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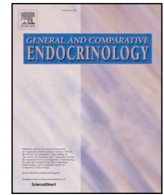

Transcription pattern of reproduction relevant genes along the brain-pituitary-gonad axis of female, male and intersex thicklip grey mullets, *Chelon labrosus*, from a polluted harbor

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ABSTRACT

The reproductive cycle of teleost fishes is regulated by the brain-pituitary-gonad (BPG) axis. The transcription profile of genes involved in the reproduction signalling in the BPG-axis differs in females and males during the gametogenic cycle. Impacts of endocrine disrupting chemicals on these signalling pathways in fish are known, but the participation of the BPG-axis in the development of the intersex condition is not well understood. Intersex thicklip grey mullets (*Chelon labrosus*) have been identified in several estuaries from the SE Bay of Biscay, revealing the presence of feminizing contaminants in the area. In previous studies, transcription patterns of genes related with steroidogenesis and gamete growth have been shown to differ among female, male and intersex mullets. However, many components of the reproduction control have not been studied yet. The aim of this study was to assess the transcription levels of target BPG-axis genes in female, male and intersex mullets captured in the polluted harbour of Pasaia, during their gametogenic cycle. After histologically examining the gonads, the transcription levels of previously sequenced target genes were measured by qPCR: *kiss2*, *gpr54* and *gnrh1* in brain, *fshβ* and *lhβ* in pituitary and *fshr* and *lhr* in gonads. In both females and males, brain genes were most transcribed in early gametogenesis, proving their relevance in the onset of both oogenesis and spermatogenesis. Pituitary gonadotropins in females showed upregulation as oogenesis progressed, reaching the highest transcription levels at vitellogenic stage, while in males transcript levels were constant during spermatogenesis. Transcription levels of gonadotropin receptors showed different patterns in ovaries and testes, suggesting differing function in relation to gametogenesis and maturation. Intersex mullets showed transcription levels of brain target genes similar to those observed in females at cortical alveoli stage and to those in mid spermatogenic males. In intersex testes the transcription pattern of gonadotropin receptor *fshr* was downregulated in comparison to non-intersex testes.

1. Introduction

Several studies have provided understanding of the brain-pituitary-gonad (BPG) axis components and their functions in relation to reproduction control in teleost fish (Levavi-Sivan et al., 2010). Environmental cues relevant for the control of gametogenesis and reproduction (temperature, circadian rhythms, food availability, social context…) are integrated in the hypothalamus, where the activation of kisspeptin neurons leads to the stimulation of downstream processes along the axis, for the first time at the onset of puberty (Roa et al., 2008; Oakley et al., 2009; Tena-Sempere, 2010). In vertebrates, two kisspeptin paralog genes, *kiss1* and *kiss2*, exists, together with the two paralogs coding for their receptors, *gpr54-1* and *gpr54-2* (Pinilla et al., 2012). Kisspeptins play a crucial role as activators of the release of gonadotropin releasing hormones, GnRHs, decapeptides synthesized in the GnRH neurons in the hypothalamus (Pinilla et al., 2012). Three forms of GnRHs are present in most fish coded by *gnrh1*, *gnrh2* and *gnrh3*. Activation of GnRHs lead to synthesis and release of the two gonadotropin hormones in the pituitary. While both gonadotropins share a common α-subunit (*gthα*), each one contains a specific β-subunit, *lhβ* in the case of the luteinizing hormone, LH, and *fshβ* in the case of the follicular stimulating hormone, FSH (Pierce and Parsons, 1981). Endogenous physiological stimuli as, for instance, metabolic state and gametogenic stage participate in the control of this event. Circulatory

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system transports gonadotropins to gonads, where they bind to their specific gonadotropin receptors; FSHR located in granulosa and theca cells in the ovaries and in Sertoli cells in the testis, and LHR located in granulosa cells in the ovaries and Leydig cells in the testis, to activate steroidogenesis. The produced sex steroids can cause a positive or negative feedback, depending on factors such as gametogenic stage, in the BPG axis (Roa et al., 2008; Levavi-Sivan et al., 2010; Zohar et al., 2010).

Kisspeptins play a crucial role in the activation of gametogenesis, reproduction and onset of puberty in several fish species belonging to distinct orders. For example, in grey mullet (*Mugil cephalus*) (Nocillado et al., 2007), fathead minnow (*Pimephales promelas*) (Filby et al., 2008), Senegalese sole (*Solea senegalensis*) (Mechaly et al., 2012), European sea bass (*Dicentrarchus labrax*) (Alvarado et al., 2013) or chub mackerel (*Scomber japonicus*) (Ohga et al., 2015), among others. Nevertheless, as reviewed by Tena-Sempere et al. (2012), slight differences in the effects of the kisspeptin system occur depending on the species. In addition, species-specific variations in transcription pattern of the different paralog kisspeptin genes exist (Tena-Sempere et al., 2012; Alvarado et al., 2013).

Regarding gonadotropins in teleosts, the first and most studied transcription pattern is that of salmonids, where at early gametogenic stages *fshβ* transcription is higher than *lhβ*, whereas the opposite occurs at the end of the gametogenic cycle (Gomez et al., 1999). FSH is thus involved in the onset and early stages of gametogenesis and germ cell proliferation while LH participates in the final gamete maturation in both sexes. Nonetheless, several studies evidenced different transcription patterns for non-salmonid species (Mateos et al., 2003; Weltzien et al., 2004; Li et al., 2005). Such differences in gonadotropin expression patterns among species are mainly due to the distinct strategies in gonad development (Swanson et al., 2003). In the case of male sea bass (*Dicentrarchus labrax*) for instance, a group-synchronous species, the mRNA levels of the three gonadotropin subunits change in parallel (Mateos et al., 2003) during spermatogenesis with no peaks of *fshβ* transcription preceding those of *lhβ*. Regarding gonadotropin receptors, their transcription profile usually coincides with the transcription profile of the gonadotropins, being in salmonids FSHR involved in gonadal growth and LHR in the final stages of gamete maturation. Furthermore, several patterns have been observed in other fish species. It is the case for example of zebrafish (*Danio rerio*), where *fshr* increases in female gonad as the oocytes enter vitellogenesis and decrease when the oocytes are fully grown (Kwok et al., 2005). In female and male sea bass, *fshr* is thought to be involved in early stages of gonadal development as well as in final gamete maturation; *lhr* transcription follows a similar pattern, but with higher transcription at final stages of maturation, related to its role at that stage (Rocha et al., 2009).

