Alteration in molecular markers of oocyte development and intersex condition in mullets impacted by wastewater treatment plant effluents

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ABSTRACT

Wastewater Treatment Plant (WWTP) discharges are an important source of endocrine disrupting chemicals (EDCs) into the aquatic environment. Fish populations inhabiting downstream of WWTP effluents show alterations in gonad and gamete development such as intersex condition, together with xenoestrogenic effects such as vitellogenin up-regulation. However, the molecular mechanisms participating in the development of intersex condition in fish are not elucidated. The aim of this study was to assess the impact of two WWTPs effluents (Gernika and Bilbao-Galindo situated in the South East Bay of Biscay) with different contaminant loads, in thicklip grey mullet (Chelon labrosus) populations inhabiting downstream, examining the presence and severity of intersex condition, during two seasons. Molecular markers of xenoestrogenicity and oocyte differentiation and development (vtgAa, cyp19a1a, cyp19a1b, cyp11b, foxl2, dmrt1 and gtf3a) were also studied. Intersex mullets were identified downstream of both WWTPs and vtgAa was upregulated in intersex and non intersex males. Sex dependent differential transcription levels of target genes were detected in mullets from Galindo. However, no such pattern was observed in mullets from Gernika, suggesting an attenuating effect over studied genes caused by a higher presence of EDCs in this site, as indicated by the elevated prevalence of intersex mullets in this population. In conclusion, no direct association between xenoestrogenic responses and intersex condition was established. Mullets from Gernika showed signs of severe EDC exposure compared to those from Galindo, as demonstrated by the higher prevalence of intersex males and the reduction in transcription profile differences between sexes of gametogenic gene markers.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are substances that interact with the endocrine system, causing alterations at different levels of biological organization, from the molecular to the individual and the population level (Brander, 2013, WHO/UNEP, 2013). Xenoestrogens are a group of EDCs with the ability to mimic oestrogen or to cause oestrogen-like responses in exposed organisms (Campbell et al., 2006). EDCs present different chemical structures and have different sources. In industrialized countries these compounds mainly arrive in the aquatic environment from the wastewater treatment plants processing municipal, industrial and hospital waste waters (Campbell et al., 2006). Wastewater treatment plant (WWTP) discharges contain complex mixtures of EDCs, such as natural and synthetic hormones, pharmaceuticals, alkylphenols, bisphenol A, phthalates and pesticides that can interact with each other thus enhancing their potency. In this way, the natural hormone oestradiol (E2) and the synthetic hormone 17α-ethinylestradiol (EE2)

can act in an additive manner (Thorpe et al., 2003). The combination of other xenoestrogenic compounds such as oestrogens, alkylphenols and pesticides are also known to have additive effects (Thorpe et al., 2001). On the other hand, it has been shown that oestrogenic and anti-androgenic substances can cause similar effects even if their mechanism of actions are different (Filby et al., 2007). Of course, other compounds in WWTP discharge mixtures can have androgenic effects, or interact with the oestrogenic response thus, acting on a suppressive manner on the xenoestrogenic effect of the above mentioned substances. In any case, the oestrogenic effects of WWTP effluents have been detected worldwide in different fish species; thicklip grey mullet (*Chelon labrosus*) in the Bay of Biscay (Bizarro et al., 2014, Puy-Azurmendi et al., 2013), wild roach (*Rutilus rutilus*) in the United Kingdom (Jobling et al., 1998, Jobling et al., 2002, Jobling et al., 2006, Tyler and Jobling, 2008, Tyler and Routledge, 1998) or Denmark (Bjerregaard et al., 2006), white sucker (*Catostomus commersoni*) in the United States and Canada (Woodling et al., 2006), rainbow darter (*Etherostoma caeruleum*) in Canada (Bahamonde et al., 2014, Bahamonde et al., 2015a, Bahamonde et al., 2015b) or brown trout (*Salmo trutta fario*) in Switzerland (Körner et al., 2005), are just some examples. One of the most widely used biomarker to indicate the presence of xenoestrogens in the environment is the induction of vitellogenin (VTG), the precursor of the female yolk protein. It is naturally expressed in mature females by oestrogen induction, but under xenoestrogenic exposure, it can also be expressed in males and juveniles (Arukwe and Goksøyr, 2003, Matthiessen, 2003, Tyler and Jobling, 2008).

Oestrogen synthesis is regulated by Cyp19 aromatase in fish, converting androgens into oestrogens (Diotel et al., 2010). Two aromatase isoforms have been described in fish, the ovarian form *cyp19a1a* and the brain form *cyp19a1b* (Diotel et al., 2010), according to their tissue expression distribution. The ovarian form is directly associated with the oocyte development, which controls the oocyte growth. The brain form has a neuroendocrine role and some authors have suggested that it can be involved in sex determination and differentiation processes in fish (Cheshenko et al., 2008, Diotel et al., 2010). Another member of the cyp family, *Cyp11b,* codes for the enzyme steroid 11β-hydroxylase, responsible for the production of 11 β-hydroxy testosterone (11-KT) in the Leydig cells of the testis (Uno et al., 2012). 11-KT is the main androgen in fish controlling the testis differentiation and spermatogenesis (Schulz et al., 2010, Piferrer, 2011). It is known that EDC exposure can alter transcription profiles of *cyp19a* and *cyp11b* gene products, altering the correct functioning of, and the development of, ovary and testis in fish (Arukwe, 2008, Kazeto et al., 2004).

Sex differentiation in fish is a complicated process which is under the control of genetic and environmental factors (Devlin and Nagahama, 2002, Piferrer et al., 2012). Due to the many reproductive strategies that can be found in fish, the process of sex differentiation differs among species. In the recent years new insights about genes that are upstream regulators of the differentiation process are being discussed. *Dmrt1* (doublesex and mab-3 related transcription factor 1) for instance is proposed as the major player in fish sex differentiation (Smith et al., 2013) and its expression has been related with testis development and spermatogenesis in several species (Herpin and Schartl, 2011). In addition, *foxl2* (forkhead box L2) is involved in ovarian development (Baron, 2004), oocyte maintenance (Alam et al., 2008) and it has been proposed to be implicated in the regulation of aromatase expression in different fish species (Baron, 2004, Guiguen et al., 2010). Alterations on the expression patterns of these genes can lead to modified sex differentiation outcomes in fish (Baron, 2004, Marchand et al., 2000).

Intersex condition, which consists in the presence of oocytes at any developmental stage within the testis of gonochoristic fishes, is the most marked feminizing effect occurring in fish populations impacted by EDCs (Bahamonde et al., 2013, Tyler and Jobling, 2008). This alteration has been described in different wild fish species belonging to a wide range of families (Bahamonde et al.,

2013, Jobling and Tyler, 2003, Ortiz-Zarragoitia et al., 2014). Intersex fish show transcription patterns of oocyte development related genes resembling those in normal ovaries (Bahamonde et al., 2013, Diaz De Cerio et al., 2012). Recently, 5S ribosomal RNA (5S rRNA) and its transcription factor (general transcription factor 3A, *gtf3a*), have been shown to present similar expression in ovaries and intersex testis of thicklip grey mullets (Diaz de Cerio et al., 2012). The 5S rRNA molecule is necessary for the oocyte development and maturation process, due to its role in the production of ribosomes. Its accumulation during oocyte growth is crucial for the successful development of the fertilized egg, as this will enable to expedite protein synthesis during early embryo development (Lubzens et al., 2010, Diaz De Cerio et al., 2012, Mazabraud et al., 1975, Ortiz-Zarragoitia et al., 2014).

In the Basque coastal region, thicklip grey mullets have been used as sentinel organisms of endocrine disruption (Bizarro et al., 2014, Diaz De Cerio et al., 2012, Ortiz-Zarragoitia et al., 2014, Puy-Azurmendi et al., 2013). Xenoestrogenic effects on mullets, such as up-regulation of vitellogenin and *cyp19a1b* transcription levels, have been related to elevated concentrations of some EDCs such as alkylphenols, pesticides, phthalates, musk fragrances, bisphenol A and oestrogenic hormones in the bile (Bizarro et al., 2014, Puy-Azurmendi et al., 2013). The main source of those EDCs detected in mullets has been associated to the effluents from the WWTPs present in the sampled sites (Ros et al., 2015). Accordingly, mullets showing intersex gonads were detected in EDC polluted estuaries, with prevalences up to 30% in the Biosphere Reserve of Urdaibai (Bizarro et al., 2014, Puy-Azurmendi et al., 2013). A distinguishable pattern between male and intersex individuals in the transcription levels of genes participating in steroidogenesis was described in mullets from the polluted harbor of Pasaia (Sardi et al., 2015). However, the molecular mechanisms leading to intersex development and its possible association with xenoestrogenic responses could not be determined (Sardi et al., 2015).

