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Molecular mechanisms in the development of the intersex condition in *Chelon labrosus* exposed to environmental xenoestrogens

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Introduction

1. Sex determination and sex differentiation in teleost fish

Sex determination is the set of molecular and cellular processes that under genetic or environmental control, or under a combination or both, initiate the responses leading to stablish the sexual characteristics of an organism (Penman and Piferrer, 2008). In teleosts, genotypic and environmental sex determination present different prevalence and characteristics depending of the species under investigation (Piferrer et al., 2012) (Figure 1). Genotypic sex determination for instance includes cases of polygenic as well as cases of sex chromosome associated determination. In this case, we can find fish that possess a XX/XY or a ZZ/ZW sex chromosome systems. During environmental sex determination, where temperature plays the leading role, we can also find cases where pH, oxygen availability, food availability and population density participate in regulating the process (Baroiller and Guiguen, 2001; Devlin and Nagahama, 2002; Piferrer et al., 2012). Regarding genetically determined sex determination, different genes, described as mainly male-specific and located in the Y chromosome (Fernandino and Hattori, 2019), have been identified as master switches or as sex determining genes in teleosts (Penman and Piferrer, 2008). Differences in such master genes have been identified even among closely related species such as *Oryzias latipes* and *Oryzias luzonensis*, with two different genes, *dmrt1Y* (doublesex and mab-3 related transcription factor 1) in *O. latipes* and *gsdfY* (gonadal soma-derived growth factor) in *O. luzonensis* (Matsuda et al., 2002; Myosho et al., 2012). In salmonids, *sdY* (sexually dimorphic on the Y-chromosome) has been characterized as the main master gene in rainbow trout (*Oncorhynchus mykiss*), while its presence and male specificity has been described in other species in the same family (Yano et al., 2013). In other species, genes related with the anti-Müllerian hormone have been described as the sex determining genes, for instance *amhY* in Patagonian pejerrey *Odontesthes hatcheri* (Hattori et al., 2013) and *amhr2* (amh receptor type II) in tiger pufferfish *Takifugu rubripes* (Kamiya et al., 2012). In the platyfish (*Xiphophorus maculatus*) and in the three-spine stickleback (*Gaterosteus aculeatus*), although a single sex determining gene has not been identified, a sex determining region has been mapped in the Y chromosome (Froschauer et al., 2002; Peichel et al., 2004). Hence, the sex determining genetic system seems quite plastic in teleosts (Penman and Piferrer, 2008).

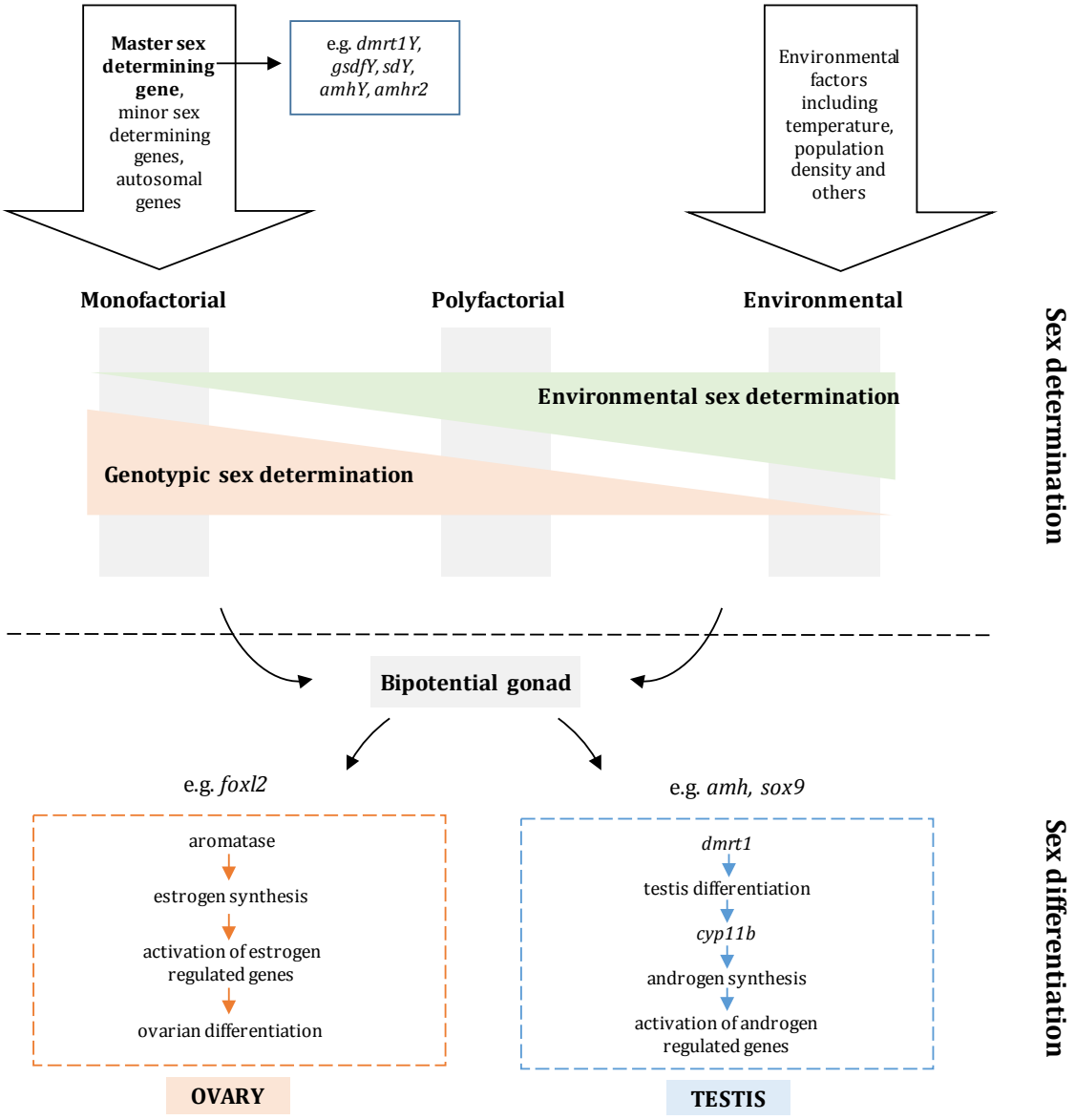

Figure 1
Diagram indicating the main sex determining and sex differentiation pathways in teleost fish, leading to the generation of a testis or an ovary from a bipotential gonad (Based on the figures by Piferrer and Guiguen (2008) and Piferrer et al. (2012)). Arrows do not reflect cause-effect events. The events described for females and males do not necessarily occur at the same time during development and are not equivalent.

Sex differentiation encompasses the genetic, cellular and physiological processes resulting from sex determination that transform an undifferentiated gonad into a functional testis or ovary (Piferrer and Guiguen, 2008). In fish, it is a labile and plastic process and can be controlled by a multitude of mechanisms. Genetic and environmental factors (e.g. temperature) or behavioral interactions can affect fish sex differentiation. Endocrine control of sex differentiation is complex as it involves various organs and biochemical, cellular and physiological pathways, that have been extensively studied (Devlin and

Nagahama, 2002; Strussmann and Nakamura, 2002; Piferrer et al., 2005). Although the genes and mechanisms underlying sex differentiation are quite conserved among species, inter-species differences have been observed in relation to the gene sequences and products involved, sex-specificity of responses and/or temporal windows of expression and activity (Piferrer and Guiguen, 2008). Many of such genes code for a set of proteins that can be grouped as steroidogenic enzymes, sex steroid receptors, and transcription factors (Piferrer and Guiguen, 2008; Piferrer et al., 2012). Steroids were proposed to be the main inducers of sex differentiation in teleosts (Yamamoto, 1969), as prior to any sign of histological sex differentiation, differences regarding steroid content and steroidogenic capacity become apparent. Many of these genes expressed differentially during sex differentiation are common for males and females, in many circumstances being their differential expression levels, or expression time windows, their main differentiating characteristic (Piferrer et al., 2012; Table 1). Some other genes, such as *foxl2* (forkhead box L2) and *dmrt1,* have been described to show a sex dimorphic transcription pattern, with higher transcriptional activity of *foxl2* in females, and of *dmrt1* in the gonads of differentiating males (Baron et al., 2004; Herpin and Schartl, 2011). However, the sex differentiation process is fish is more complicated and some mechanisms appear to be steroid independent (reviewed by Piferrer and Guiguen, 2008).

Among the approximately 30,000 known teleost species described to date, all sexuality types described for vertebrates can be found, ranging from gonochorism to hermaphroditism (simultaneous or sequential) and unisexuality (Piferrer, 2011). Many teleosts are gonochoristic, with fully differentiated mature females and males that maintain their differentiated sex during their entire life span. Two types of gonochorism exist in fish (Devlin and Nagahama, 2002) with differentiated or primary gonochoristic species being those in which the undifferentiated gonad becomes directly an ovary or a testis, and undifferentiated gonochoristic species grouping the species in which all individuals initially develop an immature ovary. There is a third case, termed secondary gonochorism, in which initially an immature intersexual gonad is formed, further maturing into one sex or the other (Devlin and Nagahama, 2002). Among gonochoristic species, intersex gonads have been described, producing both oocytes and sperm cells in their gonad in different proportions, ranging from a small proportion of cells of one sex within a fully differentiated gonad of the opposite sex, to large proportions of both coexisting gametes (Devlin and Nagahama, 2002). Nevertheless, the intersex condition is usually described as a consequence of exposure to environmental stress, most commonly exposure to contaminants either (anti)xenoestrogenic or (anti)androgenic. On the contrary, hermaphrodite fish can

naturally produce mature and functional female and male gametes, simultaneously or alternated in time during their life span. Among hermaphrodites, simultaneous or synchronous hermaphrodites produce mature oocytes and sperm at the same time, whereas sequential hermaphrodites produce one type of mature gamete and after sex reversal produce mature cells of the other type. Among sequential hermaphrodites we distinguish protandrous hermaphrodites, that first develop as functional males to later transform in functional females, and protogynous hermaphrodites with an opposite sex reversal process (Devlin and Nagahama, 2002). Very few species, all of them gobid fish, have been described as bidirectional sequential hermaphrodites, switching alternatively form one sex to the other depending on different environmental and social cues (Devlin and Nagahama, 2002). Mention also, that we can find groups of fish, notably among the perciformes (sparids, serranids, labrids…) in which some species are strictly gonochoristic while other species present different types of hermaphroditism, demonstrating the high plasticity of sex differentiation strategies among teleost fish.

Table 1
List of genes that have been proposed as sex differentiation markers in fish. M: male. F: female. Modified from Rojo-Bartolomé, 2017.

Type of cell or tissue	Genes
Primordial germ cells	*vasa, piwil1 (m), piwil2, nanos2 (f), nanos2, dnd, wt1a, dazl, buc (f), cxcr4b, sdf-1α receptor, vg1-rbp (f), foxh1 (f)*
Testis	*ccng2, hsp70, sept4, tuba7, spermatogenesis associated 4, testis-specific A-kinase anchoring protein, tektin 1, igf1, nr0b1*
Sertoli cell	*amh, sox9a, dmrt1, ar, ff1d*
Leydig cell	*cyp11b, hsd3b2, cyp17a, star, nr5a1*
Spermatic cells	*gsdf, protamines, sycp3l*
Ovary	*sox9b, zp1, zp2, zp3, vgr, 5S rRNA, tfIIIa, 42sp43, impα1, impα2, zar1, mos, gdf9, bmp15, btg3, lcal, lharm, alv, mPR*
Teca and granulosa cells	*cyp19a1a, foxl2a, foxl2b, fst, fshb, bmp4, figα, lhr, fshr, star, P450scc, 3β.hsd, cyp17, 20β-hsd*
Oocytes	*tpte, rbpms2, cx44.2, sox11b, ccnb2, activinβ, tbp, sox3, fem1c*

2. Gametogenesis

2.1. Oogenesis

Ovarian development is very different from species to species. We can find ovaries in fish in which the development is synchronous, this meaning that all oocytes present in the ovary develop at the same time, and thus only oocytes in one given stage of oogenesis can be found at one given moment. Some species are group-synchronous, when mainly two or more populations of developing oocytes are present in the ovary or, asynchronous, if oocytes at all possible developmental stages, from previtellogenic to fully mature, can be found simultaneously (Wallace and Selman, 1981).

Ovarian development is a dynamic process and although it is difficult to determine when one step has finished to begin the next one, several typical steps can be described (Tyler and Sumpter, 1996; Patiño and Sullivan, 2002; Lubzens et al., 2010; Urbatzka et al., 2011). Although differences among species exist due to the variety of reproductive strategies, the basic events of oocyte development apply to most of them (Figure 2). First, primordial germ cells (PGCs), the progenitor cells of the germ cell lineage formed in the embryo, migrate to the genital ridge and after a short proliferative stage differentiate into oogonias or spermatogonias (Yoshizaki et al., 2002; Lubzens et al., 2010). In PGCs, the transcription of *vasa*, which encodes for an ATP-dependent RNA helicase of the DEAD box family, takes place (Hay et al., 1988; Lasko and Ashburner, 1988; Yoon et al., 1997). The Vasa protein has been suggested to be important for the formation, migration and proliferation of the PGCs (Yoshizaki et al., 2002). Vasa is considered a germ cell marker (e.g. Kobayashi et al., 2000; Raghuveer and Senthilkumaran, 2010; Cao et al., 2012). In addition, its transcripts are present in the cytoplasm of oogonias and in the oocyte cortex of vitellogenic oocytes in zebrafish *Danio rerio* (Braat et al., 1999a; 1999b) and in tilapia *Oreochromis niloticus* (Kobayashi et al., 2000).

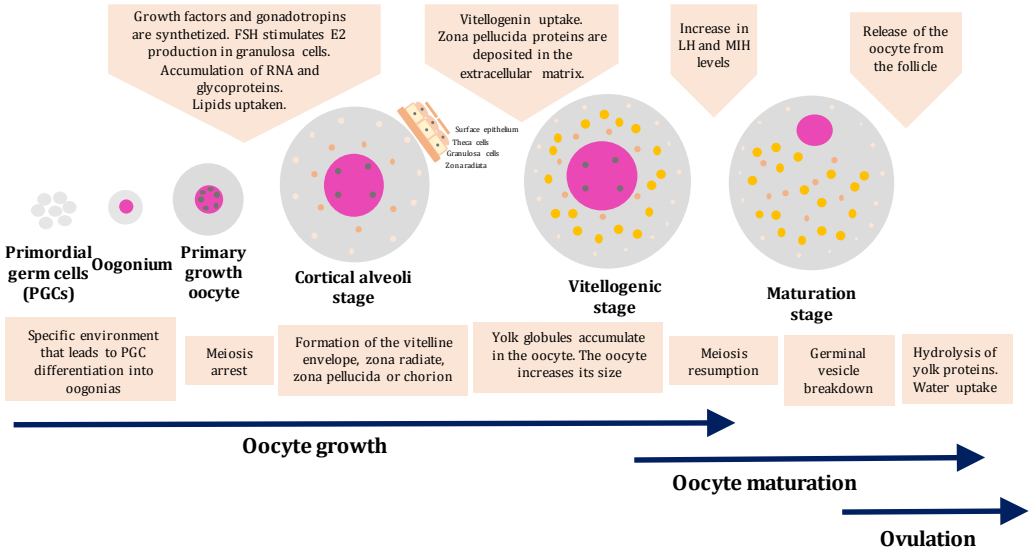

Figure 2
Schematic description of oogenesis, showing relevant events during oocyte growth and maturation. E2: 17β-estradiol. FSH: follicle stimulating hormone. LH: luteinizing hormone. MIH: maturation-inducing hormone. See text for a more detailed description. Based on a scheme by Urbatzka et al., (2011).

The upregulation and downregulation of male or female specific genes will determine the differentiation fate of PGCs. Unlike in mammals, where the presence of the *SRY* gene in the Y chromosome will trigger the development of a testis and the absence will allow the formation of an ovary (Hughes, 2001), in fish there is no universal sex determining gene (see above) and the process is more dynamic. In Nile tilapia for instance Foxl2 and Cyp19a1 aromatase are expressed during early gonad differentiation in XX females but not in males, suggesting their involvement in female sex differentiation (Ijiri et al., 2008). Such sexual dimorphic expression has also been described in rainbow trout (Vizziano et al., 2007). PGCs transform into oogonias which multiply by mitosis forming oogonial nests. For ovariogenesis, the maintenance of the expression of ovarian-specific genes is important (Lubzens et al., 2010; Table 1). The primary growth of oogonias to become previtellogenic oocytes has been suggested to be mediated by gonadotropins (Lubzens et al., 2010). Once such oocytes are formed they are enclosed by the granulosa cells that will form the inner layer and theca cells that will form the outer layer of the oocyte follicle (Urbatzka et al., 2011). Those cell layers will be especially involved in steroidogenesis and oocyte nourishment (Nagahama, 1994).

The activation of several other genes will be necessary to follow with the oocyte development. For instance, growth factors and gonadotropins are necessary for the regulation of the primary oocyte growth which is arrested in the first prophase stage (Tyler and Sumpter, 1996; Lubzens et al., 2010). In that phase, RNA molecules and glycoproteins

are actively synthetized (Wallace and Selman, 1990) as oocytes grow in size. Among the RNA molecules accumulated during previtellogenesis, 5S ribosomal RNA (rRNA) is the most abundant one (e.g. Mazabraud et al., 1975; Gornung et al., 2007; Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2016). 5S rRNA is the smallest rRNA that participates in the formation of the large ribosome subunit in eukaryotic cells (Szymański et al., 2003). It is stockpiled in the cytosol of oocytes of teleost fish so that if the oocyte is fertilized, a quick assembly of the ribosomes is facilitated to sustain early embryo protein synthesis (Diaz de Cerio et al., 2012). Lipids also start to accumulate during previtellogenesis and this progressively leads to the formation of cortical alveoli stage oocytes, which accumulate cortical alveoli in the cell cortex (Lubzens et al., 2010). Such alveoli keep migrating to the peripheral ooplasm to form one or various layers of alveoli as growth continues (Blazer, 2002). During the cortical alveoli stage a special extracellular matrix is formed around the oocyte, the vitelline envelope, also known as *zona pellucida* (ZP), *zona radiata* (ZR) or chorion. The structure and composition of the layer differs from species to species. It is formed by ZP or ZR proteins or choriogenins that are synthetized by the liver, the oocyte or both, depending on the species, under 17β-estradiol control (Patiño and Sullivan, 2002; Modig et al., 2007; Lubzens et al., 2010).

Cortical alveoli oocytes then undergo massive growth during the process called vitellogenesis. At this stage, an increase in the availability of the gonadotropin follicle stimulating hormone (FSH) in the ovary results in an increase in the production of estrogen. This increase in estrogen levels triggers vitellogenesis in the liver. Vitellogenin (Vtg), encoded by different *vtg* genes in teleosts, is the precursor of the yolk protein and is transported to the ovaries from the liver, to be incorporated into the ooplasm thanks to specific receptors (Vtgr) in the oocytes (Wallace 1985; Mommsen and Walsh 1988). The oocytes increase in size while at the same time the number of theca cells increases (Urbatzka et al., 2011). Maturational processes lead to the germinal vesicle breakdown. At this stage, the oocytes resume and complete the meiotic division. After this first meiotic division, a very small polar body that will afterwards degenerate and a large secondary oocyte at metaphase II are formed.

Then, maturational processes occur to generate a fully grown oocyte that will be competent for fertilization (Lubzens et al., 2010). When growth is completed, in many teleosts high levels of the gonadotropin luteinizing hormone (LH) arrive in the ovary (Khan and Thomas, 1998). This increase in the levels of LH triggers the production of the maturation-inducing hormone (MIH), a steroidal hormone produced by the ovarian follicle cells, and as a consequence, the oocyte proceeds with the second meiotic dividion (Patiño et al., 2001; Patiño and Sullivan, 2002). After meiosis takes place, a second polar body and the haploid female gamete are formed (Lubzens et al., 2010). Finally, and especially in marine species with pelagic eggs, oocytes undergo hydration, a process in which water enters in the oocyte increasing volume and buoyancy (Cerdà et al., 2007). Water uptake occurs as inorganic ions and free amino acids derived from the hydrolysis of yolk proteins accumulate and it is facilitated by aquaporins which are expressed in the oocyte membrane (reviewed by Cerdá et al., 2008). At the end of the maturation process, ovulation occurs with the release of the oocyte from its follicle, which is also dependent on hormonal cues such as increase in MIH, prostaglandins and their receptors (Patiño et al., 2003; Bobe et al., 2008; Lubzens et al., 2010).

At any moment during oogenesis oocytes can stop their development to undergo degeneration and reabsorption in a process called follicular atresia (Lubzens et al., 2010). Follicular atresia is a physiological and hormonally controlled process that leads to molecular and morphological changes in the oocyte and their surrounding follicular cells, and occurs in all female vertebrates (Saidapur, 1978). Nevertheless, in fish the process can be massive leading to skipped spawning and significant fecundity reduction through molecular process that still are not well understood (Lubzens et al., 2010).

2.2. Spermatogenesis

The testis is formed by two main compartments (reviewed by Schulz et al., 2010). First, the intertubular compartment that is formed by Leidig cells, blood and lymphatic vessels, macrophages, neural and connective tissue cells and then, the tubular compartment, that is defined by the basement membrane and the peritubular myoid cells, and the germinal epithelium containing the somatic Sertoli cells and the germ cells. The arrangement of the testicular germinal epithelium varies across fish taxa. Three main types have been defined (Parenti and Grier, 2004). In anostomosing tubular testes, the germinal compartments are highly branched and form anastomosing loops that do not terminate in the testis periphery.

On the contrary, if testes contain some germinal compartments that terminate blindly at the periphery, they conform a lobular testis. They are classified as unrestricted lobular if cysts containing spermatogonia are found along the lobules and as restricted lobular if they are only present in the periphery of the lobules.

Spermatogenesis is a highly organized and coordinated process, mainly controlled by FSH, LH and 11-ketotestosterone (Cerdà et al., 2008; Schulz et al., 2010; Figure 3). The germ cells within a cyst formed by Sertoli cells are originated from the same spermatogonial stem cell (Schulz et al., 2010). Two types of A spermatogonias have been described. Undifferentiated type A spermatogonias can go under self-renewal but can also give rise to differentiated type A spermatogonias, with a limited self-renewal potential. The latter mature undergoing several mitotic divisions species-specific in number. Then, A spermatogonias become type B spermatogonias, that after subsequent mitotic divisions initiate meiosis to form primary spermatocytes. After the first meiotic division, secondary spermatocytes are formed which undergo the second meiotic division to produce haploid spermatids. Such spermatids then suffer different molecular and morphological differentiation and specialization processes to mature and from sperm cells.

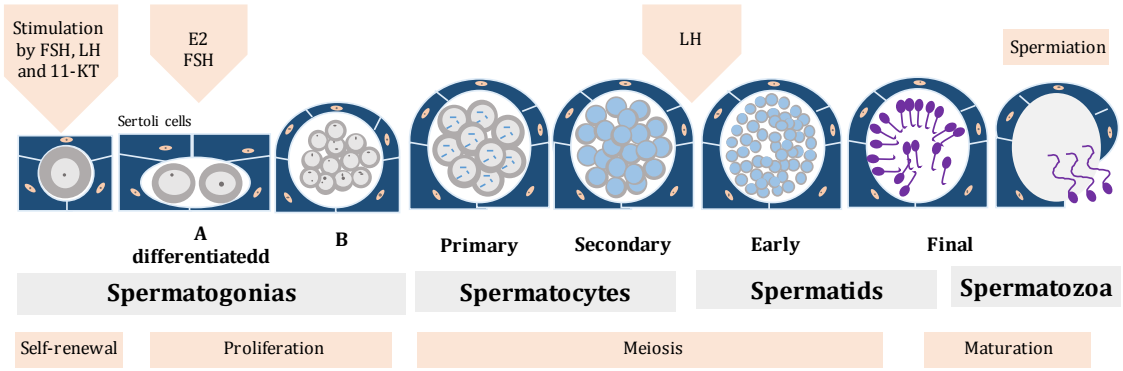

Figure 3
Simplified description of spermatogenesis, showing spermatogonias, spermatocytes, spermatids and spermatozoa and how sertoli cells form cysts around each sperm line cell type. FSH: follicle stimulating hormone. LH: luteinizing hormone. 11-KT: 11-ketotestosterone. E2: 17β-estradiol. Based on the figure by Schulz et al. (2010).

The different steps of spermatogenesis, are directed by different signaling and hormonal cues that command cell type specific gene expression pathways. Most of the signaling pathways depend on hormones produced by the germ line surrounding Leydig cells and the pituitary. Leydig cells present steroidogenic activity under the regulation of LH and FSH

produced in the pituitary. FSH mainly regulates the processes occurring during the mitotic phase or spermatogonial proliferation, thus in the early stages of spermatogenesis, while LH regulates the processes occurring in the latter stages (Schulz et al., 2010). Fish spermatogenesis is ultimately controlled by different steroid hormones, namely estrogens, androgens and progestagens (reviewed by Schulz et al., 2010). In this way, 17β-estradiol stimulates the self renewal of spermatogonias. Estrogens in general act on processes related with lipid and protein metabolism, transport and cell communication, whereas androgens are important during the whole spermatogenesis process, in general, showing an increasing trend during the process and decreasing at spermiation. Concretely, 11-ketotestosterone acts on the control of spermatogonial proliferation (Schulz et al., 2010; Knapp and Carlisle, 2011). Progestagens instead, are thought to regulate the entry of spematogonias in meiosis (Miura et al., 2006). Germ cell line maintenance and metabolic support is under the control of Sertoli cells, that in turn are also under the regulation of gonadotropins. In addition, progestagens are also important in the process of spermiation, by inducing it, increasing milt production and stimulating the mobility of spermatozoa (reviewed by Schulz et al., 2010).

