Transcription of ribogenesis genes in fish gonads: Applications in the identification of stages of oogenesis and in environmental monitoring of intersex condition

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ABSTRACT

One of the best described effects of environmental xenoestrogens in fish is the generation of intersex gonads in males. Considering 5S rRNA a marker of the presence of oocytes, a 5S/18S rRNA index was calculated in 296 thicklip grey mullets (*Chelon labrosus*) from polluted environments. In addition, qPCR analysis of transcription factors *gtf3a* and *ubtf1*, related respectively to 5S and 18S rRNA synthesis, was conducted along female-oogenesis. 5S/18S rRNA index identified sex with a threshold value of 0.4521 separating males from females. Histological analysis identified 38 intersex individuals. Intersex severity and 5S/18S rRNA indexes were correlated. 5S/18S rRNA index identified ovarian developmental stage with high 5S rRNA levels during early oogenesis and 18S rRNA relative values increasing towards maturation. *gtf3a* and *ubtf1* transcription levels followed the pattern of 5S rRNA accumulation. Thus, ribogenesis genes provide easy/quantitative methods to molecularly identify the sex, female gametogenic stage and intersex severity in mullets.

INTRODUCTION

Organisms inhabiting estuarine waters are normally exposed to complex contaminant cocktails with anthropic origin (Boehm and Bischel, 2011). Among other contaminants detected in the aquatic environments, endocrine disrupting chemicals (EDCs) have received special attention after they were first highlighted by the European Environmental Agency in 1997 (EEA, 1997). EDCs were then defined as “chemical pollutants able to interfere with the normal functioning of hormones” (EEA, 2012) and they can cause alterations at different biological organisation levels, from the molecular one to the individual or the (sub)population one (Brander, 2013, WHO/UNEP, 2013). They include a complex array of substances, with different chemical structures and sources (Tyler et al., 1998, López de Alda and Barceló, 2001, Porte et al., 2006, Casals-Casas and Desvergne, 2011, Khetan, 2014) which in industrialized countries mainly arrive to the aquatic environment through the municipal, industrial and hospital wastewater treatment plants effluents (Campbell et al., 2006). Such compounds in complex mixtures, can interact enhancing their potency and biological activity, and in some circumstances, acting in an additive manner (Thorpe et al., 2003).

Xenoestrogens are considered EDCs with the ability to mimic estrogens or to cause estrogen-like responses in exposed organisms (Campbell et al., 2006). They alter hormonal homeostasis interfering with normal sexual differentiation and gametogenesis, which in consequence affects the development and reproduction of exposed individuals/populations (Tyler et al., 1998, Goksøyr et al., 2003, Mills and Chichester, 2005). The effects of xenoestrogenic compounds in some aquatic

organisms are well known, one of the best described responses being the feminization of juvenile and male fish (WHO/UNEP, 2013, Tyler and Jobling, 2008, Goksøyr et al., 2003, Goksøyr, 2006, Bizarro et al., 2014). Sometimes this leads to the generation of intersex gonads, when oocytes differentiate within the normal testicular tissue in gonochoristic fish species (Matthiessen, 2003, Bahamonde et al., 2013, Bizarro et al., 2014, Ortiz-Zarragoitia et al., 2014). Intersex condition has been reported in both freshwater and marine fish related to chemical exposure in highly to moderately contaminated areas (Bahamonde et al., 2013, Ortiz-Zarragoitia et al., 2014). These intersex males display lower reproduction capacity than normal males, with logical consequences in population viability (Jobling et al., 2002b, Harris et al., 2011).

Xenoestrogenic effects have been reported also in mugilid fish populations from contaminated estuaries (Ferreira et al., 2004) and intersex gonads have been described in thicklip grey mullets (*Chelon labrosus*) used as pollution sentinel organisms in estuaries in the Southern Bay of Biscay (Diaz de Cerio et al., 2012, Puy-Azurmendi et al., 2013, Bizarro et al., 2014, Valencia et al., 2017). Oocytes have been found in testes of mullets from Bilbao, Pasaia and Ondarroa harbours, and in estuaries in Deba and in the Biosphere Reserve of Urdaibai in Gernika (Puy-Azurmendi et al., 2013, Bizarro et al., 2014, Valencia et al., 2017). These sites have been associated to waste water treatment plant effluents and/or to industrial activities in harbour areas. Intersex mullets have been described all along the reproductive cycle, with percentages ranging from 3% to 60% of analyzed males depending on the site and month of capture (Ortiz-Zarragoitia et al., 2014). Most intersex mullets present low to moderate intersex severity indexes following the score developed by Jobling et al. (2006) that ranks the severity according to the number and distribution of oocytes presented in the testis. Furthermore, elevated vitellogenin transcript and protein levels have been measured in these males, accompanied by an up-regulation of the aromatase coding gene *cyp19a1b* in brain (Bizarro et al., 2014). Thus, thicklip grey mullet is considered an important sentinel species for the biomonitoring of exposure to reproduction EDCs in the Southern Bay of Biscay (Ortiz-Zarragoitia et al., 2014).
In spite of the increasing number of studies describing the intersex condition in fish in the last decades, the physiological and molecular mechanisms governing the process remain unknown (Abdel-Moneim et al., 2015). In addition, and although it is known that the transcription levels of several genes related to sex differentiation are altered after exposure to xenoestrogens (Bahamonde et al., 2015b), no direct relationship with the intersex condition has been established yet and more research is needed in order to find specific molecular markers of this condition (Bahamonde et al., 2013, Ortiz-Zarragoitia et al., 2014, Abdel-Moneim et al., 2015).

In this respect, recently, accumulation of 5S rRNA and of transcripts coding for 5S rRNA accompanying proteins have been studied in mullets from polluted areas (Diaz de Cerio et al., 2012, Valencia et al., 2017). The transcription levels of ribogenesis genes enabled the identification of sex of each individual in a comparative manner, when at least one individual of each sex is present in the study, irrespective of their site of collection and their stage within the reproductive cycle. In addition, such genes were up-regulated in intersex testis in comparison to normal testis. Strong 5S rRNA transcription, specific of ovaries, can be easily identified by a simple electrophoresis of total RNA extracted from the gonads (Diaz de Cerio et al., 2012). The relative amount of 5S to 18S rRNA calculated after electrophoretic analysis (5S/18S rRNA index) identifies the presence of oocytes in gonads and allows distinguishing not only the sex but also the oogenic stage in females, as demonstrated in different commercial fish species of the Bay of Biscay (Rojo-Bartolomé et al., 2016). This is so because 5S rRNA levels relative to 18S rRNA are higher in previtellogenic oocytes than in mature ones (Rojo-Bartolomé et al., 2016). In this context, the

5S/18S rRNA index was applied in the present study for the unambiguous molecular identification of sex, female reproductive stage and intersex severity in mullets collected from polluted sites of the Southern Bay of Biscay during a complete annual reproductive cycle. In addition, transcription levels of the general transcription factor IIIA (*gtf3a*) and upstream binding transcription factor 1 (*ubtf1*), genes related to ribogenesis through regulation of 5S and 18S rRNA synthesis, were studied in ovaries of females at different developmental stages. Such analysis could elucidate the possibility of using transcription levels of ribogenesis genes in an environmental monitoring context to assess the impact of xenoestrogenic compounds and the incidence of intersex condition in pollution sentinel fish species.

