

1 **Characterization of the European sea bass (*Dicentrarchus*** 2 ***labrax*) gonadal transcriptome during sexual development**

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44 **Abstract**

45 The European sea bass is one of the most important cultured fish in Europe and has a
46 marked sexual growth dimorphism in favor of females. It is a gonochoristic species with
47 polygenic sex determination, where a combination between still undifferentiated genetic
48 factors and environmental temperature determine sex ratios. The molecular mechanisms
49 responsible for gonadal sex differentiation are still unknown. Here, we sampled fish
50 during the gonadal developmental period (110 to 350 days post fertilization, dpf), and
51 performed a comprehensive transcriptomic study by using a species-specific microarray.
52 This analysis uncovered sex-specific gonadal transcriptomic profiles at each stage of
53 development, identifying larger number of differentially expressed genes in ovaries
54 when compared to testis. The expression patterns of 54 reproduction-related genes were
55 analyzed. We found that *hsd17β10* is a reliable marker of early ovarian differentiation.
56 Further, three genes, *pdgfb*, *snx1* and *nfya*, not previously related to fish sex
57 differentiation, were tightly associated with testis development in the sea bass.
58 Regarding signaling pathways, lysine degradation, bladder cancer and NOD-like
59 receptor signaling were enriched for ovarian development while eight pathways
60 including basal transcription factors and steroid biosynthesis were enriched for testis
61 development. Analysis of the transcription factor abundance showed an earlier increase
62 in females than in males. Our results show that, although many players in the sex
63 differentiation pathways are conserved among species, there are peculiarities in gene
64 expression worth exploring. The genes identified in this study illustrate the diversity of
65 players involved in fish sex differentiation and can become potential biomarkers for the
66 management of sex ratios in the European sea bass and perhaps other cultured species.

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71 **Keywords:** genomics, transcriptomics, reproduction, sex differentiation, gonads,
72 aquaculture

73 **Introduction**

74 Many fish species exhibit sexual dimorphic growth where one sex, in many cases the
75 females, grow more than the other. Mixed sex rearing constitutes a problem for the
76 aquaculture industry because it means producing at suboptimal capacity when compared
77 to monosex culture. In addition, farming conditions results in many cases in
78 masculinization, which further aggravates the problem if the desired sex is the females.
79 Thus, the control of sex ratios is of major importance for many farmed species (Piferrer
80 2001; Budd et al. 2015). Deciphering the molecular mechanisms involved in gonadal
81 development and the establishment of population sex ratios has then not only basic
82 interest but also is of practical importance for modern fish farming.

83

84 Transcriptomic studies on gonadal tissues have been conducted in a relatively large
85 number of fish species, both cultured and not. For example, in the channel catfish
86 (*Ictalurus punctatus*), the genes differentially expressed (DEG) were described during
87 testis development (Zeng et al. 2016). In fugu (*Takifugu rubripes*) and in the spotted
88 knifejaw (*Oplegnathus punctatus*), a larger number of DEG were found in adult testes
89 when compared to ovaries (Du et al. 2017; Wang et al. 2017). However, the number of
90 studies focusing on sex-related differences precisely during sexual development and
91 with a genomics approach are much more limited, and include those carried out in Nile
92 tilapia (*Oreochromis niloticus*) (Tao et al. 2013), turbot (*Scophthalmus maximus*) (Ribas
93 et al. 2016), zebrafish (*Danio rerio*) (Ribas et al. 2017) and yellow river carp (*Cyprinus*
94 *carpio*) (Jia et al. 2018).

95

96 The European sea bass (*Dicentrarchus labrax*) stands as the third most important
97 cultured species of marine fish in Europe with a production of ~180 thousand tons per
98 year (Food and Agriculture Organization of the United Nations 2016). Its production
99 has benefited from advances in reproduction and the implementation of breeding
100 programs (reviewed in Felip and Piferrer, 2018; Wang et al. 2019). In this species,
101 females grow about 30% more than males (Saillant et al. 2001). However, temperature
102 during early development can affect sex ratios, favoring a higher number of males in the
103 populations (reviewed in Vandeputte and Piferrer, 2018). This problem can be even
104 more aggravated if males undergo precocious maturation as it slows down their growth
105 (Carrillo et al. 2015). Thus, there is interest in producing monosex female stocks.

106

107 The European sea bass is one of the richest species in terms of genomic resources
108 among cultured fish, which include the availability of the genome and Single
109 Nucleotide Polymorphism (SNP) markers, among others. The European sea bass has a
110 small genome size (675 Mb) with a total of 26,719 annotated genes (Tine et al. 2014;
111 Chaves-Pozo et al. 2017) and has 24 haploid chromosome pairs (Aref'yev 1989). It is a
112 gonochoristic species with a polygenic sex determination system with both genetic and
113 environmental influences (Piferrer et al. 2005; Vandeputte et al. 2007; Vandeputte and
114 Piferrer 2018). Although the genetic factors are still not known, efforts have been done
115 towards identifying sex-determinant markers to aid in genetic selection programs.
116 Studies using ~6,700 SNP markers showed that there are at least three loci linked to sex
117 (Palaiokostas et al. 2015) but these may be family-specific. Studies on the effects of
118 food supply during early development evidenced changes in energy balance during
119 testicular development (Díaz et al. 2014). In a similar manner, transcriptomic profiles of
120 differentiating gonads subjected to different temperatures or estrogen treatments
121 identified the involvement of steroidogenic- and epigenetic-related genes (Díaz and
122 Piferrer 2015; Díaz and Piferrer 2017). Recently, the epigenetic regulation of key sex-
123 related genes has been reported and a method based on the analysis of epimutations to
124 predict sex in the European sea bass devised (Anastasiadi et al. 2018). Nevertheless, a
125 study of the transcriptomic changes that occur during sexual development had never
126 been carried out.