3. Brain-Pituitary-Gonad axis

Steroidogenesis and gonadal development in fish of both sexes depends on the signaling pathway occurring along the brain-pituitary-gonad axis (BPG axis), being the communication among the involved organs vital for the integration of external and internal cues and control of reproduction (Devlin and Nagahama, 2002; Figure 4). The integration of environmental cues such as changes in temperature or photoperiod, alterations in food availability and modifications of the social context together with internal stimuli such as metabolic state and maturation stage occurs in the hypothalamus in the central nervous system. The most upstream participating signaling cells in the BPG-axis are kisspeptin neurons, that upon activation by external stimuli secrete neuropeptides, kisspeptins. In teleost genomes, two paralog genes exist, *kiss1* and *kiss2* with their respective receptors, *gpr54-1* and *gpr54-2* (Pinilla et al., 2012). Kisspeptins secreted by kisspetin neurons are able to activate the gonadotropin releasing hormone (GnRH) neurons present also in the hypothalamus which will then release the GnRHs. Three paralog GnRHs are present in teleosts, *gnrh1*, *gnrh2* and *gnrh3*, while only *gnrh1* and *gnrh2* are present in humans and only *gnrh1* in rodents. Their secretion into the pituitary gland leads to the synthesis and release of gonadotropins by the gonadotrope cells in the anterior pituitary of vertebrates. We have already seen that these heterodimeric glycoprotein hormones are key players on

the reproductive events in fish. Two pituitary gonadotropins are present in vertebrates, FSH (follicle stimulating hormone) and LH (luteinizing hormone). They both share a common α subunit, *gthα*, with a hormone specific β subunit, *fshβ* for FSH and *lhβ* for LH (Pierce and Parsons, 1981). Upon secretion both gonadotropins are transported through the circulatory system into the gonads, where they bind to specific gonadotropin receptors. The follicle stimulating hormone receptor FSHR is expressed in the granulosa and theca cells in the ovaries and in Sertoli cells in the testes, while the luteinizing hormone receptor LHR is located in the granulosa cells in the ovaries and Leydig cells in the testes. Typically, FSH is involved in the stimulation of vitellogenesis or sperm cell line proliferation and early gonad development, whereas LH stimulates the final maturation steps, ovulation or spermiation (Nagahama, 1994; Schulz et al., 2001).

After the interaction of the gonadotropins with the gonad via their receptors, several pathways are activated, one of the most important being steroidogenesis. Communication between the brain and the gonads is important to ensure that appropriate gonadotropin levels are synthetized at adequate developmental time-windows during gonadal maturation (Devlin and Nagahama, 2002). Feedback processes occur along the BPG axis, as gonadotropins are able to stimulate steroidogenesis in the gonads and in turn, sex steroids can modulate gonadotropin synthesis in the pituitary. The nature of this feed-back mechanisms whether positive or negative are dependent on the gametogenic stage of the individuals and are species-specific (Devlin and Nagahama, 2002; Roa et al., 2008; Levavi-Sivan et al., 2010; Zohar et al., 2010). Furthermore, the expression pattern in time and level of the above mentioned neuropeptides, hormones and receptors varies among different fish species during development and gametogenesis, according to their reproductive strategies and under the influence of environmental alterations

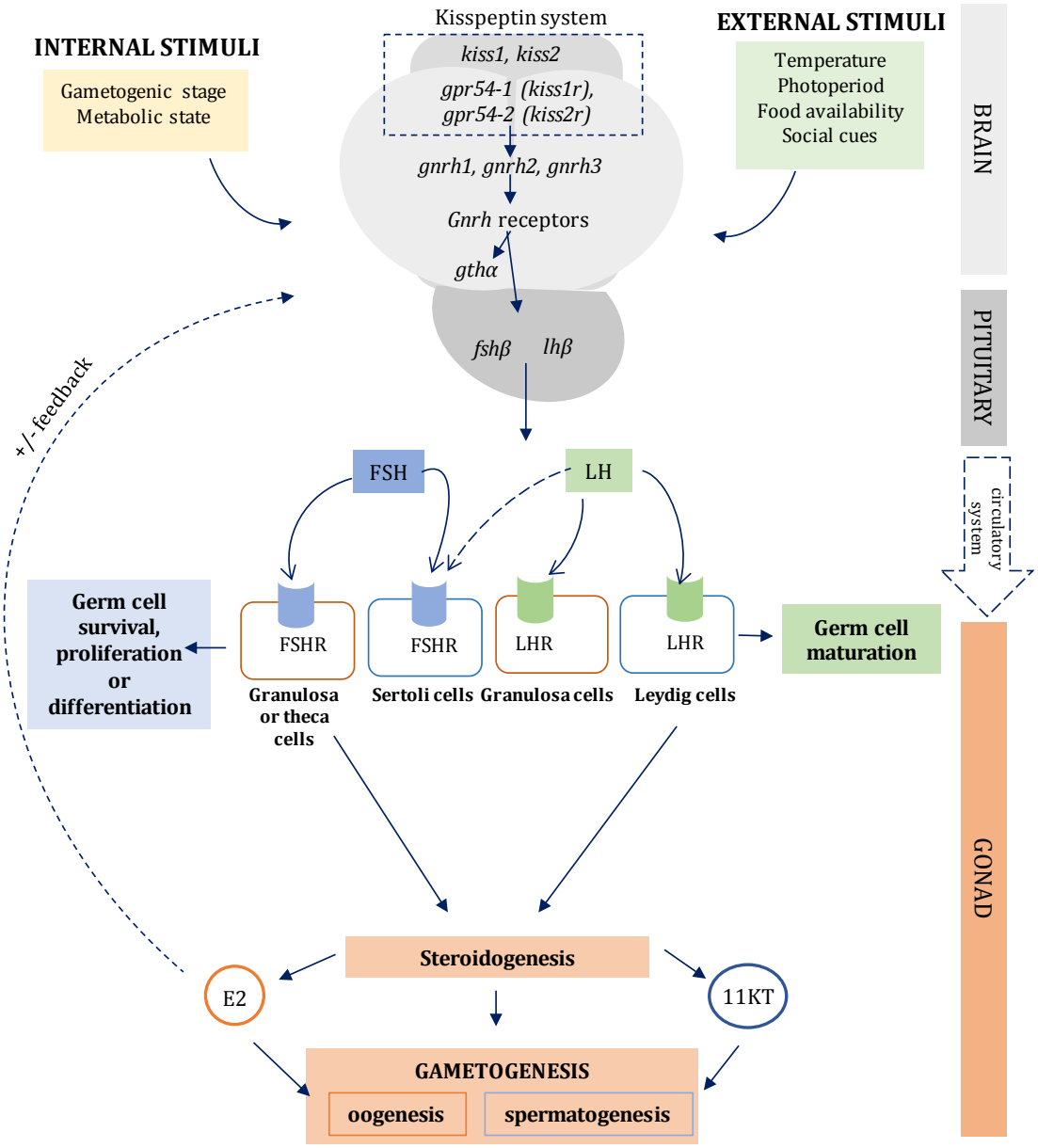

Figure 4
Schematic representation of the Brain-Pituitary-Gonad axis. Internal and external stimuli are integrated in the hypothalamus where, after the activation of kisspeptin system genes, *gnrh*s are secreted to activate the synthesis and release of gonadotropins in the pituitary. Gonadotropins (LH and FSH) are transported to the gonads and by binding to their specific receptors induce processes involved in steroidogenesis and gametogenesis. The discontinuous arrow between LH and FSHR indicates that promiscuous binding can occur in fish. E2 is synthetized in the gonads, and interacts with the brain. *kiss1/2*: kisspeptin1/2. *gpr54-1/2*: kisspeptin receptor 1/2. *gnrh*: gonadotropin releasing hormone. *gnrhr*: gonadotropin releasing hormone receptor. *gthα*: gonadotropin α subunit. *fhsβ*: follicle stimulating hormone β subunit. *lhβ*: luteninzing hormone β subunit. FSH: follicle stimulating hormone. LH: luteinizing hormone. FSHR: follicle stimulating hormone receptor. LHR: luteinizing hormone receptor. E2: 17β-estradiol. 11KT: 11-ketotestosterone. See text for more information.

4. Steroidogenesis and its involvement in sex differentiation

Sex steroids play an important role in the early sex differentiation of the gonads in vertebrates (Devlin and Nagahama, 2002), as well as being implicated in the onset of puberty and the first gametogenic cycle and also in the subsequent reproductive cycles during adulthood (Piferrer and Guiguen, 2008). Estrogens are fundamental for female sex differentiation whereas androgens are crucial for male sex differentiation (Devlin and Nagahama, 2002; Guiguen et al., 2010). Nevertheless, they are involved in other physiological processes not directly linked to gametogenesis such as pheromone signaling and osmoregulation (Tokarz et al., 2015). Gonads are the primary producers of steroid in vertebrates, however, other organs and tissues such as adrenal glands, adipose tissue and brain produce and biotransform steroid hormones (Arukwe, 2008; Piferrer, 2011), while liver participates in the catabolism of steroids and their conjugates (Devlin and Nagahama, 2002).

The molecular pathway for the synthesis of sex steroids starts with cholesterol. It is thought that the rate-limiting step in the process of steroidogenesis is the transport of cholesterol across the mitochondrial membrane, by the steroidogenic acute regulatory protein, StAR (Arukwe, 2008). Once in the inner mitochondrial membrane, the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc), converts cholesterol into the 21 carbon atom steroids progestogen, pregnenolone, progesterone and 17α-hydroxyprogesterone, by the activity of several enzymes (Figure 5). The latter is then converted into the 19 carbon atom androgen, androstenedione. Androstenedione is in turn converted into testosterone via the activity of 17β-hydroxysteroid-dehydrogenase (17β-HSD), or into 11β-hydroxyandrostenedione via the enzyme 11β-hydroxylase (Cyp11b). Both, testosterone and 11β-hydroxyandrostenedione, can be further converted by Cyp11b and 17β-HSD respectively, into 11β-hydroxytestosterone. Then, the latter is converted into 11-ketotestosterone by 11β hydroxysteroid-dehydrogenase. 18 carbon atom estrogens are synthetized via the conversion of androstenedione into estrone, or the conversion of testosterone into 17β-estradiol. In both cases the cytochrome P450 aromatase (Cyp19) is the responsible enzyme (reviwed by Piferrer, 2011 and Tokarz et al., 2015).

The main active androgens in most vertebrates are testosterone and 5α-dihydrotestosterone, although in fish 11-ketotestosterone is the main endogenous androgenic sex hormone (Le Page et al., 2010). In fish, 17β-estradiol and 11-ketotestosterone are thus considered the major sex steroid hormones necessary for gonad

development. Although species-specific and sex dependent differences are observed, both types of steroids are produced in gonads of both sexes. Ovarian theca cells contain the necessary enzymes for the production of androgens that are necessary substrates for estrogen production (Devlin and Nagahama, 2002). The ovarian granulosa cells are not able to synthetize steroids, however, conversion of testosterone into estrogen takes place in such cells (Nagahama, 1997). In the testis, androgens are secreted by the Leydig cells (Devlin and Nagahama, 2002). In addition, estradiol production is also common in testes (Pasmanik and Callard, 1988) playing a necessary role in some steps of spermatogenesis (reviewed by Schulz et al., 2010).

Being lipophilic, steroid hormones diffuse through cell membranes and bind to the steroid hormone receptors in the cytosol of competent cells, although they can also interact with membrane steroid receptors (Hardy and Valverde, 1994). Within the superfamily of nuclear receptors different kinds of steroid hormone receptors are found, and this means that in most teleost genomes at least two androgen receptors (AR), ARα and ARβ (Piferrer, 2011), and three estrogen receptors (ER), ERα, ERβ1 and ERβ2 can be found (Hawkins et al., 2000). These receptors are expressed in cells of various organs including brain and gonads.

Although similarities can be observed in both sexes, the levels of steroid hormones and thus enzymes are characteristic of each sex, before and during sex differentiation. Sex specific expression of the aromatase enzymes, due to their involvement in female sex differentiation and of *cyp11b*, due to its role in male sex differentiation, have been described (Piferrer, 2011). Teleosts show two *cyp19a* paralog genes as a consequence of the teleost specific round of genome duplication, *cyp19a1a* (gonadal aromatase) which is primarily expressed in the gonads and *cyp19a1b* (brain aromatase) which is expressed in the brain (Blázquez and Piferrer, 2004). The functions of aromatase during sex differentiation are quite conserved among fish (Guiguen et al., 2010). Gonadal *cyp19a1a* is of great importance for ovarian differentiation, especially as it is required to maintain the balance between androgens and estrogens. In fact, during ovarian differentiation following sex determination, the overexpression of *cyp19a1a* has been described in many fish species (reviewed by Guiguen et al., 2010). *cyp19a1a* is thus considered one of the first markers of ovarian differentiation in fish. Regarding the steroid hormone receptors, whereas both AR in males and ER in females have been related with sex differentiation the role of ER counts with more experimental support (Piferrer, 2011). During female sex differentiation, ERs are expressed prior to the steroidogenic enzymes, which suggests their important role in sex differentiation (Piferrer, 2011).

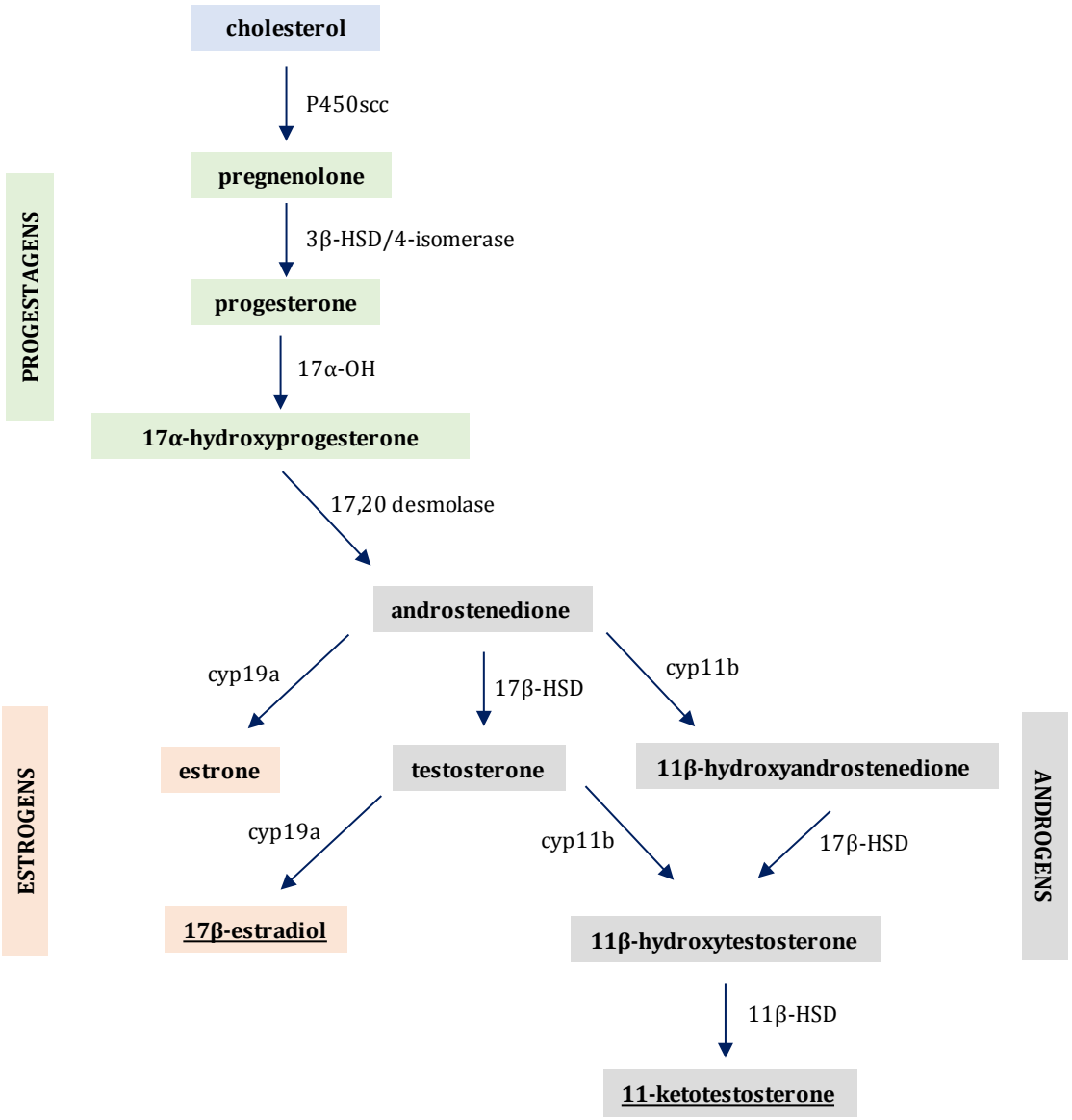

Figure 5
Simplified teleostean steroidogenic pathway showing conversion of cholesterol into sex steroids. Green boxes show progestagens with 21 carbon atoms. Grey boxes contain androgens with 19 carbon atoms, while soft orange boxes depict estrogens with 18 carbon atoms. Underline sex steroids are representative of the most potent or active sex steroids in fish. The enzymes that are responsible for each reaction are indicated with their acronyms. P450scc: cytochrome P450 side-chain cleavage. 3-HSD/4 Isomerase: 3-hydroxysteroid-dehydrogenase/4-isomerase. 17-OH: 17-hydroxylase. 17-HSD: 17-hydroxysteroiddehydrogenase. Cyp19a: aromatase. Cyp11b: 11-hydroxylase. 11-HSD: 11-hydroxy-steroid-dehydrogenase.

Other transcription factors apart from nuclear receptors are also involved in the whole process of sex differentiation with a sex dimorphic role (Piferrer, 2011) and showing ability to regulate the expression of steroidogenic enzymes. Foxl2 (forkhead box L2), which is mainly expressed in granulosa cells (Wang et al., 2004; Nakamoto et al., 2006) is thought to be the transcriptional activator of the ovarian form of Cyp19a (Diotel et al., 2010; Guiguen et al., 2010; Baron et al., 2004; Alam et al., 2008). Concomitant transcription of *foxl2* and *cyp19a1a* genes has been observed in different fish species (Vizziano et al., 2007; Wang et al., 2007; Ijiri et al., 2008), and a positive feedback loop in the expression control between both genes has been proposed (Guiguen et al., 2010). On the contrary, Dmrt1, doublesex and mab-3 related transcription factor 1, is involved in very early steps of male sex differentiation and testis formation (Herpin and Schartl, 2011; Smith et al., 2013). In addition, it has been suggested that Dmrt1 is able to repress *cyp19a* transcription, resulting in decreased estradiol levels and contributing to the promotion of the male differentiation process (Piferrer, 2011). In any case, it has also been associated with ovarian differentiation in some species (Guo et al., 2005).

Other genes are also involved directly or indirectly in the process by inhibiting or enhancing the transcription of the above mentioned genes. For instance, Amh, the anti-Müllerian hormone, is expressed in immature and in mature gonads in Sertoli cells and granulosa cells (Rodriguez-Marí et al., 2005). Amh has a negative effect on *cyp19a1a* transcription, at least in some fish species (Rodriguez-Marí et al., 2005). In addition, Amh is regulated by Sox9 (Siegfried, 2010), which is a transcription factor that belongs to the Sry-related high-mobility group box family (Schepers et al., 2002). In males, Sox9 is expressed in cells surrounding the germ cells (Rodriguez-Marí et al., 2005; Vizziano et al., 2007). Feedback processes involving cytochrome P450 genes, *amh* and *sox9* have been described in fish, at least related with testis development (Zhai et al., 2017). Moreover, the influence that other tissues such as the brain have on gonad development upon sex determination should be taken into consideration, although brain sex differences might be caused by differences at gonadal level once differentiation process has been initiated, rather than the other way around (Senthilkumaran et al., 2015). Foxl2 is also expressed in the brain and it has been proposed to be involved in the control of brain aromatase transcription (Sridevi et al., 2012).

Adverse effects in the sex differentiation related process can arise due to inappropriate exposure to steroid hormones or their mimics (Tyler et al., 1998). Hormonal treatment during early stages of sex determination can result in alteration, and even complete

reversion, of the sexual phenotype, regardless of genotypic sex, that can last permanently (Devlin and Nagahama, 2002). Incomplete sex reversal after hormonal treatment can result in the development of coexisting testicular and ovarian tissue in the same gonad. Feminization has been most often described than masculinization and it can occur after estrogen (mainly 17β-estradiol) treatment, especially during concrete developmental time windows (Devlin and Nagahama, 2002). Estrogen treatment additionally induces alterations in endogenous steroidogenesis in several species (reviewed by Guiguen et al., 2010). Masculinization of fish has been observed after androgen treatment, for instance with 17α-methyltestosterone (Devlin and Nagahama, 2002), although its mechanisms are less understood and masculinization has been very rarely described under real environmental conditions. In this sense, some androgenic treatments inhibit aromatase transcription and thus we should refer to antiestrogenic effects (reviewed by Guiguen et al., 2010). The dose of the hormonal treatment is relevant in any case, as for instance, excessive androgen treatment has been reported to have a feminizing effect. Anyhow, not all species are equally susceptible (Lange et al., 2012) and the critical time window in which sex differentiation is labile and more responsive to external hormonal treatment may differ from one fish species to another.

5. Endocrine disruption in the aquatic environment

Impairment of the normal endocrine functions of wildlife began to be documented in the 1940's, nevertheless, it is since the 1990's that the number of studies has increased specially in regard to exposure to chemical compounds that interfere with the endocrine system (Matthiessen, 2003; Hotchkiss et al., 2008). Endocrine disrupting chemicals (EDCs) are substances that have the potential to disrupt normal endocrine processes, although EDCs appear in the environment from different sources and display diverse chemical structure. In 1998, the Working Group on Endocrine Disruptors under the European Commission´s Scientific Committee on Toxicity, Ecotoxicity and the Environment, agreed on the following definition for an endocrine disruptor: "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (IPCS, 2002). Nevertheless, other definitions have also been proposed, and one of the most used ones (Ankley et al. 2009) states that endocrine disruptors are "agents that cause alterations in reproduction or development through direct effects on the vertebrate hypothalamic-pituitary-thyroidal or hypothalamic-pituitary-gonadal (HPG) axes" (USEPA, 1998).

EDCs comprise a wide range of manufactured substances as well as synthetic or natural hormones. Such chemicals include polycyclic aromatic hydrocarbons, several persistent organochlorines such as some pesticides and polychlorinated biphenyls, herbicides, fungicides, plasticizers such as alkylphenols and phthalates, organotin compounds, dioxins, brominated flame retardants, heavy metals, synthetic and natural hormones including myco- and phytoestrogens (e.g. Tyler et al., 1998; Sanderson, 2006). Some of those compounds are very persistent in the environment and can be easily uptaken and bioaccumulated (Singleton and Khan, 2003). Upon uptake effects may be observed on various tissues involved in reproduction, from the brain and pituitary to the liver and gonads (Van der Oost et al., 2003). The deleterious effects of such substances can be noted at the molecular, cellular, physiological and population levels. In some circumstances these molecules do behave directly as mimics of endogenous hormones while in other circumstances they interfere with the synthesis, availability and action of endogenous hormones. In this way, they can interfere in many different ways with sex determination, sex differentiation, gamete maturation and reproduction (Crisp et al., 1998; Sanderson, 2006; James, 2011). The effects of such compounds are difficult to foreseen and to quantify once they occur, due to the complexity of the endocrine system (Denslow and Sepúlveda, 2007). In ultimate instance exposure to such compounds has been associated with reduced fertility (Jobling et al., 2002a), sex reversal (Jobling et al., 1998) and reproductive failure (Kidd et al., 2007; Nash et al., 2004). The direct link to population level effects is always difficult to stablish (Mills and Chichester, 2005; Harris et al., 2011; Santos et al., 2013; Johnson and Chen, 2017), but in some cases exposure to typical estrogenic compounds has been linked to population declines (Hamilton et al., 2014). In any case, there are some contradictory examples, that have shown a reduced reproductive capacity of fish without a decline in the population (Harris et al., 2011).

Although fish are not the only organisms affected by EDCs (Tyler et al., 1998), they are strongly exposed to them due to their environmental niche and the multiple uptake routes of EDCs that include aquatic respiration, osmoregulation, dermal contact and ingestion (Weber and Goerke, 2003; Kwong et al., 2008; Söffker and Tyler, 2012). In addition, related with the lipophilic nature of many of these EDCs, maternal transfer of contaminants accumulated in lipids stockpiled inside eggs has been described (Van Der Kraak et al., 2001). EDCs can reach the aquatic environment (freshwater, estuarine and marine) through several sources and routes, for instance via wastewater treatment plant discharges, industrial, urban or agricultural runoff, refuse dumps and groundwater discharges (Mills and Chichester, 2005; Söffker and Tyler, 2012). Subsequently, they can be accumulated in

the sediment, water and biota (van der Oost et al., 2003). It has been suggested that it is important to stablish connections between the contamination levels in the different environmental compartments, to understand their possible effects on ecosystem health (van der Oost et al., 2003). In relation to their possible biological effects in addition, species-specific differences should be considered, as different species may have different sensitivity. For instance, different species may show different capacity to metabolize and biotransform such substances before they can exert any deleterious molecular level effect (Gibson et al., 2005). Thus, some teleost species are more tolerant to contaminated environments, including contamination by EDCs, than others. Likewise, the higher sensitivity of early life stages of teleost fish has been described, as there is evidence of a critical window of time where EDCs exert permanent effects with reproductive deleterious outcomes (Tyler et al., 1998).

The most studied EDCs display estrogenic activity, conforming a special group of compounds termed collectively xenoestrogens. But not all feminizing EDCs are xenoestrogens *sensu lato*, as some may interfere with the physiological steroidogenic pathway increasing the levels of endogenous estrogens. Xenoestrogens in general, present the ability to mimic endogenous estrogens and bind to the estrogen receptors. There are also some EDCs that on the contrary show androgenic effects, although they represent a far more reduced group of chemicals among the EDCs (reviewed in Söffker and Tyler, 2012). Xenosteroids in general have structural features that make them similar to the natural steroids (Tyler et al., 1998) and although they bind to the receptors with less affinity than the natural ligands, they can still activate them. On the other hand, there are some EDCs that sometimes are able to block the receptor-steroid binding (Tyler et al., 1998). This is the case of environmental anti-androgens or anti-estrogens (Filby et al., 2007). In addition, the variety of EDCs is such, that some are able to interact with both estrogen and androgen receptors (e.g. Filby et al., 2007). Some estrogenic EDCs have also shown the ability to bind to non-steroidal receptors (Tyler et al., 1998), while some can cause alterations through non-receptor mediated mechanisms (Denslow and Sepúlveda, 2007). Thereby, mechanisms of xenoesteroids are assorted.

In a polluted environmental context, we have to take into account that complex chemical mixtures prevail in the environment, due to the high amount of variety of chemicals that we use during our daily life in association with natural chemicals derived from animals and plants (Sumpter, 1998). This complexity of EDC mixtures, as the ones present in the effluents of paper mills or wastewater treatment plants, makes it difficult to relate the

observed effects with exposure to single compounds. Feminization of fish exposed to wastewater treatment plant effluents has been documented worldwide and in a wide range of fish species and generally showing accumulation of hundreds of different chemical compounds.