The aim of this study was to compare the transcription profiles of genes related with sex differentiation, oogenesis and steroidogenesis (*vtgAa, cyp19a1a, cyp19a1b, cyp11b, foxl2, dmrt1 and gtf3a*) on female, male and intersex male mullets inhabiting downstream of two different WWTPs. One such WWTP processes the waters of the Bilbao metropolitan area and the second one processes the waters of the area of Gernika in the Biosphere Reserve of Urdaibai and both are known to cause intersex condition in local mullets. We wanted to compare whether the qualitatively and quantitatively different discharge of both WWTPs could be reflected in differences in the xenoestrogenic response of mullets in two different seasons of the year (winter and late spring).

MATERIAL AND METHODS

Study area

The Wastewater Treatment Plant (WWTP) of Gernika (43°19’01”N, 2°40’36”W) is located in the Oka river in the Biosphere Reserve of Urdaibai (Bay of Biscay, Northern Iberian Peninsula) in the upper limit of tidal influence. This WWTP receives urban waters from the town of Gernika and its surrounding area (about 26,000 inhabitants). The main activities that are held around the river basin are agriculture and limited metallurgy, surface treatments, dye, cutlery and plastic manufacture. The treatment plant has a biological treatment capacity of 0.4 m^3/s and it consists of a first and a secondary biological treatment. Previous studies showed xenoestrogenic effects in the local mullet population (Puy-Azurmendi et al., 2013, Bizarro et al., 2014).

The WWTP of Galindo (43°18'17"N, 2°59'45"W) is located in the Bilbao estuary (Bay of Biscay). The surrounding area is densely populated (1,000,000 inhabitants) and very industrialized. The WWTP has a first, secondary and tertiary biological treatment where a water volume of 6 m^3/s is treated (García-Barcina et al., 2006). Since the year 1990 the WWTP has played a key role in the recovery of the ecological status of the estuary thanks to the reduction on the untreated sewage discharge (García-Barcina et al., 2006). No previous analysis on the possible xenoestrogenic effects of the effluent area has been published.

Fish and tissue collection

Adult (>20 cm) thicklip grey mullet (*Chelon labrosus*) individuals were collected by fishing-rod in both sampling sites in June 2013 and in February 2014 (n = 20 per site and season). Fish were anaesthetized in a saturated ethyl-4-aminobenzoate water bath. All fish handling and procedures were performed following the local and national regulations (procedure approval CEBA/152/2010/ORTIZ ZARRAGOITIA). Length (cm) and weight (g) of the individuals were measured. Dissection was immediately performed *in situ*, and liver, brain and gonads were collected. For histological analysis, a portion from the middle part of the gonad from each fish was fixed in 4% neutral buffered formalin. For gene transcription analysis, the full brains and a small portion of the gonad and liver were placed in RNAlater solution (Ambion; Life Technologies, Carlsbad, USA). After 10 min incubation for a correct embedding of the protective solution, samples were frozen in liquid nitrogen and then stored at −80 °C until processing.

Histological analysis of the gonad

After fixing gonad samples for 24 h in neutral buffered formalin, they were dehydrated in a graded ethanol series (70%, 90% and 96%). Afterwards, samples were embedded in methacrylate resin (Technovit 7100; Heraeus Kulzer GmbH & Co. KG, Wehrheim, Germany) following manufacturer's instructions. Then, sections 5 µm in thickness were cut in a Leica RM 2125 RT manual microtome (Leica Microsystems, Nussloch, Germany) and placed on Superfrost microscope slides. Tissue sections were stained with hematoxylin/eosin (Gamble and Wilson, 2002) using the Leica Autostainer XL and mounted with the aid of the Leica CV 5030 workstation. The slides were microscopically examined under an Olympus BX61 light microscope (Tokyo, Japan). Two slides with three sections each were analyzed per individual, for a total of 6 sections per individual. The criteria by McDonough et al. (2005) for flathead grey mullet *Mugil cephalus*, were followed to rank the reproductive stage of the individuals analyzed: 1 = immature; 2 = developing; 3 = ripe; 4 = spawning or atretic; and 5 = inactive or resting. Within stage 2, we distinguished ovaries with previtellogenic (PV) or mid vitellogenic (MV) oocytes and testes during early (ES), mid (MS) and late spermatogenesis (LS). When intersex testis was identified its severity was calculated using the Intersex Index described by Jobling et al. (2006), where the severity increases as the number of oocytes present in testis increases. The intersex index ranges from 0 to 7, with normal male testis scoring 0; presence of up to 5 oocytes scattered through the testis = 1; testis showing 6–20 clustered oocytes = 2; presence of 21–50 oocytes = 3; 50–100 oocytes = 4; a mosaic of testicular and ovarian tissue with more than 100 oocytes = 5; more than 50% of the gonad is ovarian and it is clearly separated from the testicular tissue = 6; and with a completely feminized and ovarian like gonad scoring 7 (Jobling et al., 2006).

Gene transcription analysis

Six individuals of each gender per site and season, and all identified intersex individuals found during the study, were selected for the transcription analyses in gonads and brain. Transcription levels of *vtgAa* in liver were only carried out in male and intersex individuals.

Extraction and capillary electrophoresis of total RNA

Total RNA was extracted from each gonad, liver and brain sample using TRI Reagent® Solution (Ambion; Life Technologies) following the manufacturer's instructions. 80–100 mg of tissue was 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). Quality and RNA concentration was measured by spectrophotometry in a biophotometer (Eppendorf, Hamburg, Germany) and only A_{260}/A_{280} absorbance ratios between 1.8 and 2.2 were considered for further analysis. Agilent RNA 6000 Nano Kits (Agilent Technologies, Santa Clara, California, USA) were used to determine RNA integrity and to analyze ribosomal rRNA concentration in gonad RNA in a Bioanalyzer 2100 (Agilent Technologies).

cDNA synthesis and quantification

First strand cDNA synthesis was performed from 2 µg of RNA per sample 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 concentrations in the gonad, brain and liver 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).

Sequencing of selected genes

Partial cDNA sequences belonging to the genes *foxl2* and *dmrt1* were cloned for *C. labrosus*. For that purpose degenerate primers, designed in domains especially well conserved among the sequences available for other teleosts in the NCBI database, were used (Table 1). Sequences of interest were amplified through PCR using cDNA pooled from gonads of mullets in different stages of development. 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 59 °C for *foxl2* and 57 °C for *dmrt1*) and an elongation step (70 °C for 30 s) and a final step of 72 °C for 8 min. The specificity 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 Service of the University of the Basque Country. The obtained sequences were annotated and submitted to the NCBI database.

Table 1. Degenerate primers and PCR condition used for the cloning of the target sequences of *Chelon labrosus*. The specific mullet fragment length obtained with the used primers and identities of the deduced aminoacid sequences in comparison with the most similar ortholog sequences available in Genbank is provided.

Gene	Forward (5'–3')	Reverse (5'–3')	Fragment size (bp)	Tm (°C)	Amino acid identity (blastx)
foxl2	CCCAGAAACCRCCGTACTC	GTGTTTGSTCTCGTGWTCCCA	600	59	98% Dicentrarchus labrax (ACW83540); 2e−128
dmrt1	TTCTGCAACTGGAGGGACTG	CCRTAGTAGGTGGGGTARC	380	57	95% Odontesthes hatcheri (ACG69837); 7e−25

Real-time qPCR

Specific primers were designed using the mRNA sequences available for *Chelon labrosus* in GenBank for the target genes (*gtf3a, cyp19a1a, cyp19a1b* and *vtgAa*) and the just sequenced genes (*foxl2* and *dmrt1*) (Table 2). Primers were designed using IDT and Eurofins online tools.

