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

Ribas L[1+], Crespo B[2+], Sánchez-Baizán N[1], Xavier D[3], Kuhl H[4], Jose Manuel Rodriguez[5,9], Díaz N[2], Boltañá S[6,7], MacKenzie S[6,7], Reinhardt R[4,8], Moran F[3], Zanuy S[2], Gómez A[2*], Piferrer F[1]*

[1] Institute of Marine Sciences (ICM), Spanish National Research Council (CSIC), Barcelona, Spain
[2] Institute of Aquaculture of Torre de la Sal (IATS-CSIC), Castellón, Spain
[3] Dept. of Biochemistry and Molecular Biology I, Complutense University, Madrid, Spain
[4] Max Planck Institute for Molecular Genetics, Berlin, Germany
[5]Spanish National Bioinformatics Institute, Madrid, Spain
[6]Autonomous University of Barcelona, Barcelona, Spain
[7]Current address: Institute of Aquaculture, University of Stirling, Scotland, UK
[8]Current address: Max Planck Institute for ……, Cologne, Germany
[9]Current address: Cardiovascular Proteomics Laboratory, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain

[+]These authors contributed equally
*Correspondence: Dr. Francesc Piferrer, Institute of Marine Sciences, Spanish Council for Scientific Research (CSIC), Barcelona, Spain. Tel. +34-932309567; fax, +34-932309555; e-mail: piferrer@icm.csic.es. Dr. Ana Gómez. Institute of Aquaculture Torre la Sal (CSIC). Ribera de Cabanes s/n. 12595 Torre la Sal. Castellón. Spain. Tel. +34 964 319500; fax, +34 964 319509; e-mail: a.gomez@csic.es

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Abstract

European sea bass, one of the most important cultured fish species in Europe, has a marked sexual growth dimorphism in favor of females. It is a gonochoristic fish with a polygenic sex determination system, in which several still unidentified loci and environmental temperature play a role in establishing sex ratios. Further, the underlying mechanisms responsible to convert a sexually undifferentiated gonad into a differentiated ovary or testis are still globally unknown. To better understand sexual development in this cultured fish species, we sampled fish during the gonadal developmental period (110 to 350 days post fertilization, dpf), and performed a comprehensive transcriptomic study by using a species-specific reproduction-enriched microarray. Microarray analysis uncovered sex-specific gonadal transcriptomic profiles at each stage of development, identifying larger number of differentially expressed genes in ovaries rather than in testis. By focusing on a curated list of canonical reproduction-related genes, the expression pattern of 49 genes with significant differences between females and males in at least one of the studied stages was quantified. Two early ovarian markers were identified in the European sea bass: *cyp19a1a* and *17hsdb10*. On the other hand, three genes not previously related to sex differentiation were tightly associated with testis development: *pdgfb, snx1* and *nfy*. Regarding signaling pathways, lysine degradation, bladder cancer and NOD-like receptor signaling were required for ovarian development while eight (including amino sugar and nucleotide sugar metabolism, basal transcription factors and steroid biosynthesis) were required for testis development. The expression of transcription factors during gonadal development occurred earlier and in a more pronounced manner in females than in males. Our results not only provide the first comprehensive data that explored the transcriptomes along gonadal development in the European sea bass, reporting the but also directs sheds some light on sex differentiation in fish.

Keywords: genomics, transcriptomic, reproduction, sex differentiation, gonads, aquaculture

Introduction

Many cultured fish species present sexual dimorphism in growth, which constitutes a problem in their production. This is the case of the European sea bass (*Dicentrarchus labrax*) in which females grow about 30% more than males (Saillant et al. 2001). European sea bass is the third most important cultured species of marine fish in Europe with a production of ~180 thousand tons per year (Food and Agriculture Organization of the United Nations 2016). Thus, there is an evident interest in the aquaculture industry to produce stocks of sea bass with a high percentage of females in order to increase biomass and consequently to increase productivity. However, environmental factors, like temperature, can affect the final sex of the fish gonads increasing the number of males in the populations (reviewed in Felip et al. 2018). In fact, the appearance of undesired masculinization in fish stocks grown for market purposes is a major problem in fish farms across the world (Piferrer 2001; Budd et al. 2015). In the European sea bass, elevated temperatures can generate populations of 80-100% males, which consequently decrease drastically productivity (Blázquez et al. 1998). This problem can be even more aggravated if males present precocious maturation as it slows down their growth (Carrillo et al. 2015) and diminishes productivity. Given the concern of the aquaculture industry in controlling the sex ratio in fish populations, there is interest in deciphering the molecular events involved in gonadal development.

In the last few years, the development of genomic and genetic technologies has exponentially grown and consequently large number of genomic and transcriptomic data of commercial fish species have become available. This is the case of European sea bass, as is one of the richest species in terms of genomic resources among cultured fish, which include the availability of the genome and Single Nucleotide Polymorphism (SNP) markers, among others. The European sea bass has a small genome size (675 Mb) with a total of 26,719 annotated genes (Tine et al. 2014; Chaves-Pozo et al. 2017) and has 24 haploid chromosomes pairs (Aref'yev 1989).

European sea bass is a gonochoristic species with a polygenic sex determination system in which both genetic and environmental factors influence the final sexual phenotype (Piferrer et al. 2005; Vandeputte et al. 2007). Although the genetic factors are still not

known, efforts have been done towards identifying sex-determinant markers to develop genotyping selection programs. Recent studies using ~6,700 SNP markers showed that there are at least three loci responsible to determine the final sex in European sea bass (Palaiokostas et al. 2015) but these may be family-specific and thus research on European sea bass sexual development deserves further attention.

Transcriptomic studies on gonadal tissues are available in a relatively large number of cultured and non-cultured fish species. For example, in the channel catfish (*Ictalurus punctatus*), a description of genes differentially expressed (DEG) along testis development has been described (Zeng et al. 2016), and in fugu (*Takifugu rubripes*) and in the spotted knifejaw (*Oplegnathus punctatus*), a larger number of DEG were found in testis when compared to ovary (Du et al. 2017; Wang et al. 2017). Yet, the number of studies focused on gonadal transcriptomes during sexual development are just a few. Aside from some studies using qPCR on selected genes, the first studies with a genomic approach were those carried out in the Nile tilapia (*Oreochromis niloticus*) (Tao et al. 2013), followed by turbot (*Scophthalmus maximus*) (Ribas et al. 2016), zebrafish (Ribas et al. 2017) and more recently in the yellow river carp (*Cyprinus carpio*) (Jia et al. 2018). In all these studies, the molecular complexity of generating a testis or an ovary from a bipotential undifferentiated tissue has been reported.