5.1. Biomarkers of xenoestrogenicity in fish

The exact mechanisms underlying xenoestrogenicity are not fully known, and a great effort has been done to try to understand the molecular mechanisms responsible of the observed effects. The aim of the research carried out during the last decades has focused partly on stablishing early warning signals, or biomarkers, of exposure to EDCs and most specifically to xenoestrogens. Pollutant stress results in a sequential order of responses within a biological system (Bayne et al., 1985) and biomarkers are useful to determine if a toxicant has entered the organism and/or if it has exerted any kind of biological effect (McCarhy and Shugart, 1990). Although several definitions of pollution biomarkers have been povided (van der Oost et al., 2003), in general, it can be defined as any change in a biological endpoint, that could occur at any level of biological organization ranging from the molecular to the behavioral or population levels, and which can be related with the exposure or the effects caused by an environmental toxicant.

Biomarkers of estrogenic exposure in fish are commonly used as tools at the molecular and cellular level to analyze the presence of xenoestrogens in the environment and to analyze their effects on fish health, due to the fact that assessing the effects at the population level is more complex and less chemical specific (Gunnarsson et al., 2009). Transcription and expression levels of estrogen-responsive genes in fish organs, mainly liver and gonads are studied in order to assess the estrogenic potency of chemicals and of environmental water samples. A well stablished and widely used biomarker of exposure to xenoestrogens is the up-regulation of vitellogenin (Vtg) expression in the liver of males and immature fish (Sumpter and Jobling, 1995; Tyler et al., 1998; Thorpe et al., 2000; Arukwe and Goksoyr, 2003). Such up-regulation has been thoroughly reported under laboratory exposure to a wide array of estrogenic compounds in multiple species. Mentioning a few examples of laboratory experiments, for instance, four alkylphenolic chemicals, 4-nonylphenol, 4-tert-octylphenol, nonylphenoxycarboxylic acid and nonylphenoldiethoxylate separately, at a concentration of 30 μg/L, were proved to induce Vtg synthesis in adult male rainbow trout *Onchorrinchus mykiss* (Jobling et al., 1996). In male sea bass (*Dincentrarchus labrax*) exposed to 17β-estradiol (E2), 17α-ethynylestradiol (EE2) and bisphenol A (BPA),

separately and in different concentration ranges, plasma Vtg was induced in all tested concentrations, reaching an induction of more than a 100% in the highest tested concentrations (2 μg/L EE2, 1 μg/L E2, 1600 μg/L BPA) (Correia et al., 2007). Fathead minnow (*Pimephales promelas*) adult males exposed separately to estradiol (10, 32 and 100 ng/L) and estrone (34, 98 and 307 ng/L) showed induction of Vtg plasma levels in both cases (Thorpe et al., 2007). In the case of estrone exposure fish survival was also affected. In the same exposure experiment but in the case of females, Vtg plasma levels were observed to be suppressed (Thorpe et al., 2007). Also with female fathead minnows, an experiment was performed to test five different chemicals, 17β-trenbolone, 17α-trenbolone, prochloraz, fenarimol and fadrozole, at different concentrations (Miller et al., 2007) that showed a correlation between the decrease in Vtg plasma levels and fecundity rates. In addition, such alterations were linked with possible adverse effects at population level.

Field studies have also shown Vtg induction in male fish exposed to xenoestrogens in their natural habitat. It is the case for instance of breams (*Abramis brama)* inhabiting the polluted Elbe river in Germany, where up to 1200 ng/mL median concentration of plasma Vtg was reported (Hecker et al., 2002). Male wild roach (*Rutilus rutilus*) from several British estuaries (e.g. Jobling et al., 1998; Jobling et al., 2002b), have also shown detectable plasma Vtg protein levels, with a mean concentration of 1μg/mL in several estuaries (Jobling et al., 1998). Also in the United Kingdom, Vtg was detected in plasma of male flounders (*Platichthys flesus*) sampled in estuaries and offshore, with a maximum of 59 mg/mL in plasma in the Mersey estuary and with around 10 mg/mL in flounders from the Irish Sea (Allen et al., 1999). In offshore waters, plasma Vtg has been detected also in dab (*Limanda limanda*) from the Irish Sea and the North Sea, where plasma levels ranged from less than 0,01 μg/mL to 8,6 μg/mL, with the two highest concentration being of 21 and 750 μg/mL (Scott et al., 2007). Vtg protein has been also detected in the liver of swordfish (*Xiphias gladius*) from the Mediterranean Sea and in South African marine waters (Desantis et al., 2005). Also in the serum of swordfish (Fossi et al., 2002; Fossi et al., 2004), bluefin tuna (*Thunnus thynnus thynnus*) and in Mediterranean spearfish (*Tetrapturus belone*) from the Mediterranean Sea (Fossi et al., 2002).

Other biomarkers of xenoestrogenic exposure are those related with changes at different levels of the steroid synthesis and metabolism. This can be identified at the transcript, enzyme (protein content or activity) or hormone levels. In teleosts, xenoestrogens and other endocrine disruptors can modify aromatase activity and transcription levels in brain

and gonads (Cheshenko et al., 2008). In laboratory experiments, exposure of juvenile zebrafish to different concentrations of EE2 during three days, gonad aromatase gene was downregulated, whereas EE2 and nonlyphenol induced brain aromatase transcription (Kazeto et al., 2004). In adult male zebrafish exposed separately to BPA, EE2 and several E2 concentrations during 21 days, *cyp19a2* (*cyp19a1b*) was upregulated in all cases (Kallivretaki et al., 2006). Upregulation of *cyp19a1a* was observed in medaka *Oryzias latipes* testis after exposure to EE2 (Scholtz and Gutzeit, 2000). In juvenile turbot (*Scolphtalmus maximus*) exposed to nonlyphenol, decreased gonad aromatase activity was observed (Martin-Skilton et al., 2006). In juvenile Atlantic salmon (*Salmo salar*) exposed to 4-nonylphenol (NP) aromatase activity and transcription levels were suppressed in gonads but not in brain (Kortner et al., 2009). In an *in vitro* experiment performed with cunner (*Tautogolabrus adspersus*), E2 and EE2 caused an increase in brain aromatase activity in males, whereas in females brain activity was unaffected and in ovaries the activity decreased (Mills et al., 2014). In the same experiment, octylphenol was tested, with no significant changes in brain aromatase activity in both sexes and in ovarian aromatase in females (Mills et al., 2014). In field studies, effects on aromatase have also been described. In carp (*Ciprinus carpio*) collected along the Ebro river in Spain, females from a relatively clean site showed higher ovarian aromatase activity than those collected in sites receiving agricultural and industrial contaminants (Lavado et al., 2004). Similarly, in female roach collected along the Seine river in France, a reduction in ovarian aromatase activity was detected in females from polluted sites when compared with a reference site (Gerbron et al., 2014), whereas an induction of brain aromatase activity was reported for males and females from two polluted sites (Geraudie et al., 2011). In killifish (*Fundulus heteroclitus*) from two bays in Massachusetts, accordingly, higher brain aromatase transcription levels were measured in female and males, whereas higher ovarian aromatase levels in females from the polluted compared to the reference site were described for one season (Greytak et al., 2005).

Altered plasma hormone levels have also been described as a consequence of exposure to xenoestrogens in several laboratory and field studies. Such alterations can be a consequence of direct effect of steroidogenic enzymes or indirect effects depending on feedback loops (Arukwe, 2001). Changes in sex steroid levels after exposure to xenoestrogens differ among fish species, being dependent also on their developmental stage and sex, as well as the exposure duration and xenoestrogen concentration (Kortner et al., 2009). In the laboratory experiment performed with female zebrafish exposed to EE2, a decrease in both testosterone and estradiol levels was observed (Hoffmann et al., 2006). In juvenile Atlantic

salmon exposed to NP and EE2, no changes were observed in plasma testosterone levels, whereas increased estradiol levels were described after both treatments (Kortner et al., 2009). The opposite, decreased E2 plasma levels, were observed in female rainbow trouts exposed to NP at a concentration of 85,6 μg/L (Harris et al., 2001). Comparing juvenile turbot and Atlantic cod exposed to 30 μg/L of NP, reduced E2 and T plasma levels were only observed in the case of turbot (Martin-Skilton et al., 2006). In field studies, suppression of plasma sex steroid concentrations have been described together with altered vitellogenin plasma concentrations. It is the case for instance of the above mentioned field study in Germany, where suppressed plasma levels of E2 in females and 11-ketetosterone in males was observed in breams from the most polluted sites (Hecker et al., 2002).

Although less studied and very seldom used as biomarkers in a pollution biomonitoring context, transcription levels of other genes related with steroidogenesis and/or sex differentiation have been measured. Filby et al. (2006), for instance, when studying fathead minnows exposed to E2 analyzed the transcription levels of a batch of gonadal genes among which, several estrogen receptors, gonad and brain aromatase, *amh*, *vasa* and *dmrt1,* and observed alterations in their transcription pattern. In this way, the observed results showed the downregulation of *amh* and *dmrt1* in the exposed males and females, although only the first was significant. In the last years, high throughput techniques such as next generation sequencing and the use of microarrays have also been applied to analyze to toxicological pathways of exposure to xenoestrogens and decipher gene expression related biomarkers. Expression microarrays to detect altered transcription in the gonads or liver after exposure to EE2 have been widely applied. In fathead minnow testes, the suppression of genes related with the steroid metabolism, sperm motility and sex determination was observed (Feswick et al., 2016). Among such genes, the downregulation of genes belonging to the *dmrt1* network, for instance *sox9*, *cyp19a1*, *cyp11a1* and *cyp17a1* was described. In contrast, increased transcription of genes related with granulosa cell development was detected, with altered transcription in genes such as *foxl2* and several other growth factors (Feswick et al., 2016). Similarly, in three-spined stickleback (*Gasterosteus aculeatus*), microarray revealed the downregulation of steroid biosynthesis pathway genes in testis, together with an altered transcription pattern of genes related with sperm development (Prokkola et al., 2016). In an experiment with female zebrafish, genes belonging to hormone metabolism, steroid binding and sterol metabolism were found to be significantly regulated (Hoffmann et al., 2006).

RNAseq has been applied to assess the effects of EE2 exposure in the pituitaries of coho salmon (*Oncorhynchus kisutch*) subadult females, showing altered transcription of genes related with the circadian rhythm signaling, peroxisome proliferator activated receptor and gonadotropin releasing hormone, together with upregulation of *lhβ* and upregulation of *fshβ* (Harding et al., 2013). RNAseq also identified enriched gene pathways in the liver of wild sardine (*Sardinops sagax*) and mackerel (*Scomber japonicus*) after exposure to EE2 (Renaud et al., 2019). Pathways related with carbon, retinol, glyoxilate and dicarboxylate metabolism, steroid hormone biosynthesis and drug metabolism were shown to be altered (Renaud et al., 2019). In zebrafish males exposed to EE2, RNAseq performed in the testes, showed differential expression of genes related to cell death, differentiation and proliferation, genes related to steroid synthesis, testis development and function and spermatogenesis (Porseryd et al., 2018).

Histopathological analysis of several tissues is also common in the biomonitoring of aquatic pollution, also when studying exposure to xenoestrogens (van der Oost et al., 2003). Hepatic histopathological alterations have been extensively studied in fish (reviewed by Vethaak and Rheinalt, 1992). In the particular case of British estuaries impacted by several contaminants, especially polycyclic aromatic hydrocarbons, Stentiford et al. (2003) reported higher rates of histological lesions in liver, gill, kidney and gonads in flounder (*Platichthys flesus*), goby (*Pomatoschistus minutus*) and blenny (*Zoarces viviparus*) captured in polluted locations than in those collected in control sites. Such lesions included the increased presence of melanomacrophage centers, hepatocellular fibrillary inclusions, basophilic adenoma and cholangioma in the liver, aneurysms, hyperplasia and fusion of secondary lamellae in gills and accumulation of fibrous material in the kidney (Stentiford et al., 2003). Alterations in gonad histology are commonly described as a consequence of exposure to EDCs. Increased ovarian atresia, especially in previtellogenic oocytes, has been described as a pathological response to exposure to environmental contaminants, and a possible biomarker to be applied in pollution biomonitoring campaigns (Blazer, 2002; Thomé et al., 2009). Increased oocyte atresia was observed for instance in white croaker (*Genyonemus lineatus*) inhabiting waters with high concentrations of chlorinated hydrocarbons (Cross and Hose, 1988) and English sole (*Parophrys vetulus*) from a similar environment (Johnson et al., 1988). In male fish, intersex condition, the appearance of oocytes scattered through the testes of gonochoristic males, is the most striking and most widely reported gonad histopathological alteration, which has strictly been related with EDC (xenoestrogen) exposure (e.g. Tyler and Jobling, 2008; Bahamonde et al., 2013). Intersex males have been observed in many wild freshwater and marine fish species

worldwide, related with exposure to chemicals and their mixtures of different origin. In many circumstances the appearance of intersex testes in fish has been linked to wastewater treatment plant effluents (see section 6), but also to industrial discharges or agricultural runoff waters (Bahamonde et al., 2013).

5.2. Wastewater treatment plant effluents and EDCs

High variety of chemicals can be found in the effluents of wastewater treatment plants (WWTPs) as it has been confirmed for instance by the chemical analysis of the effluents of 90 WWTPs from around Europe (Loos et al., 2013). In their study, they found a high prevalence of organophosphate ester flame retardants, pesticides, plasticizers together with a high variety of pharmaceuticals and personal care products, among others (Loos et al., 2013). In studies comparing the concentration and distribution of target contaminants along the estuaries, a high contribution of the WWTPs to the contaminant load has been claimed (Mijangos et al., 2018). Among the chemicals present in WWTPs and classified as endocrine disruptors, alkylphenols, bisphenol-A, pesticides, phthalate esters, polycyclic aromatic hydrocarbons and musk fragrances can be found, in concentrations of ng/L (e.g. Bizkarguenaga et al., 2012; Bueno et al., 2012; Ros et al., 2015). In addition, differences regarding the concentrations discarded by each WWTP have been described. For instance, when comparing the effluent waters from three WWTPs located in estuaries from the Basque coast (South East Bay of Biscay) (Bizkarguenaga et al., 2012) the maximum BPA concentrations were 248 ng/L, 974 ng/L and 2615 ng/L respectively (Bizkarguneaga et al., 2012). For the nonlyphenol mixture instead concentrations of 516, 1230 and 28917 ng/L were reported while for E2 274 ng/L, 54 ng/L and concentrations below the detection limit were reported (Bizkarguenaga et al., 2012).

During the last decade, several studies have been conducted with fish from waters receiving WWTP effluents of different kinds and sources in varied environmental scenarios, showing impairment of their endocrine function (Blazer et al. 2007; Tyler and Jobling 2008; Vajda et al. 2008; Tetreault et al. 2011). The estrogens that are present in such effluents have been considered as the major contributors to their possible xenoestrogenic effects, and 17α-ethinylestradiol (EE2), 17β-estradiol (E2) and estrone (E1) can be highlighted (Jobling et al., 2002b; Katsu et al., 2007; Lange et al., 2008; Lange et al., 2009). The synthetic hormone EE2 is known to be a potent xenoestrogen with activity at even very low concentrations (Lange et al., 2001; Lange et al., 2012). In fathead minnow, 0,2 ng/L EE2 were able to induce Vtg synthesis in males (Lange et al., 2001). In rainbow trout the effective concentration was

even lower, 0,1 ng/L EE2 (Purdom et al., 1994). In the luciferase activity assay performed by Katsu et al. (2007), EE2 was described as the most potent activator of both ERα and ERβ in roach, in comparison to natural hormones, being able to activate ERα at a concentration of 10^{-10} M and ERβ at 10^{-11} M. Instead, a higher concentration was needed to activate the receptors using natural hormones, E2 and E1, 10^{-9} M for ERα and at 10^{-10} M for ERβ. In the case of E3 (estriol) 10^{-8} M were needed to activate both receptors (Katsu et al., 2007). In a similar study, diethylstilbestrol (DES) was also tested, and shown to be a more potent activator of ERα than EE2 for roach, fathead minnow, zebrafish, three-spined stickleback, common carp and medaka, with concentrations ranging from 0,007 nM up to 0,051 nM (Lange et al., 2012). In juvenile female trout, the median effective concentration of EE2, E2 and E1 needed to induce plasma Vtg showed that EE2 was the most potent activator, followed by E2 and E1, with median effective concentrations of 0,95-1,8 ng/L, 19-26 ng/L and 60 ng/L respectively (Thorpe et al., 2003). Thus, the natural hormones E2 and E1, are less potent than synthetic hormones (Thorpe et al., 2003; Lange et al., 2012), but are also estrogenic and are present in higher concentrations than EE2 in the effluents of WWTPs (Rodgers-Gray et al., 2000; Khanal et al., 2006). In the effluent of a WWTP from the United Kingdom, which receives primarily domestic waters, E1 was detected in the range of 15-220 ng/L, E2 4-88 ng/L and EE2 in a range of 1,7-3,4 ng/L (Rodgers-Gray et al., 2001).

Other contributors to the estrogenicity of the WWTP effluents, but with a more reduced estrogenic activity are alkylphenols, which are used as surfactants, and bisphenol-A the most widely used plasticizer (Snyder et al., 2001; Brian et al., 2005). Alkylphenols (nonylphenol and its ethoxylates) were detected in higher concentrations than steroid hormones in the effluent of a WWTP in the UK, in a range of 0,75-8 μg/L (Rodgers-Gray et al., 2000). Nonylphenol (NP) and octylphenol (OP) have been shown to induce roach ERα activity at a concentration of 10^{-6} M, but unable to induce ERβ, showing less induction potency than natural and synthetic hormones (Katsu et al., 2007). Likewise, when their Vtg induction capacity was tested in male fathead minnows, both NP and OP showed less capacity than E2 and EE2, although higher than BPA (Brian et al., 2005). Alkylphenols on the other hand can act in an additive manner, as it has been proven in laboratory conditions, showing that in mixture they are able to induce estrogenic effects at lower concentrations than individually (Brian et al., 2005; 2007).

In addition, WWTPs effluents can also display anti-androgenic effects as pointed out in relation to compounds such as fungicides, pesticides and also BPA and alkylphenols (Sohoni and Sumpter, 1998; Urbatzka et al., 2007; Tollefsen et al., 2007; Jobling et al., 2009; Hill et

al., 2010). The feminizing effects of British WWTPs has been proposed to be related in part to the anti-androgenic activity of some of the chemicals present in the effluents (Jobling et al., 2009; Hill et al., 2010). Moreover, although in general the effects of androgens from the effluents have been less studied (Kirk et al., 2002), androgenicity of the effluents has been reported using the yeast andogen screenin method (YAS), suggesting that steroid androgens can also affect the exposed fish (Hill et al., 2010).

The accumulation of chemicals with probable origin in the WWTP discharges has been described in some fish species. This is the case for instance of four fish species collected in a river in Canada (Mikaelian et al., 2002). The concentration of heavy metals, PCBs and several organochlorines such as pesticides, were measured in the liver of channel catfish (*Ictalurus punctatus*), walleye (*Stizostedion vitreum*), white sucker (*Catostomus commersoni*) and lake whitefish (*Coregonus clupeaformis*), showing concentrations in the level of ng/g (Mikaelian et al., 2002). Similar chemicals were measured at the same concentration level in the livers and muscle of dab (*Limanda limanda*) from the Dublin Bay, location that also receives discharges from a WWTP (Giltarp et al., 2017).

The estrogenicity of each WWTP effluent can vary, depending of the received substances (type and concentration) and type of treatment applied in the different facilities (Kirk et al., 2002). The kind of treatments applied during the wastewater treatment process will show more or less capacity to reduce the xenoestrogenic load of the waters (Johnson and Sumpter, 2001; Nakada et al., 2007; Koh et al., 2008; Gunnarsson et al., 2009). When comparing the efficiency of the different treatments, a reduction in the estrogenic activity of the effluent compared to the influent was observed after secondary treatment in one WWTP in the UK compared (Kirk et al., 2002). In the study by Gunnarson et al. (2009), a wastewater influent was treated in parallel applying different processes, and ozonation was proved to be the most effective method to reduce the concentration of xenoestrogens in the discarded effluent. In addition, fish exposed to ozonized effluents did not present any effect (Gunnarsson et al., 2009). In the study by Korner et al. (2000) performed in a WWTP in Germany, where the treatment consisted of mechanical purification, activated sludge treatment, biological nitrate and phosphate removal and settlement, about 90% of reduction of the estrogenic activity was measured when comparing the estrogen equivalents in the influent and the effluent (Korner et al., 2000). Nevertheless, incomplete elimination of alkylphenols and BPA was reported, with a reduction from 2,13 µg/L to 0,320 µg/L (85%) and from 0,542 µg/L to 0,162 µg/L (70%) in total nonylphenol and BPA respectively (Korner et al., 2000). In addition, differences regarding the sampling season

were observed (Korner et al., 2000). In fact, the efficiency of the treatments can vary among days and seasons. In the case of hormone elimination rates, differences have been observed when comparing WWTPs. For instance, Johnson et al. (2000) reported an elimination of the 88% of the influent E2 and 74% of E1, in a WWTP from the UK, while in the work by Baronti et al. (2000), different WWTPs from Rome showed different elimination rates for E2, EE2 and E1, that ranged from 76-92%, 83-87% and 19-94% for each of them respectively.

5.3. Wastewater treatment plants and their effects on river and estuarine fish

The feminizing effects of exposure to WWTP effluents reported in male fish range from alterations recorded at the level of the molecular and gene transcription pattern in different organs and tissues, alterations at the levels of produced endogenous sex steroid hormones (Folmar et al., 2001), induction of hepatic Vtg expression (Folmar et al., 1996, 2001; Jobling et al., 1998; Harries et al., 1999; Larsson et al., 1999), to histopathological effects (Jobling et al., 1998; Rodgers-Gray et al., 2001). The most evident histopathological effects are recorded in gonads and involve impaired testis development, which in some circumstances can lead to the production of oocytes in the testes (Jobling et al., 1998; Rodgers-Gray et al., 2001).

The effects of WWTP effluents on the estuaries from the United Kingdom have been extensively studied since the 1990's (Sumpter, 1998), especially since their estrogenicity was confirmed (Purdom et al., 1994). Several endpoints related with reproduction (already reported above) have been studied in wild roach (*Rutilus rutilus*) (e.g. Jobling et al., 1998; Jobling et al., 2002a,b; Jobling et al., 2006) and flounder (*Platichthys flesus*) populations (Allen et al., 1999, Stentiford et al., 2003; Kirby et al., 2004). In addition, caging experiments have also been performed using rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) (Purdom et al., 1994; Harries et al., 1997; Harries et al., 1999) as sentinel organisms. Male flounders collected during several years (1996-2001) and male roach collected in several estuaries showed detectable plasma Vtg levels, although the Vtg concentrations varied among the sampled sites (Kirby et al., 2004, Jobling et al., 2006). Intersex male roach have been identified downstream some of these WWTPs (Jobling et al., 2002a and 2002b; Jobling et al., 2006). In some cases, plasma Vtg levels correlated with the prevalence of intersex condition in each site (Jobling et al., 1998). When reproduction success was studied, intersex males showed lower reproductive capacity than non-intersex males (Harris et al., 2011). In the case of roach females, the authors reported less affection of the ovaries, although ovarian atresia was reported (Jobling et al., 2002a).

Another example of an extensively studied area due to the impact of WWTP effluents can be found in the case of several Canadian rivers (Tetreault et al., 2013). As in the British case, intersex fish have also been detected in rivers in relation with WWTP activities. In the Grand River watershed, the greenside darter (*Etheostoma blennioides*) and the rainbow darter (*Etheostoma caeruleum*) have been studied (Tetreault et al., 2011; Tanna et al., 2013; Bahamonde et al., 2014; Bahamonde et al., 2015a, 2015b; Fuzzen et al., 2015). Those studies showed that in general, male darters with impaired reproductive health in impacted sites downstream WWTPs showing smaller gonad size, impaired sex steroid production and presence of intersex. In addition, Fuzzen et al. (2015) observed altered gonadal development also in female darters, showing a decreased egg size. Transcription levels *vtg* in the liver were inhibited in females exposed to the effluents and up-regulated in males from one of the impacted locations (Bahamonde et al., 2014). The transcription levels of other genes related with gonadal development were also measured. Although some of the genes did not show significant alterations, *dmrt1* was downregulated in exposed males (Bahamonde et al., 2014). In the particular case of the WWTP of Kitchener located in the Grand river, the improvements performed in the plant treatment procedures were monitored and a reduction of the total estrogenicity of the effluent was observed together with a decrease in the incidence of intersex darters (Hicks et al., 2017).

In addition to darters, other fish species from Canadian rivers have also been studied and intersex males have been identified among lake whitefish (*Coregonus clupeaformis*) and wild white perch (*Morone americana*) in populations influenced by industrial and domestic effluents (Mikaelian et al., 2002 and Kavanagh et al., 2004, respectively). On the other hand, no intersex male fathead minnow (*Pimephales promelas*) and brook stickleback (*Culaea inconstans*) were detected in areas downstream the WWTP effluents where they were collected, although altered gonad development and delayed spawning was observed in both species (Tetreault et al., 2012). In addition, fathead minnow males from the impacted area showed induction in liver Vtg and a reduction of the ability to produce gonadal sex steroids (Tetreault et al., 2012).

6. Intersex condition in fish under exposure to xenoestrogens

The intersex condition is described as the atypical simultaneous presence of testicular and ovarian tissue in the gonad of a gonochoristic species (Tyler and Jobling, 2008), normally occurring under exposure to EDCs, although it does not distinguish between a possible

xenoestrogenic or xenoandrogenic etiology. In any case, in most of the cases when this condition has been described authors have referred to a pathological alteration related to exposure to xenoestrogens, describing the apparition of oocytes within a differentiated testis and referring to intersex males (Bahamonde et al., 2013). Occasionally the appearance of testicular tissue within mature ovaries, intersex females, has also been described. In some cases, authors described intersex fish as intersex females, due to a clear masculinization of the ovaries, as it is the case of pikes (*Escox lucius*) from several samplings preformed upstream and downstream of a WWTP in the United Kingdom. In such study, among the total 58 female pikes that were sampled, 15 showed patches of male germ cells within the ovary, against one out of 54 males showing patches of female germ cells within their testes (Vine et al., 2005). In addition, in some cases authors have preferred not to classify the observed intersex, neither as males or as females, ignoring whether the feminization of the male gonad or the masculinization of the female gonad had occurred (van Aerle et al., 2001).

