ResearchGate

See discussions, stats, and author profiles for this publication at: http://www.researchgate.net/publication/275277978

Characterization of the annual regulation of reproductive and immune parameters on the testis of European sea bass

ARTICLE *in* CELL AND TISSUE RESEARCH · APRIL 2015

Impact Factor: 3.33 · DOI: 10.1007/s00441-015-2172-1 · Source: PubMed

DOWNLOADS
23

VIEWS
43

6 AUTHORS, INCLUDING:

Miriam Sánchez-Hernández
Instituto Español de Oceanografia
9 PUBLICATIONS **17** CITATIONS

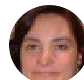
SEE PROFILE

Alfonsa García-Ayala
University of Murcia
86 PUBLICATIONS **1,213** CITATIONS

SEE PROFILE

Alberto Cuesta
University of Murcia
97 PUBLICATIONS **2,036** CITATIONS

SEE PROFILE



Elena Chaves-Pozo
Instituto Español de Oceanografia
52 PUBLICATIONS **796** CITATIONS

SEE PROFILE

Available from: Elena Chaves-Pozo
Retrieved on: 08 September 2015

1 **Characterization of the annual regulation of reproductive and immune parameters**

2 **on the testis of European sea bass.**

3 **Short title:** Reproductive cycle of one year old sea bass

4 **Authors**: Yulema Valero[1], Miriam Sánchez-Hernández[1], Alicia García-Alcázar[1],

5 Alfonsa García-Ayala[2], Alberto Cuesta[2], Elena Chaves-Pozo[1*].

6 [1]Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera

7 de la Azohía s/n. Puerto de Mazarrón, 30860 Murcia, Spain

8 [2]Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of

9 International Excellence "Campus Mare Nostrum", University of Murcia, 30100

10 Murcia, Spain

11 **Correspondence and reprint requests**: *To whom correspondence should be

12 addressed to Centro Oceanográfico de Murcia, Instituto Español de Oceanografía,

13 Carretera de la Azohía s/n. Puerto de Mazarrón, 30860 Murcia, Spain.

14 elena.chaves@mu.ieo.es, fax: +34-968153934, tel: +34-968153339.

15 **Keywords:** European sea bass, cell renewal, testis, hormone levels, immune factors

16

17 **Footnote[1]**: The genetic nomenclature used in this manuscript follow the guidelines of

18 Zebrafish Nomenclature Committee (ZNC) for fish genes and proteins and the HUGO

19 Gene Nomenclature committee for mammalian genes and proteins.

20

0. Abstract

The European sea bass, *Dicentrarchus labrax* L., is a seasonal gonochoristic species, which males are generally mature during the second year of life. It has been demonstrated that cytokines and immune cells play a key role in the testicular development. This reproductive-immune interaction might be very important in the sea bass since several pathogens are able to colonize the gonad and persist in this tissue, altering further reproductive functions and spreading the disease. This study aims to investigate the reproductive cycle of one year European sea bass males by analyzing cell proliferation and apoptosis and expression profile of some reproductive and immune-related genes in the testis as well as the serum sex steroid levels. Our data demonstrate that in one year old European sea bass males, the testis undergoes the spermatogenesis process and that the reproductive and immune parameters analyzed varied during the reproductive cycle. In the testis, the highest proliferative rates were recorded at spermatogenesis stage, while the highest apoptotic rates were recorded at spawning stage. We have also analyzed, for the first time in European sea bass males, the serum levels of 17β-estradiol (E_2) and dihydrotestosterone and the gene expression profile of the enzymes implied in their production, determining that at least E_2 might be involved in the regulation of the reproductive cycle. Some immune relevant genes, including cytokines, lymphocyte receptors, anti-viral and antibacterial molecules were detected in the testis of naïve European sea bass specimens and their expression profile was related with the stages of the reproductive cycle, suggesting an important role for the defence of the reproductive tissues.

45 **1. Introduction**

46 Fish are the most diverse and numerous group of vertebrates. However, our
47 knowledge on fish spermatogenesis is limited to a few species used in basic research
48 and/or in aquaculture biotechnology. In the case of the European sea bass
49 (*Dicentrarchus labrax* L.) most of the studies, as far as we are concern, dealing with
50 gonad physiology are focussed on early hatch specimens until 300 days post-hatching
51 (dph) or on mature specimens from two years old onwards. However, the events that
52 occur in the gonad of one year old fish have been scarcely studied. Interestingly, data
53 obtained in the gilthead seabream (*Sparus aurata* L.), a hermaphrodite species, points to
54 the fact that captive specimens quickly differentiated the testicular area and produced
55 spermatozoa even when the specimens are not spermiogenic active males (Chaves-Pozo
56 et al., 2009).

57 The reproductive cycle of fish can be divided into four stages: spermatogenesis,
58 spawning, post-spawning, and resting stages, according to the gonadosomatic index
59 (GSI), the magnitude of some processes such as cell apoptosis and proliferation and the
60 percentage of some cell types. In general, spermatogenesis stage is a complex
61 developmental process, in which spermatogonia divide (renewal of spermatogonia stem
62 cells and mitotic proliferation of spermatogonia), reduce their chromosome content by
63 meiosis, and differentiate into spermatozoa. During post-spawning stage, an intensive
64 remodelling of the testis occurs, while the enhancement of proliferation and the absence
65 of apoptosis permit the repopulation of the testis by spermatogonia and Sertoli cells
66 during resting stage (Chaves-Pozo et al., 2005; Liarte et al., 2007). In teleost fish,
67 spermatogenesis is regulated by the interplay of systemic and intragonadal factors and
68 the importance of each type of regulation vary depending on the developmental stage of
69 the gonad (Schulz et al., 2010). Thus, the pituitary-derived gonadotropins, follicle-

70 stimulating hormone (FSH) and luteinizing hormone (LH) regulate spermatogenesis by
71 acting directly on germ cells and indirectly by stimulating steroid hormone secretion
72 (Nagahama et al., 1994). In fact, plasma levels of sex steroids show important variations
73 during the maturation of the male gonad. In fish, testosterone (T) and 11-
74 ketotestosterone (11KT) are generally considered the major and most potent circulating
75 male androgens triggering spermatogenesis (Borg, 1994); however, recent data point to
76 dihydrotestosterone (DHT) as another potent androgen, involved in regulating
77 spermatogenesis in several fish species (Margiotta-Casaluci and Sumpter, 2011;
78 Margiotta-Casaluci et al., 2013; Martyniuk et al., 2013). In the other hand, 17β-estradiol
79 (E_2) has been considered the main sex steroid of female fish, although some studies
80 have suggested that estrogens are "essential" for normal male reproduction and might be
81 involved in the recrudescence of the testis (Chaves-Pozo et al., 2007; Hess, 2003; Miura
82 et al., 1999). Taken together, these data suggest that sex steroids have important and
83 distinct roles in controlling fish spermatogenesis.

84 Recent studies have also demonstrated the importance of leukocytes and several
85 immune factors in the regulation of the reproductive functions of the gonad. In fact, in
86 teleosts, the presence of immune cells and the expression of several relevant immune
87 genes in the gonad guarantees and modulate the reproductive functions (Chaves-Pozo et
88 al., 2008; Chaves-Pozo et al., 2009; Chaves-Pozo et al., 2003) and also conditioned the
89 immune response in the gonad allowing the persistence of several pathogens in those
90 tissues (Chaves-Pozo et al., 2010a; Chaves-Pozo et al., 2010b). Although the immune
91 responses triggered in the European sea bass gonad has not been well documented, it is
92 true that sea bass is very susceptible to several pathogens able to colonize the gonad,
93 persist on this tissue and be transmitted (Gómez-Casado et al., 2011; Sitja-Bobadilla
94 and Álvarez-Pellitero, 1993). Moreover, some of those pathogens alter the efficiency of

95 European sea bass reproduction and even block it (Sitja-Bobadilla and Álvarez-
96 Pellitero, 1993). Therefore, the characterization of the immune response, at both cellular
97 and molecular levels, in the sea bass gonad under naïve conditions is mandatory and
98 necessary to understand how those pathogens interact or block it.

99 Bearing all this in mind, we have analyzed, throughout the reproductive cycle of
100 one year old European sea bass males, the testis morphology, the serum levels of some
101 sex steroid hormones and the expression profile of some reproductive genes. Moreover,
102 we have analyzed the expression profile of some immune-relevant genes in the testis to
103 demonstrate its presence and potential role in the defence of the reproductive tissues.
104 We also made an effort to correlate all these data in order to provide a wide overview of
105 the physiology of the testis in order to determine the more susceptible stages to
106 pathogens and to develop successful preventive treatments.

107

108 2. Materials and Methods

109 *2.1. Animals and experimental design*

110 Healthy European sea bass (*Dicentrarchus labrax* L., *Actinopterygii*,
111 *Perciformes*, *Moronidae*) specimens of one year old were bred and kept at the *Centro*
112 *Oceanográfico de Murcia* (IEO, Mazarrón, Murcia). The fish were kept in 12.5 m^3
113 tanks with natural water temperature ranging from 14 to 26 ºC, a flow-through circuit, a
114 suitable aeration and filtration system and a natural photoperiod. The specimens were
115 fed with a commercial dry pellet diet (Skretting) *ad libitum*. The environmental
116 parameters, mortality and food intake, were daily recorded.

117 The fish were sampled once a month (n=8 males/month) from September 2,010
118 (121 ± 42 g mean of body weight, bw) to May 2011 (222 ± 70 g mean of bw). The

119 specimens were anesthetized with 40 µl/l of clove oil and blood was obtained from the
120 caudal peduncle and the serum samples, obtained by centrifugation (10,000 g, 1 min, 4
121 ºC), were immediately frozen in liquid nitrogen and stored at -80 ºC until use for sex
122 steroid levels analysis. Then, specimens were weighed and decapitated, and the gonads
123 were removed, weighed and processed for light microscopy and gene expression
124 analysis as described below. Some specimens were intraperitoneally injected with 50
125 mg/kg bw of 5-bromo-2'-deoxyuridine (BrdU, Sigma) 2 h before sampling. The
126 experiments described comply with the Guidelines of the European Union Council
127 (86/609/EU), the Bioethical Committee of the *Instituto Español de Oceanografía*
128 (Spain) and of the University of Murcia (Spain) for the use of laboratory animals.

129 *2.2. Light microscopy and immunocytochemical staining*

130 The gonads (n=8/month) were fixed in 4% paraformaldehyde solution,
131 embedded in paraffin (Paraplast Plus; Sherwood Medical), and sectioned at 5 µm. Some
132 sections were stained with hematoxylin-eosin to determine the sex and the reproductive
133 stage of each fish. Other sections were used to determine cell proliferation being
134 subjected to an indirect immunocytochemical method using a monoclonal antibody anti-
135 BrdU (Becton Dickinson) at the optimal dilution of 1:100, that revealed the proliferative
136 cells which have incorporated the BrdU, previously injected, during their DNA
137 synthesis phase as described previously (Chaves-Pozo et al., 2005). Negative controls
138 have been done by omitting the first antiserum or by using tissue sections from fish that
139 had not been injected with BrdU. Other sections were subjected to *in situ* detection of
140 DNA fragmentation (TUNEL) assay to identify apoptotic cells (*in situ* cell death
141 detection kit; Roche) as described previously (Chaves-Pozo et al., 2007). Negative
142 controls were processed in an identical manner except that the TdT enzyme was
143 omitted. Positive controls were performed treating the sections with DNase I (3–3,000

144 U/ml, Sigma) in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, and 1 mg/ml BSA for 10 min
145 at room temperature to induce DNA strand breaks before labelling. All slides were
146 examined with a Nikon Eclipse E600 light microscope.