The objective of this study was to gain knowledge on the gonadal transcriptome of European sea bass along gonad sexual differentiation. We cover the process of sex differentiation from undifferentiated gonads to differentiated testes or ovaries and we evaluated the gene expression patterns by using a European sea bass reproduction-enriched microarray. This custom microarray was based on sequences generated by next generation sequencing technologies (i.e., 454 FXL Titanium). Studying transcriptomes by using a microarray platform provides accuracy and reproducibility of the performed analysis at a reasonable cost and allows to study a broad range, if not all, of the transcripts of the genome (Shi et al. 2006). In particular, our custom microarray contains 78.5% of the fully annotated European sea bass genome, thus providing a powerful molecular tool to study expression patterns of this species.

Materials and Methods

European sea bass gonad sampling

In order to obtain the widest possible range of expressed transcript sub-sets, gonad tissues were dissected in fish at different stages of gonad development: at 110 dpf (1.5 ± 0.5 cm of standard length, SD), at 250 dpf (12.7 ± 5.7 cm of SD and 11.2 ± 0.6 cm of SD in females and males, respectively) and 350 dpf (16 ± 1.3 cm and 14.8 ± 1.1 cm, in females and males, respectively). The set of samples consisted of eleven gonadal tissues at 110 dpf, six testes and six ovaries at 250 dpf and nine testes and nine ovaries at 350 dpf. Gonads were fully isolated in fish at 250 and 350 dpf and thus gonadal tissue was devoid of any other tissue. The isolation of the gonads alone was not feasible in younger fish (110 dpf) due to their extremely small size, and thus some epithelial tissue was likely attached to the gonads.

Microarray design

The microarray platform consists of 1,417 Agilent control probes and a total of 43,803 transcript probes that represent 20,977 genes of which 20,028 have two probe copies and the rest have between 1 to 6 copies per gene (Supplementary Table S1). Genes with reproduction-related functions were represented at least four times. Microarray design was based on sequences obtained by two 454 FLX Titanium runs on European sea bass gonad tissues, a former custom European sea bass microarray platform (GPL13443) available in our laboratory, a battery of selected reproduction key genes and a published European sea bass microarray (Ferraresso et al. 2010). Redundancies on all the sequences were analyzed and removed and so only annotated sequences were selected (a total of 20,918) to be printed on the microarray platform. The designed microarray platform was submitted to Gene Expression Omnibus (GEO) database (Edgar et al. 2002) with the platform number GPL16767 and its functionality was reported in a previous work using European sea bass larva (Schaeck et al. 2017). In the present work, the microarray has been re-annotated by using the European sea bass genome (Tine et al. 2014) and further validated to study the transcriptomes of the gonadal development in the European sea bass. Microarray data of the present study were submitted to GEO and are accessible through GEO Series accession number GSE115841.

Microarray hybridization

The total RNAs of a total of 41 gonad samples (between 11 and 18 replicate samples per stage of development and sex) were individually extracted by RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Quantity was determined by using a Nanodrop spectrophotometer (Nanodrop Technologies, USA) and quality (RNA integrity number, RIN) by using a Bioanalyzer (Agilent Technologies, USA). Only RNA samples with a mean RIN of ≥ 8.4 were further processed for microarray analysis. RNA was labelled using the Low Input Quick Amp Labelling Kit, One-Color (Cy3; Agilent Technologies) and cRNA was prepared for overnight hybridization with the corresponding buffers during 17 h at 65°C and washed on the following day. Samples were hybridized individually in our European sea bass custom 4x44K Agilent platform described above at the Parc d'Investigació Biomédica de Barcelona (PRBB) and slides were scanned using an Agilent G2565B microarray scanner (Agilent Technologies, USA). Agilent software was used to avoid saturation and feature extraction generated the raw data for further pre-processing.

Data analysis

Statistical analyses were carried out with the statistical language software R (2.13.1 version; www.R-project.org). Array normalization was implemented using the Quantile method in the Linear Models for Microarray Analysis (Limma) R package (Wettenhall et al. 2004; Ritchie et al. 2015). A False Discovery Rate (FDR) p-value < 0.01 and P < 0.001 thresholds were applied to identify genes with statistically significant differences in gene expression between comparisons of interest. Potential batch effects were removed by ComBat correction, a bioinformatic tool based on Empirical Bayes algorithms (Chen et al. 2011). The Principal Component Analysis was built in R software for data visualization, identification of clusters and outliers (two samples at 350 dpf were detected as outliers and excluded from further analysis). Next, the microarray analysis software TIGR Multiexperiment Viewer version 4.9 (TMeV) (Saeed et al. 2003) was used to determine the number of differentially expressed genes (DEG) between sexes at a given stage of development or between stages of development within the same sex by the Significance Analysis of Microarrays (SAM) statistical test. The analysis generated lists of DEG at each age (110, 250 and 350 dpf) in the same sex or in comparison to the other sex, including the $\log_2$ transformation of fluorescence intensity measured for each gene. In particular, we selected 55 canonical

genes to study sex differentiation and reproduction that were also reported in literature in previous studies in turbot (Ribas et al. 2016) and zebrafish (Ribas et al. 2017).

Gene ontology terms and Kegg pathway analysis

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

We used the Gene Set Variation Analysis (GSVA) from Pathway Processor 2.0 to study the signalling pathways involved in gonadal development. GSVA transforms the gene expression values into a normalized expression matrix with enrichment scores of differentially regulated pathways (DRP) with the corrected *P* value between males and females at each developmental stage (Beltrame et al. 2013). In particular, we also studied a selection of four pathways involved in sex differentiation (Ribas et al. 2017). The lists of genes that compose these pathways were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG), using the zebrafish as a background. Next, the numbers of DEG from these pathways as well as expression values (in $\log_2$ Fold Change, FC) were plotted together; upregulated in male *versus* (*vs.*) upregulated in female over time.

Transcription factors analysis

We calculated the percentage of transcription factors (TFs) identified among the DEG between females and males at each time point. To identify the total TFs present in the microarray, we used the TFcheckpoint database that is a curated collection of TFs from nine databases based on experimental evidence (Chawla et al. 2013). This list served as a background to determine the percentage of the number of TFs differentially expressed in each sampling point.