As already said, in the majority of circumstances we are referring to the intersex condition as a consequence to xenoestrogenic feminization. The severity of the intersex condition can be assessed, and several approaches to account for this have been described in order to rank the level of xenoestrogen exposure. According to the review on the topic of intersex fish by Bahamonde et al. (2013), one of the most complete approaches might be the one by Jobling et al. (1998). This index takes into account the number of oocytes present in the gonad and the score increases as the number of oocytes rises. The dissected gonads are transversally sectioned and then sectioned again to obtain a portion from the posterior, anterior and mid part of the gonad (Jobling et al., 1998). After processing the sections histologically, by embedding them in paraffin, 3 μm sections are made, stained with hematoxylin-eosin and examined under a light microscope. The mean number of oocytes present in each of the six section is measured and the severity score is calculated using their numerical scale (Jobling et al., 2006). The scale ranges from an intersex severity index of 0, that means that no oocytes have been detected in the examined sections (normal testis), up to a score of 7, when 100% of the examined tissue is ovary. Scores (S) 1, 2 and 3 are considered to be typical of a multifocal ovotestes. S1 is assigned when 1 to 5 oocytes appear scattered through the testis, S2 when 6 to 20 oocytes are present and S3 when up to 50 oocytes are quantified. S4 comprises testes that have from 50 to 100 oocytes. S5 is for testes with more than 100 oocytes and S6 when more than half of the gonadal tissue is ovarian (Jobling et al., 2006). In addition to the number of oocytes, other commonly characteristics that contribute to the ranking, are whether the oocytes appear in clusters (S2 and S3) or whether the gonads

appears likea mosaic of testis and ovary (S4). In S5 and S6 a clear separation between the testicular and the ovarian tissue is common (Jobling et al., 2006).

The intersex condition has been described in several fish species in habitats impacted by a variety of pollutants of different origins, very typically in the proximity of WWTP discharges (Abdel-Moneim et al., 2015), as described above. A high variability can be observed regarding the incidence of intersex condition among different species and also sampling locations (Figure 6). Although in most of the cases intersex fish have been detected in rivers, estuaries or coastal areas, a few cases of intersex captured in the sea have been reported, for instance, Mediterranean swordfish (*Xiphias gladius*) in the Western Mediterranean and North Ionian Seas with an intersex prevalence of 40 intersex individuals identified in a total of 162 analyzed males (25%) (De Metrio et al., 2003). Also in the Mediterranean, 2 intersex little tunny (*Euthynnus alletteratus*) from a total of 449 males were reported (Macías et al., 2014). In the North Sea, 2 intersex male dabs (*Limanda limanda*) out of 14 males were reported (Stentiford and Feist, 2005).

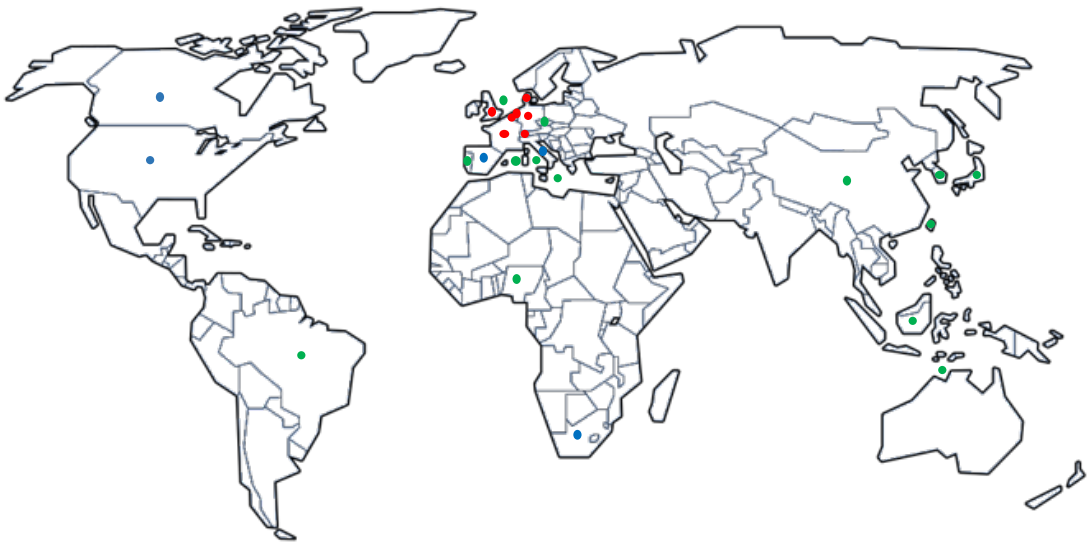

Figure 6
Map indicating countries and marine areas where cases of intersex male fish have been reported. The color of the dot indicates if fish were under the influence of wastewater treatment plant effluents (red), under other type of contamination source such as agriculture, industry or urban run-off waters (green) or both (blue). Dots do not indicate the exact position where the study was performed. For more details about intersex fish prevalence and the affected species see Chapter 4.

The incidence of intersex males can vary among samplings and in some studies, the prevalence can reach 100% of the collected males. This was the case for instance of white suckers (*Catostomus commersoni*) captured downstream two WWTPs in the district of Denver (United States) (Woodling et al., 2006). No intersex were detected in the upstream waters of the two sampled WWTPs, whereas in one of the downstream sites from the total 20 fish captured, all 4 captured males were intersex. Also in the United States, in a survey done with smallmouth bass (*Micropterus dolomieu*) from the Potomac river, high intersex prevalences were recorded, for instance, 9 out of 10 males, 9 out of 12 or 11 out of 13 (Blazer et al., 2007). In the survey performed in several rivers from the United Kingdom, Jobling et al. (1998) found intersex wild roach (*Rutilus rutilus*) in all sampled sites that comprised upstream and downstream sites where effluents of WWTPs were present, along several rivers and also in the control sites, that were selected as they did not receive effluents from WWTPs, although estrogenic diffuse contamination derived from rural areas could not be excluded. The incidence ranged from 100% of males in two localities downstream WWTPs to 4% in one of the control sites (Jobling et al., 1998).

For instance, it has to be taken into consideration that not all males captured on a site, and thus exposed to the same concentration of EDCs develop the intersex condition (Bahamonde et al., 2013). One of the possibilities for such intra-poppulation difference has been proposed to be a differential capacity to metabolize accumulated xenobiotics and another, the age of fish (Bahamonde et al., 2013). In addition, some species are more sensitive than others, and different species collected in the same site can show different intersex prevalence rates, This was the case of several rivers in the USA, where only four species among the fourteen that were sampled showed intersex condition (Hinck et al., 2009). Bahamonde et al. (2013) suggested that the possibility of a natural background occurrence of the intersex condition should be taken into account. Intersex fish have been reported in reference sites, which were thought to be free from environmental influences. Such cases have been reported in roach (Jobling et al., 1998; Bjerregaarda et al., 2006), bream (Hecker et al., 2002), brown trout (Korner et al., 2005) or darter (Tetreault et al., 2011). Nevertheless, taking these into account, the presence of intersex males in pristine environments is much scarcer than in polluted sites (Bahamonde et al., 2013). Another factor to consider is the possibility of a seasonal dependent change in the prevalence of intersex fish. The sampling period has been suggested to be important in smallmouth bass that showed higher intersex prevalence during the prespawning season (Blazer et al., 2007). The development of the intersex condition is considered to be affected by the timing and length of the exposure to xenoestrogens (Bahamonde et al., 2013). The plasticity of the

gonad development is lower in adults than in immature and the effects exerted on immature and adult fish by xenoestrogens can be different.

Intersex condition in fish has been induced under laboratory conditions in various fish species, mainly after exposure of juvenile fish before maturation, but also in fully mature males. In medaka fries exposed to EE2 (2 ng/L) until adulthood, intersex males were observed (Balch et al., 2004). Medaka exposed to EE2, E1, E2, E3 and BPA separately during their early life also developed intersex condition although with different efectivity (Metcalfe et al., 2001). Nevertheless, intersex induction has also been reported after exposure of adults. Exposure of adult medaka to E2 at concentrations ranging from 29,3 ng/L up to 463 ng/L resulted in high prevalence of intersex, and in some cases all males showed signs of intersex gonads (Kang et al., 2002). Intersex males have also been observed among adult medakas exposed to 4-tert-octylphenol (Gray et al., 1999; Gronen et al., 1999) and to a fungicide (Kiparissis et al., 2003). In addition, intersex males were generated in sxulaly mature carps (*Carassius carassius*) after exposure to a WWTP effluent (Diniz et al., 2005).

In intersex induction laboratory experiments gene transcription analyses have not been common. For instance, in the one by Hirakawa et al. (2012), adult intersex medaka resulting from the exposure to estradiol benzoate (EB) and EE2 showed hepatic upregulation of vitellogenin and choriogenin transcript levels in a microarray analysis. Upregulation of zona pellucida genes and of the oocyte marker *42sp50* was reported in the testes. They proposed the zona pellucida *Zpc5* gene as molecular marker of intersex condition in male medaka (Hirakawa et al., 2012). In rainbow trout male fry exposed to EE2 (0,01 µg/L, 0,1 µg/L, 1 µg/L, 10 µg/L) for 76 days, intersex gonads were observed even at the lowest concentration tested, together with a decrease in 11-ketotestosterone but not in E2 and testosterone (Depiereux et al., 2014). In some cases even vitellogenic oocytes were observed. Upregulation of *vtg* and downregulation of the male differentiation related genes *dmrt1*, *sox9a2*, *sdY* and *cyp11b* was reported at the highest concentrations, with no direct effect on aromatase genes (Depiereux et al., 2014).

Regarding the molecular mechanisms underlying the development of the intersex testis, the transcription levels of some sex differentiation and gonad development genes have been measured in fish populations with high prevalences of intersex condition. Nevertheless, it is not clear which are the changes leading to, and which are consequences of the development of the condition. In their review Abdel-Moneim et al. (2015), compiled data about the transcription levels of potential biomarker genes that could be related to the

occurrence of the intersex condition. They concluded that no differences existed between intersex males and normal males. Only in some cases intersex individuals showed altered transcription levels becoming more similar to those observed in females. It is the case for instance of genes related with ribosomal RNA production, transport and stockpiling. Importin α1 and α2, *42sp43*, and transcription factor III A (*gtf3a*), in thicklip grey mullets (*Chelon labrosus*) from a polluted harbor in the Basque Coast presented transcriptional levels in intersex testes in between normal testes and ovaries (Diaz de Cerio et al., 2012). In rainbow darters from Canadian rivers impacted by WWTP discharges, transcription levels of genes related with female sex differentiation and reproduction such as *sox9* and *foxl2* were upregulated in intersex testes (Bahamonde et al., 2015a). Intersex male roach also showed elevated 17β-estradiol and testosterone levels compared to normal males (Jobling et al., 2002a), suggesting a disruption of steroidogenesis in such fish. In roach, a reduced capacity to produce sperm has been reported in intersex males concluding that the high incidence of intersex in the studied populations was probably compromising their reproductive success (Jobling et al., 2002a and 2002b).

In the filed-based study by Kidd et al. (2007), chronic experimental EE2 exposure of a fathead minnow population inhabiting a lake led to several effects after seven years of chronic exposure. In males intersex condition was induced in males, while altered oogenesis was observed in females. The observed effects ultimately led to reproductive failure and population-level collapse. Those results could be indicative of the significant impacts that the intersex condition can have in wild fish populations, although as pointed out by Matthiessen et al. (2018), the EE2 concentrations reached in the lake were higher than the concentrations usually found in WWTP impacted waters. In any case, severe cases of intersex have been associated with reduced reproductive success (Jobling et al., 2002a; Harris et al., 2011; Fuzzen et al., 2015), although the possible ecological impact on fish populations with high intersex prevalence remains unclear (Mills and Chichester, 2005; Sumpter and Jobling, 2013).

7. Thicklip grey mullets *Chelon labrosus* as sentinel of xenoestrogen exposure

Chelon labrosus belongs to the Mugilidae family, which is composed of about 75 species that are classified in 20 different genera, 7 belonging to the *Chelon* genus (Nelson, 2006). Nevertheless, taxonomic conflicts have been reported (reviewed by González-Castro and Ghasemzadeh, 2015). The Mugilidae family is distributed worldwide and although includes mainly coastal species from tropical, subtropical and temperate seas (Nelson, 1984),

mugilids can inhabit numerous habitats in open sea, coastal waters, estuaries, coastal lagoons and rivers. The species *Chelon labrosus* is distributed along the Mediterranean Sea, South-western Black Sea, North-eastern Atlantic coasts, North Sea, Norwegian Sea, Barents Sea, Baltic Sea, Bay of Biscay, West African coasts and the offshore islands of the Canaries, Azores and Madeira (reviewed by Turan, 2015). The diet of most of the species is based on the organic matter from the sediment they eat, feeding mainly on the microphytobenthos. In addition, they can eat benthic invertebrates, green filamentous macroalgae, plankton and suspended organic matter (Cardona, 2015). Thanks to their complex feeding apparatus, they can exploit this trophic niche as no other species is able in the habitats they inhabit (Cardona, 2015).

Mullets are gonochoristic and although there is no sexual dimorphism, they show differentiated male and female gonads, with both sexes showing internal and paired gonads. Females are synchronous, although they have also been described as group-synchronous in several studies with a dynamic oocyte growth (González-Castro and Minos, 2015). Males show unrestricted lobular type of testis (Parenti and Grier, 2004). Gonad development has been more extensively studied in female mullets (González-Castro and Minos, 2015). McDonough et al (2005) described the gametogenic stages of both sexes in *Mugil cephalus*, using the criteria used for black sea bass. Such criteria, slightly modified for *M. cephalus* by McDonough et al., (2005) have been used also for *Chelon labrosus* with slight modifications on the nomenclature (Bizarro, 2015; Sardi et al., 2015; Rojo-Bartolomé et al., 2017; Valencia et al., 2017) According to McDonough et al. (2005), immature mullets show inactive gonads, males showing spermatogonia and spermatocytic development while females show previtellogenic oocytes. Developing males show spermatocytes and signs of spermatozoa, while developing females start showing oocytes at cortical alveoli stage among previtellogenic oocytes. Ripe males show high incidence of spermatozoa and ripe females show yolk accumulation in the oocytes. Spent testes show empty lobules and residual spermatozoa while spent ovaries show empty follicles and atretic oocytes. Resting males show empty lobules and resting females show high presence of connective tissue. Within the developing stage, previtellogenic, mid vitellogenic or cortical alveoli stage females and early, mid and late spermatogenic males have been described for *Chelon labrosus* (Valencia et al., 2017). For spawning and reproduction, mullets migrate from estuarine and coastal waters to the sea where they release the gametes into the water and fertilization occurs externally. Nevertheless, that some *M. cephalus* individuals remain in the estuaries and do not migrate offshore to spawn has been suggested (McDonough et al., 2003). This event has not been studied in depth in mugilids (González-Castro and Minos, 2015) and spawning

grounds in the Basque coast are completely unknown. Depending on the species, fry return to the estuaries at a different age. In *Chelon labrosus* from the Mediterranean Sea, it has been described that such migration occurs at the age of 2 months (Mickovic et al., 2010). Nothing is known for the process through which they are incorporated into estuaries after their reproductive migration occurring in December-February (Bizarro, 2015). In the Basque coast, the structure of the mullet population has not been studied and it is not known if they form a panmyctic population or wether different populations can be distinguished and linked to specific estuaries or geographically close estuaries.

Mugilids in general have been proposed to be sentinel species of pollution by EDCs, due to their widespread distribution, feeding habits, ability to bioaccumulate pollutants and at the same time their resilience to pollution (Ferreira et al., 2004; Whitfield et al., 2012; Ortiz-Zarragoitia et al., 2014; Tancioni et al., 2015). In Europe, fathead grey mullet (*Mugil cephalus*) from the Douro estuary in Portugal and thicklip grey mullets (*Chelon larbosus*) from estuaries and harbours in the Basque Coast have been used as pollution sentinel species (Ferreira et al., 2004; Ortiz-Zarragoitia et al., 2014). Xenoestrogenicity has been detected in several mugilid populations worldwide. *M. cephalus* from the Douro estuary showed PCB accumulation in the liver and muscle and up to 21% of males showed intersex gonads (Ferreira et al., 2004). In *Chelon haematochelius* from China and *M. cephalus* from Japan and Korea (Aoki et al., 2010; Soyano et al., 2010) contamination by environmental estrogens resulted in induced of plasma Vtg levels and appearance of intersex testes. Thinlip grey mullets (*Liza ramada*) captured in sites affected by WWTP effluents in the Tiber river in Italy showed intersex condition in up to 24% of the total studied population (Tancioni et al., 2015).

7.1. Endocrine disruption in *Chelon labrosus* from the Basque Coast

In the Basque coast, *Chelon labrosus* mullets have been sampled in several estuaries and harbor areas since 2000 (e.g. Orbea et al., 2002; Bizarro et al., 2014). Nevertheless, the studies focusing on the endocrine system and the reproduction axis of mullets under exposure to EDCs did not begin until 2005 (Raingeard et al., 2009). Since then, mullets have been sampled in several sites along the coast (Figure 7). In the Nerbioi-Ibaizabal estuary, the Arriluze marina, the commercial harbor of Santurtzi and the Galindo tributary have been studied (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). The ports of Plentzia (Diaz de Cerio et al., 2012), Ondarroa and Deba (Bizarro et al., 2014), the Oka estuary in Gernika (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017)

and the harbor of Pasaia (Diaz de Cerio et al., 2013; Bizarro et al., 2014) have been sampled as well. In many of those localities, chemical analysis of water and sediment have been performed, and sometimes the chemical analysis of mullet bile has also been conducted (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Ros et al., 2015). The obtained results confirmed that several EDCs are present and that mullets are able to accumulate them. The Oka estuary and the harbor of Pasaia have been the most extensively studied sites, and among other substances, high concentrations of PAHs, musk fragrances, several alkylphenols, BPA, phthalates and E2 have been measured in bile (Puy-Azurmendi et al., 2010, 2013; Bizarro et al., 2014; Ros et al., 2015). In mullet bile, nonylphenol concentrations of up to 7482 ng/g were recorded in Gernika in 2007, and up to 95 ng/g of octylphenol (Puy-Azurmendi et al., 2013). In the same locality, high concentrations of BPA (177,5 ng/mL in average) and some pesticides such as β-HCH (β-hexachlorocyclohexane) (1508 ng/mL in average) were reported (Ros et al., 2015). In addition, in the localities of Galindo, Arriluze, Gernika, Ondarroa and Pasaia intersex males have been detected repeatedly (Diaz de Cerio et al., 2012; 2018; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Sardi et al., 2015; Rojo-Bartolomé et al 2017; Valencia et al., 2017).

Table 2
Summary of the transcription pattern of genes related with sex differentiation, gonad development and steroidogenesis in *Chelon labrosus* intersex males compared with normal males in samplings performed in the Basque coast (Pasaia and Gernika). *5S rRNA*: 5S ribosomal RNA. t*fiiia/gtf3a*: transcription factor 3a. *piwil1* and *piwil2*: piwi like protein coding genes. *er*: estrogen receptor. *rxr*: retinoid x receptor. *cyp11b*: 11β-hydroxylase. *cyp19a1a* and *cyp19a1*: gonad aromatase. *sult*: sulfotransferase. *ugt*: UDP-glucuronosyltransferase. *cyp19a1b* and *cyp19a2*: brain aromatase. *vtg*: vitellogenin. *foxl2*: forkhead box L2. *dmrt1*: doublesex and mab-3 related transcription factor 1.

Locality	Tissue	Gene	Transcription pattern	Reference
Pasaia				
	Gonad	*5S rRNA*	Intersex higher transcript levels than males, more similar to females	Diaz de Cerio et al., 2012
		tfiiia	Intersex higher transcript levels than males, more similar to females	
		42sp43	Intersex higher transcripti levels than males, more similar to females	
		importin α1	No differences in transcription levels. High variability in males	
		importin α2	No differences in transcription levels	
		importin β2	No differences in transcription levels	
		piwil 1	No differences in transcription levels	
		piwil 2	No differences in transcription levels	
		er	No differences in transcription levels	Bizarro, 2015
		rxr	No differences in transcription levels	
		star	No differences in transcription levels	Sardi et al., 2015
		cyp11b	No differences in transcription levels	
		cyp19a1a	No differences in transcription levels	
		sult	No differences in transcription levels	
		ugt	No differences in transcription levels	
	Brain	*cyp19a1b*	No differences in transcription levels. High variability in intersex	Bizarro, 2015
		er	No differences in transcription levels	
		rxr	No differences in transcription levels	
	Liver	*vtg*	Detectable transcription levels. No differences in transcription levels	Bizarro, 2015
Gernika				
	Gonad	*cyp19a1*	No differences in transcription levels	Puy-Azurmendi et al., 2013
		er	No differences in transcription levels	
		rxr	No differences in transcription levels	
		gtf3a	No differences in transcription levels	Valencia et al., 2017
		foxl2	No differences in transcription levels	
		cyp19a1a	No differences in transcription levels	
		cyp11b	No differences in transcription levels. High variability in males	
		dmrt1	No differences in transcription levels. High variability in males	
	Brain	*cyp19a2*	No differences in transcription levels	Puy-Azurmendi et al., 2013
		er	No differences in transcription levels	
		rxr	No differences in transcription levels	
		cyp19a1b	Intersex higher transcript levels than males, but not statistically significant	Bizarro et al., 2014
		cyp19a1b	Intersex higher transcript levels than males, more similar to females	Valencia et al., 2017
		foxl2	No differences in transcription levels	
	Liver	*vtg*	Detectable transcription levels. No differences in transcription levels	Puy-Azurmendi et al., 2013
		er	No differences in transcription levels	
		rxr	No differences in transcription levels	
		vtg	Detectable transcription levels. No differences in transcription levels	Bizarro et al., 2014
		vtg	Detectable transcription levels. No differences in transcription levels	Valencia et al., 2017

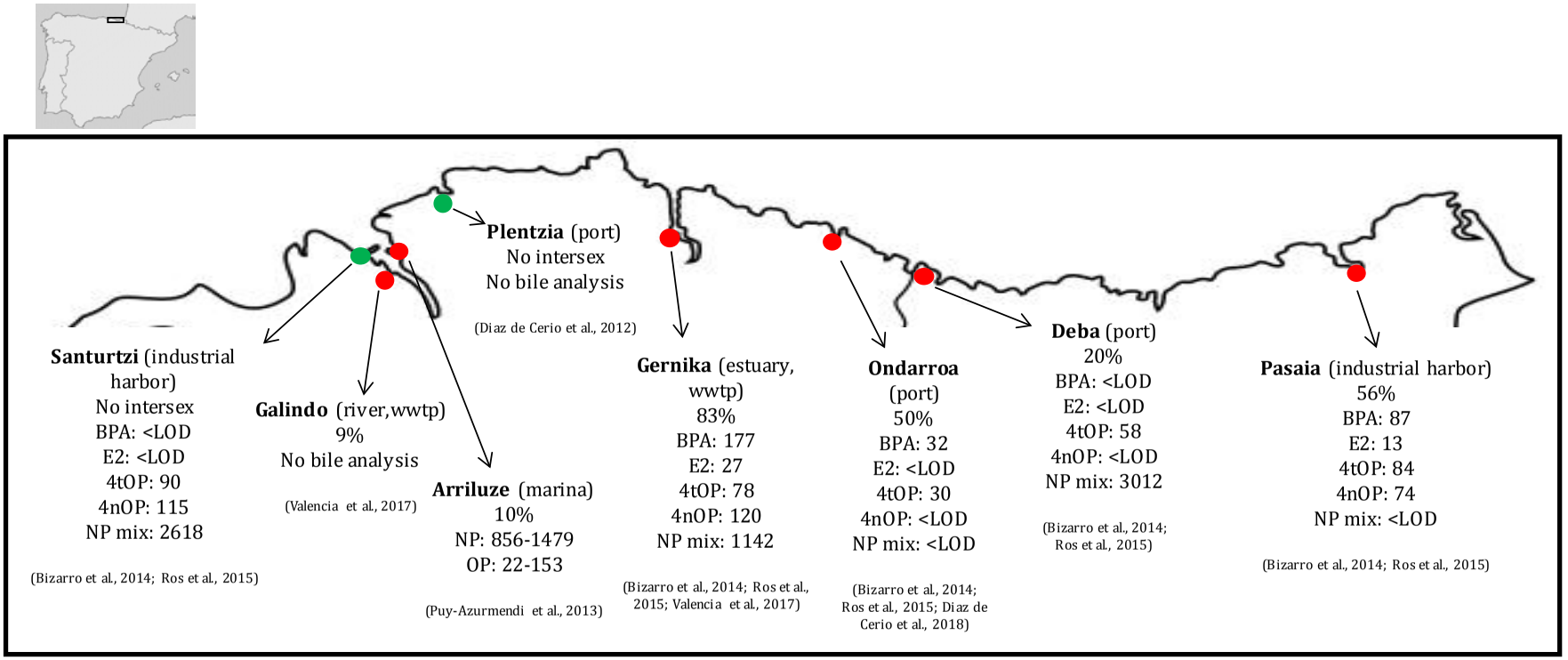

Figure 7

Map showing the locations were samplings of *Chelon labrosus* mullets have been performed along the Basque Coast, in the South East Bay of Biscay. Inside the brackets the type of location and if it is near a wastewater treatment plant (wwtp) is indicated. Percentages indicate the maximum prevalence of intersex males detected. Mean concentrations of some of the chemicals measured in mullet bile are indicated in ng/mL, except for the case of Arriluze, where average ranges are indicated in ng/g. Dots in green indicates that no intersex mullet has been detected in the location and dots in red indicates that at least once, intersex mullets have been detected. BPA: bisphenol-A. E2: 17β-estradiol. 4tOP: 4-tert-octylphenol. 4nOP: 4n-octylphenol. NP: nonylphenol.

The transcription pattern of a variety of gametogenesis and steroidogenesis related genes and the plasma levels of sex steroids has been measured along the gametogenic cycle of female, male and intersex mullets from Pasaia (Diaz de Cerio et al., 2012; Bizarro et al., 2014; 2015; Sardi et al., 2015; Rojo-Bartolomé et al., 2017; Table 2). In general, results showed altered transcription patterns of the measured genes. For instance, *vtg* transcription was detected in liver of male mullets (Bizarro, 2015). For males and females, transcription levels were measured during the whole gametogenic cycle and in cases such as *42sp43*, no differences were observed between females collected in different months (Diaz de Cerio et al., 2012). No marked season dependent differences were observed either for gonadal *cyp19a1a* and *cyp11b* (Bizarro, 2015). Although no clear pattern could be observed, transcription levels of *ugt*, *star* and *sult*, genes involved in the phase II steroid metabolism, showed differential transcription pattern among some of the studied female and male gametogenic stages (Sardi et al., 2015). The lack of a clear gametogenic stage dependent pattern was suggested to be the consequence of endocrine disruption caused by the several EDCs present in the waters where those mullets were living. Moreover, in Gernika, gene transcription analyses conducted in April and October, showed seasonal differences only for estrogen receptor and retinoid X receptor in the brain of females, whereas, a lack of variability between seasons was observed for the majority of genes analyzed; for instance brain and gonad aromatase. Such pattern, together with the lack of differences between females and males was also linked to the effect of EDCs in male mullets from that site (Puy-Azurmendi et al., 2013).