MATERIAL AND METHODS

Study area and biological samples

From September 2010 to September 2011 twelve to thirty adult (> 20 cm length) thicklip grey mullets (*Chelon labrosus*) were monthly collected by fishing-rod in the harbour of Pasaia (43°19'35"N, 1°55'9"W), located on the Basque coast (SE Bay of Biscay). Mullets were also sampled in June 2013 and in February 2014 in the estuaries of Gernika (43°19'26"N, 2°40'26"W) and Galindo (43°18'11"N, 2°59'55"W) close the points of discharge of the waste water treatment plants of Gernika and of the Bilbao metropolitan area. The total amount of individuals collected for this study was of 296.

All animal manipulations conducted were authorised by competent regional authorities after the evaluation and approval of all protocols by the Ethics and Animal Welfare Commission of the University of the Basque Country (CEBA/152/2010). After capture, individuals were immediately anaesthetized in a saturated ethyl 4-aminobenzoate, sacrificed by decapitation and gonads were removed. A portion of each gonad was embedded in RNA later (Ambion, Life Technologies, Carlsbard, USA), frozen in liquid nitrogen and then stored in the laboratory at − 80 °C until further used.

For histological analysis, a portion was taken from the middle part of the gonad from each fish (around 1 cm in length across the whole gonad). This was then fixed in 4% neutral buffered formalin and transported at 4 °C to the laboratory for further processing.

Histological analysis of the gonads

After 24 h in the fixative, gonads were dehydrated in a graded series of ethanol (70%, 90% and 96%) and embedded in methacrylate resin according to the manufacturer's instructions (Technovit 7100; Heraeus Kulzer GmbH & Co. KG, Werheim, Germany). 6 to 9 resin sections (5 µm) were cut in a 2065 Supercut microtome (Leica Instruments GmbH, Wetzlar, Germany) and stained with hematoxylin/eosin. Sex and the developmental stage of the oocytes in ovaries were determined microscopically, following the gametogenic stage grading described by McDonough et al. (2005) for *Mugil cephalus*. The stages were as follows. Resting (R), with the presence of atretic oocytes (> 20%) in an otherwise empty ovary with scarce oogonia and lamellae presenting some muscle and connective tissue bundles while ovarian wall look thickened. Perinucleolar (Pn), with inactive ovary containing perinucleolar oocytes and a very thin ovarian wall. Cortical alveoli (Ca), with oocytes at cortical alveoli stage some of them starting vitellogenesis (< 50%). Vitellogenesis (V), with oocytes full of yolk globules and enlarged plasma membrane. Mature/spawning (M), with hydrated oocytes showing coalescence of lipid droplets and very thick oocyte envelope, and presence of some atretic oocytes (< 20%). No full mature or spawning individuals could be identified as spawning occurs in the open sea (Ortiz-Zarragoitia et al., 2014) and samplings were always carried out in estuarine areas.

In the case of identified intersex individuals, the intersex severity was established microscopically. For that, up to 9 non-consecutive 5 µm histological sections were completely examined with a 20X objective, dividing each section in several fields of view. To determine the number of oocytes in each histological section, the field of view with the maximum oocyte amount recorded was considered, following the methodology described by Blazer et al. (2007). Then, the intersex severity for each fish was established depending on the mean number of oocytes within all histological sections analyzed per individual and following the index developed by Jobling et al. (2006).

Extraction of total RNA, capillary electrophoresis and 5S/18S rRNA index

Total RNA was extracted from 50 to 100 mg of tissue using TRIzol® (Invitrogen, Life Technologies) and following the manufacturer's instructions. Obtained RNA was purified using Qiagen RNeasy kit (Qiagen, California, USA) after a DNase digestion step (RNase-Free DNase Set, Qiagen). After purification, the same amount of RNA (250–500 ng), as estimated through absorbance at 260 nm (good quality RNA established at absorbance ratios of 260/280 and 260/230 around 2), was loaded in an Agilent RNA 6000 Nano Kit Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Electropherograms provided by the Bioanalyzer were used to quantify the concentration of the bands corresponding to 5S rRNA and 18S rRNA in each sample using the "Time Corrected Area" of each peak to calculate the 5S/18S rRNA ratio (Rojo-Bartolomé et al., 2016). When the presence of one of the rRNAs was below the levels of detection of the machine a 0.1 value was given to each sample instead of 0 (the lowest recordable value was 0.2). The binary logarithm of the ratio was calculated in order to develop a 5S/18S rRNA index that allowed clear visualization of the differences between testes and ovaries.

cDNA synthesis and PCR analysis in ovaries

2 µg of total RNA from each individual gonad were used for cDNA synthesis with the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies) using random primers, according to manufacturer's instructions. 5S rRNA is a very small transcript and random primers may not adequately retrotranscribe such small nucleic acids. For that reason, and for comparative purposes, an additional retrotranscription assay was performed with RNA from ovaries with perinucleolar (Pn) and vitellogenic (V) oocytes using a reverse primer specific for mullet 5S rRNA (5'-AAGCTTACAGCACCTG-3').

Primers to obtain mullet 45S pre-rRNA sequence fragments were designed through alignment (Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/) of piscine 45S pre-rRNA sequences available in NCBI (http://www.ncbi.nlm.nih.gov/) and searching for highly conserved nucleotide regions. Properties of designed primers were checked employing the IDT OligoAnalyzer Tool (https://eu.idtdna.com/calc/analyzer). Conventional PCRs were run with 0.8 nM of forward 5'-GAGGCCCTGTAATTGGAATGAG-3', reverse1 5'-CAAAGTGCGTTCGAAGTGTCGA-3' or reverse2 5'-AGAGAAGGCGCGAGGACAC-3' primers. The amplification was run with commercial Taq DNA Polymerase, recombinant Kit and 100 mM dNTP Mix (Invitrogen) for 35 cycles in a 2720 Thermal Cycler (Applied Biosystems, Carlsbard, California, USA). PCR procedure was as follows: 94 °C for 2 min, and 35 cycles with a denaturation step at 94 °C for 30 s, annealing at 61 °C for 30 s and elongation at 72 °C for 30 s. A final step at 72 °C was added for 8 min. Obtained PCR products were sequenced in the SGIker Sequencing Service of the University of Basque Country. Once sequenced, fragments were aligned to obtain a consensus sequence and analyzed using CAP3 (http://pbil.univlyon1.fr/cap3.php) and ClustalW2 tools. Sequences obtained have been published in GenBank (KX358060, KX358061).