Validation of the microarray

The microarray analysis results were validated by studying the gene expression of twelve genes by quantitative real time polymerase chain reaction (qPCR). The gene

selection for validation was based on a representation of FC range and an equal representation of upregulated and downregulated genes. The reference genes were chosen based on bibliography recommendation of using the house-keeping genes Elongation factor-1 alpha (*ef-1α*) and 40S ribosomal protein (*fau*) for expression quantification of genes previously validated in the European sea bass (Mitter et al. 2009). For the chemical reaction, 100 ng of total RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and 100 ng of random hexamer primers (Sigma) following the manufacturer's instructions. The reaction was carried out with SYBR Green chemistry (Power SYBR Green PCR Master Mix; Applied Biosystems). qPCR reactions contained 1X SYBR green master mix (Applied Biosystems), 10 pmol of each primer and 1 µl of the RT reaction. Samples were run individually and in triplicate in optically clear 384-well plates. Cycling parameters were: 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 s and 60ºC for 1 min. Finally, a temperature-determining dissociation step was performed at 95ºC for 15 s, 60ºC for 15 s and 95ºC for 15 s at the end of the amplification phase. qPCR data were collected by SDS 2.3 and RQ Manager 1.2 software and relative quantity (RQ) values for each reaction replicate were calculated by the 2ΔΔCT method (Schmittgen et al. 2008). Primer sequences used for gene expression study are shown in Supplementary Table S2.

Results

Microarray design and validation

To ensure the reliability of microarray results, we studied the hybridization repeatability and consistency for a selection of the 55 genes related to reproduction (Ribas et al., 2016, 2017) (Supplementary Table S3). Probe copy tendency for 52 of these genes were the same for all the copies and only three of them (representing 0.05% of the studied probes) showed different probe behavior in at least one of the copies. We represented the expression patterns throughout the printed probe copies of the same gene that presented upregulated (Supplementary Fig. S1 a, c, e, g) and downregulated (Supplementary Fig. S1 b, d, f, h) or variable (i) expressions in all the samples analyzed in the microarrays. Since most probes had two or four copies (Supplementary Fig. 1j) to further evaluate the hybridization accuracy we determined the magnitude of variation between technical replicates. The mean of the standard deviations for all probes ranged between 0.205 and 0.347 (Supplementary Fig. 1j). Thus, given the low standard error

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

Furthermore, microarray results were validated by qPCR for twelve DEG between males and females at 350 dpf, representing a wide range of FC values and including up- and downregulated genes. Results showed good correlation between the two techniques ($R^2 = 0.748$, $P = 0.0003$; Supplementary Fig. S2a). Additionally, gene expression values for aromatase (*cyp19a1a*) for samples at 110, 250 and 350 dpf determined either by using microarray or qPCR further validated our results (Supplementary Fig. S2b).

Gonadal transcriptomes overview

The microarray data was spatially distributed in two clear clusters between female and male groups in the PCA, with females showing a less disperse cluster when compared to males (Fig. 1a). At each cluster, three subgroups were observed corresponding to the age of the samples for each of the sex (F, females; M, males): F 110 dpf, F 250 dpf, F 350 dpf and M 110 dpf, M 250 dpf M 350 dpf. Component 1 already contributed to 87.14% of the variance while component 2 and 3 contributed to 8.35% and 1.98% of variance, respectively. Thus, the first three components together explained 97.47% of variance.

The six sex-age groups described above were used for gene expression comparison between sex (F *vs.* M) and developmental stages (3 stages), resulting in 7 comparisons analyzed with the SAM statistical test (Fig. 1b). Among the 20,978 genes included in the microarray, 64.93% were differentially expressed at one or several developmental stages. The study of expression differences between ovaries and. testes resulted in a total of 708; 7,639 and 6,926 DEG with a P value < 0.01, at 110, 250 and 350 dpf, respectively (Fig. 1b, Dataset 1). When compared between sexes, a larger number of genes were upregulated in F, particularly at 110 and 350 dpf. However, among the few genes that were upregulated in M higher FC values were found (Supplementary Table S4). At 110 dpf there were only 23 upregulated genes in M and up to 685 upregulated in F. At 250 dpf 3,769 showed male-biased expression and 3,870 genes were upregulated in F. At 350 dpf, less genes were upregulated in M (829 DEG) than in F (6,097 DEG).

Considering the same statistical threshold, there were 451 upregulated genes in M and 3,564 upregulated genes in F between 110 and 250 dpf. However, lower number of genes were found in the comparison between the second and third stages: 309 upregulated genes in M and 1,100 upregulated genes in F between 250 and 350 dpf. The higher fold change values were observed at 250 dpf ($P < 0.001$), indicating important sex-related differences in expression levels (Supplementary Table S4). The highest FC was found to be 7.5 and 6.7 Log_2 in M and F at 250 dpf, respectively (Dataset 1).

Discovery of gene ontologies and gene pathways along gonadal development

The GO-term enrichment analysis of the DEG between F and M revealed several related categories of biological processes (BP), molecular function (MF) and cellular component (CC) categories throughout development (Dataset 2). In Fig. 2a the 15 significant upregulated BP common to 110, 250 and 350 dpf ($P < 0.01$) required for ovary formation are shown. The GO terms of the three developmental stages included genes related to metabolic processes (GO:0008152, GO:0071704, GO:0044237), catalytic activity (GO:0003824), oxidoreductase activity (GO:0016491), coenzyme and cofactor binding (GO:0050662; GO:0048037), and biosynthetic processes (GO:0009058, GO:1901576). Among the upregulated GO terms required for testis formation, there were 30 significant terms in all the three categories that were common at 250 and 350 dpf, since there were very few regulated genes at 110 dpf. Fig. 2b shows GO terms found in males in BP category ($P < 0.05$) which were related to catabolic processes (GO:0000956, GO:0006402, GO:0006401), regulation of ion transmembrane activity (GO:1904427, GO:0032414, GO:0034767), regulation of calcium ion (GO:0010524, GO:0050850, GO:0051281, GO:0060316, GO:1901021, GO:1904427) and positive regulation of growth (GO:0045927).