Intersex mullets from Pasaia showed transcription levels more similar to females for some of the measured genes. This was the case of genes related with the synthesis and storage of 5S ribosomal RNA (5S rRNA) (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2017). 5S rRNA itself, its transcription factor g*tf3a* and *42sp43* which upon 5S rRNA binding forms the 42S ribonucleoprotein particles(RNP) were upregulated in intersex males compared with normal males (Diaz de Cerio et al., 2012). In addition, among the genes related with steroid metabolism, the transcription levels of *ugt* (UDP-glucuronosyltransferase) were similar in intersex and females (Sardi et al., 2015). Despite those findings, the molecular mechanism underlying the development of intersex conditionin male thicklip grey mullets remains unclear and specific molecular markers of intersex condition are needed.

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State of the art, Hypothesis and Objectives

State of the Art

Among the more than 30,000 known fish species, gonochorism, with fully differentiated mature females and males, is the most usual strategy for reproduction, although several instances of hermaphroditism and even gynochorims can also be found (Piferrer, 2011). In general, fish show high plasticity during their sex differentiation process, as many external environmental factors can interfere with the fate of gonadogenesis that is in general terms genetically determined. Sex differentiation can be altered by the environmental presence of endocrine disrupting chemicals (EDCs), which have been described as "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations" (IPCS, 2002). Some of such chemicals show estrogenic activity and are usually called xenoestrogens. They are able to interfere with sex determination, sex differentiation, gamete maturation and/or reproduction. Alterations in such molecular, cellular and physiological processes have been described in fish populations in contact with xenoestrogens in the environment (Crisp et al., 1998; Jobling et al., 1998; 2002; Sanderson, 2006; James, 2011). It has to be considered that such chemicals appear in complex mixtures and that it is not easy to link the observed biological effects with specific compounds.

The most widely described histopathological alteration in fish gonads related with exposure to xenoestrogens is the intersex condition recorded in males, which is defined as the appearance of oocytes scattered through the testis in gonochoristic males (e.g. Tyler and Jobling, 2008; Bahamonde et al., 2013). This condition has been reported worldwide and in wild fish species belonging to different families (Bahamonde et al., 2013; Abdel-Moneim et al., 2015). Many questions related with the development of the intersex condition remain unanswered. The timing and length of the exposure to xenoestrogens is thought to be an important factor in the development of the intersex condition (Bahamonde et al., 2013). Nevertheless, it is not clear which is the time window when fish are more susceptible to xenoestrogen exposure. This time window is thought to be species dependent. In laboratory experiments, the intersex condition has been triggered in adult males of different species, such as adult medaka (*Oryzas latipes*) exposed to estradiol (Kang et al., 2002) or mature carp (*Carassius carassius*) exposed to a WWTP effluent (Diniz et al., 2005). In the field, differences on the prevalence of intersex males within a population depending on the sampling season have been reported in some species, suggesting that seasonality, in relation with the gametogenic stage of fish, could be an important factor (reviewed by Bahamonde et al., 2013). In addition, inter species differences have been noted, as in samplings where more than one species have been collected in the same environment, some species have

displayed intersex condition while others have not, suggesting different susceptibility to xenoestrogen effects (e.g. Hinck et al., 2009). Moreover, not all males from the same species and population show intersex gonads, thus showing intra species differences in the susceptibility or exposure history.

Although not all of the reported cases of intersex condition have been linked to exposure to WWTP effluents, a correlation between both circumstances has been stablished in many cases (e.g. Jobling et al., 1998; 2006; Bejerregaard et al., 2006; Tetreault et al., 2011; Woodling et al., 2006). Such effluents show EDC concentrations in the level of ng/L (e.g. Bizkarguenaga et al., 2012; Bueno et al., 2012; Ros et al., 2015). Populations inhabiting waters downstream WWTPs have been repeatedly studied, as it is the case of wild roach (*Rutilus rutilus*) in the United Kingdom (e.g. Jobling et al., 1998; 2006). The monitoring of different roach populations since the 1990's, has shown effects also at population level, as the most contaminated sites have shown reduced population sizes (Hamilton et al., 2014).

Classically, female specific hepatic vitellogenin synthesis or *vtg* gene transcription levels (Sumpter and Jobling, 1995; Tyler et al., 1998; Thorpe et al., 2000; Arukwe and Goksoyr, 2003) and activity or transcription levels of ovarian and brain aromatase (Arukwe, 2008; Cheshenko et al., 2008) have been used as biomarkers of xenoestrogen exposure in juvenile or male fish. Nevertheless, in the last years, transcription levels of other genes mainly related with sex differentiation and steroidogenesis have been studied as pollution biomarker genes, also in intersex males. For instance *foxl2,* which is related with female sex differentiation, and *dmrt1* or *sox9*, related with male sex differentiation have been proposed as valid biomarkers of exposure to xenoestrogens (Bahamonde et al., 2015b). Nevertheless, the molecular mechanism underlying the intersex condition remains unclear.

In the Basque Coast, which is located in the South East Bay of Biscay, the presence of intersex fish has been extensively reported in the mugilid species *Chelon labrosus*. The first report dates back to samplings performed in Gernika in 2007 (Puy-Azurmendi et al., 2013). Thereafter, in Gernika, periodic sampling campaigns have consistently identified intersex male mullets (Bizarro et al., 2014). Intersex mullets have also been detected in the Bilbao estuary, in samplings carried out in the harbor of Santurtzi and the Arriluze marina, the port of Ondarroa and the harbor of Pasaia (Diaz de Cerio et al., 2012; 2018; Bizarro et al., 2014). These fish inhabit the estuaries and harbor areas where the presence of xenoestrogens such as alkylphenols, natural and synthetic hormones and BPA have been detected in water and sediments (e.g. Ros et al., 2015). In the case of Gernika, the effluent of the WWTP discharged into the estuary has been proved to contain high concentrations of xenoestrogens (Bizkarguenaga et al., 2012). The bioavailability of such chemicals has been confirmed

through analytical methods to identify bioaccumulated chemicals in mullet bile (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Ros et al., 2015). Vitellogenin transcription in males and intersex from the studied locations certified the effects of such xenoestrogens (Puy-Azurmendi et al., 2013; Bizarro et al., 2014). In addition, the altered gene transcription pattern of genes related with gametogenesis and steroidogenesis have been characterized in mullets from the harbor of Pasaia (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Sardi et al., 2015). Some of the studied genes showed differences in transcription levels between intersex and non-intersex males, especially in the case of genes related with oocyte development. This is specially the case of genes related with the synthesis and stockpiling of 5S ribosomal RNA that function as markers of the presence of oocytes, *gtf3a* and *42sp43* (Diaz de Cerio et al., 2012; Rojo-Bartolomé, 2017; Rojo-Bartolomé et al., 2017). Such genes have been proposed to be suitable biomarkers for intersex identification (Diaz de Cerio et al., 2012; Rojo-Bartolomé, 2017). Intersex males showed higher transcription levels than males also in the case of brain aromatase (Bizarro et al., 2014). Nevertheless, in some cases it is not clear if the observed transcription patterns in intersex males are a consequence of the intersex condition itself or if they are indicative of exposure to xenoestrogens, or both. Thus, more information is needed regarding the transcription pattern of genes related with oogenesis, spermatogenesis and reproduction in intersex thicklip grey mullets for the development of specific molecular markers of intersex condition.

In this study we wanted to focus our attention on new possible biomarkers of intersex condition, related with oogenesis and spermatogenesis. In relation with male sex differentiation we have focused in genes such as *dmrt1* (doublesex and mab-3 related transcription factor), *sox9* (Sry-related high mobility group box) or *amh* (anti-Müllerian hormone), and related with female sex differentiation in genes such as *foxl2* (forkhead box L2). In addition, the transcription pattern of genes related with the BPG-axis were of special interest due to their involvement in reproduction signaling and also because they had not been previously studied in *Chelon labrosus*. On the other hand, the effects on female mullets were also of interest, as studies in relation to intersex condition displaying populations had previously focused only in male individuals, and no information had been gathered on the possible effects of xenoestrogen exposure in females.

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HYPOTHESIS

The characterization of the transcription pattern of genes related with reproduction processes such as hormonal signaling, steroidogenesis and gametogenesis along the brain-pituitary-gonad axis should be useful to understand the molecular mechanisms underlying the intersex condition in thicklip grey mullets *Chelon labrosus* exposed to environmental estrogens and to develop biomarkers of intersex condition to be utilized in xenoestrogen biomonitoring programs in the Basque Coast.

OBJECTIVES

The following objectives were stablished to test this hypothesis:

1. To compare two thicklip grey mullet (*Chelon labrosus*) populations inhabiting waters downstream of two wastewater treatment plants, Galindo and Gernika, at two different seasons, winter and late spring.
    a. To characterize during the gametogenic cycle the transcription levels of genes related with sex differentiation in the gonads of mullets.
    b. To analyze the intersex prevalence and characterize the transcription profile of genes related with sex differentiation, oogenesis and steroidogenesis in the brain and gonads in mullets from Galindo and Gernika.
2. To analyze the transcription pattern of genes involved in development and reproduction signaling in the brain-pituitary-gonad axis (*kiss2*, *gpr54* and *gnrh1* in the brain, *fshβ* and *lhβ* in the pituitary and *fshr* and *lhr* in the gonads) during the gametogenic cycle of female and male thicklip grey mullets from the harbor of Pasaia and in comparison to the pattern exhibited by intersex males.
3. To analyze the transcription pattern of genes involved in development and reproduction signaling in the brain-pituitary-gonad axis outside their archetypal and functional organ of transcription (*fshr* and *lhr* in the pituitary and *kiss2*, *gpr54*, *gnrh1*, *gthα*, *fshβ* and *lhβ* in the gonads) during the gametogenic cycle in female and male thicklip grey mullets from the Pasaia harbor and in comparison to the pattern exhibited by intersex males.
4. To analyze the evolution and trends of the yearly prevalence and severity of the intersex condition in thicklip grey mullets (*Chelon labrosus*) captured in the localities of Gernika from 2007 to 2018 and Pasaia from 2010 to 2018.
5. To analyze the prevalence of the ovarian atresia in female thicklip grey mullets (*Chelon labrosus*) exposed to environmental xenoestrogens in Gernika and Pasaia

trying to elucidate the molecular mechanisms underlying this histopathological alteration by measuring the transcription levels of genes involved in apoptosis and autophagocytosis in atretic and non-atretic ovaries.

Results and Discussion

Chapter 1

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

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European Society for Comparative Physiology and Biochemistry (ESCPB), Glasgow (United Kingdom) 2014, oral communication. Bizarro, C., Ros, O., Quesada, A., **Valencia, A.,** Vallejo, A., Prieto, A., Etxebarria, N., Navas, J.M., Cajaraville, M.P., Ortiz-Zarragoitia, M. "Characterization of xenoestrogenic effects in two Waste Water Treatment Plants with different pollutant complexity using in vitro and in vivo bioassays together with determination of emerging pollutants in fish bile and water samples".

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Valencia, A., Rojo-Bartolomé, I., Bizarro, C., Cancio, I., Ortiz-Zarragoitia, M. 2015. "Ugalketako disrupzio endokrinoa Gernika eta Galindoko araztegien inguruko korrokoietan (*Chelon labrosus*)". "I. Ikergazte: Nazioarteko ikerketa euskaraz. Kongresuko artikulu-bilduma", edited by I. Alegria, A. Latatu, MJ. Ormaetxebarria. Udako Euskal Unibertsitatea, UEU. ISBN: 978-84-8438-540-0, 335-341.

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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 waters downstream of WWTP effluents show alterations in gonad and gamete development such as intersex condition, together with molecular xenoestrogenic effects such as *vitellogenin* gene up-regulation. However, the molecular mechanisms participating in the development of intersex condition in fish have not been elucidated yet. 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 local thicklip grey mullet (*Chelon labrosus*) populations, examining the presence and severity of intersex condition, during two seasons. Molecular markers of xenoestrogenicity and gonad differentiation and development (*vtgAa, cyp19a1a, cyp19a1b, cyp11b, foxl2, dmrt1, sox9, amh, vasa* and *gtf3a*) were also studied in liver, brain and gonads. Intersex mullets were identified in the proximity of both WWTPs and *vtgAa* was transcribed in intersex and non-intersex males. Sex dependent differential transcription levels of target genes were detected in mullets from Galindo, with female marker genes showing higher transcription levels in females (*cyp19a1a*, *cyp19a1b, foxl2, sox9, and gtf3a*) and the other way around in males (*cyp11b, dmrt1* and *amh*). However, no such pattern was observed in mullets from Gernika, suggesting a modulatory effect on 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, and although 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. This was demonstrated by the higher prevalence of intersex males and the reduction in the gametogenic gene markers transcription profile differences between sexes.

Laburpena

Araztegietako isurpenak ur ingurunera iristen diren disruptore endokrinoen (DE) jatorri garrantzitsuak dira. Araztegietatik ur behera bizi diren arrain populazioek aldaketak pairatzen dituzte gonada eta gametoen garapenean, esaterako, intersex-egoera eta xenoestrogenoen maila molekularreko efektuen eraginez eragindako bitelogenina genearen gain erregulazioa. Hala ere, arrainen intersex egoeraren garapenean parte hartzen duten mekanismo molekularrak argitu gabe daude oraindik. Ikerketa honen helburua kutsatzaile karga desberdina duten bi araztegiren efluenteen (Gernika eta Bilbo-Galindo, Bizkaiko Golkoko hego ekialdean kokatuak) eragina aztertzea zen, bi garai desberdinetan, ur behera bizi diren korrokoi (*Chelon labrosus*) populazioetan, intersex egoeraren presentzia eta larritasuna aztertuz. Xenoestrogenizitatearen eta gonaden desberdintzapen zein garapenaren markatzaile molekularrak (*vtgAa, cyp19a1a, cyp19a1b, cyp11b, foxl2, dmrt1, sox9, amh, vasa* eta *gtf3a*) ere aztertu ziren gibelean, burmuinean eta gonadatan. Intersex korrokoiak bi araztegietatik ur behera aurkitu ziren, intersex eta ar ez intersexetan *vtgAa* genearen transkribapenarekin batera. Galindoko korrokoietan itu geneen transkribapen-mailetan sexuaren araberako desberdintasunak ikusi ziren, emeen gene markatzaileak (*cyp19a1a*, *cyp19a1b, foxl2, sox9 eta gtf3a*) emeetan transkribapen altuagoa erakutsiz eta alderantziz arretan (*cyp11b, dmrt1* eta *amh*). Gernikan aldiz, ez zen horrelako patroirik antzeman, bertako ED presentzia altuagoak aztertutako geneengan eragindako efektu leungarriaren adierazgarri, populazio horretako korrokoi intersexen prebalentzia altuak adierazi bezala. Ez zen hala ere erantzun xenoestrogenikoen eta intersex egoeraren arteko erlaziorik finkatu. Ondorioz, esan genezake Galindoko korrokoiekin alderatuz, Gernikakoek ED esposizio larriago baten zantzuak aurkeztu zituztela, ar intersexen prebalentzia altuagoan eta gametogenesiaren gene markatzaileen transkribapen perfilean sexu arteko desberdintasun murrizketan frogatu daitekeen bezala.

1. 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). EDCs present different chemical structures and have different sources. 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). In industrialized countries EDCs mainly arrive in the aquatic environment from the wastewater treatment plants processing municipal, industrial and hospital wastewaters (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, oestrogenic and anti-androgenic substances can cause similar effects even if their mechanisms of action 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 (Puy-Azurmendi et al., 2013; Bizarro et al., 2014), wild roach (*Rutilus rutilus*) in the United Kingdom (Jobling et al., 1998, 2002, 2006; Tyler and Routledge, 1998; Tyler and Jobling, 2008) 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, 2015a, 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 Goksoyr, 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 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 et al., 2011). It is known that EDC exposure can alter transcription profiles of *cyp19a* and *cyp11b* gene products, altering the correct functioning and the development of the ovary and the 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. Transcription factor *Sox9*, which is present in all vertebrate types and is well conserved, belongs to the Sry-related high mobility group box (SOX) proteins, which play important roles in developmental and tissue-specific processes (Schepers et al., 2002). In teleosts, as in mammals, the involvement of *sox9* in adult fish gonadal development has been stated (Uhlenhaut et al., 2009). Two forms of the *sox9* gene have been described in teleosts, *sox9a* and *sox9b* (Koopman et al., 2004; Postlethwait et al., 2004; Klüver at al., 2005). *Sox9* is able to stimulate the expression of *amh*, the anti-Müllerian hormone (a transforming growth factor β-like hormone) in mammals (de Santa Barbara et al., 1998; Arango et al., 1999, Lasala et al., 2011). Teleosts lack Müllerian ducts, but *amh* orthologs have been identified in several species (Pfenning et al., 2015). *Amh* has a negative effect on *cyp19a1* expression in both ovaries and testes in zebrafish (Rodríguez-Marí et al., 2005). Interestingly, Wang and Orban (2007) indicated the importance of *amh* in the ovary-to-testis transformation of juvenile gonads of zebrafish, as result of its role in inhibiting *cyp19a1a* expression. High *amh* transcript levels were also detected in the sex inversion process in the rice-field eel (Hu et al., 2015). Another gene related to Sry is *dmrt1* (doublesex and mab-3 related transcription factor 1). In humans, the haploinsufficiency of this gene leads to male-to-female sex reversal (Raymond et al., 1999). In fish, it is proposed as the major player in sex differentiation (Smith et al., 2013) and its expression has been related with testis development and spermatogenesis in several species (Herpin and Schartl, 2011). Furthemore, in the protandrous black porgy (*Acanthopagrus schlegelii*) the downregulation of *dmrt1* precedes the male-to-female sex

change (Wu et al., 2012). *foxl2* (forkhead box L2) is involved in ovarian development (Baron et al., 2004), oocyte maintenance (Alam et al., 2008) and it is implicated also in the regulation of aromatase expression in different fish species (Baron et al., 2004; Guiguen et al., 2010). Alterations on the expression patterns of all described genes can lead to modified sex differentiation outcomes in fish (Baron et al., 2004; Marchand et al., 2000).

Regarding gamete production in differentiated gonads, VASA is an ATP-dependent RNA helicase belonging to the DEAD-box family (Hay et al., 1988; Lasko and Ashburner, 1988). It is considered the first molecular marker for primordial germ cells (PGCs) in species such as zebrafish (Yoon et al., 1997). Maternal inheritance of *vasa* transcripts seems common to all teleost, as shown for zebrafish (Braat et al., 1999a; 1999b), medaka (*Oryzias latipes*) (Shinomiya et al., 2000), rainbow trout (Yoshizaki et al., 2000), gibel carp (*Carassius auratus gibelio*) (Xu et al., 2005) rare minnow (Cao et al., 2012), sea bass (Blázquez et al., 2011), Japanese flounder (Wu et al., 2014), a neotropical catfish (*Rhamdia quelen*) (Ricci et al., 2018).

Intersex condition, which consists in the presence of oocytes at any developmental stage within the testis of gonochoristic fish, 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 (Jobling and Tyler, 2003; Bahamonde et al., 2013; Ortiz-Zarragoitia et al. 2014). Intersex fish show transcription patterns of oocyte development related genes resembling those in normal ovaries (Diaz de Cerio et al., 2012; Bahamonde et al., 2013). 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; Rojo-Bartolomé et al., 2017). The 5S rRNA 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 (Mazabraud et al., 1975; Lubzens et al., 2010; Diaz de Cerio et al., 2012; Ortiz-Zarragoitia et al., 2014).

In the Basque coastal region, thicklip grey mullets have been used as sentinel organisms of endocrine disruption (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Puy-Azurmendi et al., 2013; Ortiz-Zarragoitia et al., 2014). Xenoestrogenic effects on mullets, such as up-regulation of *vtg* 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 (Puy-Azurmendi et al., 2013; Bizarro et

al., 2014). The main source of those EDCs detected in mullets has been associated to the effluents from the WWTPs (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 (Puy-Azurmendi et al., 2013; Bizarro et al., 2014). A pattern distinguishing male and intersex individuals in relation to 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, gtf3a, sox9, amh and vasa*) in female, male and intersex male mullets inhabiting downstream of two different WWTPs. One such WWTPs 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 linked to intersex condition identified 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).

2. Materials and methods

2.1. Study area

The Wastewater Treatment Plant (WWTP) of Gernika (43°19'01"N, 2°40'36"W) is located in the Oka estuary 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 around the river basin are agriculture and limited metallurgy, surface treatments, dye, cutlery and plastic manufacture. The plant has a biological treatment capacity of 0,4 m^3/s and 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.

Before accomplishing the site comparison analysis, levels of some of the gonad transcripts (*folx2*, *dmrt1*, *cyp19a1a*, sox9, *amh* and *vasa*) were studied in mullets at different gametogenic stages. For this purpose, gonad samples of mullets collected in the inner part of the Pasaia harbor (SE Bay of Biscay, 43°19'18'' N; 1°55'53'' W) were analyzed. This population was selected due to the availability of mullets all along the year. Discharges of urban and industrial origin arrive periodically to the area.

2.2. Fish and tissue collection

Adult (>20 cm) thicklip grey mullet (*Chelon labrosus*) individuals were collected monthly by fishing-rod in Pasaia between September 2010 and September 2011, and between October 2016 and January 2017. In Gernika and Galindo, samplings were performed in June 2013 and in February 2014. Fish were anaesthetized in a saturated ethyl-4-aminobenzoate water bath. 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. All fish handling and procedures were approved by the UPV/EHU Ethical Committee for Experimental Animals and by the regional authorities.

2.3. Histological analysis of the gonad

After fixing gonad samples for 24 hours 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 or oocytes at cortical alveoli stage and testes during early , mid and late spermatogenesis . 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 to 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.

2.4. Gene transcription analysis

The number of individuals of each sex and gametogenic stage analyzed in the developmental stage analysis using mullets from Pasaia is indicated in figure 1. The number of individuals of each sex per site and season in the case of Gernika and Galindo is indicated in figure 4 for the gonads and figure 5 for the brain. Transcription levels of *vtgAa* in liver were only carried out in male and intersex individuals and the number of individuals used for the analysis is indicated in figure 3.

2.4.1. 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 to 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 RNA concentration in gonad RNA in a Bioanalyzer 2100 (Agilent Technologies).

2.4.2. 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).

2.4.3. Sequencing of selected genes

Partial cDNA sequences belonging to the genes *foxl2*, *dmrt1, sox9, amh* and *vasa* were cloned for *C. labrosus*. Transcripts of *sox9, amh* and *vasa* were obtained by Next Generation Sequencing. For *foxl2*, *dmrt1* instead, 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 94ºC for 2 minutes, 35 cycles of denaturalizing step (94ºC for 30 seconds), annealing step (30 seconds at 59ºC for *foxl2* and 57ºC for *dmrt1*) and an elongation step (70ºC for 30 seconds) and a final step of 72ºC for 8 minutes. 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 conditions used for the cloning of gene 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, are provided (last revision performed in October 2018).

Gene	Forward Primer (5'-3') Reverse Primer (5'-3')	Fragment size (bp)	Tm (ºC)	Amino acid identity (blastx)
foxl2	CCCAGAAACCRCCGTACTC GTGTTTGSTCTCGTGWTCCCA	600	59	*98% Dicentrarchus labrax (ACW83540.1) 2e-128*
dmrt1	TTCTGCAACTGGAGGGACTG CCRTAGTAGGTGGGGTARC	380	57	*75% Odontesthes hatcheri (ACG69837.1) 3e-24*

2.4.4. Real-time qPCR

Specific primers were designed using the mRNA sequences available for *Chelon labrosus* in GenBank for the target genes (*gtf3a, cyp19a1a, cyp19a1b, cyp11b* and *vtgAa*) and the just sequenced genes (*foxl2*, *dmrt1*, *sox9, amh,* and *vasa*) (Table 2). Primers were designed using IDT and Eurofins online tools.

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 50ºC for 2 minutes and 95ºC for 10 minutes, 40 cycles of a denaturing step at 95ºC for 15 seconds and annealing step at Tm (Table 2) for 1 min, finally a dissociation stage of 95ºC for 15 seconds, 60ºC for 1 min and again 95ºC for 15 seconds. 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. For a more detailed description of the procedure, see Appendix. 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).

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 Primer (5'-3')	Reverse Primer (5'-3')	Fragment size (bp)	Tm (ºC)
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
amh	MH251321	GTAGGTTCCGTGCTCATTAAAATC	CGCCGTCCTGCTCAACTT	118	58
sox9	MH251318	GGGTGAGCTGAGTAGCGAT	GTAGGTGCTGGTAAAGGCAC	130	58
vasa	MH251319	GATGTCTTCGTTCAAGGAGAAGA	GTCCTCGTCTTCAGGCAG	108	58

2.5. Statistical analysis

Statistical analyzes were performed with the aid of the SPSS.22 statistical package (SPSS Inc., Microsoft Co., Redmond, USA). Normality was assessed with Shapiro-Wilk test. Multiple comparisons between experimental groups were analyzed with the non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test for multiple comparisons. Pairwise comparisons were performed with Mann-Whitney U-test. A Spearman correlation analysis with gonad transcripts at all gametogenic stages was performed. Significant differences were established at $p<0.05$.

3. Results

3.1. Sequencing of *foxl2*, *dmrt1*, *sox9*, *amh* and *vasa* genes

Fragments of *foxl2* and *dmrt1*, 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 sequences for *sox9*, *amh* and *vasa* were obtained after Illumina Miseq analyses of mullet gonads (data not shown). Obtained sequences were submitted and published in the NCBI database. The fragments cloned for *C. labrosus* genes represent 54% of the total coding sequence of *foxl2*, 37% of that of *dmrt1*, 21% of that of *sox9*, 11% of that of *amh* and 10% of that of *vasa*. The partial sequences of *foxl2*, *dmrt1* and *amh* obtained corresponding to domains typical of their gene families, FH superfamily, Dmrt1 superfamily and TGF-beta superfamily, respectively.

3.2. Transcription pattern of *foxl2*, *dmrt1*, *sox9*, *amh*, and *vasa* at different gametogenic stages of mullets

Females at different gametogenic stages showed quite a homogenous transcription pattern for *foxl2,* although higher, but statistically not significant, transcription levels could be observed at vitellogenesis (Figure 1). Males at early spermatogenesis showed higher *foxl2* levels transcription than males at more advanced stages, similar to those observed in immature individuals (Figure 1).