127

128 The objective of this study was to fill this gap. To do so, we transcriptomically analyzed
129 sexually undifferentiated, differentiating and differentiated gonads. Gene expression
130 levels were evaluated by using a homologous custom-made microarray enriched with
131 reproduction-related genes. Studying transcriptomes by using a microarray platform
132 provides accuracy and reproducibility of the performed analysis and allows studying a
133 broad range, if not all, of the transcripts of the genome (Shi et al. 2006). In particular,
134 our custom microarray contained 78.5% of the annotated coding genes of the European
135 sea bass genome, thus providing a powerful molecular tool to study gene expression
136 patterns of this species.

137 **Materials and Methods**

138 *European sea bass gonad sampling*

139 In order to obtain the widest possible range of expressed transcripts, gonads were
140 dissected from fish (1.5 ± 0.5 cm of standard length, SD) at 110 days post fertilization
141 (dpf), when they are still morphologically undifferentiated but can be sexed by
142 analyzing the expression of sex-markers (i.e., *cyp19a1a* (Blázquez et al. 2008), when
143 differentiating at 250 dpf (12.7 ± 5.7 cm of SD and 11.2 ± 0.6 cm of SD in females and
144 males, respectively) and when differentiated at 350 dpf (16 ± 1.3 cm and 14.8 ± 1.1 cm,
145 in females and males, respectively). The set of samples consisted of eleven gonads at
146 110 dpf, twelve gonads (six testes and six ovaries) at 250 dpf and eighteen gonads (nine
147 testes and nine ovaries) at 350 dpf. When possible, gonads were fully isolated from fish
148 at 250 and 350 dpf and thus gonadal tissue was devoid of any other tissue. However, a
149 clean gonad isolation was not feasible in younger fish (110 dpf) due to their extremely
150 small size, and thus some epithelial contamination could not be ruled out.

151

152 *Microarray platform*

153 The microarray platform used in the present study consists of 1,417 Agilent control
154 probes and a total of 43,803 transcript probes that represent 20,978 genes of which
155 20,028 have two probe copies each while the rest have between 1 to 6 copies per gene
156 (Supplementary Table S1). Genes with known reproduction-related functions had at
157 least four copies. Microarray was based on sequences obtained from two 454 FLX
158 Titanium runs on European sea bass gonad tissues at different ages (from 40 dpf up to 6
159 years), a former custom European sea bass microarray platform (GPL13443) available
160 in our laboratory (Díaz et al. 2014; Díaz and Piferrer 2015), a previously published
161 European sea bass microarray (Ferrareso et al. 2010) and a battery of selected
162 reproduction canonical genes. Only non-redundant and annotated sequences were
163 selected. This microarray was submitted to Gene Expression Omnibus (GEO) database
164 (Edgar et al. 2002) with the platform number GPL16767 and its functionality was
165 reported in a previous work using European sea bass larvae (Schaeck et al. 2017). In the
166 present work, this microarray has been re-annotated by using the European sea bass
167 genome (Tine et al. 2014) and used to study the gonadal development in the European
168 sea bass. Microarray data of the present study were submitted to GEO and are
169 accessible through GEO Series accession number GSE115841. For a complete list of
170 gene names and abbreviations, see Dataset 1.

171

172

173 *RNA isolation and microarray hybridization*

174 Total RNAs was extracted from 41 gonad samples using RNeasy Mini Kit (Qiagen,
175 Germany) following the manufacturer's instructions. Quantity was determined by a
176 Nanodrop spectrophotometer (Nanodrop Technologies, USA) and quality (RNA
177 integrity number, RIN) measured with a Bioanalyzer (Agilent Technologies, USA).
178 Only RNA samples with a mean RIN ≥ 8.4 were further processed for microarray
179 analysis. RNA was labelled using the Low Input Quick Amp Labelling Kit, One-Color
180 (Cy3; Agilent Technologies) and cRNA was hybridized overnight with the
181 corresponding buffers during 17 h at 65°C and washed on the following day. Samples
182 were hybridized individually in the European sea bass custom 4x44K Agilent
183 microarray described above at the Parc d'Investigació Biomèdica de Barcelona (PRBB)
184 and slides were scanned using an Agilent G2565B microarray scanner (Agilent
185 Technologies, USA). Agilent software was used to avoid saturation and the extraction
186 feature generated the raw data for further pre-processing.