Then, we determined the significantly differentially regulated cellular pathways (DRP) between males and female along gonadal development. A total of 41, 151 and 106 DRP were found between males and females at 110, 250 and 350, respectively (Dataset 3, $P < 0.05$). Some of these DRP were related to sex differentiation, for example, at early gonadal development (110 dpf): p53 signaling pathway, steroid hormone biosynthesis or erbβ signaling pathway; at 250 dpf: wnt signaling pathway, oocyte meiosis or steroid biosynthesis and at 350 dpf: MAPK signaling pathway or cytokine-cytokine receptor interaction among others. A total of 16 DPR were consistently regulated at the three

developmental stages in both sexes (Table 1). The lysine degradation, bladder cancer and the nucleotide-binding oligomerization (NOD)-like receptor signaling pathways were upregulated in F at the three stages. The dorso-ventral axis formation pathway was significantly downregulated at 110 but upregulated at 250 and 350 dpf. The p53 signaling pathway and the Chagas disease (American trypanosomiasis) pathways showed significance, being upregulated at 110 and 350 dpf in females but downregulated at 250 dpf. There were two pathways, phosphatidylinositol signaling system and the ErbB signaling pathway, that began upregulated in females at 110 dpf and in males at 250 and 350 dpf. Finally, there were up to eight pathways upregulated in males throughout the studied period: butirosin and neomycin biosynthesis, basal transcription factors, amino sugar and nucleotide sugar metabolism, type II diabetes mellitus, glycine, serine and threonine metabolism, steroid biosynthesis, ribosome, and folate biosynthesis pathways.

Expression of canonical genes and pathways related to sex differentiation

Among a compilation of 55 canonical genes known to be relevant for reproduction and sex differentiation in fish according to the literature, 49 genes presented significant differences between males and females at least at one of the studied stages; the majority (80.5%) were DEG between males and females at 250 dpf (Supplementary Table S3). The hierarchical clustering analysis and the corresponding heatmaps of the 25 pro-female genes (Fig. 3a) and the 24 pro-male genes (Fig. 3b) showed that gene expression data mostly matched according to gender. Fig. 4 provides the insight of the gene expression profiles of twelve important and significant canonical genes (Fig. 4). Six of them are related to the steroidogenic pathway (*cyp19a1a, hsd17β10, hsd3β, cyp11β, arα, fshr*, Fig. 4a-f) and the remaining six genes are transcription factors related to sex differentiation (*foxl2*, *sox3*, *figα*, *sf1a*, *sox9b* and *dmrt1*, Fig. 4g-l). The genes *cyp11β, arα, hsd3β* and *fshr* were upregulated in males at 250 dpf and onwards while *cyp19a1a* and *hsd17β10* were upregulated in females already at 110 dpf. In all these genes, sex-specific significant differences in expression were observed at least in one of the three sampling ages. All genes except *cyp19a1a* and *hsd3β* had maximal sex-related expression differences at 250 dpf. Regarding the expression profile of the six canonical transcription factors studied they were upregulated towards the expected gender: the genes *foxl2*, *sox3* and *figα* were upregulated in females while *sf1a*, *sox9b* and *dmrt1* were upregulated in males during gonadal development. All of them were differentially

expressed between sexes at least in 250 dpf and some also at 350 dpf (*sox3, figα, sf1a and sox9b*).

Next, we looked specifically at the four signaling pathways known to be associated with sex differentiation from previous studies: two (fanconi anemia and wnt signaling pathways) associated with female differentiation (Rodríguez-Marí et al. 2011; Sreenivasan et al. 2014) and two with male differentiation (p53 signaling and cytokine-cytokine interaction receptor pathways) (Yasuda et al. 2012; Ribas et al. 2017). Among genes that constitute these signaling pathways, we looked at the number of DEG and the magnitude of the gene expression values. The two selected pathways related to ovarian development, fanconi anemia (Fig. 5a, b) and wnt (Fig. 5c, d) signaling pathways, had larger number of DEG and higher gene expression (FC) values in females. Similarly, when looking among pathways related to testis development, p53 signaling pathway (Fig. 5e, f) and cytokine-cytokine interaction receptor pathway (Fig. 5g, h), had larger number of DEG and higher gene expression (FC) values in males.

Presence of transcription factors during gonadal development

The present microarray included 2,822 TFs in total, which means 13.5% of all probes represented in the microarray. The proportion of differentially expressed TFs was variable between sexes and across time (Fig. 6). At 110 dpf 8.0% of the DEG were upregulated TFs in ovaries, while no TFs were upregulated in testis. At 250 dpf, the percentage of differentially upregulated TFs increased up to 13.0% in ovaries and 9.6% in testis. Up to 25.6% of the DEG at 350 dpf were identified as TFs; 14.5% upregulated in ovaries and 11.1% were upregulated in testis.

Discussion

Robustness of the microarray

This study represents a comprehensive transcriptomic analysis of gonad differentiation in the European sea bass. We used a custom species-specific microarray previously used in European sea bass larvae (Schaeck et al. 2017) and here we further validate its use for the analysis of gonadal tissues. The microarray is completely annotated representing almost 80% of the European sea bass genome. The performed quality control analyses showed that the reproduction-related microarray was a reliable platform with high reproducibility and accuracy. The repeatability of the microarray probes was very robust

as the mean of standard deviations of the probe replications were very low (0.276 as a mean value among 20,029 duplicated probes), confirming the high reproducibility of RNA analysis using the Agilent oligo-array (Shi et al. 2006). Although in the last years the RNA sequencing-based projects have gained favor over array platforms for studying the RNA expression, it has been shown that analyzing exactly the same samples throughout the two different techniques similar results are obtained (Zhao et al. 2014). Thus, both techniques can be used indistinctly. Here we provide a validated tool for aquaculture research to study at a reasonable cost the expression pattern of reproduction-related gene patterns (useful for all stages of gonadal development) but also to other non-reproduction-related projects performed in European sea bass (Schaeck et al. 2017).

Transcriptomic differences between females and males during gonadal development

PCA classified the individuals in well-defined clusters apart from the other groups which were in accordance with their gonadal developmental stage. Thus, at 110 dpf, when gonads were still not histologically differentiated, transcriptomic analysis was already capable to classify samples according to phenotypic sex. Statistical analysis showed that the highest number of DEG were found in differentiated females when compared to differentiated males, in particular at 350 dpf, probably explained by the fact that sex differentiation in the European sea bass starts earlier in females (Piferrer et al. 2005) as it also occurs in many other fish species (Saillant et al. 2001). These results contrast to those found in other fish species such as zebrafish (Small et al. 2009), tilapia (Tao et al. 2013) or turbot (Ribas et al. 2016) in which male-related genes were enriched with respect to female-related genes and so this research deserves further attention. For testis development, we found a downregulation of genes in a certain stage when compared to the previous developmental stages. This tendency was also observed in turbot (Ribas et al. 2016), indicating that, in addition to the upregulation of some genes, active gene repression is also important for testis development. This is in accordance with the current view stating that positive and negative regulatory loops are required for sex differentiation in vertebrates (Munger et al. 2013; Capel 2017).