Table 2. List of target genes, their GenBank accession number and the specific forward and reverse primers used for transcription level analysis by qPCR.

Gene	Accession number	Forward (5'–3')	Reverse (5'–3')	Fragment size (bp)	Tm (°C)
vtgAa	EF535843	CGAGAGCCGGACTCAAAGT	CCACAAGCTTCAGCAGGTATTTG	78	59
gtf3a	JN257141	CCAGGAGAAGCGATATAAATGTGA	TCGTGATGCTTCAGTTTTCCATG	168	59
cyp19a1a	EF535845	TCCAAAGCCCTGACGGGTC	AGCCAAACTGTCCAAGTCGTC	93	60
cyp19a1b	EF535846	CGTGGTTCTGGGCAAAGATGA	CTGTCGCAAAATCAC	162	59
dmrt1	KT248846	GAGAGGCATAGAGTCATGGC	GGAGGCACTGGCAGGTGT	177	59
foxl2	KT248845	CAGGGAAATCCTTGTTCGGAG	GTGTAGGACATCGGTGTTGG	121	59
cyp11b	JX294416	GGAGGGGTCGACACGACAGC	ATCATGGGCAAAGGCTGGCGG	172	60

Real time qPCR analysis was performed in a 7300 Real-Time PCR system thermocycler (Life Technologies) using a SYBR Green fluorescent dye master mix (Roche Diagnostics, Indianapolis, USA). Each sample was run in triplicates in a total volume of 20 µL containing 10 µL of water, 7.88 µL of mix and 0.12 µL 12.5 pmol primer pair. A control without template was run (also in triplicates) 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. Reaction efficiency of each plate was calculated using a standard curve consisted of serial dilutions of pooled cDNA. The specificity of the reaction was determined by confirming the presence of a single peak in the dissociation curve, which served also to confirm that no primer dimmers had been formed. cDNA concentration obtained by fluorescent quantification method was used for normalization of target genes in brain, gonad and liver, as performed by Rojo-Bartolomé et al. (2016).

The gonad transcription pattern of the newly sequenced *C. labrosus foxl2* and *dmrt1* along male and female gametogenesis was characterized in samples available from an annual campaign, carried out in the harbor of Pasaia. Gonads of 6 individuals at each of the distinguished gametogenic stages were analyzed.

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. Transcript levels of *foxl2* and *dmrt1* during the gametogenic cycle of mullets were analyzed with the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. Bootstrap resampling techniques (Efron and Tibshirani, 1993) followed by ANOVA and Bonferroni's post hoc test was performed for multiple comparisons in the WWTP study. The same procedure but using Studenťs T-test was used for pair comparisons. For each experiment N = 2000 repetitions of the same size of the original sample were selected. Significant differences were established at $p < 0.05$.

RESULTS

Histological and histopathological analysis of gonads

Female mullets from the four different samplings showed immature gonads, with ovaries containing only previtellogenic oocytes. Males showed gonads at different gametogenic stages, depending on the sampling season. In June 2013, all of the males showed immature (inactive) testes with the presence mainly of spermatogonia in spermatic ducts. In February 2014, the predominant gametogenic stage in males was mature (late spermatogenesis), where spermatids and spermatozoa could be distinguished in the middle of the ducts. Nevertheless, some of the males (22.2% at Galindo and 37.5% at Gernika) presented testes which were at immature stage.

Intersex males were identified in all samplings independent of their gametogenic stage (Fig. 1). The prevalence of intersex condition was 36% and 90% at Gernika in June and February, respectively, it was 9% at Galindo, on both samplings. Intersex mullets showed testes at the same gametogenic stage of the corresponding non-intersex males from the same sampling (Fig. 1). Oocytes in intersex males were interspersed within the testes and in the previtellogenic stage (Fig. 1A-B), except for a single individual from Gernika in February, which showed vitellogenic oocytes (Fig. 1C). According to the intersex scoring index developed by Jobling et al. (2006), in all cases the index was in the range 1–3 (Fig. 1). Thus, a low to moderate severity was established for intersex condition in both of the sampled mullet populations.

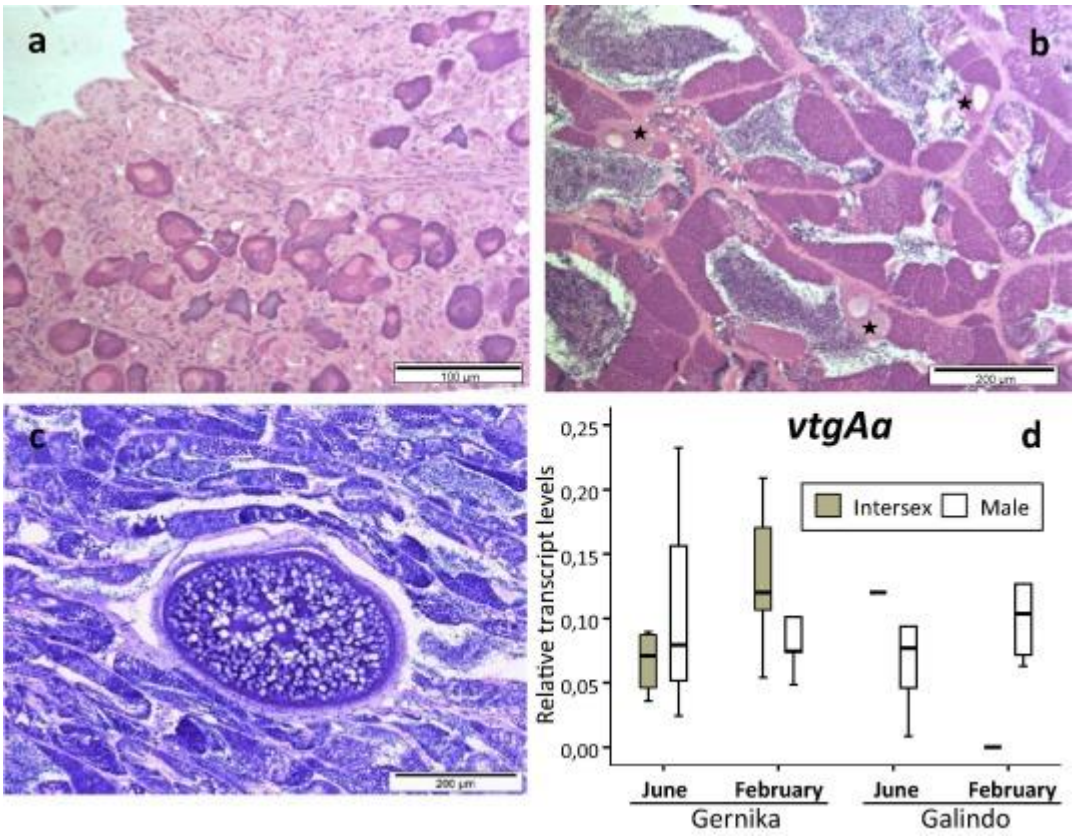

Fig. 1. Light micrographs illustrating the gonads of intersex male thicklip grey mullets with their intersex Severity Index. a) Immature (stage 1) male from Galindo with previtellogenic oocytes, Intersex Index 3. b) Mature male (stage 2) from Gernika with previtellogenic oocytes (stars), Intersex Index 2. c) Mature male (stage 2) from Gernika with a vitellogenic oocyte, Intersex Index 1. d) Relative transcription levels of liver vitellogenin (*vtgAa*) in intersex and male individuals from Gernika and Galindo captured in two samplings. Box-plots represent median value (line), 25–75% quartiles (box) and standard deviations (whiskers).

Sequencing and transcription pattern of foxl2 and dmrt1 in gonads during the gametogenic cycle

Fragments of *foxl2* (542 bp, Genbank accession number KT248845) and *dmrt1* (317 bp, Genbank accession number KT248846) were amplified and sequenced in gonads of thicklip grey mullet that, upon blastx analysis, showed a high degree of deduced amino acid identity in comparison to existing piscine sequences (Table 1). The fragments cloned for *C. labrosus* genes represent 54% of the total coding sequence of *foxl2* and 37% of that of *dmrt1*.