187

188 *Data analysis*

189 Statistical analyses were performed with R software (2.13.1 version; [www.R-](http://www.R-project.org)
190 project.org). Array normalization was implemented using the Quantile method in the
191 Linear Models for Microarray Analysis (Limma) R package (Wettenhall and Smyth
192 2004; Ritchie et al. 2015). Potential batch effects were removed by ComBat correction,
193 a bioinformatic tool based on Empirical Bayes algorithms (Chen et al. 2011). Data
194 visualization, Principal Component Analysis of the variance and identification of
195 clusters and outliers (two samples at 350 dpf were detected as outliers and excluded
196 from further analysis) were performed using R. TIGR Multiexperiment Viewer version
197 4.9 (TMeV) software (Saeed et al. 2003) was used to determine the number of
198 differentially expressed genes (DEG) between sexes at a given stage of development or
199 between stages of development within the same sex. Significance was assessed by
200 Significant Analysis of Microarrays (SAM) statistical test with a False Discovery Rate
201 (FDR) and adjusted P values < 0.01 and < 0.001 were applied to identify genes with
202 statistically significant differences in expression. The above-mentioned analysis
203 generated lists of DEG at each stage (110, 250 and 350 dpf) in the same sex or in

204 comparison to the other sex, including the \log_2 transformation of fluorescence intensity
205 measured for each gene.

206

207 *Gene ontology terms and KEGG pathway analysis*

208 The over-represented gene ontology (GO) functional categories of the DEG between
209 females and males at each stage were obtained by GO-terms enrichment analysis using
210 GO.db and topGO packages from the Bioconductor Project (Gentleman et al. 2004;
211 Alexa and Rahnenfuhrer 2016; Carlson 2017) in R software (R Core 2017). The graphs
212 and heatmaps were produced using gplots and ggplot2 packages (Wickham 2009;
213 Warnes et al. 2016).

214

215 We used the Gene Set Variation Analysis (GSVA) from Pathway Processor 2.0 to study
216 the signalling pathways involved in gonadal development. GSVA transforms the gene
217 expression values into a normalized expression matrix with enrichment scores of
218 differentially regulated pathways (DRP) with the corrected P value between males and
219 females at each developmental stage (Beltrame et al. 2013).

220

221 Four pathways involved in sex differentiation: fanconi anemia and wnt signaling
222 pathways, associated with female differentiation (Rodríguez-Marí and Postlethwait
223 2011; Sreenivasan et al. 2014), p53 signaling and cytokine-cytokine interaction receptor
224 pathways, associated with male differentiation (Yasuda et al. 2012; Ribas et al. 2017)
225 were also studied. The lists of genes making-up these pathways were obtained from the
226 Kyoto Encyclopedia of Genes and Genomes (KEGG), using zebrafish as background.
227 The numbers of DEG from these pathways as well as expression values (in \log_2 Fold
228 Change, FC) were plotted together; upregulated in male *vs.* upregulated in female over
229 time.

230

231 *Transcription factors analysis*

232 The transcription factors (TFs) present in the microarray were identified with the aid of
233 the TF checkpoint database, a list of TFs compiled from nine databases (Chawla et al.
234 2013). Using this list as background, the percentage of TFs present in the DEG between
235 sexes and differentially expressed at each age was calculated.

236

237 *Validation of the microarray*

238 Microarray results were validated by quantitative real time polymerase chain reaction
239 (qPCR) analyzing the expression of twelve genes selected with a wide range of FC
240 values and equal amount of upregulated and downregulated genes when ovaries and
241 testes were compared. Two house-keeping genes were chosen as reference: Elongation
242 factor-1 alpha (*ef-1 α*) and 40S ribosomal protein (*fau*) that were previously validated in
243 the European sea bass (Mitter et al. 2009). One hundred nanograms of total RNA were
244 reverse transcribed into cDNA using Superscript III (Invitrogen) and 100 ng of random
245 hexamer primers (Sigma) following the manufacturer's instructions. The reaction was
246 carried out with SYBR Green chemistry (Power SYBR Green PCR Master Mix;
247 Applied Biosystems). qPCR reactions contained 1X SYBR green master mix (Applied
248 Biosystems), 10 pmol of each primer and 1 μ l of the RT reaction. Samples were run
249 individually and in triplicate in optically clear 384-well plates in Applied Biosystems
250 7900 machine. Cycling parameters were: 50°C for 2 min, 95°C for 10 min, followed by
251 40 cycles of 95°C for 15 s and 60°C for 1 min. Finally, a temperature-determining
252 dissociation step was performed at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s at the
253 end of the amplification phase. qPCR data were collected by SDS 2.3 and RQ Manager
254 1.2 software and relative quantity (RQ) values for each reaction replicate were
255 calculated by the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Primer sequences used
256 for gene expression study are shown in Supplementary Table S2.

257

258 **Results**

259 *Microarray platform and validation*

260 Hybridization repeatability and consistency of results was verified in 55 genes related to
261 reproduction and previously selected in turbot (Ribas et al. 2016) and zebrafish (Ribas
262 et al. 2017) to study sex differentiation and reproduction (Supplementary Table S3).
263 Probe copy tendency for 52 of these genes were the same for all the copies and only
264 three of them (representing ~5% of the 52 tested probes) showed different probe
265 tendency in at least one of the copies. Some examples of upregulated (Supplementary
266 Fig. S1 a, c, e, g), downregulated (Supplementary Fig. S1 b, d, f, h) or variable (i) gene
267 expression are shown. Since most probes had two or four copies (Supplementary Fig.
268 1j) to further evaluate the hybridization accuracy, we determined the magnitude of
269 variation between technical replicates. The mean of the standard deviations for all 54
270 probes ranged between 0.205 and 0.347 (Supplementary Fig. 1j). Thus, given the low

271 standard error among probe copies of the same gene, the average FC value of all probe
272 copies was used for each gene.