There were 15 GO terms overexpressed along ovarian development (from 110 dpf to 350 dpf), and all of them were related to metabolic functions. In this regard, the metabolic process category was the most upregulated one found in the differentiating

ovary of the protogynous ricefield eel (*Monopterus albus*) (Cai et al. 2017) and was required for ovarian development in turbot (Ribas et al. 2016). In contrast, we did not find any common GO term in the three developmental stages studied in testis. Nevertheless, 30 common GO terms were found between 250 and 350 dpf and were related to catabolic processes, regulation and positive regulation of growth, among others.

Next, we studied sex-biased pathways that were required exclusively for ovarian or testis development (from 110 to 350 dpf). By performing these analyses, we found three pathways (i.e., lysine degradation, bladder cancer and NOD-like receptor signaling) that showed a consistent upregulation in the ovaries in all stages. Lysine is an essential aminoacid required for protein synthesis, enzyme catalysis and L-carnitine biosynthesis and so essential for energy metabolism in all body tissues, including ovaries (Ramseyer et al. 1994; Hallen et al. 2013). The NOD-like receptor signaling pathways is activated in response to host defense and inflammatory disease response (Caruso et al. 2014) and it was upregulated in human polycystic ovaries (Wang et al. 2014). In testes, we identified eight pathways that were essential for testicular differentiation all along their development, which included pathways related to metabolism (e.g., butirosin and neomycin biosynthesis, amino sugar and nucleotide sugar metabolism), to genetic information processing (e.g., basal transcription factors, type II diabetes mellitus ribosome) and to steroid biosynthesis. The representation of these molecular pathways in a sex-biased manner might also be dependent on the species as, for example, in Japanese flounder (*Paralichthys olivaceus*) it was found an upregulation of metabolic-related pathways in ovaries rather than testes (Fan et al. 2014) and in tilapia, steroidogenic genes were more expressed in females than in males, particularly at early stages of development (Tao et al. 2013).

Sex-biased expression of canonical genes during sex differentiation

To identify reproduction-related genes, we selected 55 pro-female or pro-male genes to study their expression along gonadal development. We found 49 DEGs whose expression matched the expected sex bias described in previous studies of other fish species. However, six of the genes upregulated in male European sea bass had been previously described as upregulated in female zebrafish (Ribas et al. 2017), namely, *hsd3β, cyp19b1, tradd, erb1*, *fshr* and *esr2*.

In females, there were two steroidogenic genes whose expression was upregulated at the early stage of 110 dpf: *cyp19a1a* and *hsd17b10*. The *cyp19a1a* gene, a key enzyme responsible for converting androgens into estrogens (Guiguen et al. 2009) is considered an early marker of ovarian differentiation in several fish species, including sea bass (Blázquez et al. 2008) and also Atlantic halibut (*Hippoglossus hippoglossus*) (Matsuoka et al. 2006) and turbot (Ribas et al. 2016) among others. *Hsd17b10* is a mitochondrial enzyme involved in multiple cellular functions, which include fatty acid oxidation, aminoacid degradation and steroid metabolism (Yang et al. 2007; Zschocke 2012). In humans, the mutation of this gene has been related to neurodegeneration and Alzheimer disease (Zschocke 2012). In fish, there is a lack of information about the roles of this gene as it has been only described in amphioxus (*Branchiostoma belcheri*) (Zhang et al. 2008) and zebrafish (He et al. 2009). However, information does exist for *hsd17b1* and *hsd17b3* in fish, which are genes of the same family involved in sex steroid biosynthesis. *Hsd17b1* is responsible to convert inactive estrone to active estradiol and leads to female sex differentiation while *hsd17b3* is required for 11-ketotestosterone synthesis (Tokarz et al. 2015). *Hsd17b1* has been identified in some fish species such as Nile tilapia (Zhou et al. 2005) Atlantic cod (*Gadus morhua*) (Breton et al. 2014) and olive flounder (Fan et al. 2014) while *hsd17b3* in zebrafish and medaka only (Mindnich et al. 2004; Kim et al. 2014). *Hsd17b1* was already detected at early stages of development in pre-differentiated fathead minnow (*Pimephales promelas*) embryos, although its expression was not correlated to any sex in particular (Wood et al. 2015). Recently, *hsd17b1* has been suggested as the sex determining gene in the California yellowtail (*Seriola dorsalis*), which seems to have a ZW sex determination system (Purcell et al. 2018). In our data, we did not find any differential expression in *hsd17b1* and *hsd17b3* genes but in *hsd17b10*. To our knowledge, this is the first time that the *hsd17b10* steroidogenic enzyme gene is described in European sea bass and it is detected early in the ovaries and so, it should be considered as a novel early ovarian marker in this fish species.

In males, the first signs of sex-biased expression of canonical reproduction-related genes were detected at 250 dpf onwards and not earlier indicating that their expression starts somewhere between 110 to 250 dpf. For example, we found genes involved in the steroidogenic pathway such as *hsd3β* or *cyp11b* (Tokarz et al. 2015) and in androgenic

action such as *ar*α and *fshr*, both previously reported in European sea bass (Blázquez et al. 2005; Mazon et al. 2014). When looking for DEG ($P < 0.001$) in testes compared with ovaries at 110 dpf, a total of 15 genes were found, although none were considered as canonical reproduction-related genes. Among them, we identified three genes that were previously described to be involved in reproduction: platelet-derived growth factor beta polypeptide (*pdgfb*), sorting nexin 1 (*snx1*) and nuclear transcription factor Y, beta (*nfy*), although few data has been documented on the role of these genes in testis formation, not only in fish, but also in mammals. *Pdgfb* has roles in the regulation of many biological processes including embryonic development and reproduction phenotype as alteration of this gene generated male and female infertility in several species, including humans (Donnem et al. 2010). *Snx* is a gene involved in cellular endocytosis functions and its role in oogenesis was described in the gibel carp (Wen et al. 2003) but never in spermatogenesis. *Nfy* is a pleiotropic transcription factor that can bind together with the orphan nuclear receptor steroidogenic factor-1 (*sf1*) to the promoter of *fshb* gene in mouse cells showing its implication in the gonadotropic pathway (Jacobs et al. 2003).