Regarding *cyp19a1a*, high transcript levels were detected in females at vitellogenic stage, although statistical differences along oogenesis were absent due to the high interindividual variability (Figure 1). Males showed low transcript levels and similar to non-vitellogenic females. A significant up-regulation was quantified in males at resting stage, in comparison to other spermatogenic stages.

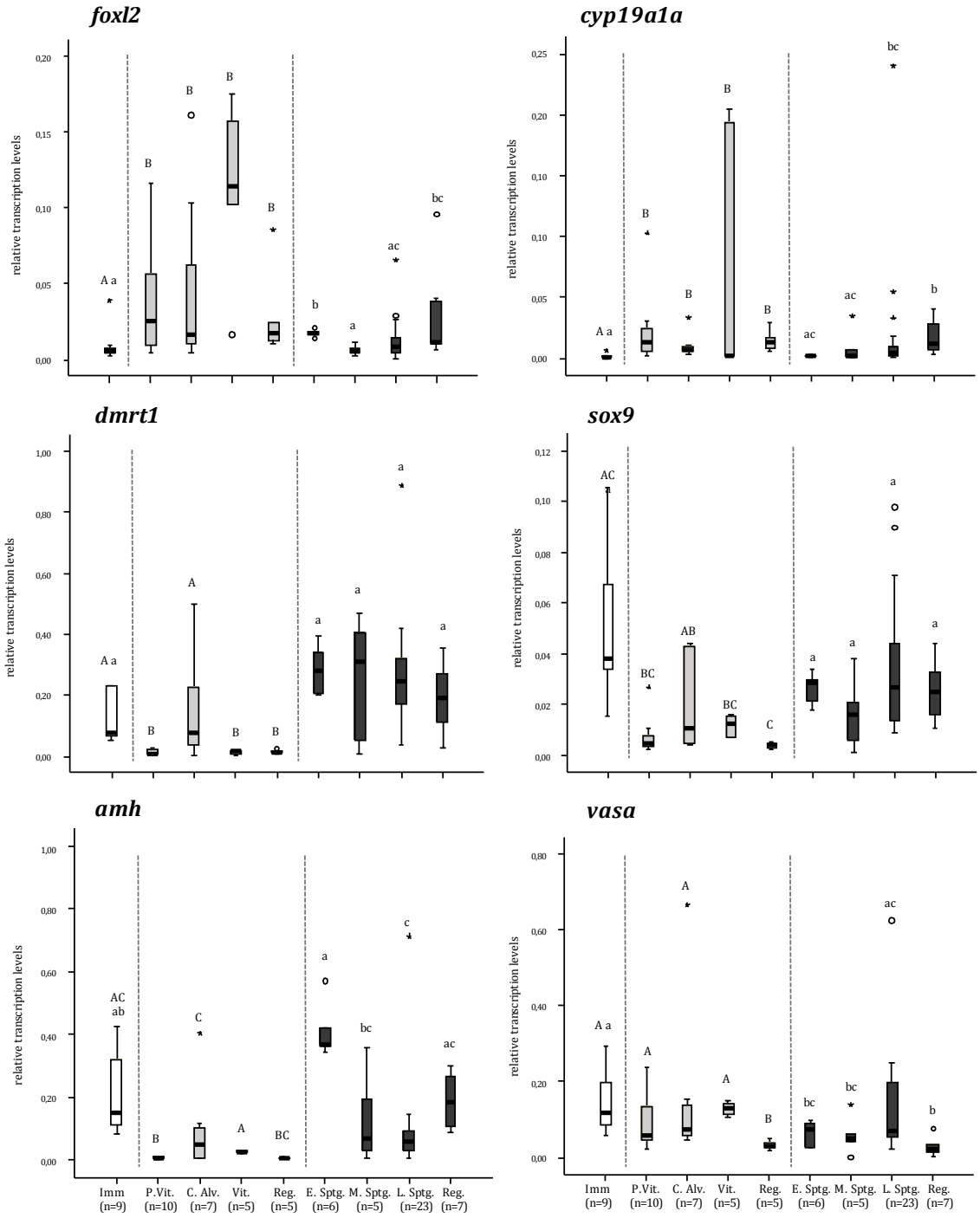

Figure 1
Transcription levels of *foxl2*, *dmrt1*, *cyp19a1a*, *sox9*, *amh* and *vasa* in gonads of immature, female (previtellogenesis, cortical alveoli stage, vitellogenesis, regressing) and male (early, mid and late spermatogenesis and regressing) thicklip grey mullets captured in the Pasaia harbor during their reproductive cycle. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots denote outlayers. Capital letters denote statistical significant differences between immatures and females and lower case letters denote statistical significant differences between immatures and males ($p \leq 0,05$, after Kruskal-Wallis test followed by Dunn's post hoc test). Number between brackets indicates the number of individuals from each group.

dmrt1 transcription levels were higher in testes at any gametogenic stage than in ovaries, with the exception of cortical alveoli stage ovaries. Such females showed the highest transcription levels, while very low levels were quantified at other oogenic stages. Males showed high *dmrt1* transcript levels at all studied spermatogenic stages, although a decreasing trend could be observed from mid spermatogenesis onwards.

The highest transcription levels of *sox9* were observed in immature mullets (Figure 1). Transcript levels during oogenesis peaked at cortical alveoli stage and remained low at other oogenic stages. During spermatogenesis *sox9* transcription did not change but remained higher than in females, with the exception of cortical alveoli stage.

Females showed the lowest transcription levels of *amh* (Figure 1). At cortical alveoli stage higher *amh* transcript levels than at other oogenic stages were detected. In males, early spermatogenic males showed the highest levels in comparison to other stages, but similar to immature individuals (Figure 1). A decrease in transcript levels occurred at mid spermatogenesis, remaining low at late spermatogenesis and slightly increasing at regressing stage (Figure 1).

vasa transcript levels were similar inimmature, female and male fish at all gametogenic stages except at regressing stage, when *vasa* levels dropped in both sexes (Figure 1). Nevertheless, a trend suggesting higher *vasa* transcript levels at vitellogenesis and late-spermatogenesis was observed.

The correlation analysis (Table 3) showed that *foxl2* and *cyp19a1a* were positively correlated. Instead negative correlationbetween *foxl2* and *dmrt1* and *cyp19a1a* and *amh* was detected. *dmrt1*, *amh* and *sox9* were positively correlated. *vasa* and *sox9* showed also a positive correlation.

Table 3
Table showing the results obtained by Spearman's correlation analysis between target gene transcription levels at gametogenesis. Statistically significant correlations are highlited in bold (p<0,05).

		foxl2	***amh***	***sox9***	***vasa***	***cyp19a1a***
dmrt1	correlation coeficient	**-0,305**	**0,706**	**0,624**	-0,044	**-0,282**
	bilateral significance	**0,004**	**0,000**	**0,000**	0,705	**0,011**
	n	**85**	**77**	**79**	78	**80**
foxl2	correlation coeficient		-0,130	-0,071	-0,044	**0,518**
	bilateral significance		0,260	0,534	0,705	**0,000**
	n		77	79	78	**80**
amh	correlation coeficient			**0,677**	-0,099	**-0,313**
	bilateral significance			**0,000**	0,395	**0,006**
	n			**77**	76	**77**
sox9	correlation coeficient				**0,330**	-0,183
	bilateral significance				**0,003**	0,106
	n				**78**	79
vasa	correlation coeficient					-0,213
	bilateral significance					0,062
	n					78

3.3. Histological and histopathological analysis of gonads from Galindo and Gernika

All analyzed female mullets 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 (Figure 2). The prevalence of intersex condition was 36% and 83% at Gernika in June and February, respectively, and 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 (Figure 2). Oocytes in intersex males were interspersed within the testes and in the previtellogenic stage (Figure 2A-B), except for a single individual from Gernika in February, which showed vitellogenic oocytes (Figure 2C). According to the intersex

scoring index developed by Jobling et al. (2006), in all cases the index was in the range 1 to 3 (Figure 2). Thus, a low to moderate severity was established for intersex condition in both mullet populations.

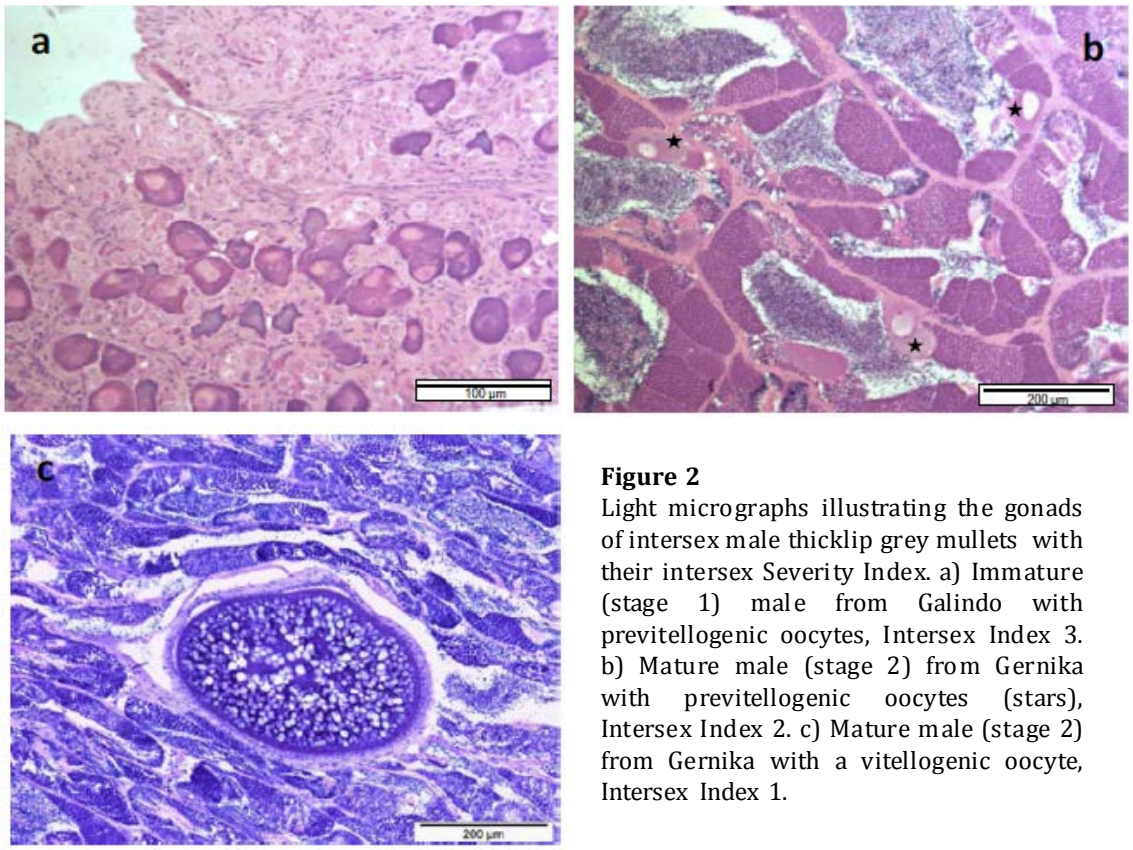

Figure 2
Light micrographs illustrating the gonads of intersex male thicklip grey mullets with their intersex Severity Index. a) Immature (stage 1) male from Galindo with previtellogenic oocytes, Intersex Index 3. b) Mature male (stage 2) from Gernika with previtellogenic oocytes (stars), Intersex Index 2. c) Mature male (stage 2) from Gernika with a vitellogenic oocyte, Intersex Index 1.

3.4. 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 (Figure 3). High variability between individuals was noted and no significant differences could be observed between normal and intersex males.

Female, male and intersex mullets from Gernika showed low transcription levels of *cyp19a1a* in gonads, with the exception of males captured in February (Figure 4). These males, showed high *cyp19a1a* levels similar to those in females captured in Galindo at both seasons. Testes of males from Galindo showed low *cyp19a1a* levels in comparison to females from the same place (Figure 4). Intersex mullets showed overall low levels of *cyp19a1a* transcription and only the intersex individual found in Galindo in February showed transcript levels higher than males and similar to February females. The analysis for *cyp11b* showed low transcript levels for all studied females, both in Gernika and Galindo. However, males from Gernika in February and from Galindo at both samplings showed high *cyp11b* transcript levels. This was not detected in the testes of males obtained in Gernika in June, which showed low *cyp11b* transcript levels, similar to females. Regarding intersex

males, *cyp11b* transcript levels were similar to females of the same sampling and lower than males, except in Gernika in June.

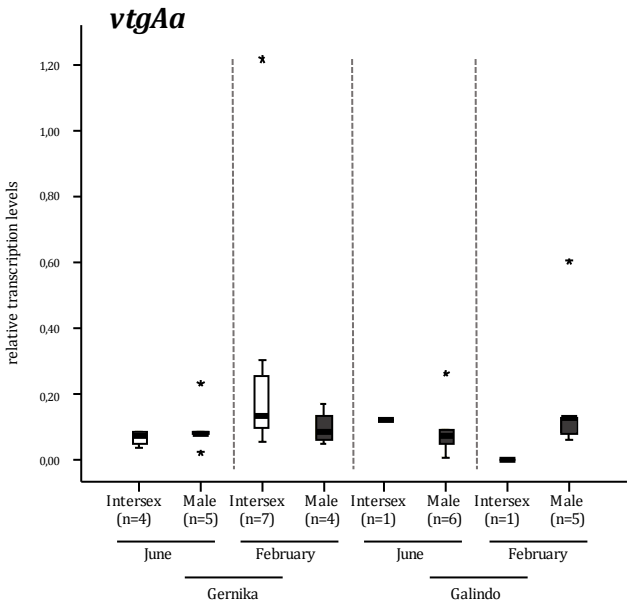

Figure 3
Gene transcription levels of liver vitellogenin (*vtgAa)* intersex and male thicklip grey mullets from the two studied populations. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Number between brackets indicates the number of individuals from each group.

Transcription levels of *foxl2* were overall higher in females than in males and intersex mullets in both sampling seasons and sites, but only females from Gernika in June showed statistically significant differences. The exception was an extremely high transcription level detected in one intersex mullet from Galindo, in February (Figure 4). Males collected in June in Gernika showed significantly lower transcription of *foxl2* than males collected in February in the same locality. Male mullets showed the highest *dmrt1* transcription levels at all of the sampling sites and during all seasons (Figure 4). Ovaries showed very low transcription levels and intersex testes showed values in between those of ovaries and normal testes, with the exception of intersex mullets from Gernika in June. On the other hand, although at Galindo only two intersex individuals were identified, those individuals showed ovary-like transcription levels (Figure 4).

Ovaries of mullets from both sites and sampling periods showed higher *gtf3a* transcription levels, than testes of normal males and intersex individuals (Figure 4). Females from Gernika showed lower transcription levels than those from Galindo, at both sampling seasons. No seasonal dependent statistical differences between females of the same site were observed. Intersex males from Gernika showed non-significantly higher transcription

levels than normal males, but below the levels observed in females. Only mullets from Galindo showed quantifiable levels of *sox9* in their gonads (Figure 4). Males showed higher *sox9* transcription levels than females at both seasons, being June the season with the highest transcription for males and females. The intersex from June showed *sox9* transcript levels similar to females from the same month. On the contrary, the intersex individual studied in February showed the highest transcription levels, such transcription being similar to that of males from the same season.

Transcript levels for *amh* were higher in males than in females at the two sampling seasons and sites (Figure 4). Intersex males from Gernika showed transcription levels between females and males (Figure 4). Males captured in February in Gernika showed higher transcription of *amh* than males from June. This was not detected in the testes of males from Galindo, with high and non-variable transcript levels for *amh* at both samplings (Figure 4). The two intersex individuals from Galindo showed very low transcription levels, similar to females.

Regarding *vasa*, female and males from Gernika showed higher transcription in February than in June (Figure 4). This pattern was not observed in Galindo, with a trend to higher levels in June than in February. In addition, females from Galindo showed always higher values than males, but this trend was not observed in Gernika (Figure 4). Intersex individuals showed always the lowest transcription levels at both sites and samplings. The exception was the intersex group from Gernika in June that showed similar levels to both females and males of the same site, which were at the same time the lowest values detected for each sex.

In brains, transcription levels for *cyp19a1b* were higher in males than in females at both sites in June while the opposite was detected in Galindo in February (Figure 5). In Gernika, no differences were detected between males and females in February. Intersex mullets did not show differences when comparing to males from the same site and sampling. Transcription levels of *foxl2* were similar between females, males and intersex mullets in both seasons and at both sampling sites. In mullets from Gernika, brain *foxl2* levels were higher in February than in June. A similar trend was also observed in Galindo. (Figure 5). Intersex individuals showed levels similar to males.

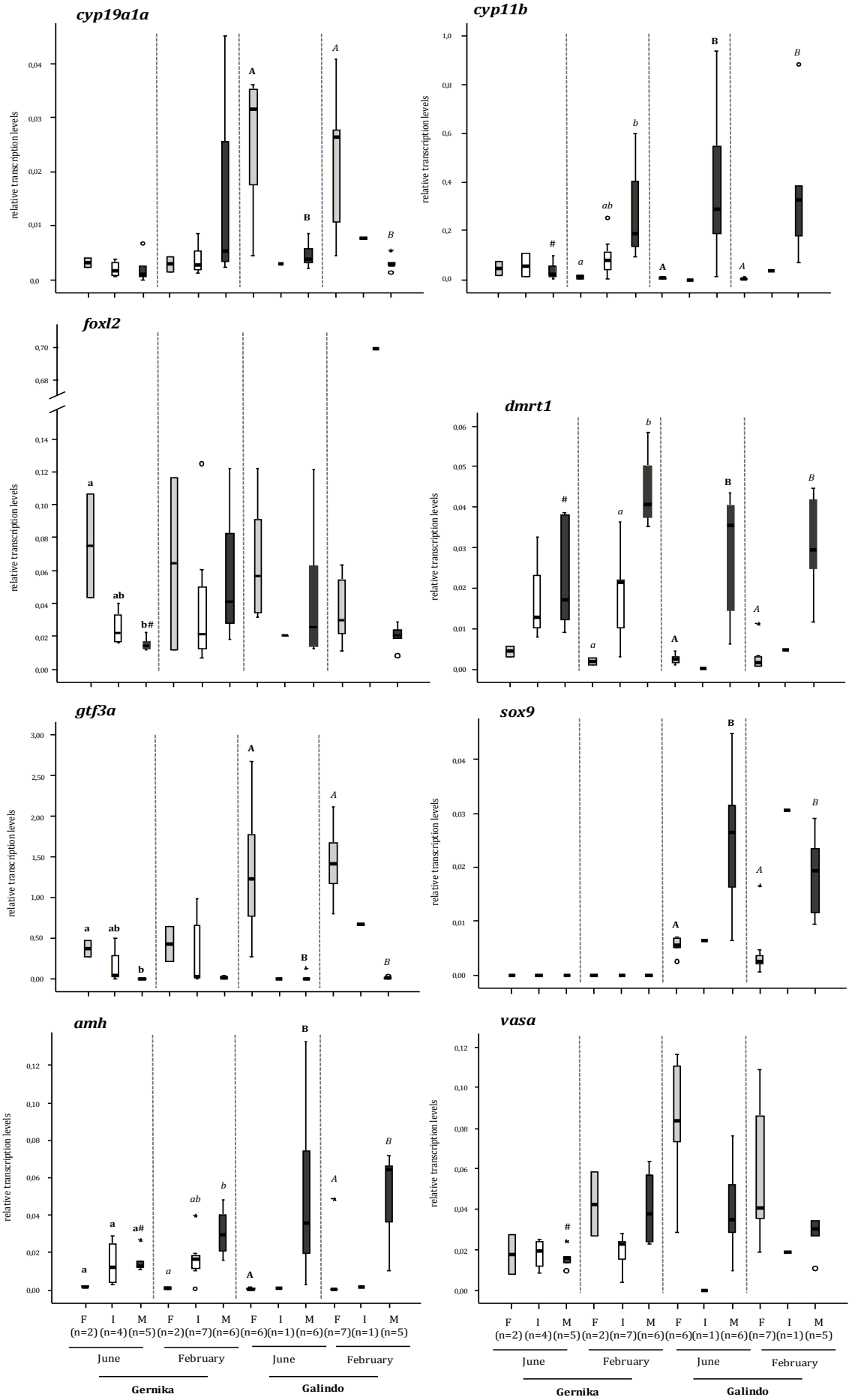
cyp19a1a
cyp11b
foxl2
dmrt1
gtf3a
sox9
amh
vasa
relative transcription levels
F I M F I M F I M F I M
(n=2) (n=4) (n=5) (n=2) (n=7) (n=6) (n=6) (n=1) (n=6) (n=7) (n=1) (n=5)
June February June February
Gernika
Galindo

Figure 4
Gene transcription levels of *foxl2*, *dmrt1*, *cyp19a1a*, *gtf3a*, *cyp11b*, *sox9*, *amh* and *vasa* in gonads of female (F), intersex (I) and male (M) thicklip grey mullets from the two studied populations. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical significant differences ($p \leq 0,05$, after Kruskal-Wallis test followed by Dunn's post hoc test) between sexes at Gernika at each sampling season (bold for June and italics for February). Hash symbol denotes statistical significant differences ($p \leq 0.05$, after Mann-Whitney U test) between males at different seasons in Gernika. Upper case letters denote statistical significant differences ($p \leq 0,05$, after after Mann-Whitney U test) between sexes at Galindo at each sampling season (bold for June and italics for February). Intersex individuals from Galindo are only shown for representative purposes and are not included in the statistical analysis. Number between brackets indicates the number of individuals from each group.

4. Discussion

In this study, we characterized the transcriptional levels of several genes related with gametogenesis and candidate target genes to EDC exposure effects. Six genes (*foxl2*, *cyp19a1a*, *dmrt1*, *sox9*, *amh* and *vasa*) were assessed at different gametogenic stages of mullets. Mullet sequences for *foxl2*, *dmrt1*, *sox9*, *amh* and *vasa* had to be previously obtained and they were published in Genbank. All of them showed correspondence with ortholog gene sequences in teleost genomes.

Foxl2, *cyp19a1a*, *amh* and *dmrt1* showed a sex dependent transcriptional profile in mullets. *Foxl2* and *cyp19a1a* transcript levels were high in ovaries while *amh* and *dmrt1* levels were high in testis. These trends are common to most teleost species (Baron et al., 2004; Diotel et al., 2010; Herpin and Schartl, 2011; Pfenning et al., 2015). *Foxl2* participates in oocyte growth by regulating *cyp19a1a* transcription, and this in trun producing the estrogens required to stimulate oogenesis (Baron et al., 2004; Guiguen et al., 2010). The highest *foxl2* and *cyp19a1a* transcription levels were detected in ovaries at vitellogenic stage and lowest values were determined at immature stage. In testes, *dmrt1* plays a crucial role in sex determination and its downregulation promotes ovarian development (Wu et al., 2012). Similarly, *amh* stimulates spermatogonia differentiation, by inhibiting *cyp19a1a* transcription (Rodríguez-Marí et al., 2005; Wang and Orban, 2007). Highest *amh* levels in mullet testes were determined at early spermatogenesis, when spermatogonia differentiation occurs.

Transcript levels for *sox9* and *vasa* were similar in ovaries and testes. Immature individuals showed the highest transcription levels for both genes, suggesting that *sox9* and *vasa* are crucial for early gametogenic cell development in mullets, as shown for other teleost (de Santa Barbara et al., 1998; Notarnicola et al., 2006; Uhlenhaut et al., 2009; Úbeda-Manzanaro et al., 2014). Transcription levels of *sox9* and *vasa* in mullet gonads maintained

constant during oogenesis and spermatogenesis and lowest values were determined at resting stage, when gonad resorption occurs.

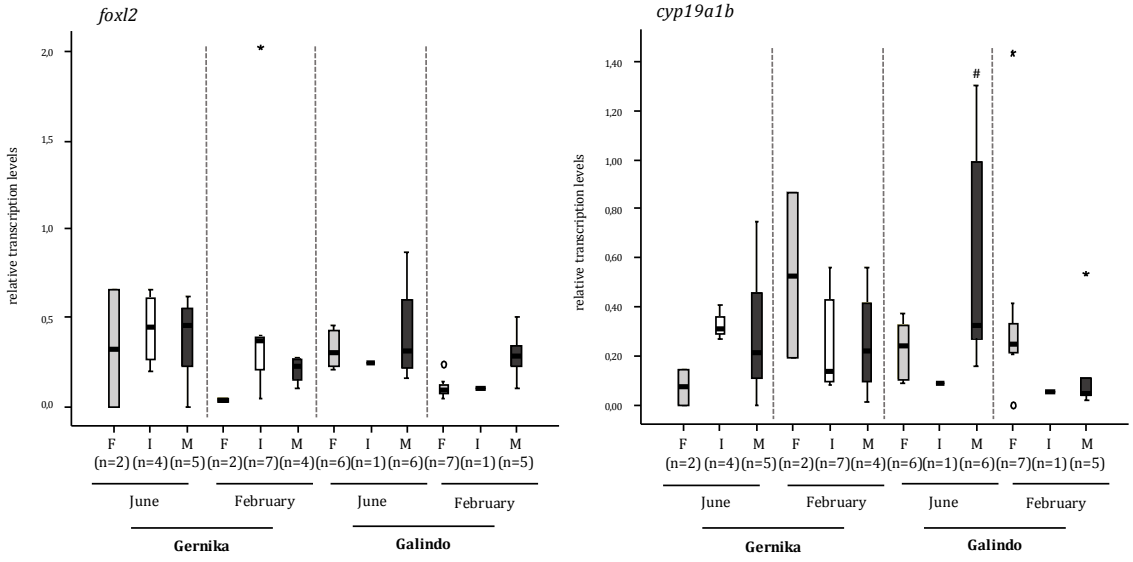

Figure 5
Gene transcription levels of *foxl2* and *cyp19a1b* in brain of female, intersex and male thicklip grey mullets from the two studied populations. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Hash symbol denotes statistical significant differences ($p \leq 0.05$) between males at different seasons in Galindo. Intersex individuals from Galindo are only shown for representative purposes and are not included in the statistical analysis.

Most of the cases of intersex condition in wild fish have been reported in relation to WWTP effluent exposure (Tyler and Jobling, 2008; Bahamonde et al., 2013; WHO/UNEP, 2013; Abdel-Moneim et al., 2015). In the present study, the intersex prevalence in mullets captured downstream the WWTP of Gernika ranged between 36% and 83%. 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 discharges to the Oka estuary, which in low tide is quite shallow and with little water volume. Consequently, chemicals are concentrated, and previous studies have shown high to moderate levels of xenoestrogenic chemicals; mainly alkylphenols (APs),

phthalates, bisphenol A (BPA) and lindane, in fish bile, sediment and water samples (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, only one intersex mullet was identified in 2013 (Puy-Azurmendi et al. 2013). The WWTP of Galindo processes the wastewater of the city of Bilbao and surrounding towns, receiving also the waters of four hospitals and discharges from industrial activities. However, Bizkarguenaga et al, (2012) concluded that the concentrations of APs, BPA, phthalates, hormones and pesticides is higher in the water discharged by the Gernika WWTP than in that discharged by the Galindo WWTP. A more modern, and thus more 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 the Galindo WWTP, more samplings are needed, as in the present study only a very limited amount of individuals could be analyzed.