273

274 Microarray validation by qPCR for 12 DEGs showed a good correlation between the
275 results obtained either by microarray and qPCR techniques ($R^2 = 0.748$, $P = 0.0003$;
276 Supplementary Fig. S2a). Additionally, gene expression values of aromatase
277 (*cyp19a1a*) from samples at different stages of gonadal development (110, 250 and 350
278 dpf) determined either by using microarray and by qPCR, further validated our results
279 (Supplementary Fig. S2b).

280

281 *Gonadal transcriptomes overview*

282 Samples clustered in two distinctive groups according to sex and within each group
283 samples tended to group according to age, except for males at 350 dpf, which showed
284 more variation in the PCA (Fig. 1a). The component 1 of the PCA alone explained
285 87.14% of the variance, while components 2 and 3 contributed to 8.35% and 1.98% of
286 the total variance, respectively. Thus, the first three components together explained
287 97.47% of variance.

288

289 A total of seven comparisons were analyzed with the SAM statistical test with an
290 adjusted P value ≤ 0.01 : three between sexes, two within males and two within females
291 at the three different ages (Fig. 1b). Among the 20,978 genes included in the
292 microarray, 64.93% were differentially expressed at one or several of these
293 comparisons. The number of DEGs between ovaries and testes was 708; 7,639 and
294 6,926, at 110, 250 and 350 dpf, respectively (Fig. 1b, Dataset 1). A larger number of
295 genes were upregulated in females when compared to M: 685 vs. 23 at 110 dpf; 3,870
296 vs. 3,769 at 250 dpf, and 6,097 vs. 829 at 350 dpf. Between 110-250 and 250-350 dpf,
297 the number of upregulated genes was 3,564 and 1,100 in females while 451 and 309 in
298 males, respectively. The number of downregulated genes between 110-250 and 250-350
299 dpf was 3,060 and 671 in females while 1,737 and 1,179 in males, respectively. Among
300 the upregulated genes in the developing ovaries, higher FC values were found in 110
301 dpf and 350 dpf while at 250 dpf FC values were higher in testes when compared to
302 ovaries (Supplementary Table S4). The highest FC values were observed at 250 dpf (P
303 < 0.001) which was 7.5 and 6.7 Log_2 in males and females, respectively (Dataset 1),

304 indicating important sex-related differences in expression levels (Supplementary Table
305 S4).

306

307 *Gene ontology and gene pathway enrichments along gonadal development*

308 The GO term enrichment analysis of the DEGs between F and M revealed several
309 categories related to biological processes (BP), molecular function (MF) and cellular
310 component (CC) throughout development (Dataset 2). A total of 39 GO terms in the
311 three categories were enriched during ovarian formation. The 15 GO terms significantly
312 enriched in BP common at 110, 250 and 350 dpf ($P < 0.01$) are shown in Fig. 2a. The
313 three developmental stages were enriched in GO terms related to metabolic processes
314 (GO:0008152, GO:0071704, GO:0044237), catalytic activity (GO:0003824),
315 oxidoreductase activity (GO:0016491), coenzyme and cofactor binding (GO:0050662;
316 GO:0048037), and biosynthetic processes (GO:0009058, GO:1901576). Among the GO
317 terms enriched for testis formation, there were a total of 52 significantly enriched terms
318 that were common at 250 and 350 dpf, but none at 110 dpf which were related to
319 catabolic processes (GO:0000956, GO:0006402, GO:0006401), regulation of ion
320 transmembrane activity (GO:1904427, GO:0032414, GO:0034767), regulation of
321 calcium ion (GO:0010524, GO:0050850, GO:0051281, GO:0060316, GO:1901021,
322 GO:1904427) and positive regulation of growth (GO:0045927). Fig. 2b shows the
323 enriched GO terms found for testis formation in BP category ($P < 0.02$).

324

325 Then, we determined the significantly Differentially Regulated Cellular Pathways
326 (DRP) between males and females along gonadal development. A total of 41, 151 and
327 106 DRP were found between males and females at 110, 250 and 350, respectively
328 (Dataset 3, $P < 0.05$). Some of these DRP were related to sex differentiation, for
329 example, at early gonadal development (110 dpf): p53 signaling pathway, steroid
330 hormone biosynthesis or $erb\beta$ signaling pathway; at 250 dpf: wnt signaling pathway,
331 oocyte meiosis or steroid biosynthesis and at 350 dpf: MAPK signaling pathway or
332 cytokine-cytokine receptor interaction among others. A total of 16 DPR were
333 consistently differentially regulated at the three developmental stages in both sexes
334 (Table 1). The lysine degradation, bladder cancer and the nucleotide-binding
335 oligomerization (NOD)-like receptor signaling pathways were upregulated in females at
336 the three gonadal developmental stages when compared to males. The dorso-ventral axis
337 formation pathway was significantly downregulated in females at 110 dpf but

338 upregulated at 250 and 350 dpf. The p53 signaling pathway and the Chagas disease
339 (American trypanosomiasis) pathways showed significance, being upregulated at 110
340 and 350 dpf in females but downregulated at 250 dpf when compared to males. There
341 were two pathways, phosphatidylinositol signaling system and the Erbβ signaling
342 pathway that were upregulated in females at 110 dpf but at 250 and 350 dpf in males.
343 Finally, there were up to eight pathways upregulated in males when compared to
344 females throughout the studied period: butirosin and neomycin biosynthesis, basal
345 transcription factors, amino sugar and nucleotide sugar metabolism, type II diabetes
346 mellitus, glycine, serine and threonine metabolism, steroid biosynthesis and ribosome
347 and folate biosynthesis pathways.