Next, we looked for canonical KEGG pathways involved in sex differentiation and previously described in some but few fish species. This was the case of two pathways required for ovarian development, which are the fanconi anemia pathway identified in zebrafish (Rodríguez-Marí et al. 2011) and in common carp (*Cyprinus carpio*) (Jia et al. 2018) and the wnt signaling pathway, identified in zebrafish (Sreenivasan et al. 2014) and in rainbow trout (Nicol et al. 2011). In the European sea bass, we found that the number of genes differentially expressed ascribed to these two pathways increased at 250 dpf and onwards in the developing ovaries. In testes, we studied the apoptotic pathway p53 previously described in zebrafish (Rodríguez-Marí et al. 2010), medaka (Yasuda et al. 2012) and spotted knifejaw testes (*Oplegnathus punctatus*) (Du et al. 2017), and the cytokine-cytokine interaction pathway identified in Japanese flounder (Zhang et al. 2015) and in zebrafish (Ribas et al. 2017) gonads. In the European sea bass, we found an increase in the number of genes differentially expressed at 250 dpf that decreased later. Thus, these results confirmed that as occurs in other fish species, these four pathways are also involved in gonad development in the European sea bass.

Transcription factors during gonadal development

Transcription factors tightly control gene expression in a large number of processes including gonadal development (Migeon et al. 2000) and so, in the last years, many studies have revealed their importance in fish sex differentiation (Herpin et al. 2011; Nakamura et al. 2011; Shen et al. 2014; Tanaka 2016). Consequently, with the aim of deciphering the involvement of transcription factors in gonadal development in the European sea bass, we studied the expression of transcription factors already known to be sexually dimorphic. These included *foxl2* (Yamaguchi et al. 2007) and *figα* (Kanamori et al. 2008) related to ovarian development and *sox9b* (Bagheri-Fam et al. 2010), *sf1a* (Crespo et al. 2013) and *dmrt1* (Deloffre et al. 2009) related to testis development, whose expression in European sea bass was in accordance to what is described in the literature. *Sox3* was considered as a male determining gene in ricefish (*Oryzias dancena*) (Takehana et al. 2014) although its expression was related to both oocyte and testis development in other fish species. This is the case found in grouper (*Epinephelus coioides*) (Yao et al. 2007) and in Japanese flounder (Jeng et al. 2018) with an expression bias towards female development. In European sea bass, *sox3* clearly showed a female pattern.

Then, we explored the presence of transcription factors at each specific stage of gonadal development and we saw an increase in the number of differentially expressed transcription factors that was in accordance with the time of the developmental stage, thus at 350 dpf, when gonads were fully differentiated, the largest number of differentially expressed transcription factors were detected (i.e., 23% of the total DEGs). In all stages, there was a larger number of differentially expressed transcription factors in females than in males that is in concordance with the largest number of DEGs found in females in this fish species. This skewed number towards females was evident at the earliest stage of gonadal development analyzed (i.e., 110 dpf) as differentially expressed transcription factors were only detected in ovaries, probably due to sex differentiation starting earlier in females (Piferrer et al. 2005) and to the increased activity of the tissue by ovary formation and meiotic division actions (D'Cotta et al. 2001).

Conclusions

A species-specific reproduction-enriched microarray was used to study gene expression during European sea bass gonadal development. In contrast to what had been described

in other species, a larger number of DEG and DE transcription factors were observed in ovaries when compared to testis. The expression profiles of 49 genes previously associated to sex differentiation in other species were examined in the European sea bass, and two early female markers, *cyp19a1a1* and *17hsdb10*, and three novel genes for male development, *pdgfb*, *snx1* and *nfy*, were identified. Further, three and eight pathways that are consistently expressed along gonadal development in ovary or testis, respectively, were also characterized. Taken together, these results contribute to our understanding of gene expression during sexual development in non-mammalian vertebrates and emphasize the great diversity, also at the molecular level, of fish sexual development.

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Conflict of Interest

The authors declare that they have no competing interests.

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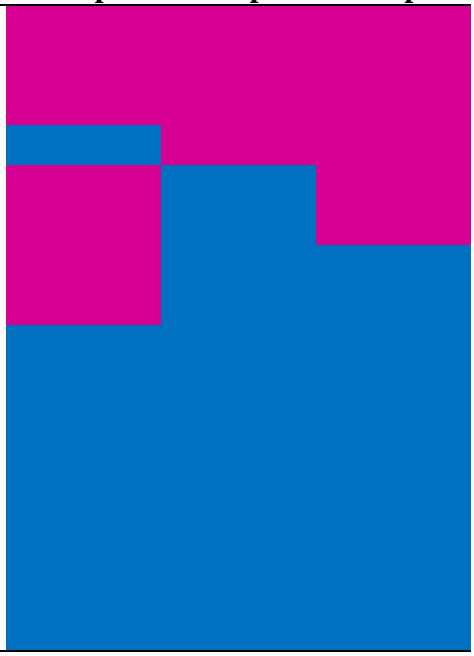
Table 1. Sixteen common differentially regulated pathways found in the microarrays along gonadal development ($P < 0.05$). Pink color means the pathways that are upregulated in females whereas blue are the upregulated pathways in males

Pathway Description	110 dpf	250 dpf	350 dpf
Lysine degradation			
Bladder cancer			
NOD-like receptor signaling pathway			
Dorso-ventral axis formation			
p53 signaling pathway			
Chagas disease (American trypanosomiasis)			
Phosphatidylinositol signaling system			
ErbB signaling pathway			
Butirosin and neomycin biosynthesis			
Basal transcription factors			
Amino sugar and nucleotide sugar metabolism			
Type II diabetes mellitus			
Glycine, serine and threonine metabolism			
Steroid biosynthesis			
Ribosome			
Folate biosynthesis			

930 **Figure Legends**

931

932 **Figure 1.** Transcriptome overview. a) Principal component analysis of microarray
933 results at three developmental stages: 110, 250 and 350 days post fertilization (dpf).
934 Samples of the different stages cluster together by gender: females (pink cluster) and
935 males (blue cluster). b) Number of differentially expressed genes found along gonadal
936 development (110, 250 and 350 dpf) in the seven comparisons analyzed by SAM test.

937

938 **Figure 2**. Common Biological Processes Gene Ontology terms at 110, 250 and 350
939 days post fertilization (dpf) of differentially expressed genes. a) Female-related genes b)
940 Male-related genes ($P < 0.05$).

941

942 **Figure 3.** Heatmap of the microarray expression data for 49 out of 55 reproduction-
943 related canonical genes: a) 25 up- and b) 24 downregulated genes in females. Each row
944 represents a gene and each column represents a group of fish by age and sex (M110 = 8
945 males at 110 days post fertilization (dpf, M250= 6 males at 250 dpf and M350 = 9
946 males at 350 dpf; F110=4 females at 110 dpf, F250= 6 females at 250 dpf and F350 = 9
947 females at 350 dpf). The key color represents the level of expression scaled by gene
948 (yellow: high expression and blue: low expression). The dendrograms inform of the
949 similarity between genes and between the different samples. Notice that all genes were
950 grouped as pro-female and pro-male as expected from studies in other species but one
951 cluster of 6 genes behaved as opposite. See Dataset 1for a complete list of gene names
952 and abbreviations.