Females showed higher transcription levels of *foxl2* in ovaries than males in testes (Fig. 2). Females in an inactive stage showed significantly higher levels than developing ones with previtellogenic (PV) oocytes. When containing oocytes in mid-vitellogenic stage (MV), *foxl2* transcription levels increased in the ovary, then decreased again in regressing females, which showed values similar to PV females. In the case of testis, the highest transcription levels for *foxl2* were observed at inactive and regressing stages.

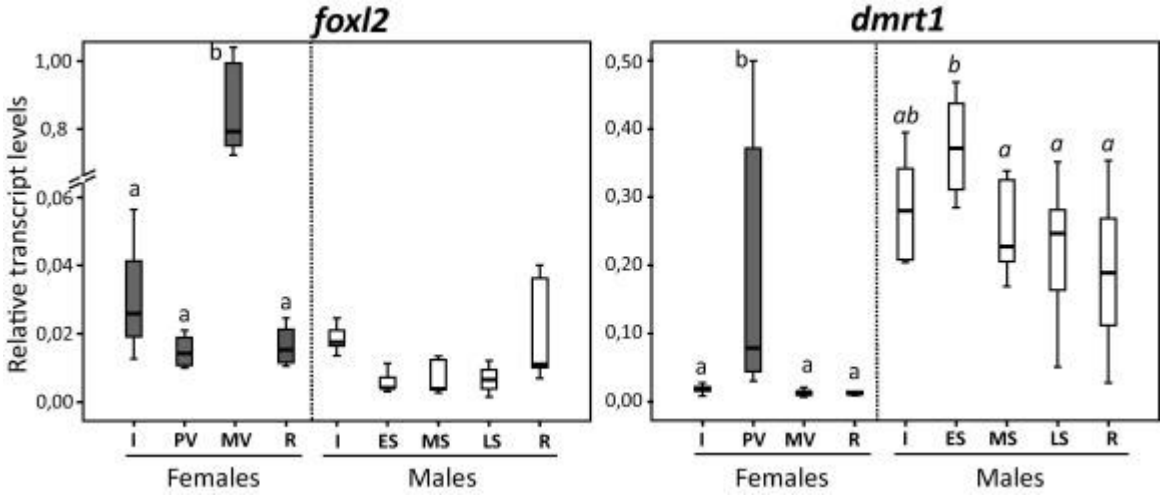

Fig. 2. Transcription levels of *foxl2* and *dmrt1* in gonads of female (I: inactive; PV: previtellogenic; MV: mid-vitellogenic; R: regressing) and male (I: inactive; ES: early spermatogenic; MS: mid spermatogenic; LS: late spermatogenic; R: regressing) thicklip grey mullets captured in the Pasaia harbor during a whole annual reproductive cycle. Box-plots show median value (line), 25–75% quartiles (box) and standard deviations (whiskers). Letters denote statistical significant differences between means ($p < 0.05$).

Regarding *dmrt1,* transcription levels were higher in testes than in ovaries (Fig. 2). Ovaries with PV oocytes showed the highest transcription levels, while very low levels were quantified at other gametogenic stages. Transcription levels of *dmrt1* in testes increased from an inactive stage to early spermatogenesis (ES). Subsequently, a decreasing trend was observed as spermatogenesis progressed.

Transcription levels of target genes at Gernika and Galindo

Liver *vtgAa* transcription was detected in all male and intersex mullets analyzed at both of the studied sites (Fig. 1). High variability between all the individuals was noted and no significant differences could be observed between normal and intersex males.

Female, male and intersex mullets from Gernika showed no statistical differences on their *cyp19a1a* transcription levels. However at Galindo, females showed higher transcription levels than males (Fig. 3). In addition, intersex males from Galindo showed higher transcription levels than normal males. Furthermore, females from Galindo showed higher ovarian *cyp19a1a* transcription levels than females from Gernika, during both sampling periods (t = −8.859; p = 0, 043 (June) and t = −7544; p = 0.041 (February); Fig. 3). *Cyp11b* showed differential transcription in ovaries, in comparison with normal testes at Galindo, during both sampling periods (Fig. 3). The same occurred at Gernika in February, but not in June. Males at Gernika in June showed down-regulated *cyp11b* levels in comparison to males at Galindo in the same season (t = −14,645, p = 0.023). Regarding intersex males, only those captured in February at Gernika showed higher *cyp11b* transcription levels, than females (Fig. 3).

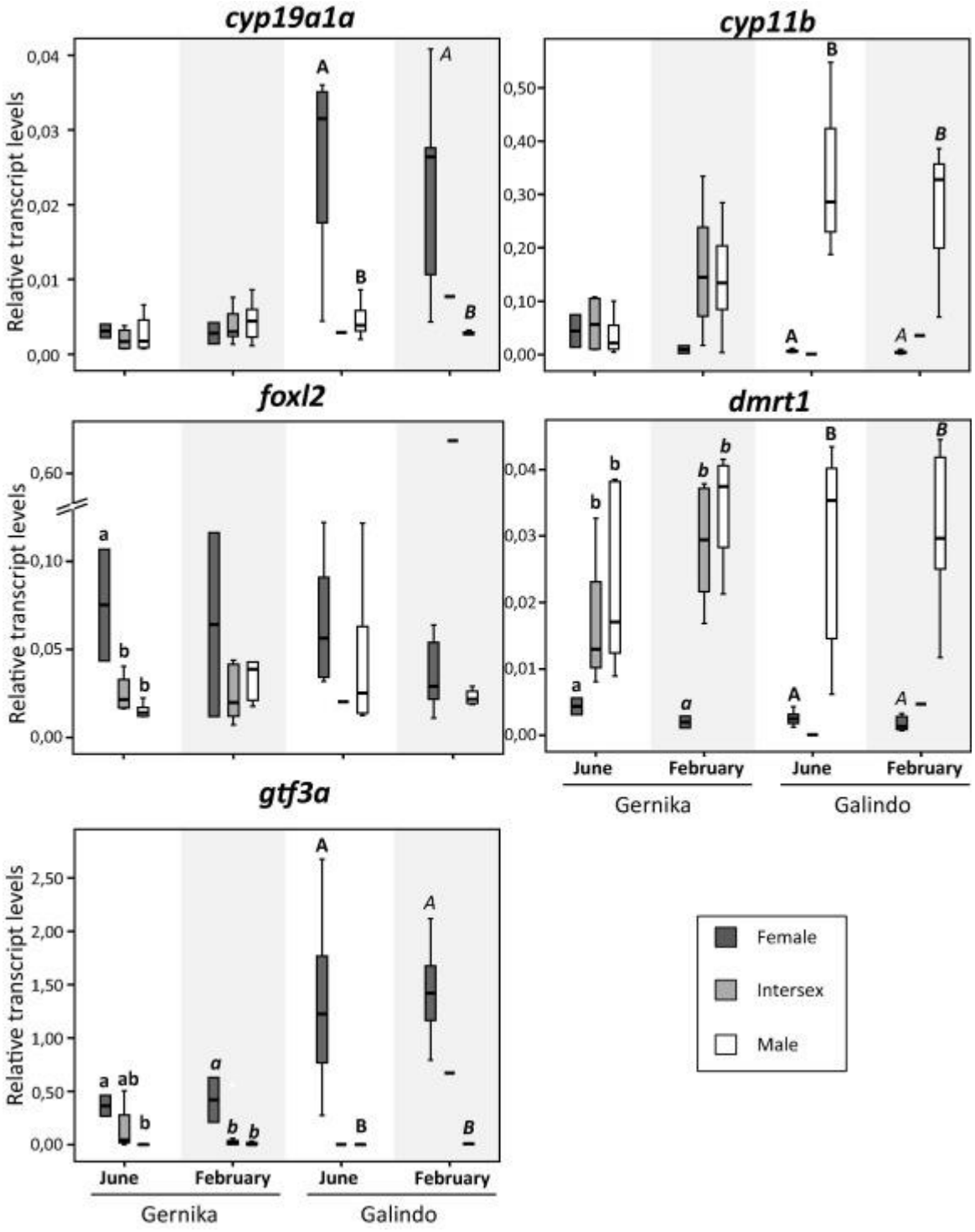

Fig. 3. Gene transcription levels of *cyp19a1a*, *cyp11b*, *foxl2*, *dmrt1* and *gtf3a* in gonads of female, intersex and male mullets from the two studied populations. Box-plots show median value (line), 25–75% quartiles (box) and standard deviations (whiskers). Lower case letters denote statistical significant differences between sexes at Gernika at each sampling season (bold for June and italics for February). Upper case letters indicate significant differences between sexes in Galindo at each sampling season (bold for June and italics for February). Intersex individuals at Galindo are only show for representative purposes and not included in the statistical analysis.