The different molecular processes explained above along the BPG axis, together with the processes occurring during steroidogenesis and gametogenesis, are potential targets to endocrine disrupting chemicals (EDCs) (Villeneuve et al., 2007; Hachfi et al., 2012). EDCs form a group of natural and designed chemical compounds of variable nature and structure that can mimic or antagonize endogenous sex steroids, and therefore trigger transcription pattern alterations. Such alterations that can vary depending on the studied species, sex and developmental stage can involve important genes in the BPG axis. Some observations include, the decrease in the levels of GnRH and GnRH receptor (GnRHR) after PCB exposure in Atlantic croaker, *Micropogonias unulatus* (Khan and Thomas, 2001); induction of *lhβ* transcription and LH plasma levels after exposure to 4-nonylphenol (NP) in the African catfish *Clarias gariepinus*, the Atlantic salmon *Salmo salar* or the masu salmon *Oncorhynchus masou* (Van Baal et al., 2000; Yadetie and Male, 2002; Maeng et al., 2005), to pesticides such as DDT in Atlantic croaker (Khan and Thomas, 1998), to 17α-ethynylestradiol (EE2) in Japanese medaka *Oryzias latipes* and coho salmon *Oncorhynchus kisutch* (Zhang et al., 2008; Harding et al., 2016) or to organophosphate flame retardants in zebrafish *Danio rerio* (Liu et al., 2013). On the contrary, negative effects on *fshβ* transcription levels have been observed after exposure to xenoestrogens such as NP in rainbow trout *Oncorhynchus mykiss* (Harris et al., 2001) or bisphenol-A in juvenile zebrafish (Chen et al., 2017).

Male thicklip grey mullets (*Chelon labrosus*) in estuaries and port areas from the Basque Coast (SE Bay of Biscay), show intersex condition, with testis-ova, (Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). Presence of EDCs, most commonly alkylphenols, phthalate ester plasticizers and some pesticides was evident in those estuaries as measured by different chemical analytical techniques in sediments, water and fish bile (Bizarro et al., 2014; Ros et al., 2015). Transcription pattern of several genes related with sex differentiation, steroidogenesis and gametogenesis were characterized in liver, brain and gonads of female, male and intersex mullets from the Pasaia harbor (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Sardi et al., 2015; Rojo-Bartolomé et al., 2017; Valencia et al., 2017). Male and intersex mullets showed detectable vitellogenin transcription levels in the liver, confirming exposure to feminizing EDCs (Bizarro et al., 2014). Intersex mullets showed also different transcription levels of both brain and gonad aromatase compared with non-intersex males (Bizarro et al., 2014; Sardi et al., 2015). In addition, gonadal 5S/18S rRNA index, normally higher in ovaries than in testis in teleosts (Rojo-Bartolomé et al., 2017), was higher, and more similar to females, in intersex individuals than in males. This was notorious in intersex individuals showing high severity index and thus high prevalence of oocytes in the testis (Rojo-Bartolomé et al., 2017). Furthermore, intersex males showed transcription levels for the liver phase II steroid conjugation enzyme gene *ugt* similar to those of females (Sardi et al., 2015).

However, transcription levels of genes directly involved in the control of gamete maturation and reproduction in the BPG axis have not been studied yet in such mullets. Therefore, the aim of this study was to characterize the transcription pattern of BPG related genes during the different stages of gametogenesis in female and male *Chelon labrosus* captured in the harbor of Pasaia. After applying a target gene sequencing approach transcriptional levels of *kiss2* (kisspeptin 2), *gpr54* (kisspeptin-1 receptor) and *gnrh1* (gonadotropin releasing hormone 1) were quantified in the brain, *fshβ* (follicle stimulating hormone β) *and lhβ* (luteinizing hormone β) in the pituitary, and *fshr* (follicle stimulating hormone receptor) and *lhr* (luteinizing hormone receptor) in the gonads. In doing so, and upon analysis of the pattern of transcription of such genes also in tissues of intersex individuals, we aimed at the definition of molecular markers that could be employed in the analysis of intersex condition and in pollution biomonitoring programs using thicklip grey mullets as sentinel organisms.

2. Material and methods

2.1. Study area, fish sampling and tissue collection

The inner part of the Pasaia harbor, located in the SE Bay of Biscay (43°19′18″ N; 1°55′53″ W), was selected as sampling area. Adult thicklip grey mullets (*Chelon labrosus*) with a size of 38.3 cm ± 9 and a weight of 760 g ± 243.4 were captured by fishing-rod. Fish included in these analyses were captured between September 2010 and September 2011, and in October 2016 and January 2017. Fish were anaesthetized upon capture in a saturated ethyl-4-aminobenzoate water bath. All fish handling and procedures were approved by the UPV/EHU Ethics Committee on Animal Experimentation and by the regional authorities. Dissections were immediately performed *in situ*. Brain and/or pituitary and a portion of the gonad of each fish were collected in RNAlater solution (Ambion; Life Technologies, Carlsbad, USA) and then frozen in liquid nitrogen until arrival at the laboratory, where they were immediately stored at −80 °C until processing. Another portion of the gonad of each fish was fixed in 10% neutral buffered formalin for histological analysis.

2.2. Histological analysis of the gonad

Gonad samples were fixed for 24 h in 10% neutral buffered formalin, then dehydrated in a graded ethanol series (70%, 90%, and 96%), and embedded in paraffin. Sections of 5 µm thickness were cut in a Leica RM 2125 RT manual microtome (Leica Microsystems, Nussloch, Germany) and then stained with hematoxylin/eosin (Gamble and Wilson, 2002) using the Leica Autostainer XL and mounted with the aid of the Leica CV 5030 workstation. Three sections per individual were microscopically analyzed with an Olympus BX61 light microscope (Tokyo, Japan). Sex and gametogenic stage of each individual were determined using the criteria by McDonough et al. (2005). The Intersex Severity Index described by Jobling et al. (2006) was used to classify intersex males.