953

954 **Figure 4.** Differentially expressed canonical genes related to sex differentiation and
955 reproduction between female and male gonads during development (* $P < 0.05$, ** $P <$
956 0.01, *** $P < 0.001$). a-f) Canonical genes of the steroidogenic pathway. g-l) Canonical
957 transcription factors.

958

959 **Figure 5.** Number of differentially expressed genes (left side) and the fold change (FC)
960 of reproduction-related pathways (right side): a) Fanconi anemia signaling pathway, b)
961 Wnt signaling pathway, c) p53 signaling pathway, d) Cytokine-cytokine interaction
962 receptor signaling pathway. The FC of upregulated genes in females are shown in pink,
963 while the FC of upregulated genes in males is indicated in blue.

Figure 6. Circle diagram that indicates the percentage of transcription factors (TFs) differentially expressed at each developmental stage of a) female-related genes, in pink or b) male-related genes, in blue.

List of Supplementary Tables

Supplementary Table S1. Total number of probes printed in the seabass microarray

no. copies	no. transcripts	no. probes
1	21	21
2	20,029	40,058
3	38	114
4	853	3,412
5	24	120
6	13	78
Total	20,978	43,803

Supplementary Table S2. List of primers used in qPCR validation of microarray results

Primers	**Sequences**	
	Forward	**Reverse**
amh	TGCAGAGCAAAGCCTGAAAG	TCAACGGGGAACAAAGACAA
cpeb1	TCCTCCCAAAGGTAATGTGG	GTTGTCCTCGGGGTGAAAC
cyp19a1a	AGACAGCAGCCCAGGAGTTG	TGCAGTGAAGTTGATGTCCAGTT
dnmt3	ACTGTCTGAACATCCTCATCGG	ATTCCTGCACACGAATGCTC
egf	CGGAGGACTTGTTTGCCTAC	TGCACATTGTACGACCAAGC
flna	GTGCTCCAGGTCTGTGTCCT	TTGGGGTCAACGATCTCTTC
hexaβ	TGAGGGTTACCCAAATGAGG	GCCTTCAGAGATGCTTGTCC
lpl	TTCCTCGACCCTCTGAAAGA	GAGTCAGCTTTGCCAGGAAC
lrap1	GAGGACGAAATCGGAAGACA	GTACTCGATCTGCGTGACCA
nr3c1	CTTCCATCCAGCCCGTTGAT	GTAGTGGAGGTCTGCGTCTG
rap1gds1	AAGGACCATCTCACGCATGT	ATGGCAACAAGACGAGGAAC
hsp70	TCACTAAGCTGTACCAGAGTGC	AATCGACCTCCTCAATGGTTGG

Supplementary Table 3. List of 55 reproduction-related genes showing probes repeatability in the microarray hybridization. ID = identification; SD = standard error; ns= no significant. * = genes with probes not showing the same tendency as the other copies. In parenthesis is shown the number of probes with the same tendency)

Gene Name	ID number	no. of probes	mean fold change			SD fold change			*P* value		
			110 dpf	250 dpf	350 dpf	110 dpf	250 dpf	350 dpf	110 dpf	250 dpf	350 dpf
amh	AM232704.1	3	-1.42	-6.47	-7.07	0.03	0.02	0.00	ns	<0.001	ns
ar	AY647256.1	3	-0.42	-1.88	-2.21	0.07	0.20	0.20	ns	<0.001	<0.001
birc5b(1/2)*	DLPD01385	2	-0.02	1.89	1.24	0.13	1.13	0.34	ns	<0.001	<0.001
bmp15	AM933668.1	5	-0.58	5.44	3.56	0.04	0.19	0.10	ns	ns	<0.001
ctnnbl1	ASSEMBLY_REP_C900	2	-0.21	-1.53	-1.11	0.03	0.33	0.31	ns	<0.001	ns
cyp11b1	AF449173.2	5	-1.72	-6.54	-6.53	0.06	0.08	0.08	ns	<0.001	<0.001
cyp19a1a	AJ311177.1	5	1.52	1.29	0.27	0.07	0.10	0.08	ns	<0.05	ns
cyp19b1a	DLPD02150	2	-0.05	-2.41	-1.91	0.21	0.38	0.20	ns	<0.001	ns
cyp26a1	ASSEMBLY_C3871	2	-1.62	3.81	2.51	0.03	0.03	0.02	ns	<0.001	<0.001
dkk3	DLPD09098	4	0.30	-0.77	-0.38	0.39	0.12	0.04	ns	<0.001	<0.001
dmrt1	Laia_2_dmrt1c	3	-0.07	-0.49	-0.95	0.14	0.03	0.31	ns	<0.01	ns
dmrt1a	AM993096.1	5	0.01	0.00	0.06	0.06	0.03	0.03	ns	ns	ns
dmrt2	ASSEMBLY_REP_C1936	2	-0.46	5.86	3.68	0.06	0.03	0.10	ns	ns	ns
dmrt3	SeabassLaia1	3	-0.32	2.62	1.45	0.09	0.19	0.27	ns	<0.001	<0.001
er2a	51100568_1_127_868	3	-0.90	-2.01	-2.31	0.08	0.30	0.41	ns	<0.001	ns
erb1	AJ489523.1	5	-0.32	-3.65	-3.67	0.06	0.18	0.19	ns	<0.001	ns
erb2	DLPD16764	2	1.32	0.18	0.58	0.05	0.04	0.02	<0.001	ns	ns
fancl	DLPD11307	2	-0.07	2.73	2.06	0.03	0.01	0.01	ns	ns	<0.001
figa	FN597057.1	3	-0.26	3.66	2.11	0.03	0.06	0.05	ns	<0.05	<0.01
foxl2	FJ705451	6	-0.26	2.37	0.58	0.05	0.05	0.05	ns	<0.001	ns
fshr	AY642113.1	5	-0.62	-3.29	-4.50	0.15	0.49	0.69	ns	<0.001	<0.001
gsdf1	JQ755271	6	-1.38	-3.27	-5.12	0.07	0.14	0.18	<0.05	<0.001	<0.001
gsdf2	JQ755272	6	-1.38	-3.01	-4.83	0.09	0.21	0.17	ns	<0.001	ns
hsd11b	AF449173.2	5	-1.72	-6.54	-6.53	0.06	0.08	0.08	ns	ns	ns
hsd17b10	ASSEMBLY_REP_C8882	2	1.08	1.88	1.33	0.04	0.04	0.04	<0.01	<0.01	<0.001
hsd3b	JQ861952	6	-1.09	-3.54	-2.92	0.03	0.04	0.04	ns	<0.001	<0.001
ift52	DLPD07363	2	-0.01	-1.24	-1.07	0.14	0.06	0.01	ns	<0.001	ns
igf2(1/2)*	DLPD06675	2	-0.22	-1.26	-0.71	0.37	1.29	1.46	ns	<0.001	<0.001
lhr(4/5)*	AY642114.1	5	-0.92	-0.50	-0.37	0.14	0.22	0.21	ns	<0.001	ns
nfkb1	DLPD04051	2	0.06	1.12	1.05	0.03	0.01	0.01	ns	<0.001	<0.001
nfkb2	ASSEMBLY_C22270	2	-0.39	0.55	0.80	0.06	0.01	0.01	ns	ns	<0.001
nfkbie	DLPD06735	2	0.44	-0.62	-0.28	0.02	0.01	0.04	ns	ns	<0.001
nr0b1 (dax1)	AJ633646.1	5	-1.02	-1.68	-2.70	0.15	0.12	0.10	ns	<0.001	ns
nr3c1 (gr)	DLPD10813	2	-0.25	-1.88	-1.32	0.08	1.26	1.14	ns	<0.001	ns
nr5a1a (sf-1)	JQ755268	6	-0.53	-0.54	-0.83	0.06	0.50	0.61	ns	<0.001	<0.01
nr5a1b (sf-1)	JQ755269	6	-0.01	-0.34	-0.54	0.04	0.05	0.05	ns	<0.001	ns
nr5a2 (cyp7)	JQ755267	6	-0.07	-1.51	-1.09	0.03	0.09	0.09	ns	<0.001	ns