Transcription levels of *foxl2* were overall higher in females than in males and intersex mullets in both sampling seasons and sites. The exception was an extremely high transcription level detected in one intersex mullet from Galindo, in February (Fig. 3). Male mullets showed the highest *dmrt1* transcription levels at all of the sampling sites and during all seasons (Fig. 3). Ovaries showed very low transcription levels and intersex testes showed values in between those of ovaries and normal testes, but more similar to the transcription levels quantified in normal testes at Gernika. On the other hand, although at Galindo only two intersex individuals were identified, those individuals showed more ovary-like transcription levels (Fig. 3).
Ovaries of mullets from both sites and sampling periods showed significantly higher *gtf3a* transcription levels, than testes of normal males and intersex individuals (Fig. 3). Females from Gernika showed lower transcription levels than those from Galindo, during both sampling seasons (t = −9.284; p = 0, 033 (June) and t = −9010; p = 0.043 (February); Fig. 3). No seasonal dependent statistical differences between females of the same site were observed. Although not significant, intersex males from Gernika showed higher transcription levels than normal males, but below the levels observed in females.
In brains, there were no statistical differences in *cyp19a1b* at Gernika. At Galindo, females showed higher transcription levels in February, than males (Fig. 4). Transcription levels of *foxl2* were similar between females, males and intersex mullets in June, at both sampling sites. In February at Gernika, males and intersex mullets showed significantly higher levels than females. The same pattern between males and females was observed at Galindo (Fig. 4).

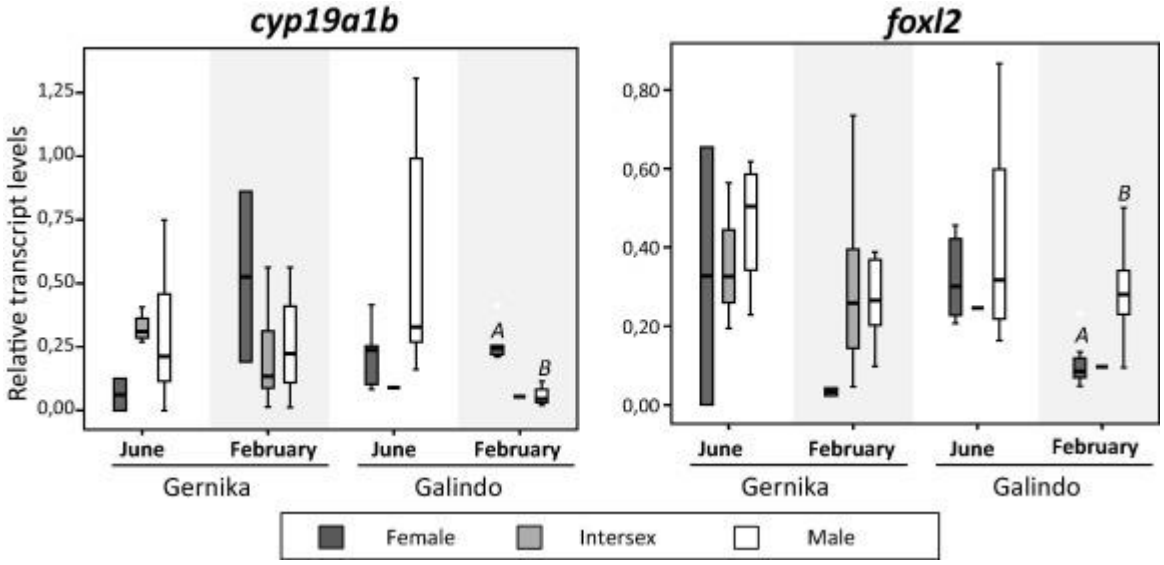

Fig. 4. Gene transcription levels of *cyp19a1b* and *foxl2* in brain of female, intersex and male thicklip grey mullets. Different letters denote significant statistical differences. Box-plots show median value (line), 25–75% quartiles (box) and standard deviations (whiskers). Lower case letters denote statistical significant differences between sexes in Gernika at each sampling season (bold for June and italics for February). Upper case letters indicate significant differences between sexes in Galindo at each sampling season (bold for June and italics for February). Intersex individuals at Galindo are only show for representative purposes and not included in the statistical analysis.

DISCUSSION

Most of the cases of intersex condition in wild fish have been reported in relation to exposure to WWTP effluents (Abdel-Moneim et al., 2015, Tyler and Jobling, 2008, Bahamonde et al., 2013, WHO/UNEP, 2013). In the present study the intersex prevalence in mullets captured downstream the WWTP of Gernika ranged between 36% and 90%. Such values are similar to those reported by Puy-Azurmendi et al. (2013) and by Bizarro et al. (2014) for the same mullet population sampled in April 2007 and June 2012. Bahamonde et al. (2013), who reviewed the information available about the intersex condition in wild fish populations, reported that the seasonal dependence of intersex condition has been rarely considered. In the light of the present and previous studies (Puy-Azurmendi et al., 2013), no seasonal dependence of intersex prevalence related to the stage within the gametogenic cycle can be considered in the thicklip grey mullets in the Basque coast.

The high incidence of intersex condition described in the mullets from Gernika, suggests that this is a site receiving large doses of xenoestrogenic/antiandrogenic compounds. The WWTP of Gernika which is about to be phased out, discharges to the Urdaibai estuary which in low tide is quite shallow and with little water volume. Consequently, chemicals are concentrated, and in this way, previous studies that have performed chemical analysis of water and sediment samples, and from bile of mullets captured in the same site, have shown high to moderate levels of xenoestrogenic chemicals; mainly alkylphenols (APs), phthalates, bisphenol A (BPA) and lindane (Cortazar et al., 2008, Bizkarguenaga et al., 2012, Bizarro et al., 2014, Puy-Azurmendi et al., 2013, Ros et al., 2015). In the case of Galindo, the present study is the first one focused on the local mullet population and thus, the first report of intersex individuals around the WWTP of the Bilbao estuary. Intersex prevalence was low, with only one individual identified in June 2013 and another one in February 2014. In previous studies in this river basin, but in the outer part of the estuary some kilometers away the Galindo WWTP effluent, another intersex mullet was identified (Puy-Azurmendi et al., 2013). High levels of APs, musk fragrances and lindane have been detected in the bile of mullets from the area, without any signs of intersex condition in studied individuals (Bizarro et al., 2014). The WWTP of Galindo processes the waste water of a much bigger population than that of Gernika, receiving also the waters of a nearby hospital and the busy industrial activity in the metropolitan area of Bilbao. In any case, Bizkarguenaga et al. (2012) concluded that the concentrations of APs, BPA, phthalates, hormones and pesticides was higher in the water discharged by the Gernika WWTP than in that discharged by the Galindo WWTP. A more modern, and thus efficient, depuration system implemented in the WWTP of Galindo in comparison to that of Gernika could explain these differences. In any case, and in relation to the xenoestrogenic effects of Galindo WWTP, more samplings are needed, as in the present study only a very limited amount of individuals could be analyzed, with only two intersex individuals identified.

When it comes to the molecular markers of exposure to xenoestrogenic compounds in fish the hepatic up-regulation of vitellogenin genes is the most widely used one (Arukwe and Goksøyr, 2003, Marin and Matozzo, 2004, Brander, 2013, Puy-Azurmendi et al., 2013, Bizarro et al., 2014). All studied male and intersex individuals showed detectable levels of *vtgAa* transcription in the liver, although interindividual differences were so high that no significant differences could be established between sampling sites, seasons or sexes. Transcription of *vtgAa* above basal levels in most individuals might indicate that both mullet populations have been exposed to xenoestrogenic chemicals. Previous studies in mullet populations in the Basque coast have reported *vtgAa* up-regulation (Puy-Azurmendi et al., 2013, Bizarro et al., 2014).