2.3. Gene transcription analysis

Individuals belonging to each sex and gametogenic stage histologically described in mullets, females at previtellogenic, cortical alveoli, vitellogenic and regressing stage and males at early, mid and late spermatogenesis and regressing stage were selected for the transcription analysis of target genes in brain, pituitary and gonads. The same analyses were performed in all the intersex individuals identified during the study. Brain and gonad samples were obtained from fish captured in 2016 and 2017. Pituitary samples were from fish captured in 2016 and 2017.

2.3.1. Extraction of total RNA

Total RNA was extracted from the pituitary, brain and gonad samples using TRI Reagent Solution (Ambion; Life Technologies) following the manufacturer's instructions. A piece of approximately 100 mg of gonad was used, while in the case of the pituitary and the brain, the whole tissues were taken. The tissues were homogenized in 1 mL TRI Reagent Solution using zirconia/silica beads (Biospec, Bartlesville, USA) in a Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France). RNA concentration and quality were measured by spectrophotometry in a biophotometer (Eppendorf, Hamburg, Germany) and only samples with A260/A280 absorbance ratios between 1.8 and 2.2 were considered for further analysis.

2.3.2. cDNA synthesis and quantification

For each sample, first strand cDNA synthesis was performed from 2 µg of RNA using the Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) with random primers, following manufacturer's protocol and using a 2720 Applied Biosystems Thermal Cycler (Life Technologies). Quant-iT OliGreen Kit (Life Technologies) was used to quantify ssDNA concentration in the pituitary, brain and gonad cDNA samples, following manufacturer's instructions. 96 well plates (Corning Incorporated, Corning, New York, USA) were used and fluorescence was measured in a Synergy HT Multi-Made Microplate Reader (Biotek, Winoosky, USA).

2.3.3. Sequencing of selected genes

With the exception of *fshr*, whose sequence was obtained from a Miseq Illumina RNASeq analysis of mullet gonads (unpublished data), partial target gene cDNA sequences were obtained from PCR amplification of total RNA from *C. labrosus* using degenerate primers. Such primers were designed for each target gene using well-conserved domains revealed after ClustalW analyses of sequences available for other teleosts in the NCBI database (Table 1). Sequences of interest were amplified through PCR using cDNA pooled from mullet target organs. Applied PCR conditions were as follows: an initial step of 94 °C for 2 min, 35 cycles of denaturalizing step (94 °C for 30 s), annealing step (30 s at Tm, see Table 1), an elongation step (70 °C for 30 s) and a final step of 72 °C for 8 min. The size of the amplicons was checked by electrophoresis in agarose gels (1.5%) stained with ethidium bromide. The obtained gene fragments were sequenced in the Sequencing and Genotyping Unit of the University of the Basque Country. Sequences were annotated upon Blastx analysis and submitted to the NCBI database for publication (Table 1).

2.3.4. Real-time qPCR

Specific primers were designed using the partial sequence obtained for each gene (Table 2). Primers were designed using IDT and Eurofins online tools. Real time qPCR analyses were performed in a 7300 Real-Time PCR system thermocycler (Life Technologies). *kiss2*, *gpr54* and *gnrh1* were analyzed in the brain, *lhβ* and *fshβ* in the pituitary and *lhr* and *fshr* in the gonads. Each sample was analyzed in triplicates in a total volume of 20 µL containing 7.88 µL of water, 10 µL of SYBR Green fluorescent dye master mix (Roche Diagnostics, Indianapolis, USA) and 0.12 µL 12.5 pmol primer pair. A control without template was run (also in triplicate) in each plate using the same reaction conditions. The qPCR conditions were as follows: an initial step at 50 °C for 2 min and 95 °C for 10 min, 40 cycles of a denaturing step at 95 °C for 15 s and annealing step at Tm (Table 2) for 1 min, finally a dissociation stage of 95 °C for 15 s, 60 °C for 1 min and again 95 °C for 15 s. The reaction efficiency of each plate was calculated using a standard curve consisting in serial dilutions of pooled cDNA. The specificity of the reaction was determined confirming the presence of a single peak in the dissociation curve, and the absence of primer dimmers. The cDNA concentration of each sample was calculated by fluorescent quantification method and used for normalization of the results obtained by qPCR of the target genes in brain, pituitary and gonad, as performed by Rojo-Bartolomé et al. (2016). The amount of cDNA loaded in qPCR reactions was adjusted to the dilution used for each gene. Then, transcription levels of each gene were normalized using an adapted ΔCt formula (*RQ*) with efficiency (*E*) correction as follows:

$$E = [10^{-1/m}] - 1$$

$$RQ = \mathrm{Log}_2[(1 + E)^{-\Delta Ct}/\mathrm{ng\,cDNA}]$$

Being *m* the slope of the standard curve of the qPCR reaction and ΔCt the difference between the mean Ct value per sample and the Ct value of the plate internal control.

2.4. Statistical analysis

Statistical analyses were performed with the aid of the SPSS.22 statistical package (SPSS Inc., Microsoft Co., Redmond, USA). Normality was assessed with Shapiro-Wilk test. Non-parametric Kruskal-Wallis test followed by Dunn's post hoc test was used for multiple comparisons and Mann-Whitney test for pairwise comparisons. Significant differences were established at $p < 0.05$.

3. Results

3.1. Histological analysis of the gonads

The histological analysis of the gonads of the mullets collected monthly from September 2010 to September 2011 and from October 2016 to January 2017 was done in order to determine the sex and gametogenic stage of each mullet. Mullets were classified as immature, if the gonad could not be identified as ovary or as testis. Regarding females, four different gametogenic stages were observed: previtellogenic, cortical alveoli, vitellogenic and regressing. Males were classified as early, mid or late spermatogenic and regressing. A total of 24 intersex males were identified in the 2010/2011 samplings; whose brains were selected for the gene transcription analysis in the brain. Only 13 gonads were selected for further transcription analysis in the gonads. Two intersex fish were identified in the 2016/2017 samplings, but unfortunately no pituitaries were collected. As a consequence, no pituitaries from intersex fish were available for further transcription

Table 1
Degenerate primers used for the cloning of each target sequence of *Chelon labrosus*, with the temperature used for the PCR and the size of the obtained fragment. GeneBank accession number and the deduced aminoacid identity when compared with the most similar ortholog sequences available in GenBank is provided (last revision made was made in October 2018).