Specific primers were designed to amplify the fragment ranging from the 18S rRNA 3'-region into the internal transcribed spacer 1 and reaching the 5.8S rRNA 5'-region in cDNA generated from RNA extracted from Pn and V ovaries. This region suffers 45S pre-rRNA internal splicing leading to the generation of the mature 18S rRNA. Thus, the amplification of the sequence in this case is only possible when 18S rRNA and 5.8S rRNA remain together. The fragment was amplified using conventional PCR and employing 0.8 mM of forward 5'-AACCTCAGTGCGTGGCGGA-3' and reverse 5'-TCCACCGCTAAGAGTAGTCATG-3' primers. Amplification was performed as described above (annealing temperature 61 °C). Finally, PCR products were analyzed by electrophoresis in ethidium bromide stained agarose gels (1.5%).

qPCR analyses in ovaries

cDNA obtained by random and specific retrotranscription from ovaries at different developmental stages was quantified in the Synergy HT Multi-Made Microplate Reader (BioTek, Winooski, USA) by Quant-itTM OliGreen® stain (Invitrogen). The quantification was performed in a reaction volume of 100 µl with a theoretical cDNA concentration range of 0.02–0.2 ng/µl, at 485/20 nm excitation and 528/20 nm emission wavelengths. Real PCR input cDNA concentration was calculated using the high-range standard curve according to manufacturer's instructions.

5S and 18S rRNA, *gtf3a* and *ubtf1* transcript levels were quantified (only in ovaries) by SYBR Green® qPCR (Roche, Basel, Switzerland). qPCR was conducted in triplicates using a 7300 Applied Biosystems Thermocycler. The PCR reaction mixtures (20 µl) consisted of 10 µl of 2 × SYBR® Green PCR master mix, appropriate concentration of primers diluted in RNase-free water (see Table 1) and 2 µl cDNA template. qPCR procedure was as follows: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles consisting of a denaturation step at95°C for 15 s and an annealing step for 30 s (temperature for each primer set in Table 1). A dissociation step was added at the end consisting of 15 s at 95 °C, 1 min at annealing temperature and a final step at 95 °C for 15 s.

Table 1. Primer sequences used for the qPCR analysis of the transcription levels of 5S rRNA, 18S rRNA, *gtf3a* and *ubtf1* in ovaries of thicklip grey mullets.

Gene	Forward sequence (5'3') Reverse sequence (5'3')	Primer (nM)	Sample dilution	Annealing T (°C)
5S rRNA[a]	Fw-CTTACGGCCATACCACCCTG Rv-GTATTCCCAGGCGGTCTCCC	6.25	1/200 & 1/12800[b]	60
18S rRNA	Fw-GAGGCCCTGTAATTGGAATGAG Rv-TAAGATACGCTATTGGAGCTGGAA	6.25	1/12800	60
gtf3a	Fw-CCAGGAGAAGCGATATAAATGTGA Rv-TCGTGATGCTTCAGTTTTCCATG	12.5	1/400	59
ubtf1	Fw-CTCTAAAGCAAAGGTCAGTCCAGA Rv-AATATGAACATGGCTGAGATGGGC	12.5	1/400	60

[A] Protected under Spanish patent P201130778.

[B] Dilution for cDNA obtained from 5S rRNA specific retrotranscription.

All amplification results were normalized with the amount of cDNA charged in the qPCR according to Rojo-Bartolomé et al. (2016) using an adapted ΔCT formula (RQ) with efficiency correction (E):

$$\mathrm{E} = \left[10^{-1/m}\right] - 1$$

m being the slope of the standard curve of the qPCR reaction.

$$\mathrm{RQ} = \mathrm{Log}_2\left[\frac{(1+\mathrm{E})^{-\Delta \mathrm{CT}}}{\mathrm{ng\ \ cDNA}}\right]$$

where Δ CT = CT sample − CT plate internal control.

5S vs. 18S rRNA transcription level indexes were calculated using qPCR results obtained for 5S rRNA from cDNA produced using both random or 5S rRNA specific primers.

Statistical analysis

To assess whether female and male individuals could be segregated into two statistically different groups according to their 5S/18S rRNA index the R 3.3.1 (2016) software version was used. To generate the clusters a logistic regression was carried out using the balance between specificity and sensitivity. Then, a maximum Area Under the Curve (AUC) was calculated using the "pROC" package version 1.8 available for R (AUC = 0.996). Finally, the 5S/18S index cutpoint value differentiating males and females was obtained. The statistical analyses of the rest of the results were undertaken using SPSS (SPSS Inc., Chicago, Illinois). Data failed in normality and variance equality after applying the Shapiro-Wilk ($n < 30$) test and Levene's test, both at a 0.05 significance level ($p < 0.05$). Significant differences between groups were then evaluated using the non-parametric Kruskal-Wallis test. When only two groups were compared (Pn vs. V), data analysis was performed with the non-parametric Mann-Whitney test for two independent samples. In all the cases, significant differences were established at $p < 0.05$. In addition, Spearman unilateral correlation analysis was performed to compare the histological intersex severity index and the 5S/18S rRNA index. Spearman significant differences were established at $p < 0.01$.

RESULTS

5S/18S rRNA index: identification of sex

5S/18S rRNA index was used to identify the sex of thicklip grey mullet individuals studied during a whole annual cycle (Fig. 1). Of all analyzed individuals (296) histological analysis was only possible in 215 (methacrylate blocks of 81 samples were lost) which were histologically sexed (colored dots in Fig. 1), identifying 91 females, 86 males and 38 intersex individuals. Then two population clusters were distinguished based on their 5S/18S rRNA index values with a cut point value of 0.4251 (Fig. S1). When the 177 histologically sexed males and females were classified using this cluster distribution 95% of females and 100% of males were correctly identified. No male individual showed a 5S/18S rRNA index value higher than the cut point and only two males out of 86 did not show negative values (one in February and another one in June). The majority of the females (86 from 91) showed an index value higher than the cut point, with the exception of only one female in November and four in January. Sample distribution revealed that while all male

individuals displayed a narrow normal distribution with an index peak around − 3.0, two populations could be distinguished within females with peaks around 5.0 and 12.0 (Supplementary Fig. S1).