pcna	DLPD00927	2	0.07	1.33	0.60	0.11	0.01	0.02	ns	<0.001	ns
piwil1	ASSEMBLY_C13668	2	0.00	1.00	0.54	0.09	0.00	0.01	ns	ns	ns
ptgs1	cDN26P0001I11.F.ab1	2	-1.81	-3.16	-2.72	0.01	0.19	0.19	ns	<0.001	ns
ptgs2b	ASSEMBLY_C21078	2	0.39	0.43	0.44	0.01	0.02	0.00	ns	<0.001	<0.001
rara	DLPD00121	2	-0.76	-3.00	-2.72	0.03	0.11	0.18	ns	<0.001	<0.001
rspo2	DLPD12564	2	-0.01	0.06	0.11	0.05	0.11	0.05	ns	ns	ns
rxra	cDN29P0003D01.F.ab1	2	-0.77	-2.09	-1.73	0.19	0.87	0.80	ns	<0.001	ns
sox17	AY247002.1	3	-0.21	0.29	0.61	0.03	0.04	0.04	ns	ns	<0.001
sox9a	AY247000.1	3	-0.62	-0.87	-0.21	0.06	0.17	0.10	ns	<0.001	ns
sox9b	AY247001.1	3	-0.41	-1.33	-1.32	0.12	0.16	0.14	ns	<0.01	<0.001
star	EF409994.1	3	-0.05	-0.40	-0.54	0.03	0.10	0.20	ns	ns	<0.05
sycp3	JQ824128	3	-0.28	1.19	-1.69	0.14	0.05	0.04	ns	ns	<0.001
tp53	ASSEMBLY_C16254	2	-0.37	0.46	0.66	0.02	0.02	0.02	ns	ns	<0.001
tp53inp2	SeabassC-CL2256Contig1	2	-0.99	-4.37	-3.28	0.01	0.03	0.08	ns	<0.001	<0.001
vasa	ASSEMBLY_REP_C21904	2	-0.66	2.42	-0.55	0.08	0.02	0.04	ns	ns	ns
tradd	DLPD08007	2	-0.74	-2.80	-2.44	0.00	0.03	0.03	ns	<0.001	ns
vtgr	FR717659.1	5	1.00	2.31	1.33	0.06	0.29	0.28	ns	ns	<0.001
wt1	ASSEMBLY_C16347	2	-0.87	-2.81	-2.89	0.05	0.03	0.04	ns	<0.001	<0.001

Supplementary Table 4. Number of genes differentially upregulated (UP) or downregulated (DOWN) in females *vs*. males. The number of differentially expressed genes in the European sea bass microarray at ($P < 0{,}001$) is shown by fold change (FC) ranges.

Fold change (FC)	110 dpf		250 dpf		350 dpf	
	UP	DOWN	UP	DOWN	UP	DOWN
FC ≤ 0,5	5	1	29	37	189	25
FC ≥ 1	79	12	370	320	1,125	55
FC ≥ 1,2	38	1	206	177	387	11
FC ≥ 1,5	52	1	285	296	422	26
FC ≥ 2	80	-	313	402	374	16
FC ≥ 4	136	-	253	620	153	16
FC ≥ 7,5	29	-	49	50	-	5

Datasets

Dataset1. List of differentially expressed genes (DEG) for the seven comparisons of the microarray experiment using the SAM test with a statistical threshold at $P < 0.001$.

Dataset2. Gene Ontology enrichment analysis results for DEG at 110, 250 and 350 dpf, as well as the common GO terms at three stages ($P < 0.05$).

Dataset3. Pathways obtained by Gene Set Variation Analysis (GSVA) with a statistical threshold at $P < 0.05$. Lists of differentially regulated pathways (DPRs) at three developmental stages between male and female gonads

Supplementary Figures

Supplementary Figure S1: Data showing low fold change variance between printed probes copies of the same gene that presented upregulated (a, c, e, g), downregulated (b, d, f, h) or variable (i) expressions. j) Mean of the standard deviations for all probes ranged showing low standard error among probe copies of the same gene.

Supplementary Figure S2: Microarray validation by RT-qPCR. a) Gene expression correlation for 12 genes measured by both techniques (R^2= 0.7479, P = 0.0003). b) Microarray validation using aromatase gene expression of each hybridized sample with standard error mean.

Supplementary Figure S3: Cellular components of the Gene Ontology (GO) terms enrichment analysis in common at the three time points of differentially expressed genes related to sex differentiation. a) Upregulated GO terms female-related genes b) Upregulated GO terms male-related genes.

Supplementary Figure S4: Molecular function of the Gene Ontology (GO) terms enrichment analysis in common at the three time points of differentially expressed genes related to sex differentiation. a) Upregulated GO terms female-related genes b) Upregulated GO terms male-related genes.