147 *2.3. Analytical techniques*

148 Serum (n=4-6 fish/reproductive stage) levels of T, 11KT and E_2 were quantified
149 by ELISA following the method previously described (Rodríguez et al., 2000). Steroids
150 were extracted from 10 µl of serum in 1.3 ml of methanol (Panreac). Then, methanol
151 was evaporated at 37º C and the steroids were resuspended in 400 µl of reaction buffer
152 [0,1 M phosphate buffer with 1mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3
153 (Sigma) and 0.1% albumin from bovine serum (Sigma)]. Samples of 50 µl were
154 dispensed into wells of microtiter plates (MaxiSorp; Nunc) so that 1.25 µl of serum
155 were used in each well for all the assays. T, 11KT and E_2 standards, mouse anti-rabbit
156 IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic
157 tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical.
158 A standard curve from 6.13×10^{-4} to 5 ng/ml (0.03-250 pg/well), a blank and a non-
159 specific binding control (negative control) were established in all the assays. Standards
160 and extracted serum samples were run in duplicate and all the measures were corrected
161 with the blank and negative control. The lower limit of detection for all the assays was
162 24.4 pg/ml. The intra-assay coefficients of variation (calculated from sample duplicates)
163 were 4.06 ± 1.02% for T, 6.00 ± 1.53% for 11KT and 1.54 ± 0.28% for E_2 assays.
164 Details on cross-reactivity for specific antibodies were provided by the supplier (T
165 cross-reacts 0.01% in the 11KT assay and 0.1% in the E_2 assay; 11KT cross-reacts 2.2%
166 in the T assay). The serum (n=4-6 fish/reproductive stage) levels of dihydrotestosterone
167 (DHT) were quantified using the 5α-Dihydrotestosterone ELISA kit (DRG diagnostics)

168 following the manufacturer's instructions. Details on cross-reactivity for specific
169 antibodies were provided by the supplier (T cross-reacts 8.7% in the DHT assay).

170 *2.4. Analysis of gene expression*

171 Total RNA was extracted from testis fragments (n=7-12 fish/reproductive stage)
172 with TRIzol reagent (Invitrogen) following the manufacturer's instructions and treated
173 with DNase I, amplification grade (1 unit/µg RNA, Invitrogen). SuperScript III RNase
174 H^{-} Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with
175 oligo-dT18 primer from 1 µg of total RNA, at 50 ºC for 60 min.

176 The expression of some genes was analyzed by real-time PCR performed with
177 an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core
178 Reagents (Applied Biosystems) as previously described (Chaves-Pozo et al., 2008).
179 These genes code for (i) steroidogenic enzymes such as steroid 11-β-hydroxylase
180 (*cyp11b1*), 3β-hydroxysteroid dehydrogenase (*dhs3b*), and aromatase (*cyp19a1a*); (ii)
181 the anti-Mullerian hormone (*amh*); (iii) hormone receptors such as follicle stimulating
182 hormone receptor (*fshr*), and estrogen receptor β 1 (*erb1*), and *erb2*; (iv) cytokines such
183 as interleukin 1β (*il1b*), *il6*, and tumour necrosis factor α (*tnfa*); (v) lymphocyte
184 receptors such as the heavy chain of immunoglobulin M (*ighm*) and the β subunit of T-
185 cell receptor (*tcrb*); (vi) viral recognition molecules such as melanoma differentiation-
186 associated 5 protein (*mda5*) and laboratory of genetics and physiology 2 protein (*lgp2*),
187 anti-viral signalling pathway molecules such as TANK-binding kinase 1 (*tbk1*) and
188 interferon regulatory factor 3 (*irf3*) and anti-viral response molecules such as interferon
189 (*ifn*), anti-viral protein kinase (*pkr*) and myxovirus (influenza) resistance proteins (*mx*);
190 and (v) anti-bacterial molecules such as lysozyme (*lyz*), complement component 3 (*c3*),
191 dicentracin (*dic*), hepcidin (*hamp*), and the histones H1 (*h1*) and H2B (*h2b*). For each
192 mRNA, gene expression was corrected by the *elongation factor 1 alpha* gene (*ef1a*)

193 content in each sample. The European sea bass specific primers used are shown in Table
194 1. Some genes were identified in the expressed sequence tags (ESTs) databases and
195 Table 2 shows their relation with the zebrafish orthologs. Before the experiments, the
196 specificity of each primer pair was studied using positive and negative samples.
197 Amplified products from positive samples were purified and sequenced. In all cases,
198 each PCR was performed in triplicate wells. The *ef1a* gene expression showed 2 % of
199 variability across stages.

200 *2.5. Calculation and statistics*

201 The gonadosomatic index (GSI) was calculated as an index of the reproductive
202 stage [100*(MG/MB) (%)], where MG is gonad mass (in grams) and MB is body mass
203 (in grams).

204 The quantification of proliferative ($BrdU^+$) and apoptotic cell areas was
205 calculated as the mean value ± SEM of the stained area/total area of 50 randomly
206 distributed optical areas at 200 x magnifications. The stained areas were measured by
207 image analysis using a Nikon Eclipse E600 light microscope, an Olympus SC30 digital
208 camera (Olympus soft imaging solutions GMBH), and Leica Qwin software (Leica
209 microsystems).

210 All data were analyzed by one-way ANOVA and a Duncan *post hoc* test to
211 determine differences between groups ($P \leq 0.05$). Normality of the data was previously
212 assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using
213 the Levene test. A non-parametric Kruskal–Wallis test was used when data did not meet
214 parametric assumptions. In addition, non-parametric Pearson correlation tests were
215 applied to test correlations among hormonal levels and gene expression levels.
216 Statistical analyses were conducted using SPPS 15.0 software (SPSS).

217

218 **3. Results**

219 *3.1. The reproductive cycle is completed in one year old European sea bass*
220 *specimens.*

221 We observed that the reproductive cycle of one year old European sea bass
222 males is divided in four stages according to the GSI (Fig. 1a) and the testicular
223 morphology (Fig. 1b-j). Thus, at resting stage, the GSI was low and the testis was
224 formed by tubules without lumen or with a very small lumen and mainly formed by
225 cysts of spermatogonia (Fig. 1b,c). At spermatogenesis stage, the spermatogonia start to
226 progress through the spermatogenetic process and cysts of more developed germ cells
227 appeared (Fig. 1d-f). As spermatogenesis progresses, the GSI progressively increased
228 (Fig. 1a) and the amount of cysts of spermatocytes, spermatids and spermatozoa and
229 the amount of free spermatozoa in the lumen of the tubules were more visible, while the
230 amount of cysts of spermatogonia were proportionally less visible (Fig. 1d-f). At
231 spawning stage, the GSI reached its maximum and sharply decreased when the
232 spermatozoa and the seminal fluid is released (Fig. 1a) and the tubules contained some
233 cysts of spermatocytes, spermatids and a few cysts of spermatogonia which delimited a
234 large lumen full of free spermatozoa (Fig. 1g) and the deferent duct is full of free
235 spermatozoa (Fig. 1h). At post-spawning stage, the GSI showed a great variability and
236 non-statistically significant difference respect the previous month was observed (Fig.
237 1a). At this stage, the tubules were formed by cysts of spermatogonia and single Sertoli
238 cells that limited the lumen of the tubules which appeared empty and reduced in size
239 (Fig. 1i,j).

240 *3.2. Proliferation mainly occurs at spermatogenesis whilst apoptosis did at*
241 *spawning.*

242 The proliferative (Fig. 2) and apoptotic (Fig. 3) rates were related with the
243 observed changes in the GSI and the testicular morphology (Fig. 1). At resting stage, the
244 proliferative rate was low as only some spermatogonia were immunostained (Fig. 2a,b).
245 However, at spermatogenesis stage, the proliferative rate reached its maximum value
246 due to the proliferation of spermatogonia and Sertoli cells (Fig. 2a,c). Later on, the
247 proliferative rates decreased to minimum levels that kept steady during spawning and
248 post-spawning stages (Fig. 2a). In these stages only spermatogonia proliferated (Fig.
249 2d,e). Regarding the apoptosis rate, the maximum value was recorded at spawning stage
250 and there were no differences on the apoptotic rates at resting, spermatogenesis or post-
251 spawning stages (Fig. 3a). The TUNEL positive cells were some spermatogonia and
252 Sertoli cells at resting stage (Fig. 3b), some Sertoli cells or interstitial cells at
253 spermatogenesis stage (Fig. 3c) and the Sertoli cells that limited the lumen of the
254 tubules at spawning (Fig. 3d) and post-spawning (Fig. 3g) stages. In addition, numerous
255 remaining spermatozoa present on the lumen of the tubules were TUNEL positive cells
256 at spawning stage (Fig. 3d,e). Interestingly, some TUNEL positive spermatozoa were
257 located in the cytoplasm of the cells that limited the efferent ducts (Fig. 3f).

258 *3.3. T, 11KT and E_2 serum levels varied through the reproductive cycle.*

259 In one year old European sea bass the serum levels of T were increased at
260 spermatogenesis stage, kept high during spawning stage and decreased at post-spawning
261 stage reaching levels similar to those observed at resting stage (Fig. 4a). However, the
262 11KT serum levels showed the greatest variation reaching its maximum level at
263 spermatogenesis stage and progressively decreasing until its minimum level at post-
264 spawning stage, which is lower than the level recorded during resting stage (Fig. 4b).

265 However, the serum levels of DHT did not show any modification during the
266 reproductive cycle analyzed (Fig. 4c). Interestingly, the levels of T were positively
267 correlated with the levels of 11KT and DHT, although 11KT and DHT serum levels did
268 not correlated (Table 3). Regarding estrogens, the serum levels of E_2 increased after
269 resting and kept steady onwards (Fig. 4d). The serum levels of E_2 did not correlated
270 with the levels of any androgen studied (Table 3).