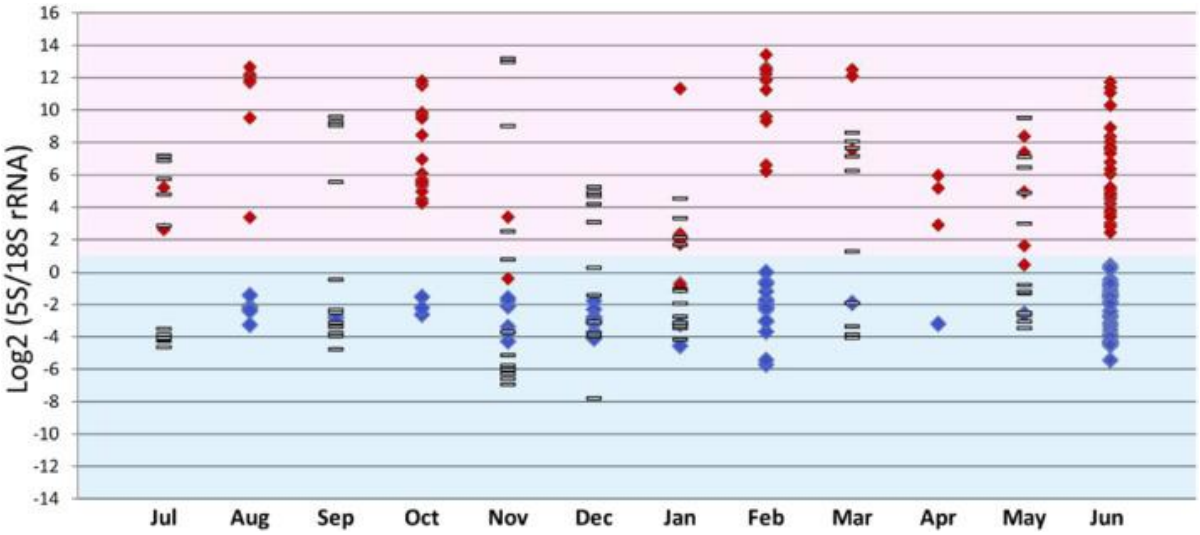

Fig. 1. 5S/18S rRNA in gonads of thicklip grey mullets collected during a complete annual cycle. Each red dot corresponds to one histologically identified female individual (*n* = 91) and each blue dot to a male (*n* = 86). Each black line corresponds to an individual whose sex was not identified histologically (*n* = 81). Box in pink groups most of female individuals, and the blue box encompasses 100% of the males. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
This sex distribution was used to classify a total of 81 individuals not sexed histologically (black lines in Fig. 1, Supplementary Fig. S2). 35 individuals displayed a 5S/18S rRNA index value above 0.4251 and thus their probability to be females was 100%. Individuals (46 out of 81) with an index value lower than 0.4251 were males with a 94.5% probability. This applies in the case that none of the 81 individuals were intersex.

5S/18S rRNA index and intersex severity

A total of 38 intersex individuals, histologically identified during our samplings in Pasaia, Galindo and Gernika, were analyzed in the present study. They all displayed low to moderate intersex severity (severity indexes 1 to 3) according to the ranking methodology used (Jobling et al., 2006, Blazer et al., 2007).
When comparing 5S/18S rRNA index from these intersex individuals and their histologically ranked intersex severity index, a positive correlation (Spearman $p < 0.01$) with a magnitude of 0.638 was observed between both indexes. As the amount of oocytes in the testes increased, the 5S/18S rRNA index increased (Fig. 2). The lowest 5S/18S rRNA index values were recorded with intersex index values of 1, where testis presented from 1 to 5 oocytes scattered within each testis section, and the highest at intersex index 3, with clusters of 21 to 50 oocytes in each testis section. When comparing intersex individuals with males and females, intersex individuals with lower severity indexes did not show significant differences in 5S/18S rRNA index values with males, while individuals with severity index 3 showed 5S/18S rRNA index values similar to females and different from males (Fig. 2, Supplementary Fig. S2).

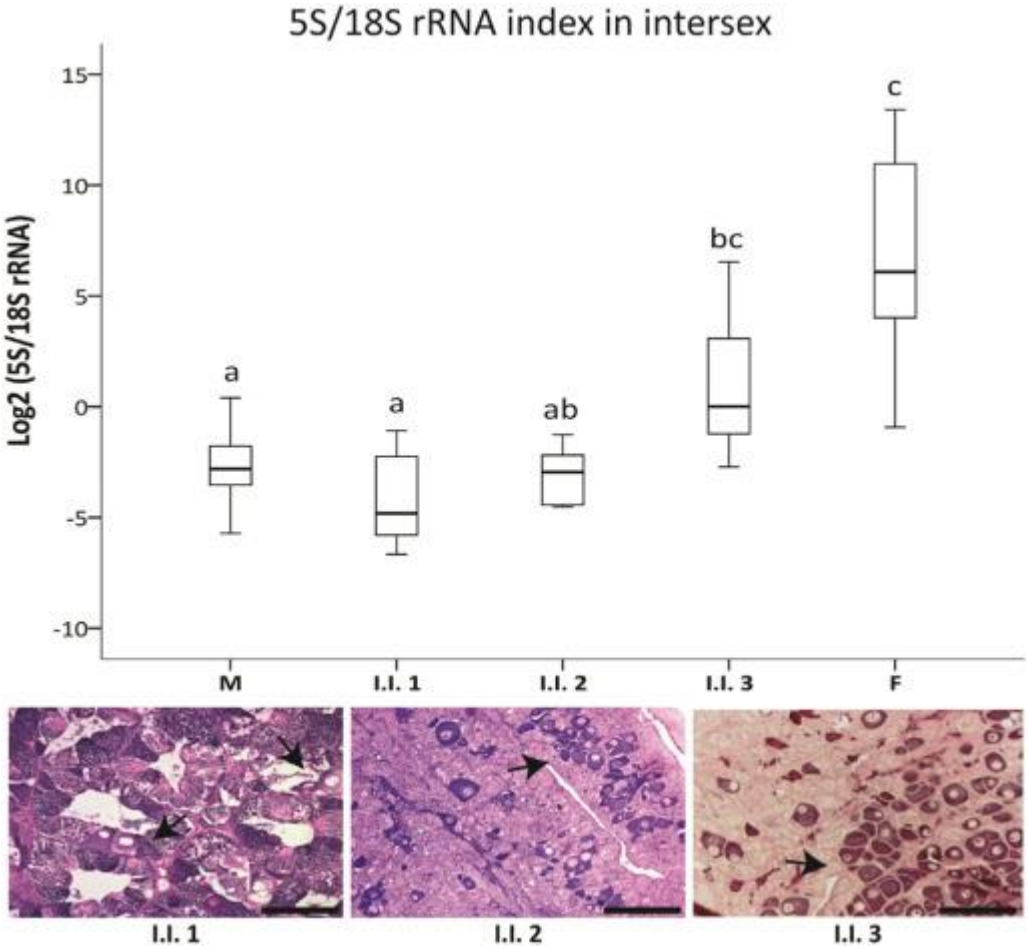

Fig. 2. Comparison between the 5S/18S rRNA index as calculated from the electropherograms and the histologically calculated intersex severity index in thicklip grey mullets. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between groups (Kruskal-Wallis, $p < 0.05$). M: male, I.I.: intersex severity index, F: female. I.I. ranked from 1 to 3 following the description proposed by Jobling et al. in 2006. The total amount of individuals in each group were; 86 in M, 22 in I.I.1, 7 in I.I.2, 9 in I.I.3 and 91 in F (total $n = 215$).

5S/18S rRNA index: identification of female reproductive stage

5S and 18S rRNA relative fluctuations allowed distinguishing ovaries at different developmental stages (Fig. 3). At early stages during oogenesis (Pn and Ca) 5S rRNA levels were very high in comparison with 18S rRNA, but as oogenesis advanced (from Pn to V), the relative amount of 5S rRNA decreased and the 18S rRNA relative amount increased in electropherograms. In consequence, the 5S/18S rRNA index that was high at the beginning of the oogenesis (Pn = 6.11 and Ca = 7.69), decreased as oogenesis advanced; the lowest index values being recorded at vitellogenesis (V = 0.90). Values increased again at regressing stage (R = 4.59).