Transcription pattern of *gtf3a* was higher in ovaries than in testes of normal males while intersex testes showed intermediate transcription patterns in mullets from Gernika. Only two intersex individuals could be studied at Galindo, and the one identified in February showed levels in between ovaries and normal testes. *gtf3a* codes for the transcription factor necessary for RNA polymerase III driven synthesis of 5S rRNA, that is accumulated in oocytes to assist quick ribosome assembly for protein production during early embryogenesis (Diaz De Cerio et al., 2012, Ortiz-Zarragoitia et al., 2014, Rojo-Bartolomé et al., 2016). Consequently, transcription of 5S rRNA and *gtf3a* mark the presence of oocytes, also when they are produced within the testis. Thus intersex individuals present higher gonad transcription levels than normal males, something that can be observed along the whole annual reproductive cycle of mullets (Diaz de Cerio et al., 2012). No differences were observed between seasons in the present study, although it must be considered that in both studied seasons gonads displayed similar gametogenic stages. However, females from Galindo showed transcript levels higher than those from Gernika. If this could suggest an alteration in normal oogenesis in female mullets from Gernika due to exposure to EDCs, needs further research.

Cyp19a1a aromatase is the responsible for the conversion of androgens into oestrogens in gonads (Devlin and Nagahama, 2002). Transcription levels of *cyp19a1a* in *C. labrosus* studied previously in previtellogenic females from Gernika did not show differences with males (Puy-Azurmendi et al., 2013). Similarly, we did not find significant differences between ovaries and testis at Gernika, within this study. Males from Galindo on the opposite did show significantly lower transcription levels than females. Sardi et al. (2015) reported that ovaries with vitellogenic oocytes present higher *cyp19a1a* transcription levels than those in earlier gametogenesis stages with a subsequent down-regulation after spawning. Females from Galindo showed higher *cyp19a1a* transcription levels than females from Gernika. This suggests a down-regulation of *cyp19a1* in females from Gernika, similar to that described for *gtf3*, which could be related with the oestrogenic impact of the WWTP effluent. The inhibitory effect of oestrogens on *cyp19a1a* transcription has been reported in several studies (Ortiz-Zarragoitia et al., 2006, Nakamura et al., 2009, Sawyer et al., 2006, Urbatzka et al., 2012). It is known that xenoestrogens can attenuate, and even inhibit, the oestrogenic regulation of oocyte growth in fish ovaries, while feminizing at the same time the testis (Piferrer, 2001, Devlin and Nagahama, 2002, Ortiz-Zarragoitia et al., 2006).

In general, *cyp11b* transcription levels should be higher in testes of normal males than in ovaries but this did not happen in mullets captured at Gernika in June. This would suggest a down-regulation of *cyp11b* in testes that cannot be attributed to their early gametogenic stage, since males from Galindo in the same period showed relatively higher transcription levels. Testes transcription levels of *cyp11b* in mullets are higher during early spermatogenesis in comparison to other spermatogenic stages (Sardi et al., 2015). Regarding females, all ovaries showed low transcription levels, as described also by Sardi et al. (2015). Intersex males at Gernika showed transcription levels similar to those in normal male testes, with low transcription levels in June that were elevated in February.

Foxl2 is an ovarian differentiation gene in vertebrates (Baron, 2004) and an upstream transcriptional regulator of *cyp19a1a* aromatase in the ovary (Guiguen et al., 2010). High transcription levels have been reported in ovaries of mullets from Pasaia harbor during vitellogenesis,. In mullets from Gernika and Galindo, *foxl2* transcription levels were also higher in females than in males; corresponding with ovaries at previtellogenic stage.

Higher *foxl2* transcription levels in ovaries than in testes has also been reported in fish populations exposed to WWTP effluents in rainbow darter, *Etherostoma caeruleum* (Bahamonde et al., 2014). In our study testes in both sampling sites and seasons showed low but still detectable transcription levels, similar to the low levels measured in testes during spermatogenesis in the gametogenic

cycle study of the harbor of Pasaia (Fig. 2). This has also been described in the Nile tilapia, *Oreochromis niloticus* (Wang et al., 2004). Baron (2004) suggested for rainbow trout, *Oncorhynchus mykiss*, that exogenous oestrogens could rapidly trigger *foxl2* transcription in testes, something necessary to trigger testis feminization, oocyte maintenance and growth. The upregulation of *foxl2* in the intersex mullets from Galindo in February could be due to a recent exposure to xenoestrogenic substances. Intersex males from Gernika showed slightly up-regulated *foxl2* in comparison to non intersex males in June.

Coming to *dmrt1,* this gene seems to play a key role in fish gonadal differentiation, being highly expressed in testis (Marchand et al., 2000, Herpin and Schartl, 2011). In fact, in most teleost fish *dmrt1* transcription has been reported in testis although in some cases it has also been detected in ovaries (Herpin and Schartl, 2011, Johnsen et al., 2010). Accordingly, *dmrt1* expression in mullet ovaries from Pasaia harbor was low with the exception of females with previtellogenic stage oocytes, suggesting that in mullets *dmrt1* could also play a role in early oogenesis. Overall male mullets showed higher *dmrt1* transcript levels than females, peaking at early spermatogenic stage. Marchand et al. (2000) described that *dmrt1* transcription was suppressed in rainbow trout males exposed to oestradiol, concluding that feminizing agents can repress *dmrt1* transcription. In male rainbow darter transcription is down-regulated after exposure to WWTP effluents (Bahamonde et al., 2014).

Regarding transcription patterns of female marker genes in brain, *foxl2* transcription levels were higher in males than in females in February. The transcription of *foxl2* shows also a sexual dimorphic pattern of transcription in several fish species (Jiang et al., 2011, Sridevi and Senthilkumaran, 2011). In the case of *cyp19a1b* aromatase, this enzyme plays the same role in brain as *cyp19a1a* does in gonads. Transcription of *cyp19a1b* in female brain is usually high (Diotel et al., 2010) but in this study differences between females and males could only be observed in mullets captured at Galindo in February. This suggests that the functioning of brain steroidogenesis in mullets downstream of the two WWTPs studied was altered.

CONCLUSIONS

The presence of intersex male mullets together with detectable levels of hepatic vtgAa transcription in males and intersex males are indicative of exposure to xenoestrogens downstream of both studied WWTPs. The presence of intersex mullets was identified for the first time at Galindo downstream of the WWTP of the Bilbao metropolitan area. Nevertheless, the prevalence of intersex males was higher at Gernika than at Galindo. Moreover, differences regarding transcription levels of cyp19a1a and gtf3a in females from both sites, suggested altered oogenesis in females from Gernika, as they showed lower transcription levels than females from Galindo. In addition, differences in cyp11b transcription in males, with a down-regulation in Gernika in comparison to Galindo, would be also indicative of altered gametogenesis in Gernika. Thus, mullets from Gernika showed signs of more severe EDC exposure than those from Galindo. Although no direct association between xenoestrogen exposure and intersex condition was established, alteration of the transcription levels of key genes for the control of gametogenesis was described in mullets from Gernika which were identical in both studied seasons. Further research is required in order to assess the early effects of EDCs complex mixtures in the intersex condition development in mullets.

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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REFERENCES

Abdel-Moneim et al., 2015

Abdel-Moneim, D.P. Coulter, C.T. Mahapatra, M.S. Sepúlveda

Intersex in fishes and amphibians: population implications, prevalence, mechanisms and molecular biomarkers

J. Appl. Toxicol., 35 (2015), pp. 1228-1240

Alam et al., 2008

M.A. Alam, Y. Kobayashi, R. Horiguchi, T. Hirai, M. Nakamura

Molecular cloning and quantitative expression of sexually dimorphic markers Dmrt1 and Foxl2 during female-to-male sex change in *Epinephelus merra*
Gen. Comp. Endocrinol., 157 (2008), pp. 75-85

Arukwe, 2008

Arukwe

Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc) regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish

Cell Biol. Toxicol., 24 (2008), pp. 527-540

Arukwe and Goksøyr, 2003

Arukwe, A. Goksøyr

Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption

Comp. Hepatol., 2 (2003), p. 4

Bahamonde et al., 2013

P.A. Bahamonde, K.R. Munkittrick, C.J. Martyniuk

Intersex in teleost fish: are we distinguishing endocrine disruption from natural phenomena?