271 *3.4. Most of the steroidogenetic, hormones and hormones receptor gene*
272 *expressions were undetected at spawning stage.*

273 The *cyp11b1* gene, codifies an enzyme (11β-hydroxylase) shared by the
274 glucocorticoid (mainly produced in the interrenal tissue) and androgen (mainly
275 produced in the gonad) pathway. This enzyme converts 11-deoxycortisol to cortisol and
276 also participates in the final steps of the synthesis of the 11-oxigenated androgens being
277 involved in the transformation of T to DHT than later on is transformed to 11KT. The
278 expression levels of *cyp11b1* in the gonad, the main synthesis site of androgens, were
279 high at spermatogenesis and post-spawning stages and very low during spawning stage
280 (Fig. 5a). However, the expression levels of *dhs3b* (Fig. 5b) and *cyp19a1a* (Fig. 5c)
281 were similar during resting, spermatogenesis and post-spawning stages, but not detected
282 at spawning stage. The expression pattern levels of *amh* gene were low during resting
283 and spermatogenesis stages and not detectable at spawning stage; however, increased
284 during post-spawning stage (Fig. 5d). Regarding the expression of the genes that code
285 for hormone receptors, the *fshr* gene expression was low during resting and
286 spermatogenesis stages, not detectable at spawning stage and increased at post-
287 spawning stage (Fig. 5e). The expression levels of *erb1* were low during resting,
288 spermatogenesis and spawning stages and increased at post-spawning stage (Fig. 5f).
289 However, the mRNA levels of *erb2* were similar during resting, spermatogenesis and

290 post-spawning stages and not detectable at spawning stage (Fig. 5g). The levels of
291 expression of *dhs3b* were positively correlated with T and negative correlated with *fshr*
292 gene expression (Table 3). As expected, the transcript levels of *cyp19a1a* showed a
293 negative correlation with T serum levels (Table 3), although this gene expression
294 showed a positive correlation with the expression of *cyp11b1* gene (Table 3). Moreover,
295 the expression level of *cyp11b1* gene was positively correlated with the serum levels of
296 E_2 and with the transcription levels of *amh, erb1* and *fshr* genes (Table 3). Additionally
297 the expression of *erb2* gene showed a positive correlation with the expression of *amh*
298 and *erb1* genes, while the transcript levels of *fshr* gene correlated with transcript levels
299 of *amh*, *erb1* and *erb2* genes (Table 3).

300

301 *3.5. Immune-related genes are expressed in the testis and changed with the*
302 *reproductive cycle.*

303 We next determined the presence and status of the immune response in the
304 gonad. For then, we analyzed the expression of several immune-relevant molecules such
305 as: cytokines (*il1b*, *il6* and *tnfa*), immune receptors (*ighm* and *tcrb*) (Fig. 6), viral
306 recognition molecules (*mda* and *lgp2*), anti-viral signalling pathway molecules (*tbk1*
307 and *irf3*), anti-viral response molecules (*ifn*, *pkr* and *mx*) (Fig. 7) and antimicrobial
308 peptides (*lyz*, *c3*, *dic*, *hamp*, *h1* and *h2b*) (Fig. 8). Thus, the *il1b* expression (Fig. 6a)
309 decreased during spermatogenesis and spawning stages and showed the same levels of
310 expression during resting and post-spawning stages. The *il6* expression (Fig. 6b) also
311 showed similar levels at resting, spawning and post-spawning stages and slightly
312 decreased during spermatogenesis stage. The expression of *tnfa* (Fig. 6c), however, did
313 not show any statistically significant change during the reproductive cycle. Regarding
314 the immune receptors (Fig. 6d,e), the expression of both, *ighm* and *tcrb*, genes increased

315 at spermatogenesis stage, however, while the expression of *ighm* decreased later on
316 showing similar levels of expression during resting, spawning and post-spawning stages
317 (Fig. 6d), the expression of *tcrb* was kept steady from spermatogenesis stage onwards
318 (Fig. 6e). Most of the anti-viral molecules analyzed (Fig. 7) showed a basal expression
319 that did not change throughout the reproductive cycle, with the exception of the single
320 stranded RNA receptor coding gene, the *lgp2* (Fig. 7b), a interferon response factor
321 coding gene, the *irf3* (Fig. 7d), and a interferon response molecule coding gene, the *pkr*
322 (Fig. 7f), which expression increased at spawning stage. Regarding the antimicrobial
323 peptides analyzed, hepcidin dicentracin, lysozyme and complement factor 3 are well
324 characterized in terms of molecular sequences and gene expression profiles that related
325 those proteins to innate immune responses (Rodríguez et al., 2006; Salerno et al., 2007;
326 Buonocore et al., 2014). In the case of histones, fish proteins highly homologous to or
327 identical to core nuclear histones have been identified and linked to innate defence
328 (Noga et al., 2011; Valero et al., 2013) but further characterization is still needed at
329 molecular and functional levels. Our data revealed different patterns of expression of
330 these genes (Fig. 8). Thus, the expression of *lyz* (Fig. 8a) and *c3* (Fig. 8b) increased at
331 spermatogenesis stage, remaining high during the rest of the reproductive cycle in the
332 case of *lyz* or decreased at spawning stage in the case of *c3*. The *dic* gene expression
333 (Fig. 8c) progressively increased from spermatogenesis stage to spawning stage when it
334 reached its maximum levels decreasing later on at post-spawning stage to the levels
335 observed at resting stage. The *h1* gene expression (Fig. 8e) decreased at
336 spermatogenesis stage, was not detectable at spawning stage and increased at post-
337 spawning stage, while the expression of *hamp* (Fig. 8d) and *h2b* (Fig. 8f) genes was
338 kept unchanged throughout the reproductive cycle.

339

340 **4. Discussion**

341 In teleost fish, spermatogenesis is regulated by the interplay of systemic and
342 intragonadal factors and the importance of each type of regulation vary depending on
343 the developmental stage of the gonad (Schulz et al., 2010). Our data demonstrate that in
344 one year old fish, the testis undergo the complete spermatogenesis process although it
345 was previously described that European sea bass males mature after two years of age
346 (Carrillo et al., 1995). Interestingly, the proliferative rates of germ cells on the testis of
347 teleosts are known to be regulated by endocrine and paracrine factors. Thus, in several
348 fish species, FSH, androgens and estrogens have been reported to trigger germ or Sertoli
349 cell proliferation, while other factors like AMH prevents this proliferation (Mazon et al.,
350 2014; Miura et al., 2002; Miura et al., 1999; Miura et al., 1991; Skaar et al., 2011). In
351 the sea bass, FSH regulates Sertoli cell proliferation (Mazon et al., 2014) and the
352 expression of *fshr* gene has also been related with early stages of the gonad
353 development and with Sertoli cell proliferation (Rocha et al., 2009). However, in one
354 year old European sea bass, the *fshr* gene expression is higher at post-spawning as also
355 occurs with other reproductive genes analyzed as *cyp11b1*, *erb1*, *erb2* and *amh*.
356 Interestingly, most of the reproductive genes analyzed showed non detectable levels of
357 transcription at spawning that could be explained due to the dilution of somatic cells
358 with respect to the high amount of spermatozoa together with a lower transcription level
359 of those genes. In fact, in two year old sea bass, a decrease in the expression levels of
360 some reproductive genes such as *fshr* and *cyp11b1* at the final stages of spermatogenesis
361 was observed (Rocha et al., 2009). Thus, the *fshr* and *cyp11b1* gene expressions showed
362 similar patterns in these two populations of fish (Rocha et al., 2009). It is worthy to note
363 that even when the levels of expression of these genes is very low and not detected in
364 our real time PCR assay, these levels could be enough to maintain serum hormone

365 levels. Moreover, the precise relation among the mRNA transcripts, enzyme activity and
366 hormone levels is unknown, an aspect that might be worthy to asses in a future.

367 Our data demonstrate that European sea bass apoptosis is involved in the
368 depletion of the remaining spermatozoa after spawning as determined by the massive
369 spermatozoa stained by TUNEL, an issue that do not occurs in all fish species.
370 Interestingly, the elimination of remained spermatozoa is a variable process in different
371 species of vertebrates independently of their taxonomical association. Thus, in a
372 seasonal breeder mammal, the Iberian mole (*Talpa occidentalis*) (Dadhich et al., 2010),
373 and in a teleostean fish, the gilthead seabream (Chaves-Pozo et al., 2005), apoptosis is
374 not involved in the depletion of spermatozoa after spawning, while in other mammalian
375 species as the mink (*Mustela vison*) (Blottner et al., 1999) and in the teleostean
376 European sea bass (as our data demonstrate) apoptosis has a prominent role. Moreover,
377 as European sea bass spermatozoa underwent apoptosis, the apoptotic spermatozoa are
378 in turn phagocytosed by the Sertoli cells that limited the lumen of the tubules, as the
379 presence of this apopototic cells, determined by the TUNEL staining, in the cytoplasm
380 of the Sertoli cells that limited the efferent ducts suggests, as also occurred in other
381 vertebrates (Carr et al., 1968; Shiratsuchi et al., 1997).

382 Serum 11KT levels were higher than T levels, however both hormone levels
383 increased at spermatogenesis stage and decreased during spawning and post-spawning
384 stages. This pattern of 11KT production has also been described in two years old sea
385 bass males (Rocha et al., 2009). DHT has been considered to be a major androgen in
386 many male vertebrates (Borg, 1994) and physiologically important in some fish species
387 (Margiotta-Casaluci and Sumpter, 2011, Margiotta-Casaluci et al., 2013) but not in
388 others (Cavaco et al., 1998). Although the function of DHT in teleosts is not clear yet,
389 there are growing lines of evidence that teleost fish are capable of synthesizing DHT

390 (Martyniuk et al., 2013; Sánchez-Hernández et al., 2013). Interestingly, we have
391 detected low levels of DHT in one year old European sea bass serum, which were kept
392 steady throughout the reproductive cycle. Moreover, our data showed that DHT serum
393 levels positively correlated with T serum levels, suggesting that the variation of T is
394 important for the maintenance of DHT levels. In addition, the mean T/DHT ratio was
395 calculated and resulted of 12 in fathead minnow males and of 20 in females (Margiotta-
396 Casaluci et al., 2013) in a similar way to the one year sea bass males which showed a
397 mean ratio of 13.22 (ranging from 4.5 to 20.3). DHT levels might be kept steady thanks
398 to its transformation into β-diol as suggested by the regulation of *dhs3b* transcription
399 levels. As occurs with DHT, the serum levels of E_2 were also very low in European sea
400 bass males although they increased slightly from spermatogenesis onwards. E_2 has been
401 related in several teleost fish species with the final events of spermatogenesis stage and
402 the regulation of germ and Sertoli cells proliferation and apoptosis (Chaves-Pozo et al.,
403 2007; Miura et al., 1999). However, high levels of E_2 triggered spermatogenesis
404 disruption and sex change in vertebrates including fish species (Chaves-Pozo et al.,
405 2007; Condeca and Canario, 1999; O'Donnell et al., 2001). Our data showed a negative
406 correlation between *cyp19a1a* gene expression and T serum levels, suggesting that the
407 regulation of *cyp19a1a* gene expression is an important point of regulation to allow the
408 maintenance of male physiology and explaining the effectiveness of estrogenic disruptor
409 pollutants to affect male physiology (Martinovic et al., 2007).

410 Interestingly, E_2 has also been related to the recruitment of leukocytes and the
411 regulation of the cytokine network of the immune response in the gonad (Cabas et al.,
412 2011; Seemann et al., 2013). The regulation of the immune response in the gonad of
413 teleost fish to avoid germ cell damage allows the persistence of several pathogens in
414 those tissues (Chaves-Pozo et al., 2010a; Chaves-Pozo et al., 2010b). The European sea

415 bass is very susceptible to several pathogens able to colonize the gonad, persist on this
416 tissue and be transmitted (Gómez-Casado et al., 2011; Sitja-Bobadilla and Álvarez-
417 Pellitero, 1993). In this framework, we have analyzed the gene expression pattern of
418 several pro-inflammatory cytokines, lymphocyte receptors and anti-viral and anti-
419 bacterial molecules in one year old European sea bass testis. In general, we observed
420 that the expression levels of the pro-inflammatory cytokines are low at spermatogenesis
421 and spawning stages while the gene expression of *ighm* and *tcrb* genes, together with
422 several anti-viral molecules (*lgp2*, *tbk1* and *pkr*) and antimicrobial peptides (*lyz*, *dic*, *c3*)
423 increased at spermatogenesis and/or spawning stages. These increments could be
424 explained due to protective reasons to infection during the sperm storage in the efferent
425 ducts until the release of spermatozoa.

