>r</i> =-0.20	<i>P</i> <0.01 <i>r</i> =-0.37
11-KT					<i>P</i> =0.13 <i>r</i> =0.22
B	<i>LHR</i>	<i>StAR</i>	<i>CYP19A1</i>	E2	LH
<i>FSHR</i>	<b><i>P</i>=0.000</b> <b><i>r</i>=0.80</b>	<b><i>P</i>=0.000</b> <b><i>r</i>=0.82</b>	<b><i>P</i>=0.000</b> <b><i>r</i>=0.43</b>	<b><i>P</i>=4.4 x 10<sup>-3</sup></b> <b><i>r</i>=0.37</b>	<i>P</i> =0.52 <i>r</i> =0.09
<i>LHR</i>		<b><i>P</i>=0.000</b> <b><i>r</i>=0.72</b>	<i>P</i> =0.110 <i>r</i> =0.21	<i>P</i> =0.06 <i>r</i> =0.24	<i>P</i> =0.25 <i>r</i> =0.15
<i>StAR</i>			<b><i>P</i>=0.000</b> <b><i>r</i>=0.43</b>	<b><i>P</i>=3.4 x 10<sup>-3</sup></b> <b><i>r</i>=0.38</b>	<i>P</i> =0.30 <i>r</i> =0.14
<i>CYP19A1</i>				<b><i>P</i>=2.4 x 10<sup>-3</sup></b> <b><i>r</i>=0.39</b>	<i>P</i> =0.94 <i>r</i> =-0.009
E2					<b><i>P</i>=0.000</b> <b><i>r</i>=0.43</b>

<sup>a</sup> Probability value.

<sup>b</sup> Correlation coefficient value.

<sup>c</sup> Values showed in bold were considered statistically significant after applying Bonferroni correction.