Gen. Comp. Endocrinol., 192 (2013), pp. 25-35

Bahamonde et al., 2014

P.A. Bahamonde, G.R. Tetreault, M.E. McMaster, M.R. Servos, C.J. Martyniuk, K.R. Mu nkittrick

Molecular signatures in rainbow darter (*Etheostoma caeruleum*) inhabiting an urbanized river reach receiving wastewater effluents
Aquat. Toxicol., 148 (2014), pp. 211-220

Bahamonde et al., 2015a

P.A. Bahamonde, M.L. Fuzzen, C.J. Bennett, G.R. Tetreault, M.E. McMaster, M.R. Servo s, C.J. Martyniuk, K.R. Munkittrick

Whole organism responses and intersex severity in rainbow darter (*Etheostoma caeruleum*) following exposures to municipal wastewater in the Grand River basin, ON, Canada. Part A
Aquat. Toxicol., 159 (2015), pp. 290-301

Bahamonde et al., 2015b

P.A. Bahamonde, M.E. McMaster, M.R. Servos, C.J. Martyniuk, K.R. Munkittrick

Molecular pathways associated with the intersex condition in rainbow darter (*Etheostoma caeruleum*) following exposures to municipal wastewater in the Grand River basin, ON, Canada. Part B
Aquat. Toxicol., 159 (2015), pp. 302-316

Baron, 2004

D. Baron

An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation

J. Mol. Endocrinol., 33 (2004), pp. 705-715

Bizarro et al., 2014

C. Bizarro, O. Ros, A. Vallejo, A. Prieto, N. Etxebarria, M.P. Cajaraville, M. Ortiz-Zarragoitia

Intersex condition and molecular markers of endocrine disruption in relation with burdens of emerging pollutants in thicklip grey mullets (*Chelon labrosus*) from Basque estuaries (South-East Bay of Biscay)
Mar. Environ. Res., 96 (2014), pp. 19-28

Bizkarguenaga et al., 2012

E. Bizkarguenaga, O. Ros, A. Iparraguirre, P. Navarro, A. Vallejo, A. Usobiaga, O. Zuloag a

Solid-phase extraction combined with large volume injection-programmable temperature vaporization-gas chromatography-mass spectrometry for the multiresidue determination of priority and emerging organic pollutants in wastewater

J. Chromatogr. A, 1247 (2012), pp. 104-117

Bjerregaard et al., 2006

L.B. Bjerregaard, B. Korsgaard, P. Bjerregaard

Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams Ecotoxicol. Environ. Saf., 64 (2006), pp. 321-328

Brander, 2013

S.M. Brander

Thinking outside the box: assessing the endocrine disruption in aquatic life

S. Ajuha (Ed.), Monitoring Water Quality: Pollution Assessment, Analysis and Remediation, Amsterdam (2013), pp. 103-107

Campbell et al., 2006

C.G. Campbell, S.E. Borglin, F.B. Green, A. Grayson, E. Wozei, W.T. Stringfellow

Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: a review

Chemosphere, 65 (2006), pp. 1256-1280

Cheshenko et al., 2008

K. Cheshenko, F. Pakdel, H. Segner, O. Kah, R.I.L. Eggen

Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish

Gen. Comp. Endocrinol., 155 (2008), pp. 31-62

Cortazar et al., 2008

E. Cortazar, L. Bartolome, S. Arrasate, A. Usobiaga, J.C. Raposo, O. Zuloaga, N. Etxebarr ia

Distribution and bioaccumulation of PAHs in the UNESCO protected natural reserve of Urdaibai, Bay of Biscay

Chemosphere, 72 (2008), pp. 1467-1474

Devlin and Nagahama, 2002

R.H. Devlin, Y. Nagahama

Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences

Aquaculture, 208 (2002), pp. 191-364

Diaz De Cerio et al., 2012

O. Diaz De Cerio, I. Rojo-Bartolomé, C. Bizarro, M. Ortiz-Zarragoitia, I. Cancio

5S rRNA and accompanying proteins in gonads: powerful markers to identify sex and reproductive endocrine disruption in fish

Environ. Sci. Technol., 46 (2012), pp. 7763-7771

Diotel et al., 2010

N. Diotel, Y. Le, K. Mouriec, S. Tong, E. Pellegrini, C. Vaillant, I. Anglade, F. Brion, F. Pak del, B. Chung, O. Kah

Frontiers in Neuroendocrinology Aromatase in the brain of teleost fish: expression, regulation and putative functions

Front. Neuroendocrinol., 31 (2010), pp. 172-192

Efron and Tibshirani, 1993

B. Efron, R.J. Tibshirani

An Introduction to the Bootstrap

Chapman & Hall, London (1993), p. 456

Filby et al., 2007

A.L. Filby, E.M. Santos, K.L. Thorpe, G. Maack, C.R. Tyler

Gene expression profiling for understanding chemical causation of biological effects for complex mixtures: a case study on estrogens

Environ. Sci. Technol., 41 (2007), pp. 8187-8194

Gamble and Wilson, 2002

M. Gamble, I. Wilson

The hematoxylin and eosin

J.D. Bancroft, M. Gamble (Eds.), Theory and Practice of Histological Techniques, Churchill Livingstone-Elsevier Science, London (2002), pp. 125-138

García-Barcina et al., 2006

J.M. García-Barcina, A. González-Oreja, A. De la Sota

Assessing the improvement of the Bilbao estuary water quality in response to pollution abatement measures

Water Res., 40 (2006), pp. 951-960

Guiguen et al., 2010

Y. Guiguen, A. Fostier, F. Piferrer, C.F. Chang

Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish

Gen. Comp. Endocrinol., 165 (2010), pp. 352-366

Herpin and Schartl, 2011

Herpin, M. Schartl

Dmrt1 genes at the crossroads: a widespread and central class of sexual development factors in fish

FEBS J., 278 (2011), pp. 1010-1019

Jiang et al., 2011

W. Jiang, Y. Yang, D. Zhao, X. Liu, J. Duan, S. Xie, H. Zhao

Effects of sexual steroids on the expression of foxl2 in *Gobiocypris rarus*
Comp. Biochem. Physiol. – B Biochem. Mol. Biol., 160 (2011), pp. 187-193

Jobling and Tyler, 2003

S. Jobling, C.R. Tyler

Endocrine disruption in wild freshwater fish

Pure Appl. Chem., 75 (2003), pp. 2219-2234

Jobling et al., 1998

S. Jobling, M. Nolan, C.R. Tyler, G. Brighty, J.P. Sumpter

Widespread sexual disruption in wild fish

Environ. Sci. Technol., 32 (1998), pp. 2498-2506

Jobling et al., 2002

S. Jobling, N. Beresford, M. Nolan, T. Rodgers-
Gray, G.C. Brighty, J.P. Sumpter, C.R. Tyler

Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents
Biol. Reprod., 66 (2002), pp. 272-281

Jobling et al., 2006

S. Jobling, R. Williams, A. Johnson, A. Taylor, M. Gross-
Sorokin, M. Nolan, C.R. Tyler, R. van Aerle, E. Santos, G. Brighty

Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations

Environ. Health Perspect., 114 (2006), pp. 32-39

Johnsen et al., 2010

H. Johnsen, M. Seppola, J.S. Torgersen, M. Delghandi, Ø. Andersen

Sexually dimorphic expression of dmrt1 in immature and mature Atlantic cod (*Gadus morhua* L.)
Comp. Biochem. Physiol. B: Biochem. Mol. Biol., 156 (2010), pp. 197-205