426 Histones package DNA in all eukaryotes and play key roles in regulating gene
427 expression. Interestingly, in mammals and birds, the linker histone H1 display tissue
428 specificity and encompass a variety of important biological processes, including cellular
429 proliferation and apoptosis. Moreover, it has been recently described their functions as
430 antimicrobial peptides (AMPs) in vertebrates linking them to the immunity. In fish,
431 proteins highly homologous to or identical to core nuclear histones have been identified
432 and linked to innate defence, and more concretely to the antimicrobial response (Noga
433 et al., 2011; Valero et al., 2013). In this framework, our data show that the *h1*
434 transcription profiles suggest that the European sea bass substitute the H1 histone of
435 germ cells with protamines or other sperm-specific histones, as spermatogenesis
436 proceeds, as occur in mammals and in contrast to what happens in other fish species
437 when histones are not fully or partially replaced (Saperas et al., 1994; Yan et al., 2003).
438 Moreover, *h2b* expression was kept steady through the reproductive cycle instead of
439 being decreased, as expected in the case that this fish species replace the core histones,

440 or increased, as expected in the case that this fish species did not replace the core
441 histones. Taken all this data together, we can suggest that H2b has a role other than
442 DNA packaging.

443 Our data demonstrate that in one year old European sea bass males, the testis
444 undergoes the complete spermatogenesis process, although these specimens are not
445 considered fully mature. Moreover, serum levels of 11KT and T and the gene
446 expression profile of the steroidogenic enzymes involved in their synthesis varied
447 during the reproductive cycle. We have described for the first time in European sea bass
448 males, the serum levels of E_2 and DHT, determining that at least E_2 might be involved
449 in the regulation of the reproductive cycle of European sea bass males as also occurs in
450 other species of teleosts. Regarding the status of the immune response on the gonad, our
451 data showed high levels of expression of several AMPs such as lysozyme, C3 or
452 dicentracin during spermatogenesis and spawning and lower levels of pro-inflammatory
453 cytokines at the same stages, suggesting an important role for AMPs in the defence of
454 the reproductive tissues.

455 **5. Conflict of interest**: The authors declare that they have no conflict of interest.

456 **6. Funding**: This work was supported by grants of the *Ministerio de Economía y*
457 *Competitividad* and FEDER (AGL2010-20801-C02-01, AGL2010-20801-C02-02 and
458 AGL2011-30264-C02-01) and *Fundación Séneca* (*Grupo de Excelencia de la Región*
459 *de Murcia* 04538/GERM/06).

460 **7. Acknowledgement**

461 Elena Chaves-Pozo thanks the *Ministerio de Economía y Competitividad* for her *Ramón*
462 *y Cajal*'s research contract and Yulema Valero and Miriam Sánchez-Hernandez thanks
463 the *Instituto Español de Ocenaografía* for their PhD grants.

464 **8. References**

465 Blázquez M., González A., Papadaki M., Mylonas C. and Piferrer F. (2008) Sex-related
466 changes in estrogen receptors and aromatase gene expression and enzymatic
467 activity during early development and sex differentiation in the European sea
468 bass (*Dicentrarchus labrax*). *Gen Comp Endocrinol* **158**, 95-101.
469 Blottner S., Roelants H., Wagener A. and Wenzel U. D. (1999) Testicular mitosis,
470 meiosis and apoptosis in mink (*Mustela vison*) during breeding and non-
471 breeding seasons. *Anim Reprod Sci* **57**, 237-49.
472 Borg B. (1994) Androgens in teleost fishes. *Comp. Biochem. Physiol.* **109 C**, 219-245.
473 Buonocore F., Randelli E., Trisolino P., Facchiano A., de Pascale D. and Scapigliati G.
474 (2014) Molecular characterization, gene structure and antibacterial activity of a
475 g-type lysozyme from the European sea bass (*Dicentrarchus labrax* L.). *Mol*
476 *Immunol* **62**, 10-8.
477 Buonocore F., Castro R., Randelli E., Lefranc M. P., Six A., Kuhl H., Reinhardt R.,
478 Facchiano A., Boudinot P. and Scapigliati G. (2012) Diversity, molecular
479 characterization and expression of T cell receptor gamma in a teleost fish, the
480 sea bass (*Dicentrarchus labrax*, L). *PLoS One* **7**, e47957.
481 Cabas I., Chaves-Pozo E., García-Alcázar A., Meseguer J., Mulero V. and García-Ayala
482 A. (2011) Dietary intake of 17alpha-ethinylestradiol promotes leukocytes
483 infiltration in the gonad of the hermaphrodite gilthead seabream. *Mol Immunol*
484 **48**, 2079-2086.
485 Carr I., Clegg E. J. and Meek G. A. (1968) Sertoli cells as phagocytes: an electron
486 microscopic study. *J Anat* **102**, 501-509.
487 Carrillo M., Zanuy S., Prat F., Cerda J., Ramos J., Mananos E. and Bromage N. (1995)
488 Sea Bass (*Dicentrarchus labrax*). In *Broodstock management and egg and*
489 *larval quality* (Edited by Bromage N. R. and Roberts R. J.), p. 138-168.
490 Blackwell Science Ltd, Oxford.
491 Casani D., Randelli E., Costantini S., Facchiano A. M., Zou J., Martin S., Secombes C.
492 J., Scapigliati G. and Buonocore F. (2009) Molecular characterisation and
493 structural analysis of an interferon homologue in sea bass *(Dicentrarchus labrax*
494 L.). *Mol Immunol* **46**, 943-952.
495 Cavaco J. E., Vilrokx C., Trudeau V. L., Schulz R. W. and Goos H. J. (1998) Sex
496 steroids and the initiation of puberty in male African catfish (*Clarias*
497 *gariepinus*). *Am J Physiol* **275**, R1793-1802.
498 Chaves-Pozo E., Liarte S., Fernández-Alacid L., Abellán E., Meseguer J., Mulero V.
499 and García-Ayala A. (2008) Pattern of expression of immune-relevant genes in
500 the gonad of a teleost, the gilthead seabream (*Sparus aurata* L.). *Mol Immunol*
501 **45**, 2998-3011.
502 Chaves-Pozo E., Liarte S., Mulero I., Abellán E., Meseguer J. and García-Ayala A.
503 (2009) Early presence of immune cells in the developing gonad of the gilthead
504 seabream (*Sparus aurata* Linnaeus, 1758). *J Reprod Dev* **55**, 440-445.
505 Chaves-Pozo E., Liarte S., Vargas-Chacoff L., García-López A., Mulero V., Meseguer
506 J., Mancera J. M. and García-Ayala A. (2007) 17Beta-estradiol triggers
507 postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.)
508 males. *Biol Reprod* **76**, 142-148.
509 Chaves-Pozo E., Montero J., Cuesta A. and Tafalla C. (2010a) Viral hemorrhagic
510 septicemia and infectious pancreatic necrosis viruses replicate differently in
511 rainbow trout gonad and induce different chemokine transcription profiles. *Dev*
512 *Comp Immunol* **34**, 648–658.

513 Chaves-Pozo E., Mulero V., Meseguer J. and García-Ayala A. (2005) An overview of
514 cell renewal in the testis throughout the reproductive cycle of a seasonal
515 breeding teleost, the gilthead seabream (*Sparus aurata* L). *Biol Reprod* **72**, 593-
516 601.
517 Chaves-Pozo E., Pelegrín P., Mulero V., Meseguer J. and García-Ayala A. (2003) A
518 role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus*
519 *aurata* L., Teleostei). *J Endocrinol* **179**, 165-174.
520 Chaves-Pozo E., Zou J., Secombes C. J., Cuesta A. and Tafalla C. (2010b) The rainbow
521 trout (*Oncorhynchus mykiss*) interferon response in the ovary. *Mol Immunol* **47**,
522 1757–1764.
523 Condeca J. B. and Canario A. V. (1999) The effect of estrogen on the gonads and on in
524 vitro conversion of androstenedione to testosterone, 11-ketotestosterone, and
525 estradiol-17beta in *Sparus aurata* (Teleostei, Sparidae). *Gen Comp Endocrinol*
526 **116**, 59-72.
527 Dadhich R. K., Real F. M., Zurita F., Barrionuevo F. J., Burgos M. and Jiménez R.
528 (2010) Role of apoptosis and cell proliferation in the testicular dynamics of
529 seasonal breeding mammals: a study in the Iberian mole, *Talpa occidentalis*.
530 *Biol Reprod* **83**, 83-91.
531 Gómez-Casado E., Estepa A. and Coll J. M. (2011) A comparative review on European-
532 farmed finfish RNA viruses and their vaccines. *Vaccine* **29**, 2657-71.
533 Halm S., Rocha A., Miura T., Prat F. and Zanuy S. (2007) Anti-Mullerian hormone
534 (AMH/AMH) in the European sea bass: its gene structure, regulatory elements,
535 and the expression of alternatively-spliced isoforms. *Gene* **388**, 148-58.
536 Halm S., Martinez-Rodriguez G., Rodriguez L., Prat F., Mylonas C. C., Carrillo M. and
537 Zanuy S. (2004) Cloning, characterisation, and expression of three oestrogen
538 receptors (ERalpha, ERbeta1 and ERbeta2) in the European sea bass,
539 *Dicentrarchus labrax*. *Mol Cell Endocrinol* **223**, 63-75.
540 Hess R. A. (2003) Estrogen in the adult male reproductive tract: a review. *Reprod Biol*
541 *Endocrinol* **1**, 1-52.
542 Liarte S., Chaves-Pozo E., García-Alcázar A., Mulero V., Meseguer J. and García-
543 Ayala A. (2007) Testicular involution prior to sex change in gilthead seabream
544 is characterized by a decrease in DMRT1 gene expression and by massive
545 leukocyte infiltration. *Reprod Biol Endocrinol* **5**, 20-35.
546 Margiotta-Casaluci L. and Sumpter J. P. (2011) 5alpha-Dihydrotestosterone is a potent
547 androgen in the fathead minnow (*Pimephales promelas*). *Gen Comp Endocrinol*
548 **171**, 309-318.
549 Margiotta-Casaluci L, Courant F, Antignac JP, Le Bizec B, Sumpter JP. Identification
550 and quantification of 5α-dihydrotestosterone in the teleost fathead minnow
551 (*Pimephales promelas*) by gas chromatography-tandem mass spectrometry. Gen
552 Comp Endocrinol. 2013 Sep 15;191:202-209. doi: 10.1016/j.ygcen.2013.06.017.
553 Martinovic D., Hogarth W. T., Jones R. E. and Sorensen P. W. (2007) Environmental
554 estrogens suppress hormones, behavior, and reproductive fitness in male fathead
555 minnows. *Environ Toxicol Chem* **26**, 271-8.
556 Martyniuk C. J., Bissegger S. and Langlois V. S. (2013) Current perspectives on the
557 androgen 5 alpha-dihydrotestosterone (DHT) and 5 alpha-reductases in teleost
558 fishes and amphibians. *Gen Comp Endocrinol* **194**, 264-74.
559 Mauri I., Roher N., MacKenzie S., Romero A., Manchado M., Balasch J. C., Béjar J.,
560 Álvarez M. C. and Tort L. (2011) Molecular cloning and characterization of
561 European sea bass (*Dicentrarchus labrax*) and Gilthead seabream (*Sparus*
562 *aurata*) complement component C3. *Fish Shellfish Immunol* **30**, 1310-1322.