Gene	Tm (°C)	Forward (5′–3′) Reverse (5′–3′)	Fragment size (bp)	Accession number	Amino acid identity (blastx)
kiss2	59	TGGTGACTCTGGTTGTGGTGT GDTGGGCACCTCCAGTTCT	243	KT248850	85% *Acanthopagrus schlegelii* (ALQ81854.1); 1e-41
gpr54	60	GTCAGCATCCCTTCCTCACAGA ACGGCGTTATTATTGTTGCCTTCCT	904	KT248849	99% *Mugil cephalus* (ABG76790.1); 0.0
gnrh1	61	TACAAAAACCTTGGCACTGTGGCT TGTTCGGTGTCCATTCTCTCTGT	171	KT248847	93% *Mugil cephalus* (AAQ83269.1); 2e-32
fshβ	58	GCTGGTTGTCATGGCAGCA GCAGTBBNTGGCCACDGG	439	KX758589	64% *Morone saxatilis* (2117355B); 2e-28
lhβ	58	TACCARCAYGTGTGCACNTAC GCAGAAGTYRGGCTGCAGGCTYT	154	MH251322	86% *Maylandia zebra* (XP_024656642.1); 2e-14
fshr			696	MH251323	82% Larimichthys crocea (KKF21850.1); 7e-75
lhr	60	GCAGAAGGACATTYAAYAACCTYC TCCCTGTGKGTGTTCCAGS	325	KX171008	73% *Epinephelus akaara* (AIW52568.1); 9e-42

Table 2
List of target genes with their specific forward and reverse primers, used melting temperature and fragment size.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Fragment size (bp)	Tm (°C)	Accession number
kiss2	ATTGGATTCCGCACAAAGGACA	CTCAGGGAGAAGCACAGGT	111	58	KT248850
gpr54	ACCGCTGTTATGTGACAGTCTA	TACATCAAAATCGGGGTGGACA	124	58	KT248849
gnrh1	GAGGGAAGAGGGAACTGGA	TGGCGAAAGGCGTGTCCT	114	58	KT248847
fshβ	CGCCAACACCAGCATCAC	CACCAATGAAGGAGAAATCCCT	104	59	KX758589
lhβ	TTCCTGGCTGTCTGCGG	TTCAAAGGTGCAGTCGGACG	103	59	MH251322
fshr	CCTTGCTCATCTTCACCGAC	CAGGACCAGGAGGACTTTAG	116	60	MH251323
lhr	GTTAATCCGACTGGGAACAACA	GCAGGCCCATAAGCAGAGTT	112	58	KX171008

level analyses. Intersex males were at different spermatogenic stages, ranging from immature or early spermatogenic stage to late spermatogenesis. All intersex individuals showed oocytes at previtellogenic stage with a very low intersex severity index that was in all cases classified as 1 in a scale of 7 (Jobling et al., 2006).

3.2. Sequencing of selected genes

Partial target gene sequences were obtained using degenerate primers and upon BlastX analysis and homology search identified as thicklip grey mullet orthologs for *kiss2*, *gpr54*, *gnrh1*, *lhβ*, *fshβ* and *lhr* (Table 1). The sequence for *fshr* was obtained after Illumina Miseq analyses of mullet gonads (data not shown). Obtained sequences were submitted and published in the NCBI database: *kiss2* (243 bp, GenBank accession number KT248850), *gpr54* (904 bp, GenBank accession number KT248849), *gnrh1* (171 bp, GenBank accession number KT248847), *lhβ* (154 bp, GenBank accession number MH251322), *fshβ* (439 bp, GenBank accession number KX758589) and *lhr* (325 bp, GenBank accession number KX171008) and *fshr* (696 bp, GenBank accession number MH251323). All partial sequences displayed high level of sequence identity with ortholog teleost sequences (Table 1). The fragments cloned for *C. labrosus* genes represent the following percentages in respect to the total coding sequence of each gene: 49% for *kiss2*, 31% for *gpr54*, 23% for *lhβ*, 63% for *fshβ*, 8% for *lhr* and 15% for *fshr*. After BlastX analysis *gpr54*, *fshβ* and *fshr* putative conserved domains were identified in the partial sequences obtained. In the case of *gpr54* and *fshr* they contained domains belonging to the transmembrane G protein coupled receptor superfamily while *fshβ* contained domains related to the glycoprotein hormone beta chain homologue superfamily.

3.3. Transcription levels of kiss2, gpr54 and gnrh1 in the brain

Females showed the same transcription pattern for *kiss2*, *gpr54* and *gnrh1* in the brain. The transcription of these genes increased from previtellogenesis to cortical alveoli stage, decreasing again at vitellogenesis and remaining low at regressing stage (Fig. 1). Males showed uniform and low transcription levels of *kiss2* during spermatogenesis with an increase, even if not statistically significant, at regressing stage (Fig. 1). For *gpr54* and *gnrh1*, transcript levels increased from early to mid spermatogenesis, followed by a decrease at late spermatogenesis to peak up again at regressing stage (Fig. 1). Thus, transcription levels of *kiss2* remained low in both female and male brain samples, except in cortical alveoli females. In the case of *grp54* and *gnrh1*, previtellogenic and vitellogenic females showed similar transcription levels to males at early and late spermatogenesis, while females at cortical alveoli stage and males at mid spermatogenesis and regressing stage showed higher transcription levels (Fig. 1). Intersex males showed low transcript levels for *kiss2* (Fig. 1). In addition, *gpr54* and *gnrh1* transcript levels were similar to mid spermatogenic and regressing males and to females at cortical alveoli stage (Fig. 1).

3.4. Transcription levels of fshβ and lhβ in the pituitary

Females showed the same transcription pattern for both *lhβ* and *fshβ*, being the transcription level of vitellogenic females higher than that of previtellogenic ones (Fig. 2). However, males did not show differences between mid and late spermatogenesis for both transcripts, although at late spermatogenesis *fshβ* transcription levels slightly increased, but not significantly (Fig. 2). Transcription levels of both genes were in the same range for females and males, with the only difference being the slightly higher transcription of *lhβ* in vitellogenic females compared with late spermatogenesis males. Pituitary samples were missed for the analyzed intersex individuals.