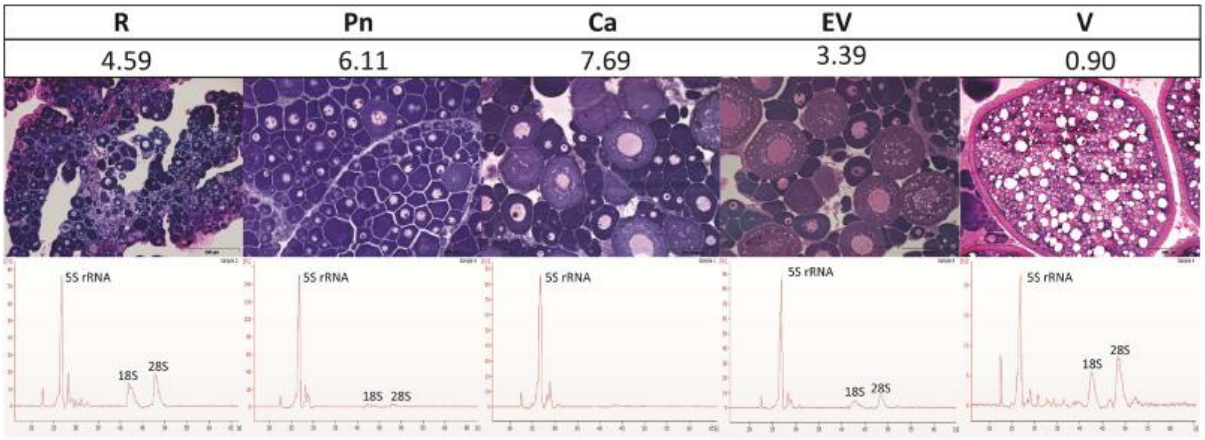

Fig. 3. Variations in the relative amount of 5S rRNA, 18S rRNA and 5S/18S index during thicklip grey mullet oogenesis. Representative micrographs of ovaries within each of the developmental stages analyzed are shown (scale bar = 200 nm): R: resting, Pn: perinucleolar, Ca: cortical alveoli, EV: early vitellogenesis and V: vitellogenesis. No individuals with mature hydrated ovaries (M) were available as mullets were never sampled at open sea. The value above each micrograph depicts the mean 5S/18S rRNA index value at each of the analyzed stages. In the total RNA electropherograms (representative of individuals in each stage) the peak at 25 s corresponds to 5S rRNA and the one at 40–45 s corresponds to 18S rRNA. The last peak corresponds to 28S rRNA. [FU]: fluorescence, [s]: time in seconds.

To prove that we were really measuring 5S and 18S rRNA levels in the electropherograms and not anything else as it could be argued by somebody, specific rRNA transcription levels were analyzed through qPCR (Fig. 4). Comparison of the 5S/18S rRNA indexes obtained from Bioanalyzer electropherograms (Fig. 4A) and after qPCR analysis (Fig. 4B) showed identical differences between groups. Anyhow, it was noticeable that 5S/18S rRNA index values calculated with qPCR analyses were always negative, this meaning that qPCR always identified higher levels of 18S rRNA than of 5S rRNA, also in Pn ovaries. In order to clarify this inconsistency with the Bioanalyzer results, 5 Pn ovaries and 5 V ovaries were randomly chosen and retrotranscription was carried out using 5S rRNA specific primers followed by a qPCR analysis of 5S rRNA transcription levels. 5S/18S rRNA index after specific 5S rRNA retrotranscription (Fig. 5C) showed the same relative profile obtained with the total cDNA (Pn > V) (Fig. 5B), but displaying positive values, as it happened with the index obtained from the electropherograms (Fig. 5A).

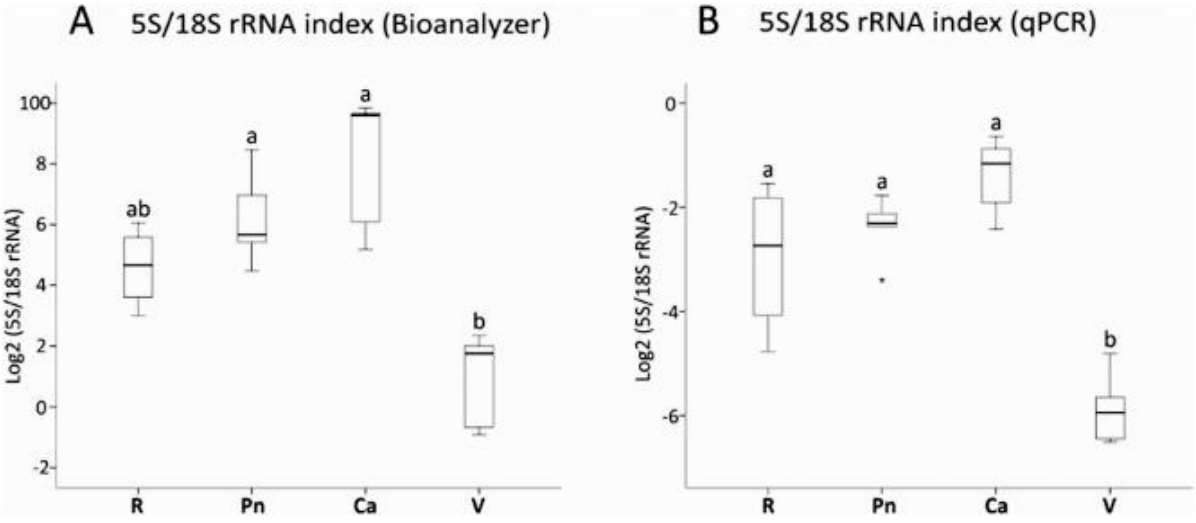

Fig. 4. 5S/18S rRNA index in the ovaries of thicklip grey mullets at different gametogenic stages. (A) 5S/18S rRNA index as quantified from total RNA electropherograms. (B) 5S/18S rRNA index after qPCR analysis of the 5S and 18S rRNA transcript levels in the same samples at different gametogenic stages: R: resting, Pn: perinucleolar, Ca: cortical alveoli and V: vitellogenesis. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between groups (Kruskal-Wallis, $p < 0.05$).

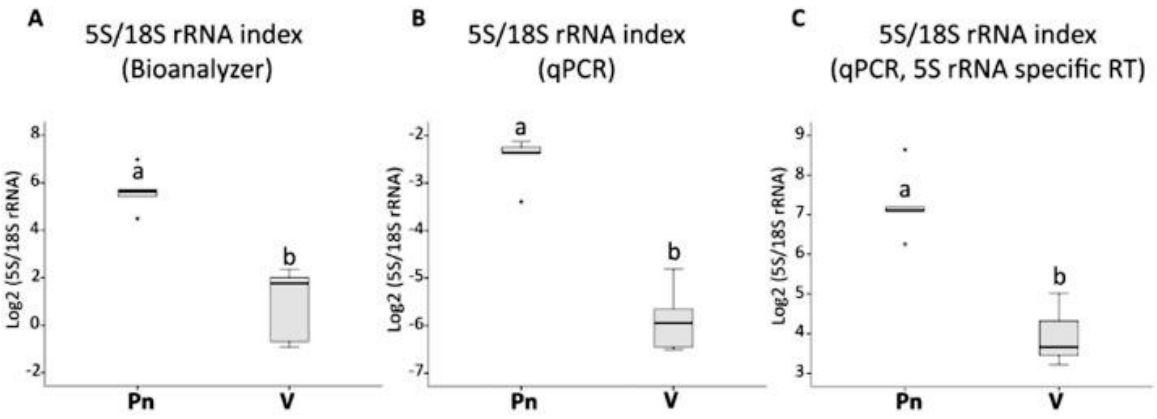

Fig. 5. 5S/18S rRNA index after specific 5S rRNA retrotranscription in thicklip grey mullet ovaries. 5S/18S rRNA index values obtained from Bioanalyzer electropherograms of total RNA (A), from qPCR analysis of total cDNA (B) and from 5S rRNA qPCR quantification of cDNA obtained after 5S rRNA specific retrotranscription (C). Pn: perinucleolar stage and V: vitellogenesis stage ($n = 5$ for each group). Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between means (Mann Whitney, $p < 0.05$).