563 Mazón M. J., Gómez A., Yilmaz O., Carrillo M. and Zanuy S. (2014) Administration of
564 follicle-stimulating hormone in vivo triggers testicular recrudescence of juvenile
565 European sea bass (*Dicentrarchus labrax*). *Biol Reprod* **90**, 1-10.
566 Miura T., Miura C., Konda Y. and Yamauchi K. (2002) Spermatogenesis-preventing
567 substance in Japanese eel. *Development* **129**, 2689-97.
568 Miura T., Miura C., Ohta T., Nader M. R., Todo T. and Yamauchi K. (1999) Estradiol-
569 17beta stimulates the renewal of spermatogonial stem cells in males. *Biochem*
570 *Biophys Res Commun* **264**, 230-4.
571 Miura T., Yamauchi K., Takahashi H. and Nagahama Y. (1991) Hormonal induction of
572 all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla*
573 *japonica*). *Proc Natl Acad Sci U S A* **88**, 5774-8.
574 Nagahama Y., Miura T. and Kobayashi T. (1994) The onset of spermatogenesis in fish.
575 *Ciba Found Symp* **182**, 255-270.
576 Nascimento D. S., Pereira P. J., Reis M. I., do Vale A., Zou J., Silva M. T., Secombes
577 C. J. and dos Santos N. M. (2007) Molecular cloning and expression analysis of
578 sea bass (*Dicentrarchus labrax* L.) tumor necrosis factor-alpha (TNF-alpha).
579 *Fish Shellfish Immunol* **23**, 701-710.
580 Noga E. J., Ullal A. J., Corrales J. and Fernandes J. M. (2011) Application of
581 antimicrobial polypeptide host defenses to aquaculture: Exploitation of
582 downregulation and upregulation responses. *Comp Biochem Physiol Part D*
583 *Genomics Proteomics* **6**, 44-54.
584 O'Donnell L., Robertson K. M., Jones M. E. and Simpson E. R. (2001) Estrogen and
585 spermatogenesis. *Endocr Rev* **22**, 289-318.
586 Rocha A., Zanuy S., Carrillo M. and Gómez A. (2009) Seasonal changes in gonadal
587 expression of gonadotropin receptors, steroidogenic acute regulatory protein and
588 steroidogenic enzymes in the European sea bass. *Gen Comp Endocrinol* **162**,
589 265-75.
590 Rocha A., Gómez A., Zanuy S., Cerda-Reverter J. M. and Carrillo M. (2007) Molecular
591 characterization of two sea bass gonadotropin receptors: cDNA cloning,
592 expression analysis, and functional activity. *Mol Cell Endocrinol* **272**, 63-76.
593 Rodríguez L., Begtashi I., Zanuy S. and Carrillo M. (2000) Development and validation
594 of an enzyme inmunoassay for testosterone: effects of photoperiod on plasma
595 testosterone levels and gonadal development in male sea bass (*Dicentrarchus*
596 *labrax*, L.) at puberty. *Fish Physiol. Biochem.* **23**, 141-150.
597 Rodrigues P. N., Vazquez-Dorado S., Neves J. V. and Wilson J. M. (2006) Dual
598 function of fish hepcidin: response to experimental iron overload and bacterial
599 infection in sea bass (*Dicentrarchus labrax*). *Dev Comp Immunol* **30**, 1156-
600 1167.
601 Salerno G., Parrinello N., Roch P. and Cammarata M. (2007) cDNA sequence and
602 tissue expression of an antimicrobial peptide, dicentracin; a new component of
603 the moronecidin family isolated from head kidney leukocytes of sea bass,
604 *Dicentrarchus labrax*. *Comp Biochem Physiol B Biochem Mol Biol* **146**, 521-
605 529.
606 Sánchez-Hernández M., Chaves-Pozo E., Cabas I., Mulero V., García-Ayala A. and
607 García-Alcázar A. (2013) Testosterone implants modify the steroid hormone
608 balance and the gonadal physiology of gilthead seabream (*Sparus aurata* L.)
609 males. *J Steroid Biochem Mol Biol* **138C**, 183-194.
610 Saperas N., Ausio J., Lloris D. and Chiva M. (1994) On the evolution of protamines in
611 bony fish: alternatives to the "retroviral horizontal transmission" hypothesis. *J*
612 *Mol Evol* **39**, 282-295.

613 Scapigliati G., Buonocore F., Randelli E., Casani D., Meloni S., Zarletti G., Tiberi M.,
614 Pietretti D., Boschi I., Manchado M., Martin-Antonio B., Jimenez-Cantizano R.,
615 Bovo G., Borghesan F., Lorenzen N., Einer-Jensen K., Adams S., Thompson K.,
616 Alonso C., Bejar J., Cano I., Borrego J. J. and Alvarez M. C. (2010) Cellular and
617 molecular immune responses of the sea bass (*Dicentrarchus labrax*)
618 experimentally infected with betanodavirus. *Fish Shellfish Immunol* **28**, 303-
619 311.
620 Scapigliati G., Buonocore F., Bird S., Zou J., Pelegrin P., Falasca C., Prugnoli D. and
621 Secombes C. J. (2001) Phylogeny of cytokines: molecular cloning and
622 expression analysis of sea bass *Dicentrarchus labrax* interleukin-1beta. *Fish*
623 *Shellfish Immunol* **11**, 711-726.
624 Schulz R. W., de Franca L. R., Lareyre J. J., Le Gac F., Chiarini-García H., Nobrega R.
625 H. and Miura T. (2010) Spermatogenesis in fish. *Gen Comp Endocrinol* **165**,
626 390-411.
627 Seemann F., Knigge T., Rocher B., Minier C. and Monsinjon T. (2013) 17 beta-
628 Estradiol induces changes in cytokine levels in head kidney and blood of
629 juvenile sea bass (*Dicentrarchus labrax*, L., 1758). *Mar Environ Res* **87-88**, 44-
630 51.
631 Sepulcre M. P., Sarropoulou E., Kotoulas G., Meseguer J. and Mulero V. (2007) *Vibrio*
632 *anguillarum* evades the immune response of the bony fish sea bass
633 (*Dicentrarchus labrax* L.) through the inhibition of leukocyte respiratory burst
634 and down-regulation of apoptotic caspases. *Mol Immunol* **44**, 3751-3757.
635 Shiratsuchi A., Umeda M., Ohba Y. and Nakanishi Y. (1997) Recognition of
636 phosphatidylserine on the surface of apoptotic spermatogenic cells and
637 subsequent phagocytosis by Sertoli cells of the rat. *J Biol Chem* **272**, 2354-2358.
638 Sitja-Bobadilla A. and Álvarez-Pellitero P. (1993) Population dynamics of
639 S*phaerospora dicentrarchi* Sitja-Bobadilla et Álvarez-Pellitero, 1992 and *S.*
640 *testicularis* Sitja-Bobadilla et Álvarez-Pellitero, 1990 (*Myxosporea: Bivalvulida)*
641 infections in wild and cultured Mediterranean sea bass (*Dicentrarchus labrax*
642 L.). *Parasitology* **106 (Pt 1)**, 39-45.
643 Skaar K. S., Nobrega R. H., Magaraki A., Olsen L. C., Schulz R. W. and Male R.
644 (2011) Proteolytically activated, recombinant anti-mullerian hormone inhibits
645 androgen secretion, proliferation, and differentiation of spermatogonia in adult
646 zebrafish testis organ cultures. *Endocrinology* **152**, 3527-3540.
647 Socorro S., Martins R. S., Deloffre L., Mylonas C. C. and Canario A. V. (2007) A
648 cDNA for European sea bass (*Dicentrachus labrax*) 11 beta-hydroxylase: gene
649 expression during the thermosensitive period and gonadogenesis. *Gen Comp*
650 *Endocrinol* **150**, 164-173.
651 Terova G., Cattaneo A. G., Preziosa E., Bernardini G. and Saroglia M. (2011) Impact of
652 acute stress on antimicrobial polypeptides mRNA copy number in several
653 tissues of marine sea bass (*Dicentrarchus labrax*). *BMC Immunol* **12**, 69.
654 Valero Y., Chaves-Pozo E., Meseguer J., Esteban M.A., Cuesta A. (2013) Biological
655 role of fish antimicrobial peptides. In *Antimicrobial Peptides: Properties,*
656 *Functions and Role in Immune Response* (Edited by Seong M.D. and Hak Y.I.),:
657 p. 31-60. Nova Science Publishers, Inc.
658 Yan W., Ma L., Burns K. H. and Matzuk M. M. (2003) HILS1 is a spermatid-specific
659 linker histone H1-like protein implicated in chromatin remodeling during
660 mammalian spermiogenesis. *Proc Natl Acad Sci U S A* **100**, 10546-10551.
661

662 **9. Figure legends**

663 **Figure 1.** GSI (a) and testicular sections stained with hematoxylin-eosin (HE; b-j) of
664 one year old European sea bass males. Resting (R) stage (b,c). Early (d), midd (e) and
665 late (f) spermatogenesis (SPG) stage. Spawning (S) stage (g,h). Post-spawning (PS)
666 stage (i,j). Letters denoted statistically significant differences between groups ($P \leq 0.05$)
667 (n=8 fish/month). SG: spermatogonia; SC: spermatocytes; SD: spermatids; SZ:
668 spermatozoa; Arrow: interstitial cells; black arrowheads: Sertoli cells enclosing a
669 spermatogonium; white arrowheads: Sertoli cells limited the lumen of the tubules;
670 asterisks: spermatogonia. Scale bars = 25 µm (c,j) and 50 µm (b,d-i).

671 **Figure 2.** The mean of the immunostaining rates (immunostaining area/total optical
672 area) with anti-BrdU (a) and testicular sections of one year old European sea bass males
673 immunostained with anti-BrdU at resting (R) (b), spermatogenesis (SPG) (c), spawning
674 (S) (d) and post-spawning (SP) (e) stages showing proliferative spermatogonia (black
675 arrows), and Sertoli cells (white arrowheads).L: lumen of the tubules. Letters denoted
676 statistically significant differences between groups ($P \leq 0.05$) (n=10 fish/reproductive
677 stage). Scale bars = 30 µm (b-e).

678 **Figure 3.** The mean of the immunostaining rates (immunostaining area/total optical
679 area) with TUNEL (a) and testicular sections of one year old European sea bass stained
680 with TUNEL at resting (R) (b), spermatogenesis (SPG) (c), spawning (S) (d-f) and post-
681 spawning (PS) (g) stages showing apoptotic spermatogonia (white arrowheads), Sertoli
682 cells (black arrows) and spermatozoa (white arrows). Notice (f) that some Sertoli cells
683 that limited the lumen of the tubules have apoptotic spermatozoa phagocyted in their
684 cytoplasm (black arrowheads). Asterisks denoted the nuclei of the Sertoli cells. ED:
685 efferent duct. Letters denoted statistically significant differences between groups
686 ($P \leq 0.05$) (n=10 fish/reproductive stage). Scale bars = 15 µm (d,e,f,g), 25 µm (c) and 50
687 µm (b).