348

349 *Expression of canonical genes and pathways related to sex differentiation*

350 Of the 54 canonical genes known to be relevant for reproduction and sex differentiation
351 in fish according to the primary literature, 49 of them had sex-related significant
352 differences in at least one of the three ages studied. The majority (80.5%) were DEG at
353 250 dpf (Supplementary Table S3). Of the 49 just mentioned above, 25 were pro-female
354 and 24 pro-male genes. Hierarchical clustering analysis and the corresponding heatmaps
355 of the 25 pro-female genes (Fig. 3a) and the 24 pro-male genes (Fig. 3b) showed that
356 gene expression results mostly matched according to their phenotypic gender. The
357 expression profiles of twelve key genes is shown in Fig. 4. Six of them are related to the
358 steroidogenic pathway (*cyp19a1a*, *hsd17β10*, *hsd3β*, *cyp11β*, *ara*, *fshr*, Fig. 4a-f) while
359 the other six genes are TFs related to sex differentiation (*foxl2*, *sox3*, *figla*, *nr5a1a*,
360 *sox9b* and *dmrt1*, Fig. 4g-l). The genes *cyp11β1*, *ara*, *hsd3β* and *fshr* were upregulated
361 in males when compared to females at 250 dpf and onwards while *hsd17β10* was
362 upregulated in females already at 110 dpf. In all these genes, sex-specific significant
363 differences in expression were observed at least in one of the three sampling ages. All
364 genes except *hsd3β* had maximal sex-related expression differences at 250 dpf.
365 Regarding the six canonical TFs, they were upregulated as expected according to sex:
366 *foxl2*, *sox3* and *figla* in females while *nr5a1a*, *sox9b* and *dmrt1* in males. All of them
367 were differentially expressed between sexes at least in 250 dpf and some also at 350 dpf
368 (*sox3*, *figla*, *sf1a* and *sox9b*).

369

370 Next, we looked specifically at the four signaling pathways known to be associated with
371 sex differentiation from previous studies (see Materials and methods). Among genes

372 that constitute these signaling pathways, we looked at the number of DEG and the
373 magnitude of the gene expression values. The two selected pathways related to ovarian
374 development, fanconi anemia (Fig. 5a, b) and wnt (Fig. 5c, d) signaling pathways, had a
375 larger number of DEG and a higher gene expression (FC) values in females. Similarly,
376 when looking among pathways related to testis development, p53 signaling pathway
377 (Fig. 5e, f) and cytokine-cytokine interaction receptor pathway (Fig. 5g, h), they had a
378 larger number of DEG and a higher gene expression (FC) values in males.

379

380 *The role of transcription factors during gonadal development*

381 Our microarray included 2,822 TFs in total, i.e., 13.5% of all the probes. The proportion
382 of differentially expressed TFs was variable between sexes and across time (Fig. 6). At
383 110 dpf 8.0% of the DEG were upregulated TFs in ovaries when compared to testes,
384 while no TFs were upregulated in testes. At 250 dpf, the percentage of differentially
385 upregulated TFs increased up to 13.0% in ovaries and 9.6% in testis. Up to 25.6% of the
386 DEG at 350 dpf were identified as TFs; 14.5% upregulated in ovaries and 11.1% were
387 upregulated in testis.

388

389 **Discussion**

390

391 *Robustness of the microarray*

392 This study provides a comprehensive transcriptomic analysis of gonad differentiation in
393 the European sea bass using a custom species-specific microarray (Schaeck et al. 2017)
394 that has been here further validated. First, the microarray was completely re-annotated
395 and includes almost 80% of the genes identified in the European sea bass genome.
396 Quality control showed that it had high reproducibility and accuracy. Transcript
397 expression values were very robust as the standard deviations of probe replicates was
398 very low (average 0.276 for 20,029 the duplicated probes), confirming the high
399 reproducibility of RNA analysis using the Agilent oligo-array (Shi et al. 2006). To date,
400 microarray analyses have been very useful in the study of fish transcriptomes, e.g.,
401 (Millan et al. 2010; Jantzen et al. 2011; Tingaud-Sequeira et al. 2013; Schaeck et al.
402 2017). Although in the last years RNA sequencing have gained favor over array
403 platforms, analysis of the same samples with the two different techniques gives similar
404 results (Zhao et al. 2014). Here we provide a validated, fast and cost-effective tool for

405 aquaculture research to study the expression patterns of genes, including all major
406 reproduction-related genes, in the European sea bass (Schaeck et al. 2017).

407

408 *Transcriptomic differences between females and males during gonadal development*
409 PCA classified individuals in well-defined and separated clusters according to sex and
410 stage. At 110 dpf, when gonads were still not histologically differentiated,
411 transcriptomic analysis was already capable of classifying samples according to
412 phenotypic sex. Statistical analysis showed that the highest number of DEG were found
413 in differentiated females when compared to differentiated males, in particular at 250
414 dpf, probably explained by the fact that sex differentiation in the European sea bass
415 starts earlier in females (Piferrer et al. 2005) as in many other fish species (Piferrer
416 2001; Devlin and Nagahama 2002). This contrasts with results found in other fish
417 species such as zebrafish (Small et al. 2009), tilapia (Tao et al. 2013) or turbot (Ribas et
418 al. 2016), where male-related genes were enriched with respect to female-related genes.
419 Testis development implied downregulation of genes in a certain stage when compared
420 to the previous developmental stages. This tendency was also observed in turbot (Ribas
421 et al. 2016), supporting the importance of active gene repression for testis development.
422 This is in accordance with the current view stating that positive and negative regulatory
423 loops are required for sex differentiation in vertebrates (Munger et al. 2013; Capel
424 2017).