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 (Arukwe and Goksoyr, 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 and seasons. Transciption 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 levels in mullets from Gernika. Of the two intersex individuals studied at Galindo, 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 previtellogenic 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* marks the presence of oocytes, also when they are produced within the testis. High *gtf3a* levels in intersex gonads was

described previously along the whole annual reproductive cycle of mullets from Pasaia (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 no show differences with males (Puy-Azurmendi et al., 2013). Similarly, we did not find significant differences between ovaries and testes at Gernika. On the opposite, in Galindo, a sexual dimorphic transcription pattern was observed, with males showing lower transcription levels than females. Sardi et al. (2015) reported that ovaries from mullets with vitellogenic oocytes present higher *cyp19a1a* transcription levels than those in earlier gametogenesis stages with a subsequent down-regulation after spawning. In this study, no seasonal variation was observed, which agrees with the fact that mullets were at the same gametogenic stage in both samplings. 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 *gtf3a*, 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; Sawyer et al., 2006; Nakamura et al., 2009; 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 the testis at the same time (Piferrer, 2001; Devlin and Nagahama, 2002; Ortiz-Zarragoitia et al., 2006; Sardi et al., 2015).

In general, *cyp11b* (11β-hydroxylase) 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. Sardi et al. (2015) also described high *cyp11b* transcript levels at early spermatogenesis than at other spermatogenic stages. Regarding intersex males at Gernika, their testes showed transcription levels of *cyp11b* similar although slightly lower than those in normal males.

foxl2 is an ovarian differentiation gene in vertebrates (Baron et al., 2004) and an upstream transcriptional regulator of *cyp19a1a* aromatase in the ovary (Guiguen et al., 2010). In

mullets from Gernika and Galindo, *foxl2* transcription levels were higher in females than in males in June, corresponding with ovaries at previtellogenic stage. Higher *foxl2* transcription levels in ovaries than in testes have also been reported in fish populations exposed to WWTP effluents, for instance in rainbow darter, *Etherostoma caeruleum* (Bahamonde et al. 2014). In our study, testes at Gernika in June and at Galindo in February 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. This has also been described in the Nile tilapia, *Oreochromis niloticus* (Wang et al., 2004). Baron et al. (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 only intersex mullet from Galindo detected 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.

dmrt1 plays a key role in fish gonadal differentiation, being highly expressed in testis (Marchand et al., 2010; 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 (Johnsen et al., 2010; Herpin and Schartl, 2011). In Gernika and Galindo, a clear difference between females and males could be observed. 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 was down-regulated after exposure to WWTP effluents (Bahamonde et al., 2014). After exposure of male adult fathead minnows to 17α-ethinylestradiol (EE2), a suppression of gene networks associated with *dmrt1* were also observed (Feswick et al., 2016). These trends were not observed in Gernika nor in Galindo, where males maintained high transcription, even if a high interindividual variation could be observed. Nevertheless, intersex mullets from Gernika, while showing the same transcription range in June and February, when compared with males, showed down-regulation of the transcription in February.

sox9 is necessary for male sex determination (de Santa Barbara et al., 1998) as well as for ovarian follicle development (Notarnicola et al., 2006). Transcripts of *sox9* were not detectable in the gonads of mullets from Gernika, something that cannot be said about males and females from Galindo. Downregulation of *sox9* is known to occur after exposure to estrogens (Barske et al., 2010; Leet et al., 2011), and this could be reflected in the observations hereby in Gernika.

A clear sexual dimorphic transcription pattern for *amh* was observed in Gernika and Galindo in the two studied seasons. Intersex males from Gernika in February showed lower transcription levels than males, indicating a downregulation that could be related to the feminization of the gonads. The two intersex individuals from Galindo showed transcription levels similar to females from the same site. The high sensitivity of this gene to xenoestrogens such as EE2 has been suggested to be involved in the disruption of gonad development in wild fish (Schulz et al., 2007).

In teleost gonads, the expression pattern of *vasa* does not show sexual dimorphic expression, except for the case of tilapia, where two *vasa* isoforms (*vas-s* and *vas*), show differential predominant transcription of one or the other in ovary and testis (Kobayashi et al., 2002). In Gernika, although no differences were observed between females and males in both seasons down regulation of *vasa* transcription was observed in intersex males captured in February. On the contrary, mullets from Galindo showed sexual dimorphic transcription of *vasa*. Although the difference between sexes was not statistically significant, females showed higher transcription than males. That *vasa* is maternally supplied has been reported in several and diverse teleost species such as zebrafish (Braat et al., 1999a; 1999b), medaka (*Oryzias latipes*) (Shinomiya et al., 2000), rainbow trout (Yoshizaki et al., 2000), gibel carp (*Carassius auratus gibelio*) (Xu et al., 2005) rare minnow (Cao et al., 2012), sea bass (Blázquez et al., 2011), Japanese flounder (Wu et al., 2014) and a neotropical catfish (*Rhamdia quelen*) (Ricci et al., 2018). The higher transcription observed in females from Galindo could be related to the stockpiling of *vasa* transcripts in oocytes. However, the values observed in the ovaries of females from Gernika, could suggest an alteration caused by exposure to xenoestrogenic chemicals.

In summary, modulation of the levels of transcription of analyzed genes in the ovaries and testes of mullets from Gernika was observed, this being more marked in the June sampling. This could suggest exposure to xenoestrogenic chemicals in mullets from Gernika, where higher prevalence of intersex condition was detected in comparison to Galindo. Intersex mullets at both sites showed transcript levels of selected genes more similar to females than to non-intersex males, indicating the crucial role of studied genes in gamete differentiation and development in mullets.

Regarding transcription patterns of female marker genes in brain, *foxl2* transcription levels were higher in males than in females in February, while no differences were observed in June. The transcription of *foxl2* also shows a sexual dimorphic transcription pattern in several fish species (Jiang et al., 2011; Sridevi and Senthilkumaran, 2011). 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, at least in June.

5. Conclusions

The studied genes play a crucial role in the gametogenesis of mullets, *foxl2, cyp19a1a* and *gtf3a* transcription being female specific and *dmrt1*, *cyp11b* and *amh* male specific. *Vasa* and *sox9* can be suggested to be required to sustain gamete development in both female and male mullets. The presence of intersex male mullets together with detectable levels of hepatic *vtgAa* transcription in males and intersex mullets 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 gonad transcription levels of all studied genes from both sites, suggested altered oogenesis and spermatogenesis in mullets from Gernika, as they showed lower transcription levels than mullets from Galindo. 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. Among studied genes *amh* and *dmrt1* could be suggested as the most powerful markers of exposure to xenoestrogens in mullets used as sentinels of exposure to chemical compounds in future monitoring programs.

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Chapter 2

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

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Abstract

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

Laburpena

Arrain teleosteoen ugalketa zikloaren erregulazioa burmuin-pituitaria-gonada (BPG) ardatzaren menpekoa da. BPG ardatzeko geneen transkribapen profila desberdina da ar eta emeen ziklo gametogenikoan. Konposatu disruptore endokrinoek bidezidor horietan duten eragina jakina bada ere, BPG ardatzak arrainetan ematen den intersex egoeraren garapenean duen eragina ez da oraindik ondo ulertzen. Intersex korrokoiak (*Chelon labrosus*) Bizkaiko golkoko hego ekialdeko hainbat estuarioetan aurkitu dira, feminizazioa eragiten duten kutsatzaileen presentzia erakutsiz. Aurretik egindako lanetan, esteroidogenesiarekin eta gametoen hazkuntzarekin erlazionaturiko geneen transkribapen patroia eme, ar eta intersexetan desberdina zela ikusi zen. Hala ere, ugalketaren erregulazioan parte hartzen duten BPG ardatzaren atal asko ez dira oraindik ikertu espezie honetan. Lan honen helburua Pasaian harrapatutako korrokoi ar, eme eta intersexetan ziklo gametogenikoan zeharreko BPG ardatzeko geneen transkribapen maila neurtzea zen. Gonadak histologikoki sexatu ziren eta aurretik sekunetziaturiko itu geneen transkribapen maila qPCR bidez neurtu zen: *kiss2*, *gpr54* eta *gnrh1* burmuinean, *lhβ* eta *fshβ* pituitaruan eta *lhr* eta *fshr* gonadetan. Eme eta arretan burmuineko geneen transkribapena altuagoa zen gametogenesi hasiera, oogenesi eta espermatogenesiaren hasieran duten garrantzia ziurtatuz. Emeetan, pituitariako gonadotropinak oogenesian zehar areagotu ziren, transkribapen mailarik altuena bitelogenesian neurtu zelarik. Arretan aldiz, spermatogenesian zehar transkribapen maila konstante mantendu zen. Ar eta emeen arteko desberdintasunak antzeman ziren gonadotropinen errezeptoreen transkribapen mailetan, horrek gametogenesi eta heltzean funtzio desberdina dutela iradokitzen duelarik. Intersex korrokoiek albeolo kortikal fasean zeuden emeen eta espermatogenesi erdialdean zeuden arren antzeko transkribapen maila erakutsi zuten burmuineko itu geneentzako. Gonadetako gonadotropinen errezeptorei dagokienez, intersexetan, arrekin alderatuz *fshr*-ren transkribapen mailen murrizketa ikusi zen, beraz, korrokoietan *fshr* intersex egoeraren biomarkatzaile egokia dela esan daiteke.

1. Introduction

Several studies have provided understanding of the Brain-Pituitary-Gonad (BPG) axis components and their functions in relation to reproduction control in teleost fish (Levavi-Sivan et al., 2010). Environmental cues relevant for the control of gametogenesis and reproduction (temperature, circadian rhythms, food availability, social context…) are integrated in the hypothalamus, where the activation of kisspeptin neurons leads to the stimulation of downstream processes along the axis, for the first time at the onset of puberty (Roa et al., 2008; Oakley et al., 2009; Tena-Sempere et al., 2010). In vertebrates, two kisspeptin paralog genes, *kiss1* and *kiss2*, exists, together with the two paralogs coding for their receptors, *gpr54-1* and *gpr54-2* (Pinilla et al., 2012). Kisspeptins play a crucial role as activators of the release of gonadotropin releasing hormones, GnRHs, decapeptides synthesized in the GnRH neurons in the hypothalamus. Three forms of GnRHs are present in vertebrates coded by *gnrh1*, *gnrh2* and *gnrh3*. The activation of GnRHs leads to the synthesis and release of the two gonadotropin hormones in the pituitary. While both gonadotropins share a common α-subunit (*gthα*), each one contains a specific β-subunit, *lhβ* in the case of the luteinizing hormone, LH, and *fshβ* in the case of the follicular stimulating hormone, FSH (Pierce and Parsons, 1981). Endogenous physiological stimuli as, for instance, metabolic state and gametogenic stage participate in the control of this event. Circulatory system transports gonadotropins into the gonads, where they bind to their specific gonadotropin receptors; FSHR located in granulosa and theca cells in the ovaries and in Sertoli cells in the testis, and LHR located in granulosa cells in the ovaries and Leydig cells in the testis, to activate steroidogenesis. The produced sex steroids can cause a positive or negative feedback, depending on factors such as gametogenic stage, in the BPG-axis (Roa et al., 2008; Levavi-Sivan et al., 2010; Zohar et al., 2010).

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

Regarding gonadotropins in teleosts, the first and most studied transcription pattern is that of salmonids, where at early gametogenic stages *fshβ* transcription is higher than *lhβ*,

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

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

observed after exposure to xenoestrogens such as NP in rainbow trout *Oncorhynchus mykiss* (Harris et al., 2001) or bisphenol-A in juvenile zebrafish (Chen et al., 2017).

Some male thicklip grey mullets (*Chelon labrosus*) in estuaries and port areas from the Basque Coast (SE Bay of Biscay) show intersex condition, with oocytes within their testicular tissue, (Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). Presence of EDCs, most commonly alkylphenols, phthalate ester plasticizers and some pesticides was evident in those estuaries as measured by different chemical analytical techniques in sediments, water and fish bile (Puy-Azurmendi et al., 2010 and 2013; Bizarro et al., 2014; Ros et al., 2015). Transcription pattern of several genes related with sex differentiation, steroidogenesis and gametogenesis have been characterized in liver, brain and gonads of female, male and intersex mullets from the Pasaia harbor (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Sardi et al., 2015; Rojo-Bartolomé et al., 2017; Valencia et al., 2017). Male and intersex mullets showed detectable vitellogenin transcription levels in the liver, confirming exposure to feminizing EDCs (Bizarro et al., 2014). Intersex mullets showed also different transcription levels of both brain and gonad aromatase compared with non-intersex males (Bizarro et al., 2014; Sardi et al., 2015). In addition, gonadal 5S/18S rRNA index, normally higher in teleost ovaries than in testes (Rojo-Bartolomé et al., 2017), was higher, and more similar to females in intersex individuals than in males. This was notorious in intersex individuals showing high severity index and thus high prevalence of oocytes in the testis (Rojo-Bartolomé et al., 2017). Furthermore, intersex males showed transcription levels for the liver phase II steroid conjugation enzyme gene *ugt* (UDP-glucuronosyltransferase) similar to those of females (Sardi et al., 2015).

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

condition and in pollution biomonitoring programs using thicklip grey mullets as sentinel organisms.

2. Material and methods

2.1. Study area, fish sampling and tissue collection

The inner part of the Pasaia harbor, located in the SE Bay of Biscay (43°19'18'' N; 1°55'53'' W), was selected as sampling area. Adult (>20 cm) thicklip grey mullets (*Chelon labrosus*) were captured by fishing-rod. Fish included in these analyses were captured between September 2010 and September 2011, and between October 2016 and January 2017, although pituitaries were only collected during the 2016/17 sampling period. Fish were anaesthetized upon capture in a saturated ethyl-4-aminobenzoate water bath. All fish handling and procedures were approved by the UPV/EHU Ethical Committee for Experimental Animals and by the regional authorities. Dissections were immediately performed *in situ*. Brain, pituitary and a portion of the gonad of each fish were collected in RNAlater solution (Ambion; Life Technologies, Carlsbad, USA) and then frozen in liquid nitrogen until arrival at the laboratory where they were immediately stored at -80°C until processing. Another portion of the gonad of each fish was fixed in 4% neutral buffered formalin for histological analysis.

2.2. Histological analysis of the gonad

Gonad samples were fixed for 24 h in 4% neutral buffered formalin, then dehydrated in a graded ethanol series (70%, 90%, and 96%), and embedded in paraffin. Sections of 5 μm thickness were cut in a Leica RM 2125 RT manual microtome (Leica Microsystems, Nussloch, Germany) and then stained with hematoxylin/eosin (Gamble and Wilson, 2002) using the Leica Autostainer XL and mounted with the aid of the Leica CV 5030 workstation. Three sections per individual were microscopically analyzed with an Olympus BX61 light microscope (Tokyo, Japan). Sex and gametogenic stage of each individual were determined using the criteria by McDonough et al. (2005). The Intersex Severity Index described by Jobling et al. (2006) was used to classify intersex males.

2.3. Gene transcription analysis

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

2.3.1. Extraction of total RNA

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

2.3.2. cDNA synthesis and quantification

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

2.3.3. Sequencing of selected genes

With the exception of *fshr*, whose sequence was obtained from a Miseq Illumina RNASeq analysis of mullet gonads (unpublished data), partial target gene cDNA sequences were obtained from PCR amplification of total RNA from *C. labrosus* using degenerate primers (Table 1). Such primers were designed for each target gene using well-conserved domains revealed after ClustalW analyses of sequences available for other teleosts in the NCBI database (Table 1). Sequences of interest were amplified through PCR using cDNA pooled from mullet target organs. Applied PCR conditions were as follows: an initial step of 94°C for 2 min, 35 cycles of denaturalizing step (94°C for 30 s), annealing step (30 s at Tm, see table1), an elongation step (70°C for 30 s) and a final step of 72°C for 8 min. The size of the amplicons was checked by electrophoresis in agarose gels (1.5%) stained with ethidium bromide. The obtained gene fragments were sequenced in the Sequencing and Genotyping Service of the University of the Basque Country. Sequences were annotated upon Blastx analysis and submitted to the NCBI database for publication (Table 1).

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

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

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

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

2.3.4. Real-time qPCR

Specific primers were designed using the partial sequence obtained for each gene (Table 2). Primers were designed using IDT and Eurofins online tools. Real time qPCR analyses were performed in a 7300 Real-Time PCR system thermocycler (Life Technologies). *kiss2*, *gpr54* and *gnrh1* were analyzed in the brain, *lhβ* and *fshβ* in the pituitary and *lhr* and *fshr* in the gonads. Each sample was analyzed in triplicates in a total volume of 20 µL containing 7.88 µL of water, 10 µL of SYBR Green fluorescent dye master mix (Roche Diagnostics, Indianapolis, USA) and 0.12 µL 12.5 pmol primer pair. A control without template was run (also in triplicate) in each plate using the same reaction conditions. The qPCR conditions

were as follows: an initial step at 50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of a denaturing step at 95°C for 15 seconds and annealing step at Tm (Table 2) for 1 minute, finally a dissociation stage of 95°C for 15 seconds, 60°C for 1 minute and again 95°C for 15 seconds. The reaction efficiency of each plate was calculated using a standard curve consisting in serial dilutions of pooled cDNA. The specificity of the reaction was determined confirming the presence of a single peak in the dissociation curve, and the absence of primer dimmers. For a more detailed description of the procedure, see Appendix. cDNA concentration obtained by fluorescent quantification method was used for normalization in brain, pituitary and gonad, as performed by Rojo-Bartolomé et al. (2016).

2.4. Statistical analysis

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

3. Results

3.1. Histological analysis of the gonads

The histological analysis of the gonads of the mullets collected monthly from September 2010 to September 2011 and from October 2016 to January 2017 was done in order to determine the sex and gametogenic stage of each mullet. Mullets were classified as immature, if the gonad could not be identified as ovary or testis. Regarding females, four different gametogenic stages were observed: previtellogenic, cortical alveoli, vitellogenic and regressing. Males were classified as early, mid or late spermatogenic and regressing. A total of 24 intersex males were identified in the 2010/2011 samplings; whose brains were selected for the genes transcription analysis in the brain. Only 13 intersex gonads were selected for further transcription analysis in the gonads. No intersex gonad was identified in the samplings carried out in the 2016/2017 sampling period, were pituitaries were collected. As a consequence, no pituitaries from intersex fish were available for further transcription level analyses. Intersex males were at different spermatogenic stages, ranging from immature or early spermatogenic stage to late spermatogenesis. All intersex individuals showed oocytes at previtellogenic stage with a very low intersex severity index that was in all cases classified as 1 in a scale of 7 (Jobling et al., 2006).

3.2. Sequencing of selected genes

Partial target gene sequences were obtained using degenerate primers and upon Blastx analysis and homology search identified as thicklip grey mullet orthologs for *kiss2*, *gpr54*, *gnrh1*, *lhβ, fshβ* and *lhr* (Table 1). The sequence for *fshr* was obtained after Illumina Miseq analyses of mullet gonads (data not shown). Obtained sequences were submitted and published in the NCBI database (Tables 1 and 2). All partial sequences displayed high level of sequence identity with ortholog teleost sequences (Table 1). The fragments cloned for *C. labrosus* genes represent the following percentages in respect to the total coding sequence of each gene: 49% for *kiss2*, 31% for *gpr54*, 23% for *lhβ*, 63% for *fshβ*, 8% for *lhr* and 15% for *fshr*. After Blastx analysis *gpr54*, *fshβ* and *fshr* putative conserved domains were identified in the obtained partial sequences. *gpr54* and *fshr* contained domains belonging to the transmembrane G protein coupled receptor superfamily, while *fshβ* contained domains related to the glycoprotein hormone beta chain homologue superfamily.

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

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

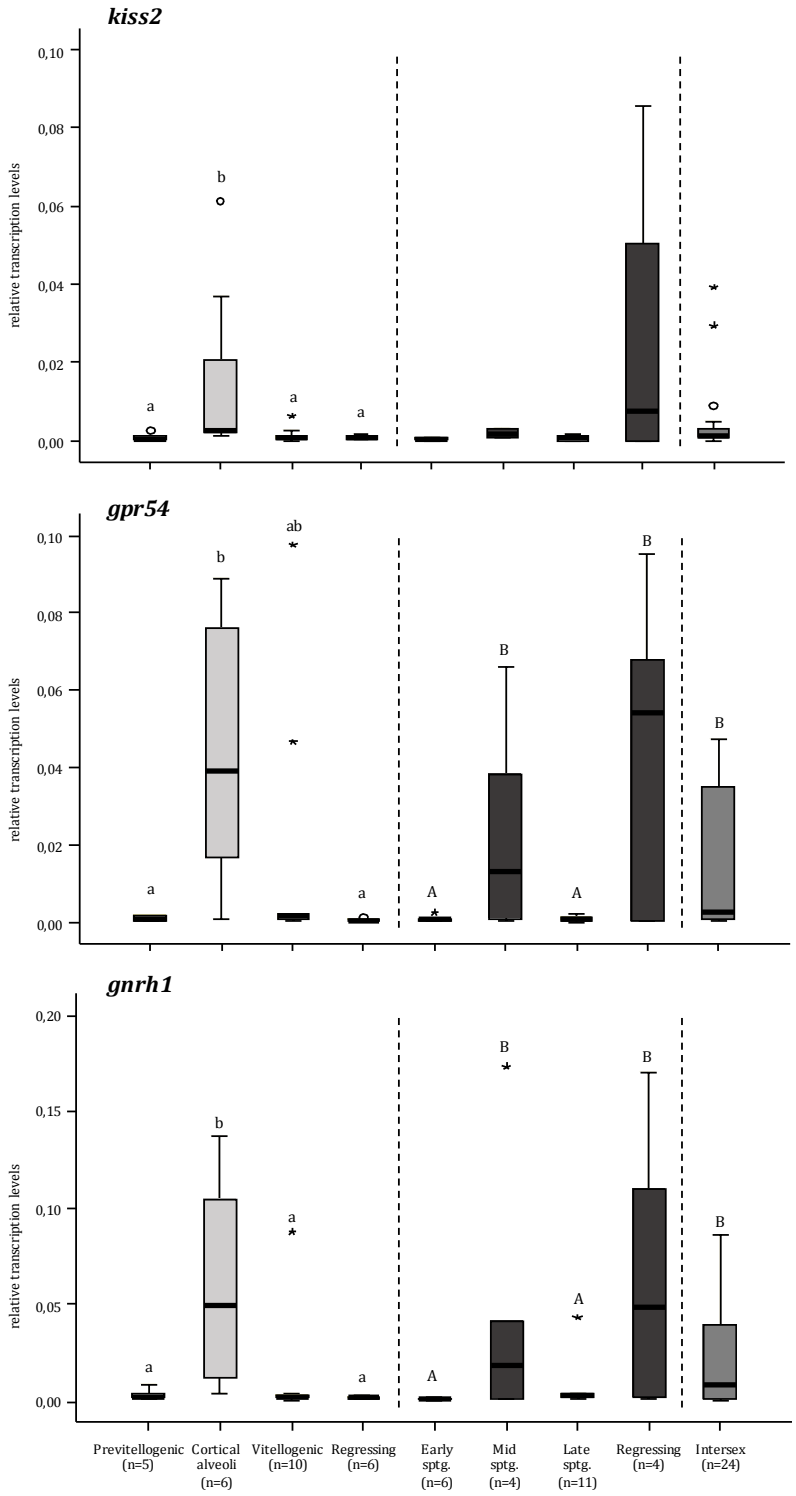

Figure 1
Gene transcription levels of *kiss2*, *gpr54* and *gnrh1* in brain of females (grey) in different gametogenic stages (previtellogenic, cortical alveoli stage, vitellogenic, regressing), males (black) in different gametogenic stages (early spermatogenesis, mid spermatogenesis, late spermatogenesis and regressing) and intersex (dark grey) thicklip grey mullets. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical differences between means ($p \leq 0,05$, after Kruskal-Wallis test followed by Dunn's post hoc test) for females; upper case letters denote statistical differences between means ($p \leq 0,05$ after Kruskal-Wallis test followed by Dunn's post hoc test) for males and intersex. Number between brackets indicates the number of individuals from each group.

3.4. Transcription levels of *fshβ* and *lhβ* in the pituitary

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

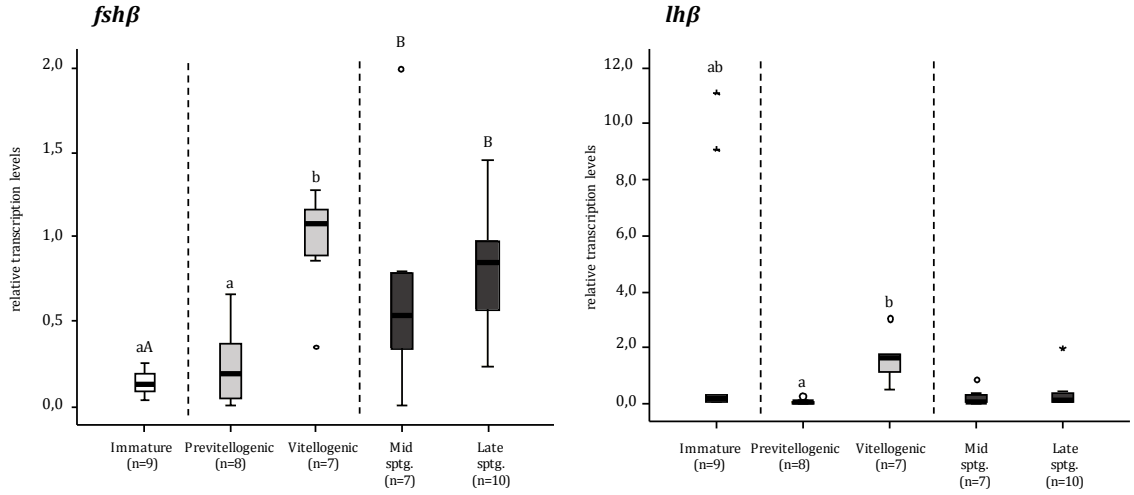

Figure 2
Gene transcription levels of *lhβ* and *fshβ* in pituitaries of, immatures (white), females (grey) in different gametogenic stages (previtellogenic and vitellogenic), males (black) in different gametogenic stages (mid spermatogenesis and late spermatogenesis) thicklip grey mullets. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical differences between means ($p \leq 0,05$, after Mann-Whitney U test) for females and immatures; upper case letters denote statistical differences between means ($p \leq 0,05$, after Mann-Whitney U test) for males, immatures and intersex. Number between brackets indicates the number of individuals from each group.