688 **Figure 4.** Sex steroid levels of testosterone (T) (a), 11-ketotestosterone (11KT) (b),
689 dihydrotestosterone (DHT) (c) and 17β-estradiol (E_2) in the serum of one year old
690 European sea bass males through the reproductive cycle. R: resting stage; SPG:
691 spermatogenesis stage; S: spawning stage; PS: post-spawning stage. Letters denoted
692 statistically significant differences between groups ($P \leq 0.05$) (n=4-6 fish/reproductive
693 stage).

694 **Figure 5.** Expression of reproductive *cyp11b1* (a), *dhs3b* (b), *cyp19a1a* (c), *amh* (d),
695 *fshr* (e), *erb1* (f) and *erb2* (g) genes in the testis of one year old European sea bass
696 males through a reproductive cycle. R: resting stage; SPG: spermatogenesis stage; S:
697 spawning stage; PS: post-spawning stage. Letters denoted statistically significant
698 differences between groups ($P \leq 0.05$) (n=7-12 fish/reproductive stage).

699 **Figure 6.** Expression of the cytokines *il1b* (a), *il6* (b) and *tnfa* (c) as well as lymphocyte
700 markers *ighm* (d) and *tcrb* (e) genes in the testis of one year old European sea bass
701 males through a reproductive cycle. R: resting stage; SPG: spermatogenesis stage; S:
702 spawning stage; PS: post-spawning stage. Letters denoted statistically significant
703 differences between groups ($P \leq 0.05$) (n=7-12 fish/reproductive stage).

704 **Figure 7.** Expression of viral recognition molecules *mda5* (a) and *lgp2* (b), anti-viral
705 signalling pathway molecules *tbk1* (c) and *irf3* (d) and anti-viral response molecules *ifn*
706 (e), *pkr* (f) and *mx* (g) coding genes in the testis of one year old European sea bass
707 males through a reproductive cycle. R: resting stage; SPG: spermatogenesis stage; S:
708 spawning stage; PS: post-spawning stage. Letters denoted statistically significant
709 differences between groups ($P \leq 0.05$) (n=7-12 fish/reproductive stage).

710 **Figure 8.** Expression of anti-microbial peptide *lyz* (a), *c3* (b), *dic* (c), *hamp* (d) *h1* (e)
711 and *h2b* (f) genes in the testis of one year old European sea bass males through a

712 reproductive cycle. R: resting stage; SPG: spermatogenesis stage; S: spawning stage;

713 PS: post-spawning stage. Letters denoted statistically significant differences between

714 groups ($P \leq 0.05$) (n=7-12 fish/reproductive stage).

715

716

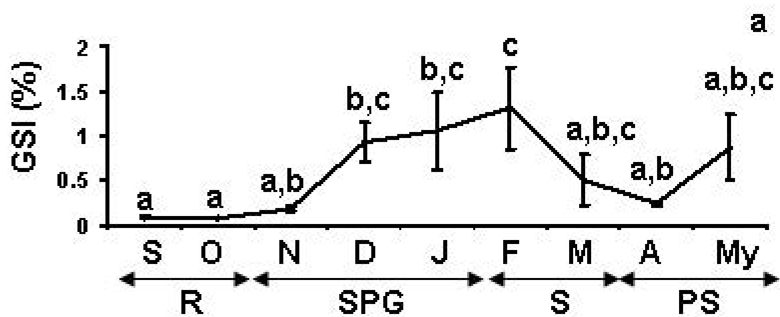
717
718
719
720

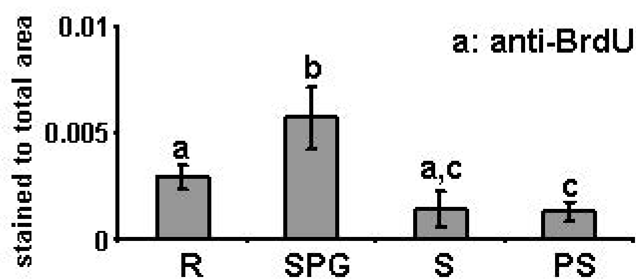
Highlights

1. In one year old European sea bass males, the testis undergoes the complete spermatogenesis process and steroidogenesis.

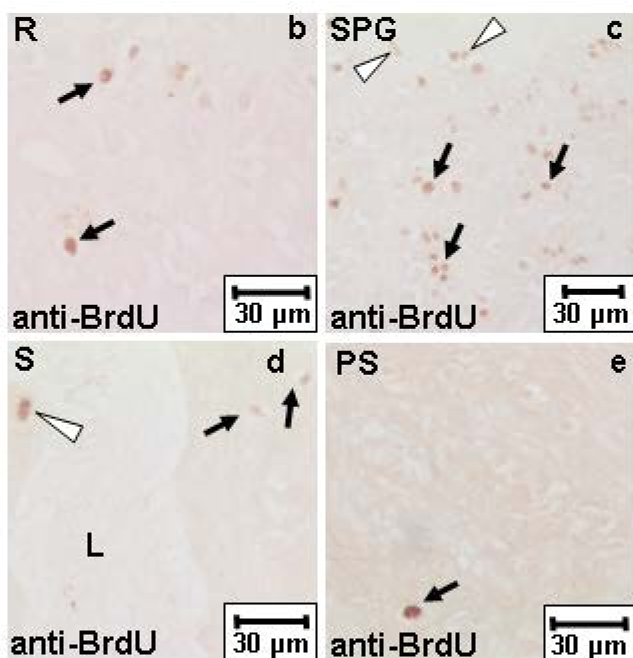
2. We have described for the first time that European sea bass males have detecting levels of E_2 and DHT in serum.

3. The levels of expression of several relevant-immune genes suggest an important role for antimicrobial peptides in the defence of the reproductive tissues.

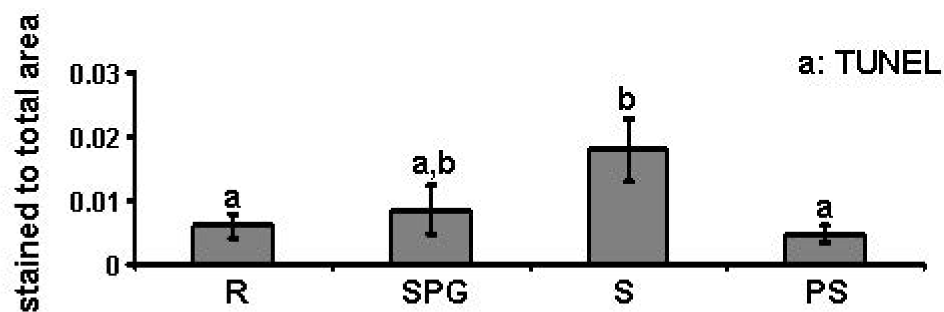

a
GSI (%)
2
1.5
1
0.5
0
a
a
a,b
b,c
b,c
c
a,b,c
a,b
a,b,c
S
O
N
D
J
F
M
A
My
R
SPG
S
PS
HE b
50 µm
R
HE c
25 µm
R
HE d
SG
50 µm
SPG
HE e
SZ
SD
50 µm
SPG
HE f
SC
SD
SZ
50 µm
SPG
HE g
SZ
SZ
50 µm
S
HE h
50 µm
S
HE i
50 µm
PS
HE j
25 µm
PS

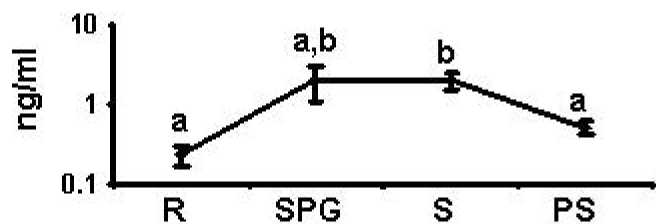


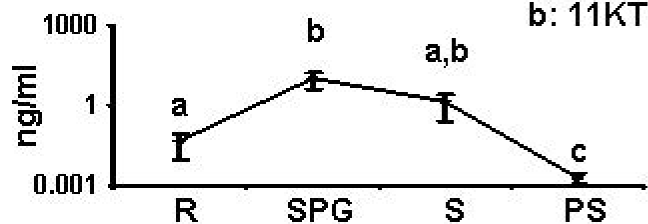
a: anti-BrdU
stained to total area
0.01
0.005
0
a
b
a,c
c
R
SPG
S
PS

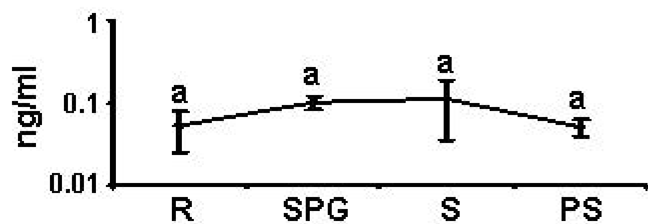


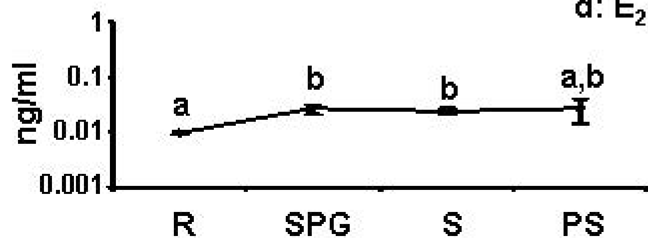
R
b
anti-BrdU
30 µm
SPG
c
anti-BrdU
30 µm
S
d
L
anti-BrdU
30 µm
PS
e
anti-BrdU
30 µm

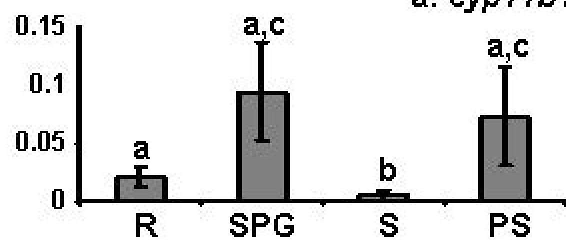

stained to total area
0.03
0.02
0.01
0
a: TUNEL
a
a,b
b
a
R
SPG
S
PS
R
b
TUNEL
50 µm
SPG
c
TUNEL
25 µm
S
d
TUNEL
15 µm
S
e
TUNEL
15 µm
S
f
*
*
*
TUNEL
ED
15 µm
PS
g
TUNEL
15 µm