3.5. Transcription levels of fshr and lhr in the gonads

Transcription levels of *lhr* in ovaries did no change throughout oogenesis (Fig. 3). Nevertheless, ovaries at cortical alveoli stage showed a slight up-regulation in comparison to the other oogenic stages (Fig. 3). Testis at early spermatogenesis showed slightly but not significant higher transcription levels for *lhr* than at mid and late spermatogenesis. Then, transcription increased at regressing stage (Fig. 3). Intersex testis showed similar transcription levels for *lhr* to corresponding non-intersex testis stages (Fig. 3). Regarding transcription levels of *fshr*, immature mullets showed high transcription levels (Fig. 3). Females

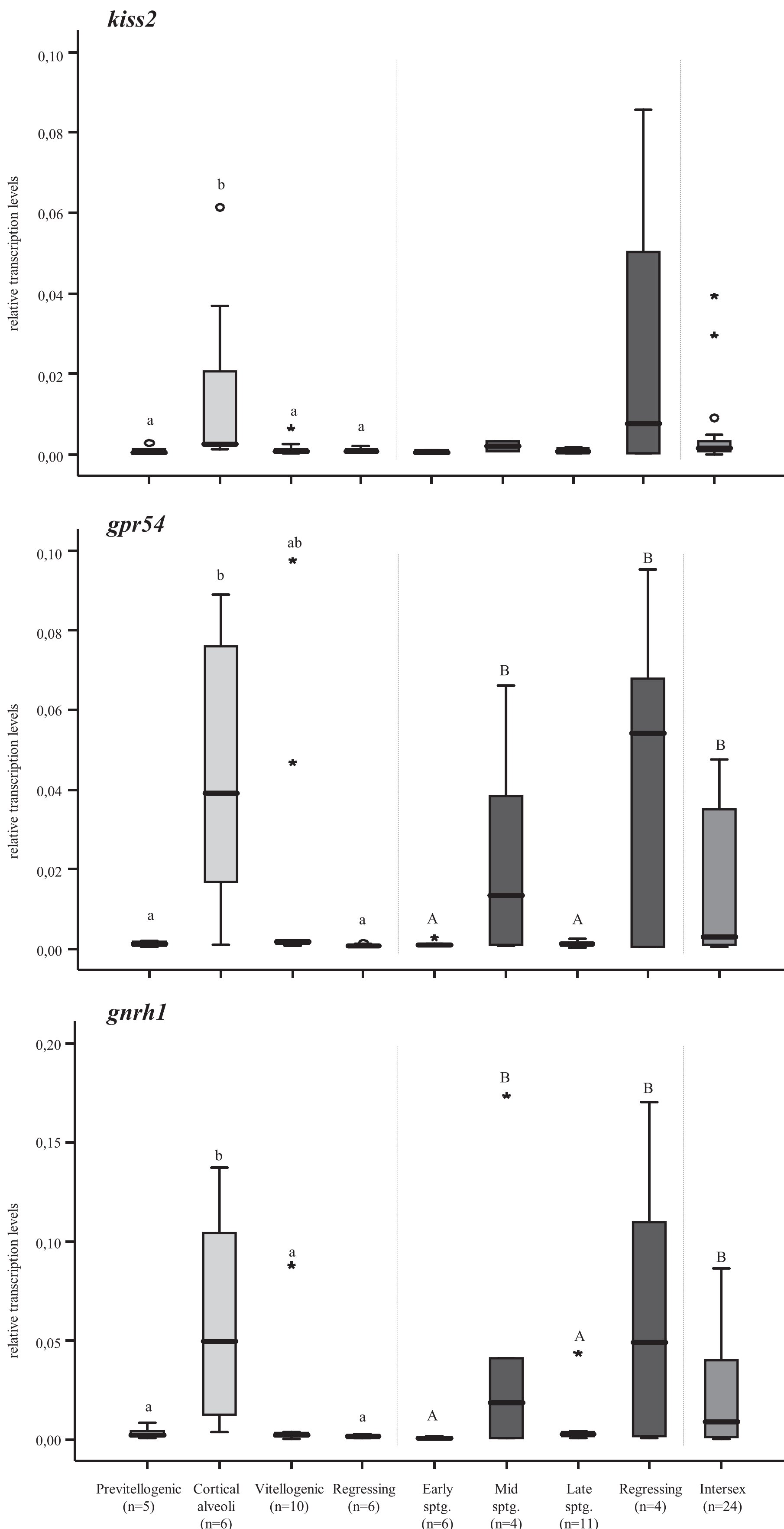

Fig. 1. Gene transcription levels of *kiss2*, *gpr54* and *gnrh1* in brain of females (grey) in different gametogenic stages (previtellogenic, cortical alveoli stage, vitellogenic, regressing), males (black) in different gametogenic stages (early spermatogenesis, mid spermatogenesis, late spermatogenesis and regressing) and intersex (dark grey) thicklip grey mullets. Box-plots show median value (line), 25–75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical differences between means ($p \leq 0.05$) for females; upper case letters denote statistical differences between means ($p \leq 0.05$) for males and intersex.

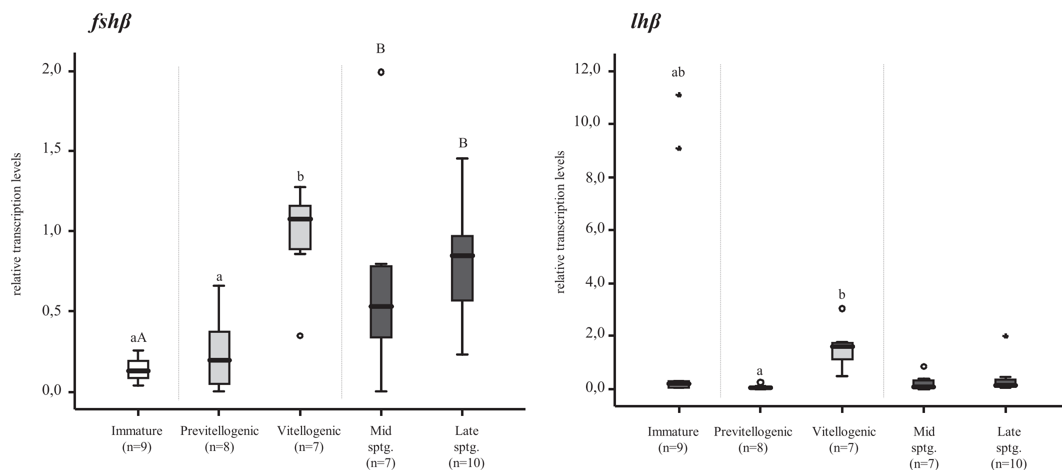

Fig. 2. Gene transcription levels of *lhβ* and *fshβ* in pituitaries of, immatures (white), females (grey) in different gametogenic stages (previtellogenic and vitellogenic), males (black) in different gametogenic stages (mid spermatogenesis and late spermatogenesis) thicklip grey mullets. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical differences between means (p ≤ 0.05) for females; upper case letters denote statistical differences between means (p ≤ 0.05) for males and intersex.