Kazeto et al., 2004

Y. Kazeto, A.R. Place, J.M. Trant

Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (Danio rerio) juveniles

Aquat. Toxicol., 69 (2004), pp. 25-34

Körner et al., 2005

O. Körner, E.L.M. Vermeirssen, P. Burkhardt-Holm

Intersex in feral brown trout from Swiss midland rivers

J. Fish Biol., 67 (2005), pp. 1734-1740

Lubzens et al., 2010

E. Lubzens, G. Young, J. Bobe, J. Cerda

Oogenesis in teleosts: how eggs are formed

Gen. Comp. Endocrinol., 165 (2010), pp. 367-389

Marchand et al., 2000

O. Marchand, M. Govoroun, H. D'Cotta, O. McMeel, J.J. Lareyre, A. Bernot, V. Laudet, Y. Guiguen

DMRT1 expression during gonadal differentiation and spermatogenesis in the rainbow trout, *Oncorhynchus mykiss*
Biochim. Biophys. Acta – Gene Struct. Expr., 1493 (2000), pp. 180-187

Marin and Matozzo, 2004

M.G. Marin, V. Matozzo

Vitellogenin induction as a biomarker of exposure to estrogenic compounds in aquatic environments

Mar. Pollut. Bull., 48 (2004), pp. 835-839

Matthiessen, 2003

P. Matthiessen

Endocrine disruption in marine fish

Pure Appl. Chem., 75 (2003), pp. 2249-2261

Mazabraud et al., 1975

Mazabraud, M. Wegnez, H. Denis

Biochemical research on oogenesis: RNA accumulation in the oocytes of teleosts

Dev. Biol., 44 (1975), pp. 326-332

McDonough et al., 2005

C.J. McDonough, W.A. Roumillat, C.A. Wenner

Sexual differentiation and gonad development in striped mullet (*Mugil cephalus* L.) from South Carolina estuaries
Fish. Bull., 103 (2005), pp. 601-619

Nakamura et al., 2009

Nakamura, M. Kusakabe, G. Young

Differential suppressive effects of low physiological doses of estradiol-17β in vivo on levels of mRNAs encoding steroidogenic acute regulatory protein and three steroidogenic enzymes in previtellogenic ovarian follicles of rainbow trout

Gen. Comp. Endocrinol., 163 (2009), pp. 318-323

Ortiz-Zarragoitia et al., 2006

M. Ortiz-Zarragoitia, J.M. Trant, M.P. Cajaraville

Effects of dibutylphthalate and ethynylestradiol on liver peroxisomes, reproduction, and development of zebrafish (*Danio rerio*)
Environ. Toxicol. Chem., 25 (2006), pp. 2394-2404

Ortiz-Zarragoitia et al., 2014

M. Ortiz-Zarragoitia, C. Bizarro, I. Rojo-Bartolomé, O.D. de
Cerio, M.P. Cajaraville, I. Cancio

Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine environments

Mar. Drugs, 12 (2014), pp. 4756-4782

Piferrer, 2001

F. Piferrer

Endocrine sex control strategies for the feminization of teleost fish

Aquaculture, 197 (2001), pp. 229-281

Piferrer, 2011

F. Piferrer

Hormonal control of reproduction and growth: endocrine control of sex differentiation in fish

A.P. Farrell (Ed.), Encyclopedia of Fish Physiology, Academic Press, London, UK (2011), pp. 1490-1499

Piferrer et al., 2012

F. Piferrer, L. Ribas, N. Díaz

Genomic approaches to study genetic and environmental influences on fish sex determination and differentiation

Mar. Biotechnol., 14 (2012), pp. 591-604

Puy-Azurmendi et al., 2013

E. Puy-Azurmendi, M. Ortiz-
Zarragoitia, M. Villagrasa, M. Kuster, P. Aragón, J. Atienza, R. Puchades, A. Maquieira, C. Domínguez, M. López de
Alda, D. Fernandes, C. Porte, J.M. Bayona, D. Barceló, M.P. Cajaraville

Endocrine disruption in thicklip grey mullet (*Chelon labrosus*) from the Urdaibai Biosphere Reserve (Bay of Biscay, Southwestern Europe)
Sci. Total Environ., 443 (2013), pp. 233-244

Rojo-Bartolomé et al., 2016

Rojo-Bartolomé, O. Diaz de Cerio, G. Diez, I. Cancio

Identification of sex and female's reproductive stage in commercial fish species through the quantification of ribosomal transcripts in gonads

PLoS ONE, 11 (2) (2016), p. e0149711

Ros et al., 2015

O. Ros, J.K. Izaguirre, M. Olivares, C. Bizarro, M. Ortiz-
Zarragoitia, M.P. Cajaraville, N. Etxebarria, A. Prieto, A. Vallejo

Determination of endocrine disrupting compounds and their metabolites in fish bile

Sci. Total Environ., 536 (2015), pp. 261-267

Sardi et al., 2015

A.E. Sardi, C. Bizarro, M.P. Cajaraville, M. Ortiz-Zarragoitia

Steroidogenesis and phase II conjugation during the gametogenesis of thicklip grey mullet (*Chelon labrosus*) from a population showing intersex condition
Gen. Comp. Endocrinol. (2015)

Sawyer et al., 2006

S.J. Sawyer, K.A. Gerstner, G.V. Callard

Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: gene specific tissue distribution, sex differences, developmental programming, and estrogen regulation

Gen. Comp. Endocrinol., 147 (2) (2006), pp. 108-117

Schulz et al., 2010

R.W. Schulz, L.R. de Franca, J.J. Lareyre, F. LeGac, H. Chiarini-Garcia, R.H. Nobrega, T. Miura

Spermatogenesis in fish

Gen. Comp. Endocrinol., 165 (2010), pp. 390-411

Smith et al., 2013

E.K. Smith, J.M. Guzmán, J.A. Luckenbach

Molecular cloning, characterization, and sexually dimorphic expression of five major sex differentiation-related genes in a Scorpaeniform fish, sablefish (*Anoplopoma fimbria*)
Comp. Biochem. Physiol. – B Biochem. Mol. Biol., 165 (2013), pp. 125-137

Sridevi and Senthilkumaran, 2011

P. Sridevi, B. Senthilkumaran

Cloning and differential expression of FOXL2 during ovarian development and recrudescence of the catfish, *Clarias gariepinus*
Gen. Comp. Endocrinol., 174 (2011), pp. 259-268

Thorpe et al., 2001

K.L. Thorpe, T.H. Hutchinson, M.J. Hetheridge, M. Scholze, J.P. Sumpter, C.R. Tyler

Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (*Oncorhynchus mykiss*)
Environ. Sci. Technol., 35 (2001), pp. 2476-2481

Thorpe et al., 2003

K.L. Thorpe, R.I. Cummings, T.H. Hutchinson, M. Scholze, G. Brighty, J.P. Sumpter, C.R. Tyler

Relative potencies and combination effects of steroidal estrogens in fish

Environ. Sci. Technol., 37 (2003), pp. 1142-1149

Tyler and Jobling, 2008

C.R. Tyler, S. Jobling

Roach, sex, and gender-bending chemicals: the feminization of wild fish in English rivers

Bioscience, 58 (2008), pp. 1051-1059

Tyler and Routledge, 1998

C.R. Tyler, E.J. Routledge

Oestrogenic effects in fish in English rivers with evidence of their causation

Pure Appl. Chem., 70 (1998), pp. 1795-1804

Uno et al., 2012

T. Uno, M. Ishizuka, T. Itakura

Cytochrome P450 (CYP) in fish

Environ. Toxicol. Pharmacol., 34 (2012), pp. 1-13

Urbatzka et al., 2012

R. Urbatzka, E. Rocha, B. Reis, C. Cruzeiro, R.A.F. Monteiro, M.J. Rocha

Effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens on steroidogenic gene expression and specific transcription factors in zebrafish

Environ. Pollut., 164 (2012), pp. 28-35

Wang et al., 2004

D.S. Wang, T. Kobayashi, L.Y. Zhou, Y. Nagahama

Molecular cloning and gene expression of Foxl2 in the Nile tilapia, *Oreochromis niloticus*
Biochem. Biophys. Res. Commun., 320 (2004), pp. 83-89

WHO/UNEP, 2013

WHO/UNEP (World Health Organization/United Nations Environmental Programme)

Bergman, J.J. Heindel, S. Jobling, K.A. Kidd, R.T. Zoelle (Eds.), State of the Science of Endocrine Disrupting Chemicals (2013), p. 260

Woodling et al., 2006

J.D. Woodling, E.M. Lopez, T.A. Maldonado, D.O. Norris, A.M. Vajda

Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams

Comp. Biochem. Physiol. C, 144 (2006), pp. 10-15