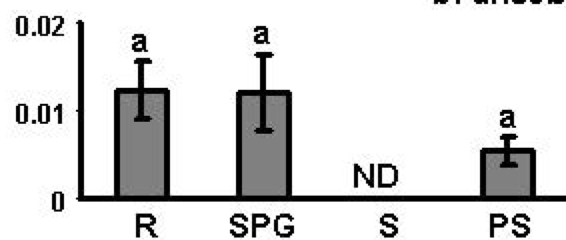

a: T
10
1
0.1
ng/ml
a
a,b
b
a
R
SPG
S
PS

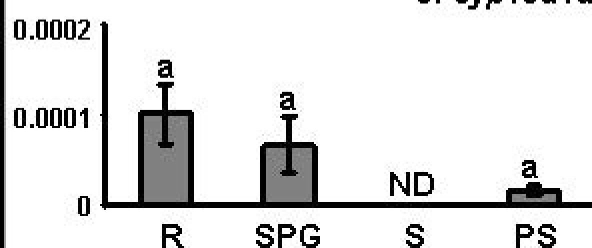

b: 11KT
1000
1
0.001
ng/ml
a
b
a,b
c
R
SPG
S
PS

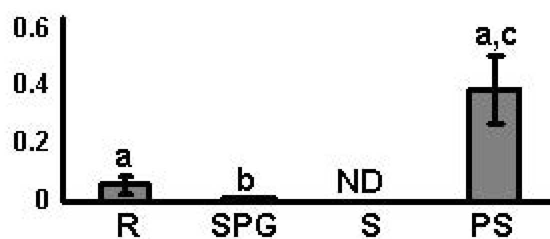

c: DHT
1
0.1
0.01
ng/ml
a
a
a
a
R
SPG
S
PS

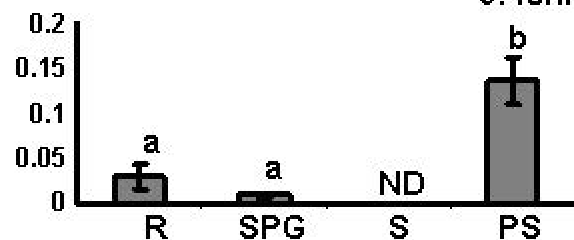

d: E2
1
0.1
0.01
0.001
ng/ml
a
b
b
a,b
R
SPG
S
PS

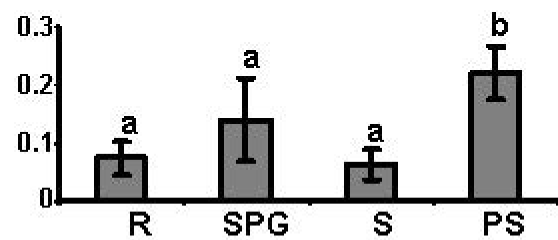


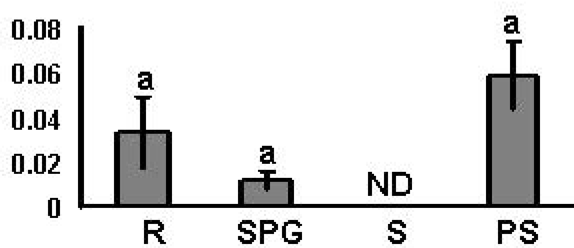




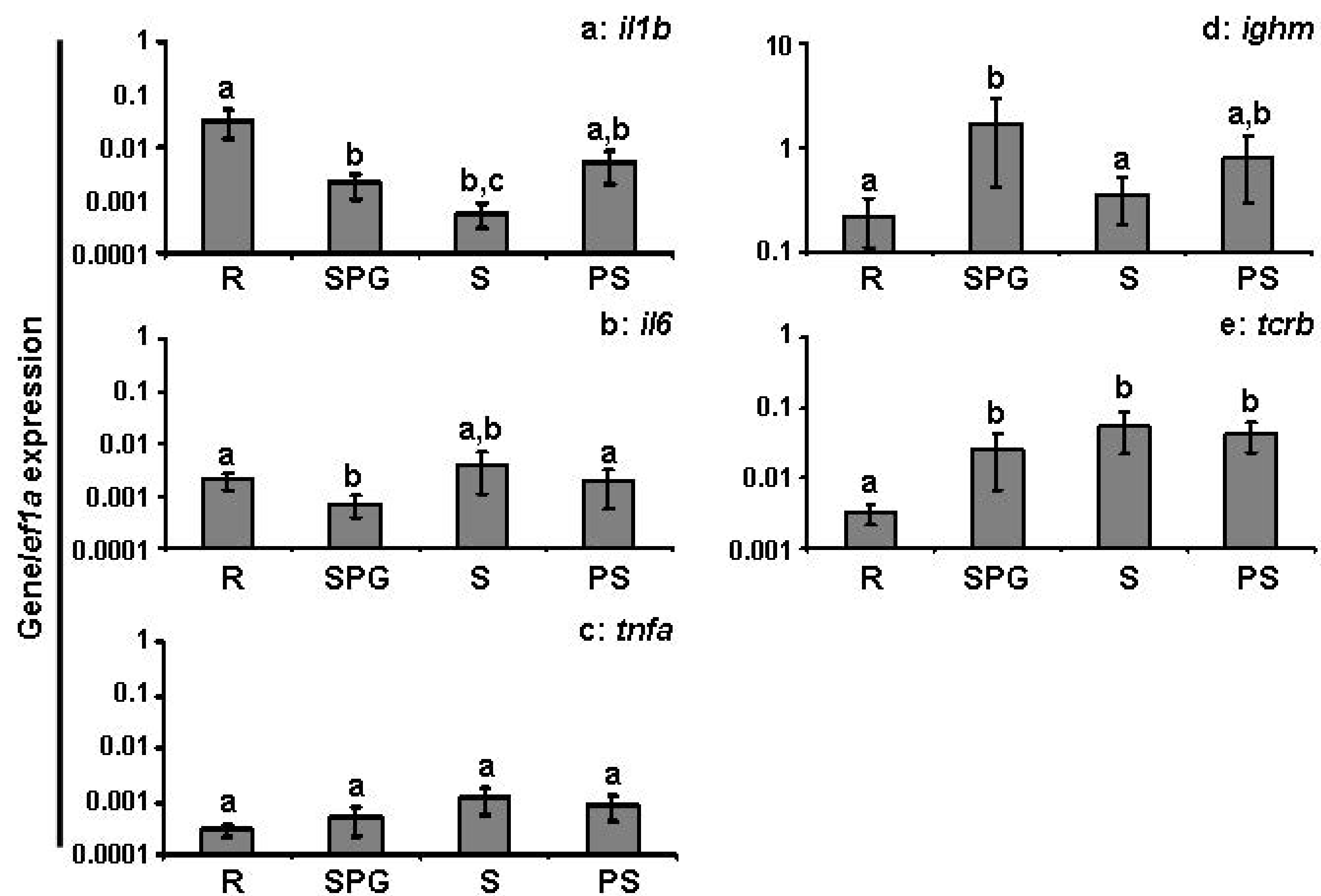


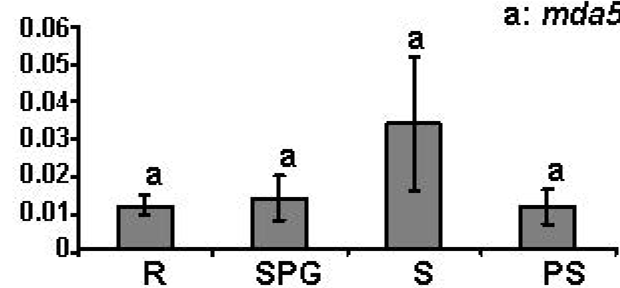


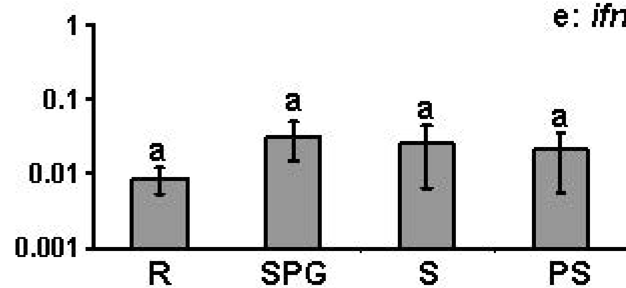


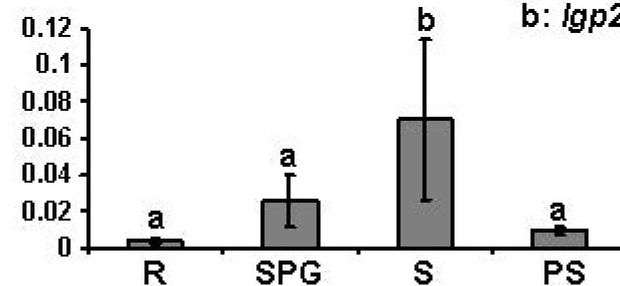


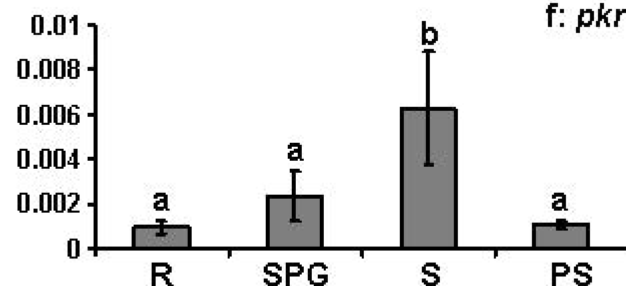
Gene/ef1a expression

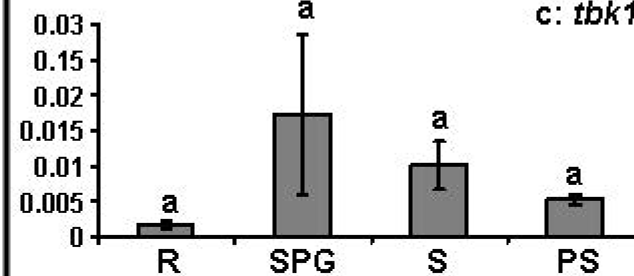

a: il1b
b: il6
c: tnfa
d: ighm
e: tcrb
Gene/ef1a expression
R
SPG
S
PS
1
0.1
0.01
0.001
0.0001
10
a
b
b,c
a,b
c