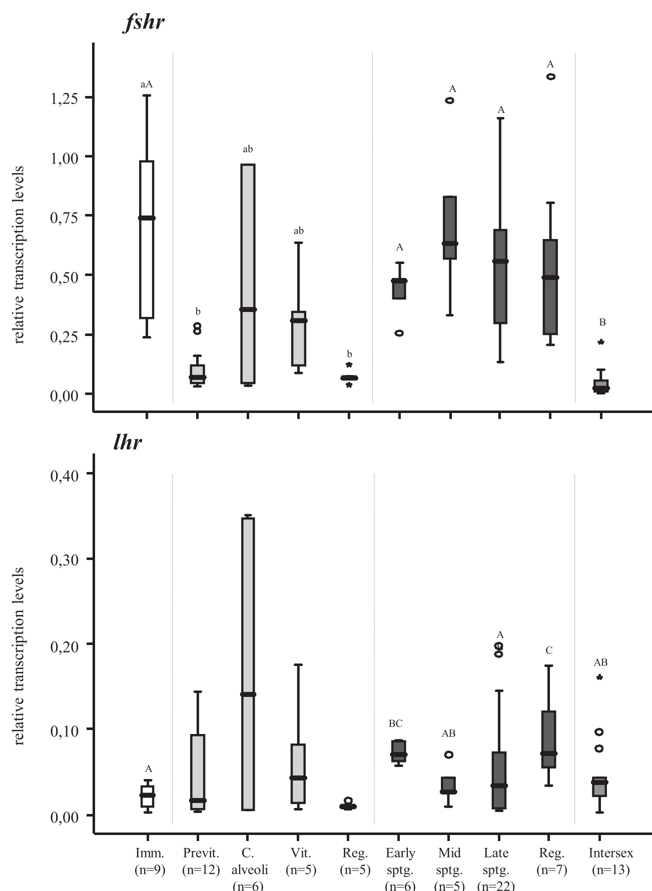

Fig. 3. Gene transcription levels of *lhr and fshr* in gonads of, immatures (white), females (grey) in different gametogenic stages (previtellogenic, cortical alveoli, vitellogenic and regressing), males (black) in different gametogenic stages (early sptg.: early spermatogenesis; mid sptg.: mid spermatogenesis; late sptg.: late spermatogenesis and regressing) and intersex (grey) thicklip grey mullets. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical differences between means (p ≤ 0.05) for immatures and females; upper case letters denote statistical differences between means (p ≤ 0.05) for immatures, males and intersex.

showed up-regulation in *fshr* transcription levels from previtellogenesis to cortical alveoli and vitellogenesis stages and decreased again at regressing stage (Fig. 3). In general, ovaries showed lower transcription levels than testis for *fshr*. In males, *fshr* transcription levels remained constant through the different stages of spermatogenesis (Fig. 3). However, intersex males showed lower transcription levels than the other males, being similar to those of ovaries at previtellogenesis (Fig. 3).

4. Discussion

In this study, we present the transcription pattern of genes involved in the control of gametogenesis at different levels of the brain-pituitary-gonad axis in female, male and intersex thicklip grey mullets from the polluted harbor of Pasaia, by focusing on *kiss2*, *gpr54*, *gnrh1* in the brain, *lhβ* and *fshβ* in the pituitary and *lhr* and *fshr* in the gonads.

4.1. Transcription pattern of kisspeptin-system genes in the brain

The kisspeptin system, formed by the kisspeptins kiss1 and kiss2, and their receptors gpr54-1 or kiss1r and gpr54-2 or kiss2r, are thought to be involved in the onset of puberty in teleost (Roa et al., 2008; Oakley et al., 2009; Tena-Sempere, 2010) and in the upstream control of the gonadotropins, via gonadotropin releasing hormone receptor (GnRH) (Pinilla et al., 2012). Conserved sensitivity of kisspeptin neurons to sex steroids among vertebrates has been suggested (Oakley et al., 2009; Kanda and Oka, 2013). Nevertheless, species-differences regarding the transcription pattern of the kisspeptin-system genes have been described (Tena-Sempere et al., 2012; Alvarado et al., 2013; Mechaly et al., 2013).

In the case of female thicklip grey mullets, *kiss2, gpr54* and *gnrh1* showed an identical transcription pattern along oogenesis, with the highest levels at the cortical alveoli stage. Other fish species also showed such a concerted transcription pattern of brain genes. In female *Mugil cephalus* association between *GnRH1* and *gpr54* was reported (Nocillado et al., 2007). In sea bass, *Dicentrarchus labrax*, *kiss2*, *gpr54-1b* and *gpr54-2b* presented the same transcription profile during oogenesis (Alvarado et al., 2013), and in female chub mackerel, *Scomber japonicus*, *kiss1*, *kiss1r* and *gnrh1* showed parallel transcription profile (Ohga et al., 2015). In addition, in the chub mackerel, these transcripts peaked at cortical alveoli stage and remained low at other oogenic stages (Selvaraj et al., 2010), similar to that observed for mullets in the present work. This transcription pattern points towards a meaningful role of *kiss2*, *gpr54* and *gnrh1* in the steps preceding brain control of

vitellogenesis and secondary oocyte growth. Wang et al. (2013) suggested the important role of the estrogen feedback in the hypothalamo-gonadotropic axis in goldfish (*Carassius auratus*), after reporting a direct action of estrogens on *kiss1* and *kiss2* transcription levels. The up-regulation of kisspeptin system mediated by estrogen exposure was described in medaka (*Oryzias latipes*) (Kanda et al., 2008), in juvenile zebrafish (Servili et al., 2011) and in goldfish (Kanda et al., 2012). Sardi et al. (2015) described in the same mullet population studied hereby, increased estradiol levels in the plasma of female mullets at cortical alveoli stage, which corresponds with the highest levels for analyzed genes in the brain. This would suggest that estrogens in female mullets also regulate the kisspeptin system.