425

426 There were 15 enriched GO terms in upregulated genes during ovarian development
427 (from 110 dpf to 350 dpf), all of them related to metabolic functions. In this regard, the
428 metabolic process category was the most enriched one found in the differentiating ovary
429 of the protogynous ricefield eel (*Monopterus albus*) (Cai et al. 2017) and was described
430 as well for ovarian development in turbot (Ribas et al. 2016). In contrast, we did not
431 find any common GO term in the three developmental stages studied in testis.
432 Nevertheless, 30 common GO terms were found enriched between 250 and 350 dpf in
433 developing males and were related to catabolic processes, regulation and positive
434 regulation of growth, among others.

435

436 Three pathways (i.e. lysine degradation, bladder cancer and NOD-like receptor
437 signaling) showed a consistent upregulation in the ovaries in all stages when compared
438 to testes. Lysine is an essential amino acid required for protein synthesis, enzyme

439 catalysis and L-carnitine biosynthesis and thus essential for energy metabolism in all
440 body tissues, including ovaries (Ramseyer and Garling 1994; Hallen et al. 2013). The
441 bladder cancer pathway includes genes involved in gonadal development such as the
442 tumor protein tp53 (Mitra et al. 2006), a gene present in germ cells and that induces
443 apoptosis and atresia in oocytes (Rodriguez-Mari et al. 2010; Sayed et al. 2018). The
444 NOD-like receptor signaling pathways is activated in response to host defense and
445 inflammatory disease response (Caruso et al. 2014) and it is upregulated in human
446 polycystic ovaries (Wang et al. 2014). On the other hand, during testicular development
447 eight pathways were identified, including pathways related to metabolism (e.g.,
448 butirosin and neomycin biosynthesis, amino sugar and nucleotide sugar metabolism), to
449 genetic information processing (e.g., basal transcription factors, type II diabetes mellitus
450 ribosome) and to steroid biosynthesis. A sex-biased representation of these molecular
451 pathways might also be species-specific. Thus, for example, in Japanese flounder
452 (*Paralichthys olivaceus*) the upregulation of metabolic-related pathways was found in
453 ovaries rather than in testes (Fan et al. 2014). Also, in the tilapia, steroidogenic
454 pathways were more expressed in females than in males, particularly at early stages of
455 development (Tao et al. 2013).

456

457 *Sex-biased expression of canonical genes during sex differentiation*

458 We selected 54 pro-female or pro-male genes to study their expression along gonadal
459 development. We found 49 DEGs whose expression matched the expected sex bias
460 described in previous studies of other fish species (see Materials and methods).

461 However, six of the genes upregulated in male European sea bass had been previously
462 described as upregulated in female zebrafish (Ribas et al. 2017), namely: *hsd3 β* ,
463 *cyp19b1*, *tradd*, *er1*, *fshr* and *er2b*.

464

465 In females, the steroidogenic gene *hsd17 β 10* was upregulated at 110 dpf. The
466 expression of *hsd17 β* , *cyp19a1a* and *star* was downregulated in European sea bass at
467 170 dpf in fish previously exposed to high temperature, due to the masculinization of
468 the ovary (Díaz and Piferrer 2017). The *cyp19a1a* gene, a key enzyme responsible for
469 converting androgens into estrogens (Guiguen et al. 2009) is considered an early marker
470 of ovarian differentiation in several fish species, including sea bass (Blázquez et al.
471 2008) and also Atlantic halibut (*Hippoglossus hippoglossus*) (Matsuoka et al. 2006) and
472 turbot (Ribas et al. 2016), among others. In the present study, *cyp19a1a* showed

473 differentially expression at 110 dpf but was not significant until 250 dpf. *Hsd17β10* is a
474 mitochondrial enzyme involved in multiple cellular functions, which include fatty acid
475 oxidation, amino acid degradation and steroid metabolism (Yang et al. 2007; Zschocke
476 2012). In humans, *hsd17β10* is related to neurodegenerative diseases such as Parkinson
477 or Alzheimer and has been fully documented (Zschocke 2012; Yang et al. 2014). In
478 fish, there is a lack of information about this isoform as it has been only described in
479 amphioxus (*Branchiostoma belcheri*) (Zhang et al. 2008) and zebrafish (He and Yang
480 2009) but no biological functions have been yet ascribed. However, information does
481 exist for other genes of the same family such as *hsd17β1* and *hsd17β3*, which are
482 involved in sex steroid biosynthesis: *hsd17β1* is responsible to convert inactive estrone
483 to active estradiol and leads to female sex differentiation while *hsd17β3* is required for
484 11-ketotestosterone synthesis (Tokarz et al. 2015). *Hsd17β1* has been identified in some
485 fish species such as Nile tilapia (Zhou et al. 2005), Atlantic cod (*Gadus morhua*)
486 (Breton and Berlinsky 2014) and olive flounder (Fan et al. 2014) while *hsd17β3* in
487 zebrafish and in medaka (*Oryzias latipes*) only (Mindnich et al. 2004; Kim et al. 2014).
488 *Hsd17β1* was already detected at early stages of development in pre-differentiated
489 fathead minnow (*Pimephales promelas*) embryos, although its expression was not
490 correlated to any sex in particular (Wood et al. 2015). Recently, *hsd17β1* has been
491 suggested as the sex determining gene in the California yellowtail (*Seriola dorsalis*),
492 which seems to have a ZW sex determination system (Purcell et al. 2018). In our data,
493 we did not find any differential expression in *hsd17β1* and *hsd17β3* genes but we did it
494 in *hsd17β10*. To our knowledge, this is the first time that the *hsd17β10* steroidogenic
495 gene is described in European sea bass and it is detected early in the ovaries. Therefore,
496 it is a candidate to be considered as a novel early ovarian marker in this species,
497 although research on its functional role during ovarian differentiation needs further
498 attention.