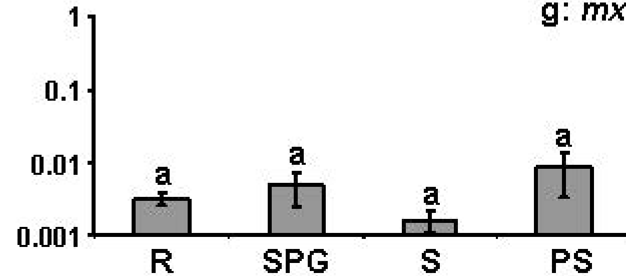

Gene/ef1a expression
a: mda5
0.06
0.05
0.04
0.03
0.02
0.01
0
a
a
a
a
R
SPG
S
PS

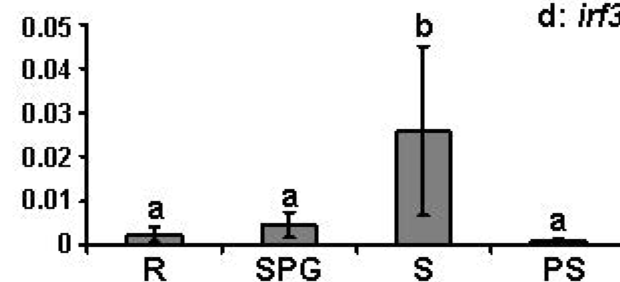

e: ifn
1
0.1
0.01
0.001
a
a
a
a
R
SPG
S
PS

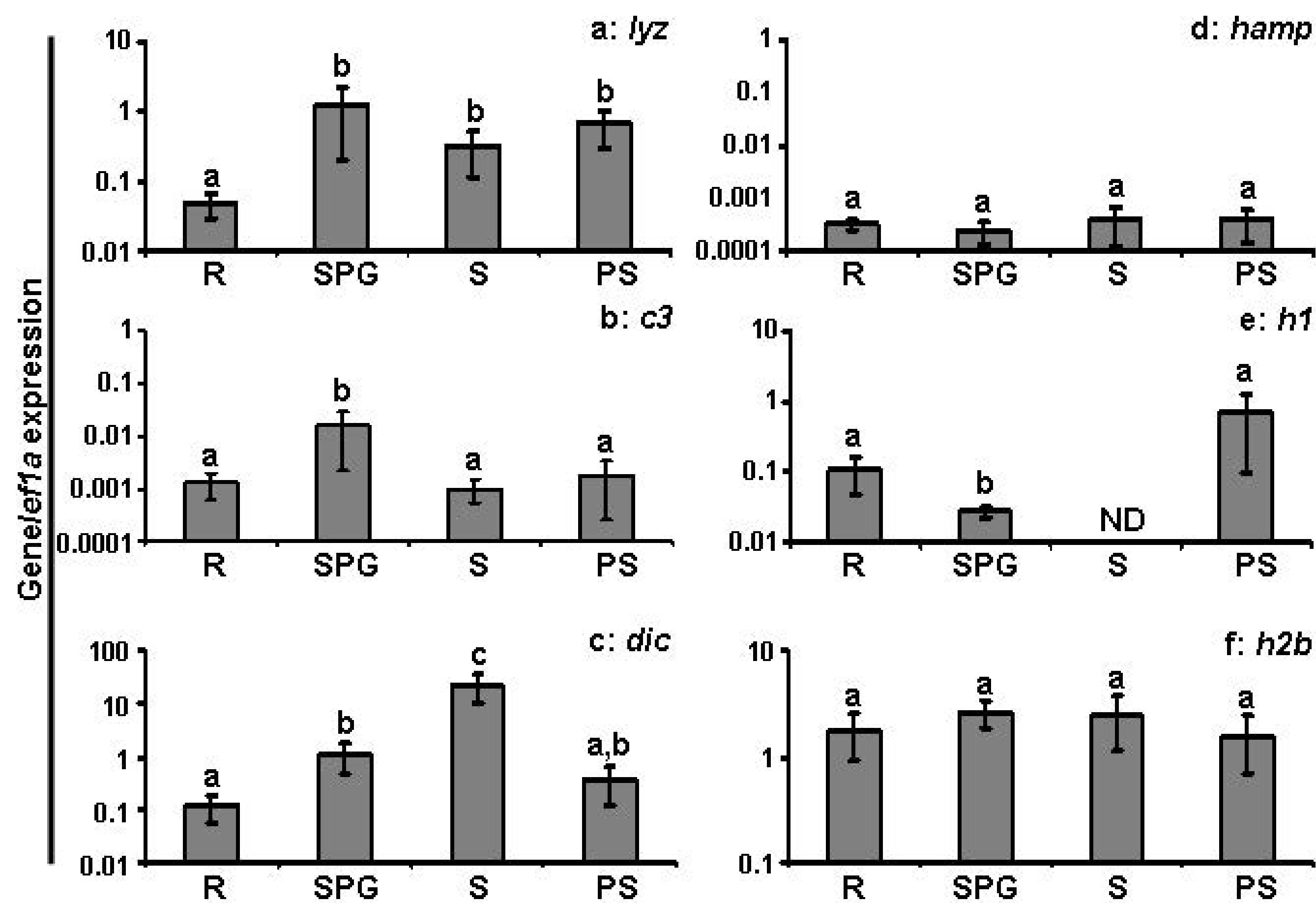

b: lgp2
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a
b
a
R
SPG
S
PS


f: pkr
0.01
0.008
0.006
0.004
0.002
0
a
a
b
a
R
SPG
S
PS


c: tbk1
0.03
0.15
0.02
0.015
0.01
0.005
0
a
a
a
a
R
SPG
S
PS


g: mx
1
0.1
0.01
0.001
a
a
a
a
R
SPG
S
PS


d: irf3
0.05
0.04
0.03
0.02
0.01
0
a
a
b
a
R
SPG
S
PS



Gene/ef1a expression
a: lyz
d: hamp
b: c3
e: h1
c: dic
f: h2b
R
SPG
S
PS
ND

Table 1: Genes and primer sequences used for gene expression analysis. ESTs, expression sequences tags. GenBank (http://www.ncbi.nlm.nih.gov/genbank).

Protein name	Gene abbreviation	Acc.number	Sequence (5'-3')	Source of the sequences
Steroid 11-β-hydroxylase	*cyp11b1*	AF449173	CCCATCTACAGGGAGCATGT GGAAGACTCCTTTGCTGTGC	(Socorro et al., 2007)
3β-hydroxysteroid dehydrogenase	*dhs3b*	FF578926	CACCTCTGGGCTTCAACATT GTGCTTCCTCCCACGTGTAT	ESTs
Aromatase	*cyp19a1a*	AJ298290, AJ311177	CTGGAGCCACACAGACAAGA AACTGAGGCCCTGCTGAGTA	(Blazquez et al., 2008)
Anti-Mullerian hormone	*amh*	AM232703	TGCAGGTCTCACAAGGACTG CTGGATGCAAAACCTCCAAT	(Halm et al., 2007)
Follicle stimulating hormone receptor	*fshr*	AY642113	ACTCCACCTCCATCATCTGC AACGGGGAACAGTCAGTTTG	(Rocha et al., 2007)
Estrogen receptor β1	*erb1*	AJ489523	GGGTGAGAGAGCTCAAGCTC AAGCTAAGGCCGGTTTTGGC	(Halm et al., 2004)
Estrogen receptor β2	*erb2*	AJ489524	AGTGGGCATGATGAAGTGCG TGCACGTGGTTCACCTGAGG	(Halm et al., 2004)
Interleukin 1β	*il1b*	AJ269472	CAGGACTCCGGTTTGAACAT GTCCATTCAAAAGGGGACAA	(Scapigliati et al., 2001)
Interleukin 6	*il6*	AM490062	ACTTCCAAAACATGCCCTGA CCGCTGGTCAGTCTAAGGAG	(Sepulcre et al., 2007)
Tumor necrosis factor-α	*tnfa*	DQ200910	AGCCACAGGATCTGGAGCTA GTCCGCTTCTGTAGCTGTCC	(Nascimento et al., 2007)
Heavy chain of immunoglobulin M	*ighm*	FN908858	AGGACAGGACTGCTGCTGTT CACCTGCTGTCTGCTGTTGT	GenBank
β subunit of T-cell receptor	*tcrb*	FN687461	GACGGACGAAGCTGCCCA TGGCAGCCTGTGTGATCTTCA	(Buonocore et al., 2012)
Melanoma differentiation-associated 5 protein	*mda5*	AM986362	AATTCGGCAATGGTGAAGTC TCATTGGTCACAAGGCCATA	ESTs
Laboratory of genetics and physiology 2 protein	*lgp2*	AM984225	TGATGGCAGTCAGTGGAGAG TGAGAGCTCAACGTGTTTGG	ESTs
TANK-binding kinase 1	*tbk1*	FM013306	ACAAGGTCCTGGTGATGGAG CGTCCTCAGGAAGTCCGTAA	ESTs
Interferon regulatory	*irf3*	CBN81356	AGAGGTGAGTGGCAATGGTC	GenBank

factor 3			GAGCAGTTTGAAGCCTTTGG	
Interferon	*ifn*	AM765847	GGCTCTACTGGATACGATGGC CTCCCATGATGCAGAGCTGTG	(Casani et al., 2009)
dsRNA-dependent protein kinase	*pkr*	FM008342	AGGGTCAGAGCATCAAGGAA GACACCTTGCTGTCCCAGTC	ESTs
Myxovirus (influenza) resistance proteins	*mx*	AM228977, HQ237501, AY424961	GAAGAAGGGCTACATGATCGTC CCGTCATTGTAGAGAGTGTGGA	(Scapigliati et al., 2010)
Lysozyme	*lyz*	FN667957	ATTTCCTGGCTGGAACACAG GAGCTCTGGCAACAACATCA	(Buonocore et al., 2014)
Complement component 3	*c3*	HM563078	ACCAAAGAACTGGCAACCAC CTAGCAGTCGGTCAGGGAAC	(Mauri et al., 2011)
Dicentracin	*dic*	AY303949	GGCAAGTCCATCCACAAACT ATATTGCTCCGCTTGCTGAT	(Salerno et al., 2007)
Hepcidin	*hamp*	DQ131605	CCAGTCACTGAGGTGCAAGA GCTGTGACGCTTGTGTCTGT	(Rodrigues et al., 2006)
Histone H1	*h1*	JN410661	AAGAAGACGGGTCCCTCAGT CTTGACCTTCTTCGCTTTGG	(Terova et al., 2011)
Histone H2B	*h2b*	JN410660	GGAGAGCTACGCCATCTACG GCTCAAAGATGTCGCTCACA	(Terova et al., 2011)
Elongation factor 1α	*ef1a*	AJ866727	CGTTGGCTTCAACATCAAGA GAAGTTGTCTGCTCCCTTGG	GenBank

Table 2. Identification of the interest genes in the expressed sequence tags (ESTs) databases and their relation with the zebrafish orthologs.

Predicted protein	Fish specie	Gene acc. number	Protein length	Protein homology[a]	E-value[b]
dhs3b	Sea bass Zebrafish	FF578926 NP_997962	185 374	72	7e-70
mda5	Sea bass Zebrafish	AM986362 XP_694124	206 997	72	1e-91
lgp2	Sea bass Zebrafish	AM984225 NP_001244086	297 679	71	2e-115
tbk1	Sea bass Zebrafish	FM013306 NP_001038213	220 727	95	3e-33
pkr	Sea bass Zebrafish	FM008342 CAM07151	304 682	41	1e-41

Percentage of homology ([a]) and E-value ([b]) of the predicted proteins respect to the zebrafish ortholog.

Table 3: Correlations observed between the sex hormone levels in serum and reproductive related gene expressions in gonad of one year old European sea bass males throughout a reproductive cycle. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated.

	11KT	DHT	E_2	*cyp11b1*	*dhs3b*	*cyp19a1a*	*amh*	*erb1*	*erb2*	*fshr*
T	**0.715** **0.002**	**0.557** **0.025**	0.345 0.190	-0.295 0.267	**0.608** **0.027**	**-0.525** **0.065**	-0.250 0.369	-0.143 0.625	0.296 0.350	-0.265 0.382
11KT		0.299 0.261	0.238 0.375	-0.221 0.411	0.228 0.453	-0.311 0.300	-0.153 0.587	-0.173 0.554	0.127 0.694	-0.202 0.508
DHT			0.328 0.215	-0.057 0.834	0.141 0.646	-0.271 0.370	-0.087 0.758	-0.039 0.895	-0.219 0.494	-0.239 0.431
E_2				**0.693** **0.003**	0.285 0.345	0.253 0.405	-0.236 0.397	0.257 0.374	0.093 0.773	-0.241 0.427
cyp11b1					-0.311 0.149	**0.445** **0.043**	**0.525** **0.008**	**0.393** **0.035**	0.161 0.485	**0.557** **0.007**
dhs3b						0.028 0.908	-0.319 0.159	-0.252 0.257	-0.133 0.576	**-0.470** **0.031**
cyp19a1a							-0.308 0.199	-0.082 0.723	0.070 0.788	-0.120 0.625
amh								0.307 0.165	**0.661** **0.002**	**0.885** **0.000**
erb1									**0.600** **0.005**	**0.628** **0.002**
erb2										**0.708** **0.001**