Transcription levels of gtf3a, ubtf1 and 45S rDNA

gtf3a was strongly transcribed during previtellogenesis with a significant downregulation at vitellogenesis. The highest transcript levels of the RNA polymerase III product, 5S rRNA, appeared later, at Ca stage, decreasing again at V stage. After spawning, at R stage, *gtf3a* was upregulated, showing transcription levels as in Pn. On the contrary, 5S rRNA transcript levels were low at R stage, similar to the levels recorded at V and close also to values at Pn (Fig. 6). In the case

of *ubtf1* related to RNA polymerase I activity and 18S rRNA synthesis (Fig. 6), transcription levels were very high early during oogenesis with a maximum at Pn stage, similar to *gtf3a*. Then, the transcription levels decreased to their lowest levels at V and R stages. *ubtf1* thus, did not follow the transcription pattern displayed by 18S rRNA with its highest transcription levels at V stage (Fig. 6).

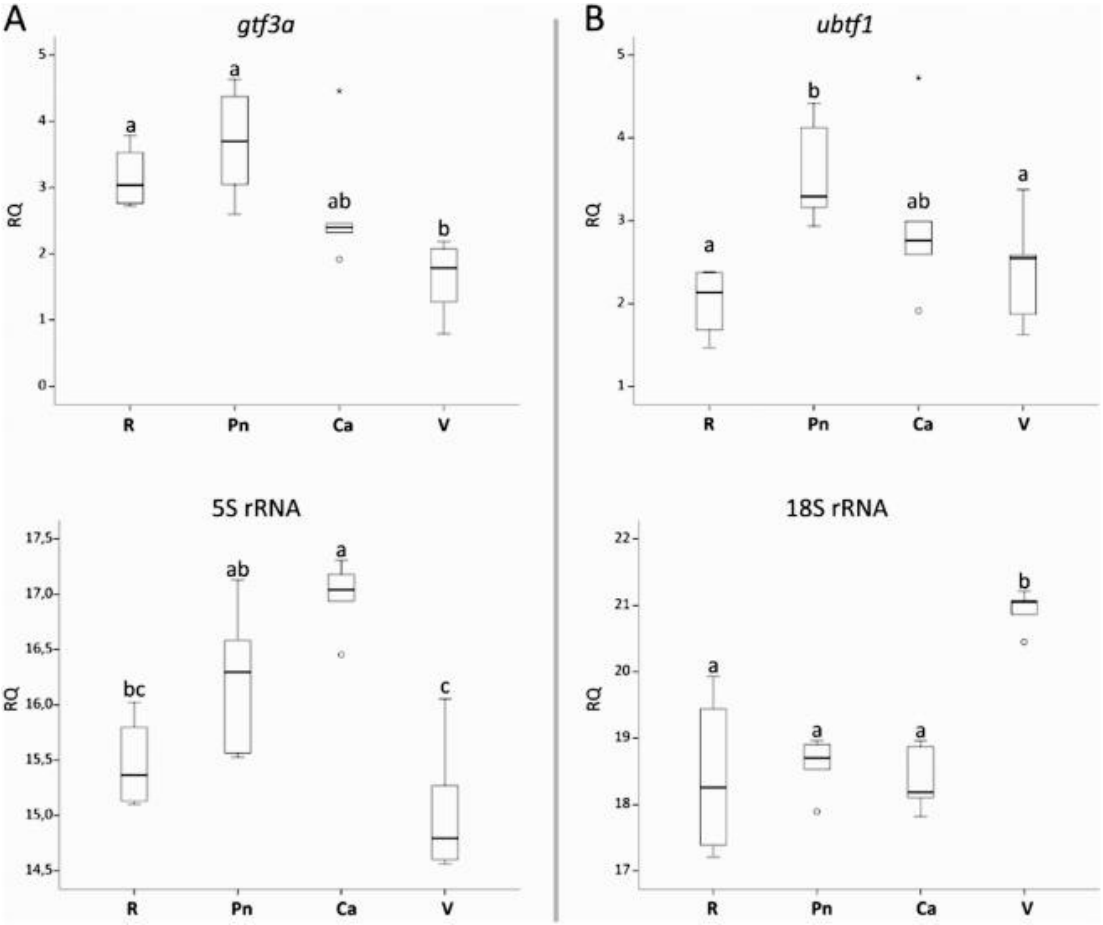

Fig. 6. Transcript levels of genes related to RNA Polimerase III (A) and I (B) activity in ovaries of thicklip grey mullets. The developmental stages of the ovaries were: R: resting, Pn: perinucleolar, Ca: cortical alveoli and V: vitellogenesis. Box plots represent the data within the 25th and 75th percentiles, with the median indicated by a line, and top and bottom whiskers indicating the minimum and maximum values. Different letters indicate significant differences between means (Kruskal-Wallis, $p < 0.05$).

The transcription of immature 45S pre-rRNA was assessed in Pn and V stage ovaries by conventional PCR, results showing that 45S pre-rRNA transcripts are present during both phases without any apparent difference in transcription levels (Fig. 7). The transcription began early during oogenesis as suggested by the high *ubtf1* transcript levels and 45S pre-rRNA levels maintained constant until vitellogenesis even under low *ubtf1* transcription.