499

500 In males, the first signs of sex-biased expression of canonical reproduction-related
501 genes were detected at 250 dpf onwards and not earlier, indicating that their expression
502 starts somewhere between 110 to 250 dpf as previously showed in fish subjected to high
503 temperature treatments at 170 dpf (Díaz and Piferrer 2015). In the present study, genes
504 involved in the steroidogenic pathway such as *hsd3β* or *cyp11β1*, in androgen action
505 such as *arα*, or in gonadotropin signaling, *fshr* and *lhr*, were differentially expressed

506 during testis development, as previously reported (Blázquez and Piferrer 2005; Mazón
507 et al. 2014). In contrast, the gonadotropin subunits *fshb* and *lhb*, detected in the gonads
508 of the sea bass in this study, as also described in other fish species (Wong and Zohar
509 2004; von Schalburg et al. 2005; Levavi-Sivan et al. 2010) were not differentially
510 expressed.

511

512 When looking for DEG ($P < 0.01$) in testes compared to ovaries at 110 dpf, a total of 15
513 genes were found, although none could be considered as canonical reproduction-related
514 genes. Among them, we identified three genes that were previously described to be
515 expressed in the reproductive system: platelet-derived growth factor beta polypeptide
516 (*pdgfb*), sorting nexin 1 (*snx1*) and nuclear transcription factor Y beta (*nfy*), although
517 there are few data on the role of these genes in testis, not only in fish, but also in
518 mammals. For example, *pdgfb* is involved in the regulation of many biological
519 processes including embryonic development and sexual phenotype, since alteration of
520 this gene generated male and female infertility in several species, including humans
521 (Donnem et al. 2010). *Snx* is involved in cellular endocytosis functions and its role in
522 oogenesis was described in the gibel carp (*Carassius gibelio*) (Wen et al. 2003) while
523 *nfy* is a pleiotropic transcription factor that participates in multiple processes such as
524 cell proliferation and development (Li et al. 2018). For example, it has been detected in
525 breast cancer cells (Lagadec et al. 2014) and it is involved in spermatogenesis (Vanwert
526 et al. 2008) but its specific role in transcriptional regulation is not fully understood
527 although several functional studies have been published so far. It is known that it can
528 bind to the *piwill* promoter in the germ cells (Chang et al. 2015), but also binds together
529 with the orphan nuclear receptor steroidogenic factor-1 (Nr5a1), to the promoter of *fshb*
530 gene (Jacobs et al. 2003).

531

532 Next, we looked for canonical KEGG pathways involved in sex differentiation and
533 previously described in some but few fish species. This was the case of two pathways
534 required for ovarian development: the fanconi anemia pathway, identified in zebrafish
535 (Rodríguez-Marí and Postlethwait 2011) and in common carp (*Cyprinus carpio*) (Jia et
536 al. 2018), and the wnt signaling pathway, identified in zebrafish (Sreenivasan et al.
537 2014) and in rainbow trout (*Oncorhynchus mykiss*) (Nicol and Guiguen 2011). In the
538 European sea bass, we found that the number of genes differentially expressed ascribed
539 to these two pathways increased at 250 dpf and onwards in the developing ovaries. In

540 testes, we studied the apoptotic pathway p53 previously described in zebrafish
541 (Rodríguez-Marí et al. 2010), medaka (Yasuda et al. 2012) and spotted knifejaw testes
542 (*Oplegnathus punctatus*) (Du et al. 2017), and the cytokine-cytokine interaction
543 pathway identified in Japanese flounder (Zhang et al. 2015) and in zebrafish (Ribas et
544 al. 2017) gonads. In the European sea bass, we found an increase in the number of genes
545 differentially expressed and associated with these pathways at 250 dpf that then
546 decreased. Thus, these results confirm that, as occurs in other fish species, these four
547 pathways are also involved in gonad development in the European sea bass.