In male mullets, *gpr54* and *gnrh1* showed parallel transcription profile during spermatogenesis, with high transcription levels at mid spermatogenesis and regressing stages. In cobia (*Rachycentron canadum*) a positive correlation between *gnrh* and *gpr54* was described, with both transcripts peaking at the same time (Mohamed et al., 2007). Studies performed with male sea bass showed no correlation between *kiss* genes and *gnrh1* (Migaud et al., 2012; Alvarado et al., 2013), which is in agreement with the differences seen in male mullets between *kiss2* and *grnh1*. In sea bass, testosterone caused a negative feedback on *kiss2*, castration resulting in downregulation of the gene (Alvarado et al., 2016). In male mullets, the increase in plasma testosterone levels (Sardi et al., 2015) matches the increase at mid spermatogenesis observed for *gpr54* and *gnrh1*. Suggesting that testosterone plasma levels could influence negatively *kiss2* transcription levels in the brain of mullets, as shown for sea bass. The high transcription level of *kiss2, gpr54* and *gnrh1* at regressing stage in males indicates a role in the retrieving of the testes after regression. Data from other teleost do not match the pattern described in mullet testis, although species-specific differences must be considered (Selvaraj et al., 2010; Alvarado et al., 2013; Saha et al., 2016; Shahi et al., 2017).

Intersex mullets showed the same transcription levels for *kiss2*, *gpr54* and *gnrh1* in brain as mid spermatogenic males and cortical alveoli stage females. Nevertheless, intersex males showed testes at different spermatogenic stages, ranging from immature or early spermatogenic stage to late spermatogenesis, and all of them presented oocytes at previtellogenic stage. Intersex males showed slightly higher transcription levels than females at previtellogenesis for *gpr54* and *gnrh1*, which could be a response mediated by exposure to estrogenic compounds. In that regard, although an induction of kisspeptin genes after E2 exposure was described in some fish species (Kanda et al., 2008; Servili et al., 2011; Kanda et al., 2012), there are no studies showing effects on the kisspeptin system after exposure to xenoestrogenic compounds. Regarding *gnrh1*, in a study performed with Japanese medaka exposed to 17α-ethynylestradiol (EE2), Zhang et al. (2008) reported dose and sex dependent differences. Induction of GnRH mRNA levels after EE2 exposure was observed in both sexes.

4.2. Transcription pattern of gonadotropins in the pituitary

In salmonids (Levavi-Sivan et al., 2010) FSH plasma levels are generally high at early stages of gametogenesis, due to its importance in the control of germ cell proliferation and vitellogenesis. On the contrary, LH plasma levels increase at latter stages as its function is related with gamete maturation and spawning. Both gonadotropins are important for the synthesis of steroid hormones in the gonads. In turn, gonadotropins are controlled by steroids, which, depending on the reproductive stage of the fish, will have a positive or negative feedback on their secretion and release (Zohar et al., 2010). Opposite to salmonids, LHβ and FSHβ mRNA levels are co-regulated in other teleost such as goldfish (*Carassius auratus*), striped bass (*Morone saxatilis*), red seabream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceus*) and European sea bass (reviewed by Weltzien et al., 2004; Levavi-Sivan et al., 2010). This pattern seems to be related with oocytes at different developmental stages co-existing in the ovary (Mittelholzer et al., 2009; Levavi-Sivan et al., 2010). Similarly, female mullets showed up-regulated mRNA levels of both *fshβ* and *lhβ* from early to late vitellogenesis in the pituitary. The up-regulation of both gonadotropins occurs after the up-regulation of the kisspeptin system at cortical alveoli stage, showing an interaction between the brain and the pituitary. This would suggest a gonadotropin activation via the kisspeptin system in mullets, as described in several other teleost.

Male mullets showed low *lhβ* transcription levels during early and late spermatogenesis. Hellqvist et al., (2006), described a similar pattern for the three – spined stickleback (*Gasterosteus aculeatus*). However, sticklebacks simultaneously showed low *fshβ* levels, which was not the case for male mullets. They showed higher, although not statistically significant, transcription levels of *fshβ* at late spermatogenesis compared to mid spermatogenesis. This trend has been reported also for male trout (*Oncorhynchus mykiss*) (Prat et al., 1996) and European sea bass (Mateos et al., 2003; Alvarado et al., 2013). Although in the latter, both gonadotropins showed parallel transcription pattern (Mateos et al., 2003; Alvarado et al., 2013). In male mullets, transcription levels were higher for *fshβ* than for *lhβ* at early spermatogenesis, which agrees with the role of FSH as an early spermatogenesis stimulator (Schulz et al., 2010).

Comparing female and male mullets, they showed a very similar pattern for *fshβ* transcription, with up-regulation as gametogenesis advanced. Regarding *lhβ*, vitellogenic females showed higher transcription levels than males at late spermatogenesis. In other species such as the Senegalese sole (*Solea senegalensis*), sex dependent gonadotropin transcription pattern has been observed (Mechaly et al., 2012). Nevertheless, the effect of exposure to xenoestrogenic compounds in the transcription pattern of the gonadotropin subunits cannot be discarded. In females, the lower transcription of *fshβ* observed at previtellogenesis could be a result of exposure to xenoestrogens. The suppressed transcription of *fshβ* has been reported in female fish exposed to E2 or BPA, for instance in Japanese eel (Jeng et al., 2007), sea bass (Mateos et al., 2002; Alvarado et al., 2016) and zebrafish (Chen et al., 2017). On the contrary, when female zebrafish were exposed to a xenoestrogen mix containing E2, EE2, BPA and alkylphenols among others, upregulation of the *fshβ* transcription levels was observed (Urbatzka et al., 2012). In male mullets, the uniform transcription of *lhβ* during the two analyzed spermatogenic stages could suggest that the low transcription observed at late spermatogenesis is an effect of the exposure to endocrine disrupting chemicals. Despite the great number of studies that report an induction of *lhβ* after exposure to estrogenic compounds, some works have described the down-regulation of *lhβ*. It is the case of male zebrafish exposed to organophosphate flame retardants (Liu et al., 2013) and Japanese medaka and rare minnow (*Gobiocypris rarus*) exposed to EE2 (Zhang et al., 2008, Qin et al., 2014). Thus, whether the low transcription levels of *lhβ* in late spermatogenic males and the low *fshβ* levels in previtellogenic females are due to a species-specific transcription differences or due to the influence of estrogenic chemicals present in the environment remains unclear. The absence of pituitaries of intersex fish complicates the interpretation of these results.