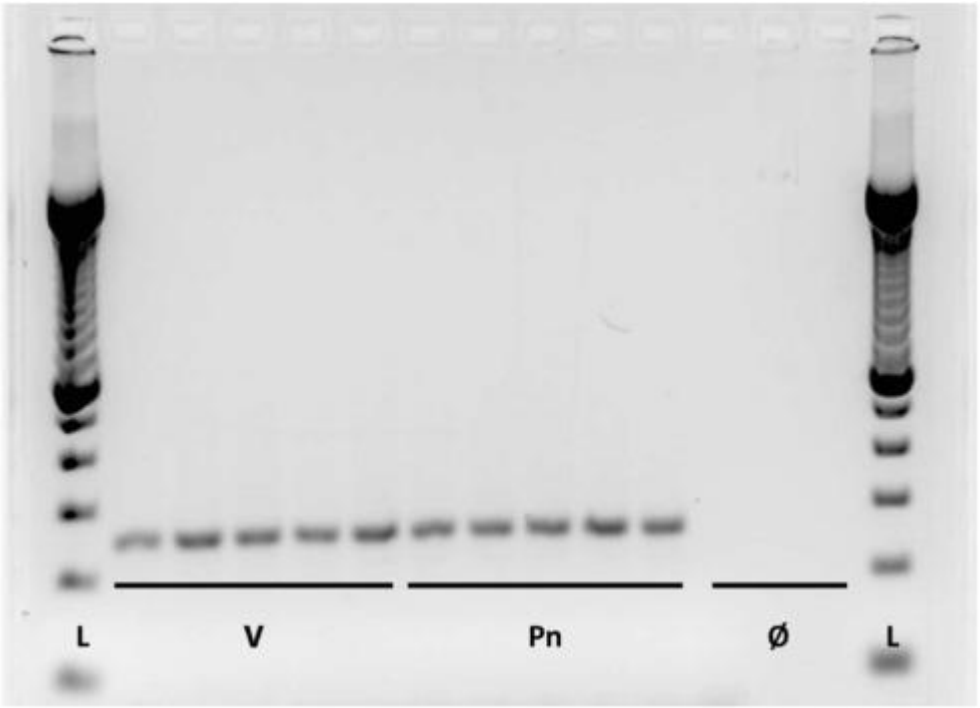

Fig. 7. 45S pre-rRNA levels in ovaries with oocytes at vitellogenic (V) and perinucleolar (Pn) stages. Agarose gel electrophoresis of 45S pre-rRNA (fragment around 257 nucleotides in length) after conventional PCR for 35 cycles in ovaries with oocytes at Pn or V stages. Ø = no template control, L = standard 100 bp (Invitrogen).

DISCUSSION

In the present study the 5S/18S rRNA index was used to molecularly identify sex in thicklip grey mullets independent of their reproductive stage. In addition, this index provided an easy and unbiased method to identify the severity of intersex condition in males captured in sites with high burdens of xenoestrogenic compounds. Moreover, this methodological approach proved to be reliable to identify the ovarian developmental stage in females due to the high index values (high 5S rRNA levels) displayed at Pn and Ca stages and the low values (high 18S rRNA) recorded in ovaries at V stage. The transcription levels of *gtf3a*, related to 5S rRNA production and stockpiling, followed the transcript pattern of 5S rRNA while those of *ubtf1* differed from the pattern of 18S rRNA accumulation. This was consequent with the accumulation of 45S pre-rRNA already observed at early oogenesis.

5S/18S rRNA index in fish gonads: identification of sex

When using fish as sentinel organisms in pollution biomonitoring, knowing the sex of each analyzed individual is crucial as many biological parameters under exposure to contaminants differ depending on the sex (Williams et al., 2003). Analyzing only one sex helps to reduce noise, but for many fish species without clear sexual dimorphism this requires histological skills that are not always in place in molecular laboratories. Moreover, histological sexing is not that easy in some reproductive stages along the reproductive cycle (Diaz de Cerio et al., 2012). In other circumstances, material for histological analysis is not collected during experimental set up and the only samples available are those devoted to molecular or biochemical analysis. On the other hand, several studies have described that exposure to EDCs alters the transcription levels of genes related to sex differentiation, hampering the reproductive success in exposed fish populations

(Bahamonde et al., 2015a). Thus, the analysis of sex ratios in pollution sentinel fish populations is in itself important to understand the possible effects of exposure to EDCs in the environment. Previous studies in our laboratory have demonstrated that 5S rRNA can be used as a molecular marker of the presence of oocytes (Diaz de Cerio et al., 2012, Ortiz-Zarragoitia et al., 2014, Rojo-Bartolomé et al., 2016). This oocyte specific accumulation of 5S rRNA has been proved in different fish species, e.g. European anchovy (*Engraulis encrasicolus*), hake (*Merluccius merluccius*), megrim (*Lepidorhombus whiffiagonis*) or zebrafish (*Danio rerio*) through a simple electrophoresis of the total RNA extracted from gonads (Rojo-Bartolomé et al., 2016). Other research groups have begun to use 5S rRNA in sea bass *Dicentrarchus labrax* for instance as an early molecular marker of sex differentiation to identify sex during the onset of puberty (Espigares et al., 2015). This approach for sex identification was used for the first time in thicklip grey mullets, comparing the 5S rRNA transcription pattern of females vs. males (Diaz de Cerio et al., 2012). Later, a 5S/18S rRNA index was developed based on the quantification of 5S and 18S rRNA peaks in the electropherograms provided by the Bioanalyzer RNA nanochips. This index identified the sex of individuals without the need to compare females to males, irrespective of the reproductive period and the fish species studied (Rojo-Bartolomé et al., 2016). Presently, this index has been proved to be useful to sex mullets from different polluted sampling sites all along the year and irrespective of their gametogenic stage. Exception were five females, 1 captured in November and 4 captured in January, that were in very advanced stages of vitellogenesis and due to the extremely high production levels of 18S classified as males. The index values obtained with the electropherograms have been proved real through qPCR, although the use of random primers does not permit proper retrotranscription of the small molecular mass 5S rRNA molecules (better results using a 5S rRNA specific primer for retrotranscription). A 5S/18S rRNA index threshold value (0.4251) has been established for this species above which any individual can be identified as female. This threshold value allows performing, a preliminary identification of sex from any mullet individual in a biomonitoring campaign; easily and without additional cost for transcriptomic studies. Obviously the presence of intersex individuals would constitute a confounding factor in such identification (see next section). In addition, the statistical analysis revealed two groups of individuals within females (Supplementary Fig. S1). The group with the highest 5S/18S rRNA index (peak in distribution plot around 12.0) would presumably belong to females with oocytes at previtellogenic stages while the one with the lowest index (peak around 5.0) would belong to females at V stage. Invariably, analysis in different fish species will identify new species specific threshold values.

5S/18S rRNA index and intersex severity

Jobling et al., 2002a, Jobling et al., 2002b demonstrated a decrease in the fertility of wild intersex roach (*Rutilus rutilus*) compared to normal males. Moreover, Kidd et al. (2007) reported a total collapse of a fathead minnow (*Pimephales promelas*) population in which, after exposure to ethinylestradiol the intersex condition was observed; and Harris et al. (2011) demonstrated that intersex roach showed a decrease in reproductive success with the increase in the severity of their intersex condition. Therefore, there is a need to differentiate intersex individuals and identify the molecular mechanisms associated to the differentiation of oocytes in fish testis in order to better predict the possible effects on affected populations (Bahamonde et al., 2015a). Gene expression analysis is becoming a commonly used approach to study the mechanisms of action of pollutants in wild fish (Garcia-Reyero et al., 2008, Ankley et al., 2009, Martyniuk and Denslow, 2012, Diaz de Cerio et al., 2012, Bahamonde et al., 2015b, Valencia et al., 2017). In this respect, vitellogenin expression in male fish liver has been implemented as a biomarker of exposure to xenoestrogenic compounds (ICES, 2005, Bahamonde et al., 2013, Abdel-Moneim et