548

549 *Transcription factors during gonadal development*

550 TFs tightly control gene expression in a large number of processes including gonadal
551 development (Migeon and Wisniewski 2000) and so, in the last years, many studies
552 have revealed their importance in fish sex differentiation (Herpin and Schartl 2011;
553 Nakamura et al. 2011; Shen and Wang 2014; Tanaka 2016). Consequently, with the aim
554 of deciphering the involvement of TFs in gonadal development in the European sea
555 bass, we studied the expression of TFs already known to be sexually dimorphic. These
556 included *foxl2* (Yamaguchi et al. 2007) and *figlα* (Kanamori et al. 2008), related to
557 ovarian development, and *sox9b* (Bagheri-Fam et al. 2010), *nr5a1a* (Crespo et al. 2013)
558 and *dmrt1* (Deloffre et al. 2009) related to testis development, the expression of some
559 being in accordance to what was previously described in the European sea bass gonads
560 fish at 170 dpf and subjected to high temperatures (Díaz and Piferrer 2015). *Sox3* was
561 considered as a male-determining gene in ricefish (*Oryzias dancena*) (Takehana et al.
562 2014) although its expression was related to both oocyte and testis development in other
563 fish species. This is the case found in grouper (*Epinephelus coioides*) (Yao et al. 2007)
564 and in Japanese flounder (Jeng et al. 2018) with an expression bias towards female
565 development. In the present study, *sox3* clearly showed a female bias in the European
566 sea bass gonads.

567

568 Then, we explored the presence of TFs at each specific stage of gonadal development.
569 The number of DE TFs increased as the gonadal development progressed. Thus, at 350
570 dpf, when gonads were fully differentiated, the largest number of DE TFs were
571 detected. In all stages, there was a larger number of DE TFs in females than in males
572 that is in concordance with the largest number of DEGs found in females in this fish
573 species. This skewed number towards females was evident already at 110 dpf. DE TFs

574 were only detected in ovaries, probably due to sex differentiation starting earlier in
575 females (Piferrer et al. 2005) and to the increased activity of the tissue by ovary
576 formation and meiotic division actions (D'Cotta et al. 2001).

577

578 **Conclusions**

579 A species-specific microarray enriched for reproduction-related genes was used to study
580 gene expression during European sea bass gonadal development. In contrast to what had
581 been described in other species, a larger number of DEG and DE TFs were observed in
582 ovaries when compared to testis. The expression profiles of 54 genes previously
583 associated to sex differentiation in other species were examined and the steroidogenic
584 gene *hsd17 β 10* is described as a promising ovarian marker capable of identifying
585 females as early as 110 dpf. Also, three genes: *pdgfb*, *snx1* and *nfy*, were identified as
586 potential markers for male development. Further, three and eight pathways that are
587 consistently enriched along gonadal development in ovary or testis, respectively, were
588 also identified. Taken together, these results contribute to our understanding of gene
589 expression during sexual development in an economically important species in
590 particular and in non-mammalian vertebrates in general, and emphasize the great
591 diversity, also at the molecular level, of fish sexual development.

592

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601

602 **Conflict of Interest**

603 The authors declare that they have no competing interests.

604

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1034 **Figure Legends**

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1036 **Figure 1.** Overview of transcriptomic changes during European sea bass sex
1037 differentiation. a) Principal component analysis of microarray results at three
1038 developmental stages: 110, 250 and 350 days post fertilization (dpf). Samples cluster
1039 together by gender: females (pink ellipse), males (blue ellipse). b) Number of
1040 differentially expressed genes found along gonadal development (110, 250 and 350 dpf)
1041 for the seven comparisons analyzed using the SAM test.

1042

1043 **Figure 2.** Common Biological Processes Gene Ontology terms at 110, 250 and 350
1044 days post fertilization (dpf) of differentially expressed genes during European sea bass
1045 sex differentiation a) Female-related genes ($P < 0.01$) b) Male-related genes ($P < 0.02$).

1046

1047 **Figure 3.** Heatmap of the microarray expression data for 49 out of 5 reproduction-
1048 related canonical genes: a) 25 up- and b) 24 downregulated genes in females. Each row
1049 represents a gene and each column represents a group of fish by age and sex: (M110 = 8
1050 males at 110 days post fertilization (dpf), M250 = 6 males at 250 dpf and M350 = 9
1051 males at 350 dpf; F110 = 4 females at 110 dpf); F250 = 6 females at 250 dpf and F350 =
1052 9 females at 350 dpf. The key color represents the level of expression scaled by gene
1053 (yellow: high expression and blue: low expression). The dendrograms inform of the
1054 similarity between genes and between the different samples. Notice that all genes were
1055 grouped as pro-female and pro-male as expected from studies in other species. See
1056 Dataset 1 for a complete list of gene names and abbreviations.

1057

1058 **Figure 4.** Changes in expression of a set of canonical genes related to sex
1059 differentiation and reproduction in female and male gonads during European sea bass
1060 sex differentiation. a-f) Canonical genes of the steroidogenic pathway. g-l) Canonical
1061 transcription factors. Abbreviations: dpf, days post fertilization; * = $P < 0.05$; ** = $P <$
1062 0.01 ; *** = $P < 0.001$).

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1064 **Figure 5.** Number of differentially expressed genes (DEG; left panels) and fold change
1065 of reproduction-related pathways (right panels) during European sea bass sex
1066 differentiation (pink, females; blue, males): a-b) Fanconi anemia signaling pathway, c-
1067 d) Wnt signaling pathway, e-f) p53 signaling pathway, g-h) Cytokine-cytokine

1068 interaction receptor signaling pathway. In the left panels data is expressed as the total
1069 number of genes (absolute values) differentially expressed at each time of development,
1070 in the right panels data is expressed as fold change using male values at 110 dpf as
1071 control group set at 0.

1072 **Figure 6.** Diagram showing at three ages during European sea bass sex differentiation
1073 the percentage of pro-male and pro-female transcription factors (TFs) differentially
1074 expressed at each developmental stage of female-related genes, in pink or male-related
1075 genes, in blue.

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