4.3. Transcription pattern of gonadotropin receptors in the gonads

Gonadotropin receptors are involved in the regulation of steroidogenesis as well as in the control of gametogenesis in response to gonadotropins (Levavi-Sivan et al., 2010). The gonads of immature mullets showed higher *fhsr* transcription levels than those of *lhr*. The high *fshr* transcription levels of immature mullets coincide with the low transcription observed for *fshβ* in the pituitary. This could indicate that the activation of the receptor occurs prior to the release of the hormone, suggesting a possible preparation of the gonad for the forthcoming FSH signaling. Different transcription pattern could be noted for *fshr* and *lhr* in mullet ovaries along oogenesis. Whereas *fshr* showed the highest transcription at cortical alveoli and vitellogenic stages, *lhr* did not show

any significant differences along oogenesis. In addition, *fshr* transcript levels were higher than those of *lhr* at all oogenesis stages. In female sea bass, unlike in mullet, both receptors showed identical transcription pattern in ovaries, nevertheless, *fshr* transcription levels were also higher than *lhr* (Rocha et al., 2009). Female sea bass showed a clear *lhr* transcription peak at late vitellogenesis, which occurred a bit earlier for *fshr*. That pattern was not observed in mullet ovaries, although cortical alveoli stage and vitellogenic females showed slightly higher *fshr* transcription levels than previtellogenic ones. The high transcription levels of *fshr* at vitellogenesis is in accordance with the high transcription recorded for the *fshβ* subunit in the pituitary of mullets at the same stage. Nevertheless, the low fluctuations of the transcription levels observed along oogenesis suggest that the transcription of the gonadotropin receptors could be under the effects of external factors such as exposure to endocrine disruptors. For instance, female zebrafish exposed to E2 and BPA showed downregulation of *lhr* and *fshr* (Chen et al., 2017). In addition, female rare minnows exposed to EE2 showed a time dependent up or downregulation of *lhr* with no significant changes in *fshr* transcription (Qin et al., 2014). These results suggest that both increase and decrease of gonadotropin receptor transcription levels can occur as a result of exposure to EDCs, making it difficult to elucidate whether the observed pattern for female mullets is a consequence of exposure to xenoestrogenic pollutants.

Gonadotropin receptors also showed a different transcription pattern during spermatogenesis in comparison to oogenesis. Although, as in ovaries, *fshr* levels were higher than those of *lhr* in testes, its transcription pattern remained constant along spermatogenesis. *lhr* transcript levels instead fluctuated. The transcription pattern observed for male sea bass *lhr* and *fshr* (Rocha et al., 2009) is similar to that observed for *lhr* in male mullets. In some fish species, both *fshr* and *lhr* levels increase at the final stage of spermatogenesis (reviewed in Levavi-Sivan et al., 2010). In male mullets, that pattern was not observed, additionally, the increase in *lhr* transcription was observed at regressing stage. The low transcription levels of *lhβ* in male mullet pituitaries together with the delayed upregulation of *lhr* in the gonad may suggest the upregulation of the receptor at regressing stage could have occurred independent to *lhβ*, due to the disruption of the gonadotropin system in mullets. In zebrafish males exposed to organophosphate flame-retardants downregulation of *lhr* was observed (Liu et al., 2013). Downregulation was also detected in male rare minnows exposed to EE2 (Qin et al., 2014). These results could explain the low *lhr* transcription levels observed in male mullets. As to *fhsr*, male rare minnows exposed to EE2 also showed decreased *fhsr* transcription levels. Although high, the constant *fhsr* transcription levels observed during spermatogenesis in mullets could be related to a xenoestrogen exposure. This can be linked to the results observed in intersex males. Intersex males showed a clear downregulation of *fshr* in comparison with males, indicating a suppression of the transcription of this gene as a consequence of the changes occurred in the gonads. In this regard, intersex males showed *fshr* transcription levels similar to those in previtellogenic females. On the contrary, *lhr* transcription levels in intersex males did not show a clear regulation when compared with males or with previtellogenic females. Unfortunately, the lack of data on gonadotropin subunit transcription levels for intersex mullets does not enable to stablish any possible correlation between the gonadotropin subunits and their receptors.

5. Conclusions

In summary, in female mullets the transcription levels of brain *kiss2*, *gpr54* and *gnrh1* peak at cortical alveoli stage, which in turn may induce from previtellogenesis to vitellogenesis the increase observed in the pituitary transcription levels of both gonadotropin subunits. Gonadotropin receptor transcription levels in ovaries increased from previtellogenesis to cortical alveoli stage, suggesting their activation previous to the production of gonadotropins in the pituitary. In male mullets transcription levels of *gpr54* and *gnrh1* in the brain increased at mid spermatogenesis, which could induce *fshβ* transcription levels observed in the pituitary from early to late spermatogenic stage. In turn, the increase in *fshβ* could be followed by the *fshr* upregulation observed in testis from early to mid spermatogenesis. The lack of a clear gametogenic transcription cycle in all analyzed genes, especially *lhβ* and *fshr* in males, could be a specific pattern of mullets or alternatively indicative of possible attenuated response mediated by external factors such as the presence of endocrine disrupting chemicals. Similar effects for steroidogenic genes in the gonads of mullets were described previously in the harbor of Pasaia (Sardi et al., 2014). The downregulation of *fshr*, as observed in intersex males, could be an effect of xenoestrogens or the consequences of testis feminisation. As such, these transcriptional alterations in *fshr* could be a potential biomarker of intersex gonads among males exposed to xenoestrogens, some of them developing oocytes and some other not. This would be in accordance with the effects observed in zebrafish exposed to EE2 (Rojo-Bartolomé et al.), where the changes observed in *gtf3ab* transcription levels were seen to be a result of oocyte differentiation rather than a result of estrogen exposure.

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Appendix A. Supplementary data

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