al., 2015). However, no statistical correlation has been found between intersex condition and vitellogenin levels in mullets (Hiramatsu et al., 2006, Bizarro et al., 2014, Valencia et al., 2017) and specific biomarkers of intersex condition in fish are lacking (Bahamonde et al., 2013).
Intersex mullets were identified using the relative expression of 5S rRNA as marker of the presence of oocytes in testis by our research group (Diaz de Cerio et al., 2012). In this study we propose the 5S/18S rRNA index as new method to rank histologically identified intersex mullets according to their intersex severity, which could be applied also in other fish species. As the severity of the condition increases (more oocytes present in the testis) the index also increases, intersex severity index 3 giving 5S/18S rRNA index values of females instead of males. It must be considered, that nearly all of the intersex males found in the Basque coast display previtellogenic oocytes, which is the stage at which 5S rRNA transcription levels are at their highest relative to 18S rRNA levels. The high degree of interindividual variability in the 5S/18S rRNA index values within each intersex group probably reflects the sensitivity of the analysis in comparison to a histological ranking. Histological analysis is based on analysis of randomly selected sections across a big and heterogeneous organ. In fact, the severity index used in the present study described by Jobling et al. (1998 updated 2006) in roach is based on histological observations of 6 testis portions. Other authors have modified the indexing strategy according to the species studied, to the observed amount of oocytes or to the oocyte distribution within the testis (van Aerle et al., 2001, Faller et al., 2003, Blazer et al., 2007, Tanna et al., 2013).
It is advisable to standardize sampling procedures when intersex male fish are studied. We strongly recommend using the histological observation to perform a first screening and to certify intersex condition and also to identify the developmental stage; including the stage of the oocytes present in the intersex testis. If only previtellogenic oocytes were present in the testis, as it is often the case, then we recommend using the 5S/18S rRNA index to rank the individuals according to their intersex severity. This provides an easy, unbiased and quantitative way to establish the coverage of oocytes in the testis. This molecular approach allows analyzing more tissue than through histology; around 100 mg per sample in each RNA extraction vs. 1–9 sections 5 μm in thickness analyzed in histology. Besides, more samples can be analyzed simultaneously in less time (around 3 h for 24 samples). Of course, this index should be tested in other fish species, where high prevalence of intersex condition has been identified, and if possible with availability of the whole range of severities. Roach could be a good candidate species (Jobling et al., 2002a, Jobling et al., 2002b, Bahamonde et al., 2013) for such additional studies. In any case, the homogeneity of gamete development within the gonad in each species should be first understood, in order to establish how much tissue should be analyzed. Should the proximal, mid and/or distal portion of the gonads be analyzed histologically/molecularly? From one or the two lobes? Once this is established, histologically it would seem advisable to range intersex severity according to the area coverage of oocytes within the testis calculated in a minimum number of complete tissue sections (Blazer et al., 2007), leaving a minimum of 10 μm of distance between analyzed sections to avoid quantifying the same oocyte twice (Bahamonde et al., 2015a). A stereological or planimetric approach would help in better estimating the importance of the ovarian tissue per area of testis.

5S/18S rRNA index: identification of female reproductive stage

Kroupova et al. (2011) analyzed in detail the stage-dependent RNA composition in roach ovaries, concluding that during primary growth and early cortical alveoli stages small-size RNAs, which they did not identify as belonging to 5S rRNA, were accumulated. 5S rRNA quantification has been later shown to provide an easy way to establish the stage of oogenesis in fish. In this way, the stage of oogenesis was unequivocally identified in megrim (group-synchronous batch spawner) and European anchovy (asynchronous batch spawner) (Rojo-Bartolomé et al., 2016). The index also

proved to be useful to monitor oogenesis during carp pituitary extract treatment in the asynchronous batch spawning European eel *Anguilla anguilla* (Rojo-Bartolomé et al., 2017). In this work, we used this index in thicklip grey mullet (synchronous spawner) ovaries to identify the maturation stage along oogenesis. Thus, 5S/18S rRNA index could constitute a useful approach to study the reproductive stage in females in an objective and quantitative manner.

Transcription levels of gtf3a, ubtf1 and 45S rDNA

Gtf3a is not only a transcription factor activating RNA polymerase III, it also binds the 5S rRNA product for its accumulation in the cytosol as small ribonucleoprotein particles (Szymański et al., 2003), and it is strongly transcribed in ovaries in contrast to testis in fish (Ortiz-Zarragoitia et al., 2014, Rojo-Bartolomé et al., 2016). Our results hereby demonstrate that, as it occurs in megrim ovaries (Rojo-Bartolomé et al., 2016), *gtf3a* is strongly transcribed in previtellogenesis and is down-regulated at vitellogenesis allowing also the identification of the entry into the secondary stage of oocyte growth.

In contrast to 5S rRNA, 18S rRNA is synthesized by Pol I (Bazett-Jones et al., 1994). The product of Pol I is the 45S pre-rRNA transcript which suffers 3 main splicing events to form mature 5.8S, 18S and 28S rRNAs (Drygin et al., 2010). No match between the profiles of *ubtf1* and 18S rRNA transcription occurs in mullets. Therefore *ubtf1* transcript levels were at their highest during early oogenesis, when, as reported in anuran frogs, Pol I activity should be very low (Roger et al., 2002). On the contrary, *ubtf1* was significantly down-regulated during secondary oocyte growth, when Pol I activity should be maximal according to 18S rRNA levels shown for roach, megrim (Kroupova et al., 2011, Rojo-Bartolomé et al., 2016) or mullets hereby. These results point to post-translational events, such as phosphorylation of Ubtf1, as responsible for Pol I machinery activation. This would allow producing 45S pre-rRNA rapidly before meiosis resumption (Rojo-Bartolomé et al., 2016). But here it became apparent that 45S pre-rRNA transcription occurs already in ovaries with oocytes at Pn stage. Thus, mature rRNA production could be boosted at vitellogenesis through splicing of accumulated 45S pre-rRNA first at the 18S–5.8S rRNA site and finally between 5.8S and 28S rRNA (Drygin et al., 2010). Surprisingly, 45S pre-rRNA transcript levels were similar in ovaries with Pn and V stage oocytes. The strong transcription at Pn stage is consequent with the fact that nucleoli, differentiating characteristic of Pn oocytes, are the structural manifestation of 45S rRNA synthesis. The whole process would all together allow rapid assembly of ribosomes in the moment of fertilization (Ortiz-Zarragoitia et al., 2014).

In conclusion, it can be said that the 5S/18S rRNA index provides an easy and inexpensive way to distinguish male and female mullets. In addition, the index proved to be a sensible and objective tool to classify intersex mullets according to the amount of oocytes present in testis (5S/18S rRNA intersex severity index). Furthermore, the index was useful to identify the ovarian developmental stage, at least those containing oocytes that had initiated secondary growth from those containing only oocytes in previtellogenesis, in a synchronous spawner (mullet). As it happened in other fish species, the 5S/18S rRNA index decreased with the onset of vitellogenesis, due to the reduction in Pol III activity and the appearance of mature 18S rRNA. In contrast of earlier hypothesis, Pol I is active in fish oocytes early during oogenesis and 45S pre-rRNA is produced in Pn stage oocytes. 45S pre-rRNA then begins cleavage during vitellogenesis together with the additional transcription of 45S pre-rRNA occurring during vitellogenesis. Further studies are needed in different fish species and under different developmental/environmental scenarios, to decipher the mechanisms that govern ribogenesis during oogenesis in females and intersex individuals and the utility of the methodology described in environmental monitoring programmes.

CONTRIBUTIONS OF AUTHORS

IR conducted the laboratory measurements and drafted the manuscript. AV conducted most of the samplings and participated in histological analysis of the samples. IC designed and supervised the study directing analysis of results and the writing process.

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