3.5. Transcription levels of *fshr* and *lhr* in the gonads

Transcription levels of *lhr* in ovaries did no change throughout oogenesis (Figure 3). Nevertheless, ovaries at cortical alveoli stage showed a slight up-regulation in comparison to the other oogenic stages (Figure 3). Testes at early spermatogenesis showed slightly but not significant higher transcription levels for *lhr* than at mid and late spermatogenesis.

Then, transcription increased at regressing stage (Figure 3). Intersex testis showed similar transcription levels for *lhr* to corresponding non-intersex testes stages (Figure 3). Regarding transcription levels of *fshr*, immature mullets showed high transcription levels (Figure 3). Females showed up-regulation in *fshr* transcription levels from previtellogenesis to cortical alveoli and vitellogenesis stages, which decreased again at regressing stage (Figure 3). In general, ovaries showed lower transcription levels than testes for *fshr*. In males, *fshr* transcription levels remained constant through the different stages of spermatogenesis (Figure 3). However, intersex males showed lower transcription levels than the other males, being similar to those of ovaries at previtellogenesis (Figure 3).

4. Discussion

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

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

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

In the case of female thicklip grey mullets, *kiss2, gpr54* and *gnrh1* showed an identical transcription pattern along oogenesis, with the highest levels at the cortical alveoli stage. Other fish species also showed such a concerted transcription pattern of brain genes. In female *Mugil cephalus*, association between *GnRH1* and *gpr54* was reported (Nocillado et al., 2007). In sea bass, *Dicentrarchus labrax*, *kiss2*, *gpr54-1b* and *gpr54-2b* presented the same transcription profile during oogenesis (Alvarado et al., 2013), and in female chub mackerel, *Scomber japonicus*, *kiss1*, *kiss1r* and *gnrh1* showed parallel transcription profile (Ohga et al., 2013). In addition, in the chub mackerel, these transcripts peaked at cortical

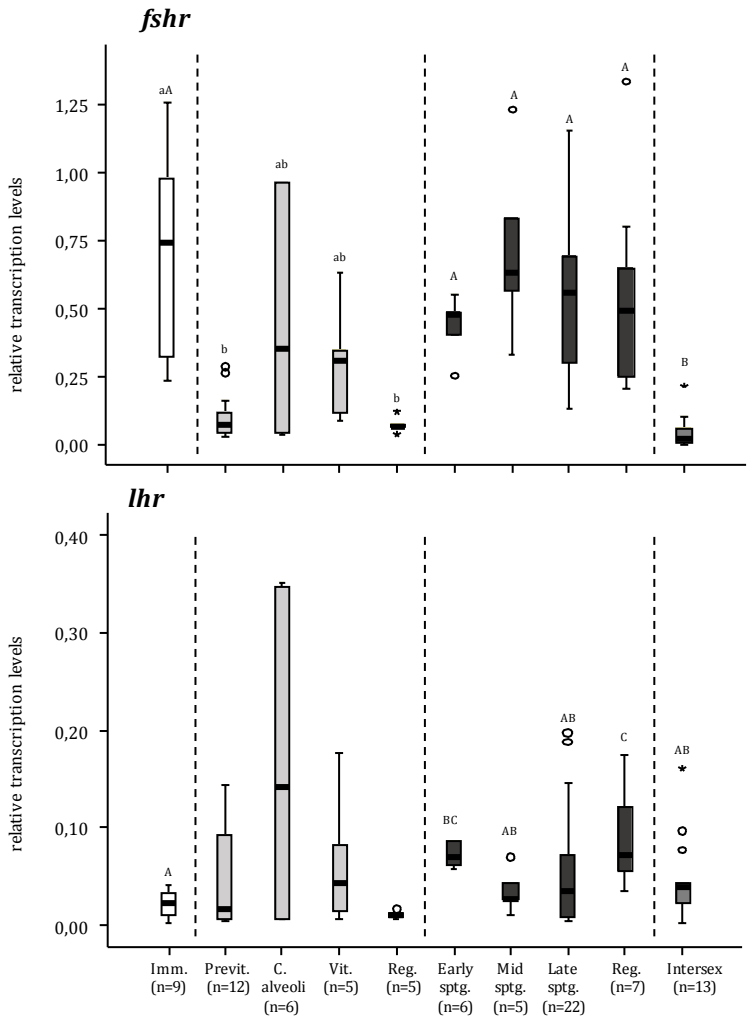

Figure 3
Gene transcription levels of *lhr and fshr* in gonads of, immatures (white), females (grey) in different gametogenic stages (previtellogenic, cortical alveoli, vitellogenic and regressing), males (black) in different gametogenic stages (early spermatogenesis, mid spermatogenesis, late spermatogenesis and regressing) and intersex (grey) thicklip grey mullets. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Lower case letters denote statistical differences between means ($p \leq 0,05$, after Kruskal-Wallis test followed by Dunn's post hoc test) for immatures and females; upper case letters denote statistical differences between means ($p \leq 0,05$, after Kruskal-Wallis test followed by Dunn's post hoc test) for immatures, males and intersex. Number between brackets indicates the number of individuals from each group.

alveoli stage and remained low at other oogenic stages, similar to that observed for mullets in the present work (Selvaraj et al., 2010). These transcription pattern points towards a meaningful role of *kiss2*, *gpr54* and *gnrh1* in the steps preceding brain control of vitellogenesis and secondary oocyte growth. Wang et al. (2013) suggested the important role of the estrogen feedback in the hypothalamo-gonadotropic axis in goldfish (*Carassius auratus*), after reporting a direct action of estrogens on *kiss1* and *kiss2* transcription levels. The up-regulation of kisspeptin system genes mediated by estrogen exposure was described in medaka (*Oryzias latipes*) (Kanda et al., 2008), in juvenile zebrafish (Servili et

al., 2011) and in goldfish (Kanda et al., 2012). Sardi et al. (2015) described increased estradiol levels in the plasma of female mullets at cortical alveoli stage, which corresponds with the highest levels for the analyzed genes in the brain. This would suggest that estrogens in female mullets also regulate the kisspeptin system.

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

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

4.2. Transcription pattern of gonadotropin subunits in the pituitary

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

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

Comparing female and male mullets, they showed a very similar pattern for *fshβ* transcription, with up-regulation as gametogenesis advanced. For *lhβ* instead, vitellogenic females showed higher transcription levels than males at late spermatogenesis. In other species such as the Senegalese sole (*Solea senegalensis*), sex dependent gonadotropin transcription pattern has been observed (Mechaly et al., 2012). Nevertheless, the effect of

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

4.3. Transcription pattern of gonadotropin receptors in the gonads

Gonadotropin receptors are involved in the regulation of steroidogenesis as well as in the control of gametogenesis in response to gonadotropins (Levavi-Sivan et al., 2010). The gonads of immature mullets showed higher *fhsr* transcription levels than those of *lhr*. The high *fshr* transcription levels of immature mullets coincide with the low transcription observed for *fshβ* in the pituitary. This could indicate that the activation of the receptor occurs prior to the release of the hormone, suggesting a possible preparation of the gonad for the forthcoming FSH arrival. Different transcription pattern could be noted for *fshr* and *lhr* in mullet ovaries along oogenesis. Whereas *fshr* showed the highest transcription at cortical alveoli and vitellogenic stages, *lhr* did not show any significant differences along oogenesis. In addition, *fshr* transcript levels were higher than those of *lhr* at all oogenesis stages. In female sea bass, unlike in mullet, both receptors showed identical transcription pattern in ovaries, nevertheless, *fshr* transcription levels were also higher than *lhr* (Rocha et al., 2009). Female sea bass showed a clear *lhr* transcription peak at late vitellogenesis, which occurred a bit earlier for *fshr*. That pattern was not observed in mullet ovaries, although cortical alveoli stage and vitellogenic females showed slightly higher *fshr* transcription levels than previtellogenic ones. The high transcription levels of *fshr* at

vitellogenesis is in accordance with the high transcription recorded for the *fshβ* subunit in the pituitary of mullets at the same stage. Nevertheless, the low fluctuations of the transcription levels observed along oogenesis suggest that the transcription of the gonadotropin receptors could be under the effects of external factors such as exposure to endocrine disruptors. For instance, female zebrafish exposed to E2 and BPA showed downregulation of *lhr* and *fshr* (Chen et al., 2017). In addition, female rare minnows exposed to EE2 showed a time dependent up or downregulation of *lhr* with no significant changes in *fshr* transcription (Qin et al., 2014). These results suggest that both the increase and the decrease of gonadotropin receptor transcription levels can occur as a result of exposure to EDCs, making it difficult to elucidate whether the observed pattern for female mullets is a consequence of exposure to xenoestrogenic pollutants or not.

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

intersex mullets does not enable to stablish any possible correlation between the gonadotropin subunits and their receptors.

5. Conclusions

In summary, in female mullets the transcription levels of brain *kiss2*, *gpr54* and *gnrh1* peak at cortical alveoli stage, which in turn may induce from previtellogenesis to vitellogenesis the increase observed in the pituitary of the transcription levels of both gonadotropin subunits. Gonadotropin receptor transcription levels in ovaries increased from previtellogenesis to cortical alveoli stage, suggesting their activation previous to the production of gonadotropins in the pituitary. In male mullets transcription levels of *gpr54* and *gnrh1* in the brain increased at mid spermatogenesis, which could induce the increase on *fshβ* transcription levels observed in the pituitary from early to late spermatogenic stage. In turn, the increase in *fshβ* could be followed by the *fshr* upregulation observed in testes from early to mid spermatogenesis. The lack of a clear gametogenic transcription cycle in all analyzed genes, especially *lhβ* and *fshr* in males, could be indicative of the effects of external factors such as the presence of endocrine disrupting chemicals. The downregulation of *fshr*, as clearly observed in intersex males, could be an effect of xenoestrogens or the consequences of testis feminization. As such, these transcriptional alterations in *fshr* should be considered as a promising biomarker of intersex condition, rather than early biomarker of xenoestrogen exposure. This would be in accordance with the effects observed in zebrafish exposed to EE2 (Rojo-Bartolomé, 2017), where the changes observed in *gtf3ab* transcription levels were seen to be a result of oocyte differentiation rather than a result of estrogen exposure.

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Chapter 3

Lost in transcription: transcription levels of kisspeptin system genes and gonadotropins in the gonads and gonadotropin receptors in the pituitary of thicklip grey mullets (*Chelon labrosus*)

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Abstract

Under exposure to xenoestrogenic chemicals, males from gonochoristic fish species can develop oocytes within their testis, developing an intersex gonad. This condition has been identified in thicklip grey mullets (*Chelon labrosus*) from several estuaries along the Basque coast (SE Bay of Biscay). Genes regulating reproduction along the brain-pituitary-gonad axis (BPG-axis) are potential targets of xenoestrogenic compounds. It has been constantly reported that some of these genes are transcribed out of place along this axis, although these observations have escaped any dedicated analysis. With the aim of understanding the molecular basis of the intersex condition, we have focused our attention on the transcription pattern of such genes outside their common expression tissue in the axis. Transcription levels of kisspeptin system genes (*kiss2, gpr54*), gonadotropin releasing hormone (*gnrh1*) and gonadotropin subunits (*gthα*, *fshβ*) were measured by qPCR in the gonads of immature, male, female and intersex mullets, while gonadotropin receptors (*fshr, lhr*) were measured in the pituitaries of females and males, all of them captured in the Pasaia harbor (SE Bay of Biscay) at different stages of the gametogenic cycle. Gametogenic stage classification of females and males and intersex identification was performed histologically. The transcription of *kiss2*, *gpr54*, *gthα*, and *fshβ,* detected at significant levels in mullet gonads, can be indicative of a local specific function. The high *gpr54* transcription levels observed in immature gonads would suggest its importance in switching on the forthcoming gametogenic cycle events, as it would for instance occur during puberty. The similar transcription levels found for both *gthα* and *fshβ* might reflect active follicle stimulating hormone (FSH) synthesis with a possible role in mullet gametogenesis locally. In contrast, the constant transcription levels of both gonadotropin receptors, *fhsr* and *lhr*, in the pituitary suggests transcription unrelated to the reproductive cycle, unless their gametogenic regulation was masked by the exposure to xenoestrogens affecting all Pasaia individuals. Intersex testes showed lower transcription levels of *gthα, fshβ* and *kiss2*, which compared with normal testes might suggest an effect linked to gonad feminization, especially on testicular FSH production. The patterns of gene transcription observed hereby during the gametogenic cycle do not resemble those observed in their putative organs of transcription and synthesis, which might reflect a disconnection from reproduction control. Functional understanding of the possible endocrine or paracrine role of the transcriptional activities described hereby should deserve further analysis.

Laburpena

Arrain espezie gonokoristikoetako arrek intersex egoera garatu dezakete, testikuloetan barren obozitoak garatuz kimiko xenoestrogenoen eraginez. Intersex egoera euskal kostaldeko hainbat estuarioetako korrokoietan (*Chelon labrosus*) aurkitu da. Ugalketa erregulatzen duten burmuin-pituitaria-gonada (BPG) ardatzeko geneak konposatu xenoestrogenikoen itu potentzialak dira. Askotan azaldu da gene horiek ardatzaren beste organo batzuetan ere transkribatzen direla, nahiz eta ez zaion atentzio berezirik eskaini. Intersex egoeraren mekanismo molekularra ulertzeko asmoz, lan honetan gene horiek ohiko organotik kanpo duten transkribapen patroietan zentratu gara. Kisspeptinen sistemako geneen (*kiss2, gpr54*), gonadotropina hormona askatzailea (*gnrh1*) eta gonadotropinen subunitate (*gthα*, *fshβ*) transkribapen mailak qPCR bidez neurtu ziren korrokoi heldugabe, ar, eme eta intersexetan. Gonadotropinen hartzaileak (*fshr, lhr*) aldiz ar eta emeen pituitarietan neurtu ziren. Korrokoiak Pasaiako portuan (Bizkaiko golkoko hego ekialdea) harrapatu ziren ziklo gametogenikoko fase desberdinetan. Ar eta emeen fase gametogenikoaren klasifikazioa eta intersexen identifikazioa histologikoki egin zen. Gonadetan detektatutako *kiss2*, *gpr54*, *gthα*, eta *fshβ*-ren transkribapena, bertan duten funtzio espezifikoaren adierazgarri izan daiteke. Korrokoi heldugabeetan *gpr54*-ren transkribapen maila altua ikusi zen, horrek, pubertaroan geratzen den moduan, hurrengo fase gametogenikoetan izan dezakeen garrantzia erakutsi dezake. *gthα* eta *fshβ*-k gonadetan transkribapen maila antzekoak zituzten, FSH-ren sintesia aktiboa eta gonadetan bertan izan dezaken eginkizuna islatu dazakelarik. Pituitarian *fshr, lhr*-ren transkribapen maila konstanteak ugalketa zikloarekin erlazionatu gabeko transkribapen basala adierazi dezake, baldin eta xenoestrogenoek berezko transkribapena ezkutatzen ez badute. Intersexen barraibilek arrek baino *gthα* eta *fshβ* transkribapen maila baxuagoak zituzten, feminizazioak eragindako disrupzioaren adierazle, batez ere FSH ekoizpenari dagokionez. Honela, gametogenesian zehar neurtutako gene transkribapen patroiek ez dute berezko organoetan ikusitakoaren antzik, ugalketaren kontrolarekiko lotura eza adierazgarri izan daitekelarik. Deskribatutako transkribapen aktibitateek izan dezaketen eginkizun endokrino edo parakrinoa ulertzeko bestelako analisiak beharrezkoak dira.

1. Introduction

Many studies have focused their attention on the characterization of the brain-pituitary-gonad axis (BPG-axis) in teleosts, especially due to its importance in the control of reproduction. In vertebrates, the most upstream reproductive molecular regulators of BPG-axis are kisspeptins, which are synthetized in the kisspeptin neurons located in the hypothalamus. Divergent evolution for these neuropeptides occurred in the tetrapod lineages (Pinilla et al., 2012), with two paralog kisspeptin genes, *kiss1* and *kiss2* present in many teleosts, in amphibians and in mammalian monotremes (Pinilla et al., 2012). Kisspeptin neurons respond to metabolic and environmental factors such as photoperiod, temperature, stress condition or food availability (Tena-Sempere, 2006), secreting kisspeptin neuropeptides, which in turn regulate the synthesis and release of the gonadotropin-releasing hormones, GnRHs, in the hypothalamus. Kisspeptins act through G protein coupled receptors located in the GnRH (gonadotropin releasing hormone) neurons. Two receptor genes exist in mammalian monotremes and in some teleosts (Pinilla et al., 2012), *gpr54-1* also known as *kiss1-r* and *gpr54-2* or *kiss2-r*. Secreted GnRH regulates the release of gonadotropin hormones in the pituitary, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH). Both hormones are formed by a common α subunit, encoded by the *gthα* gene, and a specific β subunit, *fshβ* and *lhβ,* coding for the specific subunits of each hormone. Gonadotropin hormones reach their specific receptors in the gonads, follicle stimulating hormone receptor (FSHR) and luteinizing hormone receptor (LHR). In the gonads synthesis and release of sex steroids occurs, which exert either positive or negative feedback effects on the above mentioned steps of the axis. The process is species-specific and gametogenic stage dependent (Levavi-Sivan et al., 2010; Tena-Sempere et al., 2012).

The expression of BPG-axis neuropeptides, hormones and receptors also occur in other peripheral tissues in many vertebrates (Oakley et al., 2009; Levavi-Sivan et al., 2010; Pinilla et al., 2012). In mammals, the expression of kisspeptin system genes in the ovary has been demonstrated and suggested to have implications in various ovarian processes (Castellano et al., 2006; Gaytán et al., 2009; Saadeldin et al., 2012). The kisspeptin system is less studied in the testis; however, a local role in the regulation of spermatogenesis has been suggested (Tariq et al., 2013). Other brain actors are also localized in the gonads. GnRH-like peptides have been identified in rat (Aten et al., 1986) and human (Peng et al., 1994) ovary. In teleosts, transcripts of the BPG-axis related genes have been detected in several organs, in addition to their common organ of transcription, but less attention has been dedicated to the understanding of their function, and non-reproductive roles cannot be discarded (Pinilla

et al., 2012). Species-specific transcription profiles have been noted among teleosts. *kiss1* expression was detected in the intestine of zebrafish (*Danio rerio*) but not in medaka (*Oryzias latipes*). On the contrary, medaka showed heart transcription of *kiss1*that was not detectable in zebrafish (Felip et al., 2009). In the particular case of the two GPR54 forms described in zebrafish, while the transcription of the first was detected only in brain and gonads, the second form was detected in all analyzed tissues (Biran et al., 2008). Regarding gonad transcription of these hypothalamus genes, Fairgrieve et al. (2016), characterized the gonadal kisspeptin system transcription in sablefish (*Anaplopoma fimbria*) pointing towards a possible local role of *kiss2* in oogenesis. In the same way, a role for *kiss1* and *kiss1r* in the gonad development of golden mahseer (*Tor putitora*) has been suggested (Shahi et al., 2017). In some cases, fish gonads show a sex dimorphic transcription of kisspeptin system genes (Table 1).

Localization of GnRH in gonads is also known for some teleost species (Table 2). Bogerd et al. (2002) showed that GnRH and its receptor, GnRHR, were functional in African catfish (*Clarias gariepinus*) gonads, which could indicate that they are locally involved in gonadal processes. GnRHs could be involved in the intragonadal gametogenesis regulatory mechanisms in fish (Uzbekova et al., 2001). Extrapituitary production of gonadotropin subunits receives less attention (Table 3). In the rare minnow (*Gobiocypris rarus*) tissues such as eye, gill, intestine, liver and muscle express gonadotropins and gonadotropin receptors (Qin et al., 2014). Wong and Zohar (2004), revealed that ovarian gonadotropins in gilthead seabream (*Sparus aurata*) are likely to be involved in mediating the actions of intraovarian factors. With respect to gonadotropin receptors, their transcription outside the gonads has been studied mainly in the brain and the pituitary. Once again, differences among species are detected (Table 4). While some species show transcription of these receptors in brain, as it is the case of Atlantic halibut (*Hippoglossus hippoglossus*) (Kobayashi et al., 2008), others such as channel catfish (*Ictalurus punctatus*) show absence of extragonadal transcription at all (Kumar et al., 2001a; 2001b). So far, the exact role of the BPG-axis gene products outside their commonly accepted organ of expression remains unclear.

Table 1
List of teleost species where the transcription of genes belonging to the kisspeptin system (*kiss1*, *kiss2* and their receptors) has been detected in the gonads.

kiss1	***kiss2***	**kisspeptin receptors**	**species**	**reference**
-	-	testis and ovary	Tilapia (*Oreochromis niloticus*)	Parhar et al., 2004
testis	-	testis and ovary (*kiss1ra*)	Zebrafish (*Danio rerio*)	Biran et al., 2008
testis and ovary	testis and ovary	-	Zebrafish (*Danio rerio*)	Felip et al., 2009
testis *	testis and ovary	-	Zebrafish (*Danio rerio*)	Kitahashi et al., 2009
testis and ovary	testis and ovary	-	Seabass (*Dicentrarchus labrax*)	Felip et al., 2009
testis *	-	-	Medaka (*Oryzias latipes*)	Kanda et al., 2008
testis and ovary	testis and ovary	-	Medaka (*Oryzias latipes*)	Felip et al., 2009
testis *	testis and ovary	-	Medaka (*Oryzias latipes*)	Kitahashi et al., 2009
-	-	testis and ovary	Fathead minnow (*Pimephales promelas*)	Filby et al., 2008
testis *	testis and ovary	testis and ovary (*gpr54a* and *gpr54b*)	Goldfish (*Carassius auratus*)	Li et al., 2009
-	-	testis and ovary***	Senegales sole (*Solea senegalensis*)	Mechaly et al., 2009
-	-	ovary	Grey mullet (*Mugil cephalus*)	Nocillado et al., 2008
testis and ovary	**	-	Chub mackerel (*Scomber japonicus*)	Selvaraj et al., 2010
-	testis and ovary	testis and ovary	Grass puffer (*Takifugu niphobles*)	Shahjahan et al., 2010
-	testis and ovary	testis and ovary	Orange-spotted grouper (*Epinephelus coioides*)	Shi et al., 2010
testis and ovary	testis and ovary	testis and ovary (two genes)	Sablefish (*Anoplopoma fimbria*)	Fairgrieve et al., 2016
testis and ovary	testis and ovary	-	Rohu (*Labeo rohita*)	Saha et al., 2016
testis and ovary	-	testis and ovary	Golden mahseer (*Tor putitora*)	Shahi et al., 2017

- not analyzed
* absence in ovary
** not detected in the gonads
*** *kissrv2* in testis and ovary, *kissrv1* in testis

Recently, we studied the transcription pattern of BPG-axis genes in thicklip grey mullet (*Chelon labrosus*). The transcription levels of *kiss2*, *gpr54* and *gnrh1* in brain, *lhβ* and *fshβ* in the pituitary and *lhr* and *fshr* in the gonads were quantified (see Chapter 2). *kiss2*, *gpr54* and *gnrh1* transcription peaked in the brain of females at cortical alveoli stage, associated with the increase in the transcription levels detected in the pituitary for both gonadotropin subunits (*fshβ* and *lhβ*) from previtellogenesis to vitellogenesis. Accordingly, gonadotropin receptor (*lhr* and *fshr*) transcription levels in the ovaries increased from previtellogenesis to cortical alveoli stage, suggesting its activation previous to the production of gonadotropins in the pituitary. In male mullets, transcription levels of *gpr54* and *gnrh1* in the brain increased at mid spermatogenesis, promoting the transcription of *fshβ* in the pituitary from early to late spermatogenesis. *fshr* in testis was upregulated from early to mid spermatogenesis, possibly making early testis cells sensitive to FSH signaling.

Table 2
List of teleost species where the transcription of gonadotropin releasing hormone genes (*gnrh1, gnrh2, gnrh3*) has been detected in the gonads.

gnrh1	***gnrh2***	***gnrh3***	**species**	**reference**
ovary and testis	ovary and testis	limited to the brain	Japanese eel (*Anguilla japonica*)	Okubo et al., 1999
ovary *	ovary*	limited to the brain	Grey mullet (*Mugil cephalus*)	Nocillado et al., 2003
**	ovary and testis	ovary and testis	Zebrafish (*Danio rerio*)	Kuo et al., 2005
ovary and testis	limited to the brain	ovary and testis	Pejerrey (*Odontesthes bonariensis*)	Guilgur et al., 2007
**	ovary and testis	ovary and testis	Fathead minnow (*Pimephales promelas*)	Filby et al., 2008
ovary and testis	ovary and testis	ovary and testis	Mummichug (*Fundulus heteroclitus*)	Ohkubo et al., 2010
ovary *	ovary *	ovary *	Atlantic cod (*Gadus morhua*)	Hildahl et al., 2011
ovary and testis	testis	ovary and testis	Winter flounder (*Pseudopleuronectes americanus*)	Tuziak and Volkoff, 2013
ovary and testis	ovary and testis	-	African catfish (*Clarias gariepinus*)	Bogerd et al., 2002
ovary and testis	ovary and testis	-	Rainbow trout (*Oncorhynchus mykiss*)	Uzbekoca et al., 2001

- not analyzed
* work done only with females
** no expression of *gnrh1* is detected in cyprinid fish

Table 3
List of teleost species where the transcription of gonadotropin subunit genes (*gthα, fshβ, lhβ*) has been detected in the gonads.

gthα	*lhβ*	*fshβ*	species	reference
ovary *	ovary *	ovary *	Gilthead seabream (Sparus aurata)	Wong and Zohar, 2004
ovary	ovary and testis	ovary and testis	Zebrafish (Danio rerio)	So et al., 2005
-	ovary and testis	ovary and testis	Rare minnow (Gobiocypris rarus)	Qin et al., 2014

- not analyzed
* not studied in the testis

Table 4
List of teleost species where the transcription of gonadotropin receptor genes (*fshr, lhr*) has been detected in the gonads.

fshr	*lhr*	species	reference
-	brain (*gthr*) #	Amago salmon (*Oncorhynchus rhodurus*)	Oba et al., 1999
not detected*	not detected*	Channel catfish (*Ictalurus punctatus*)	Kumar et al., 2001 (a,b)
-	brain #	African Catfish (*Clarias gariepinus*)	Vischer and Bogerd, 2003
not detected*	not detected*	Zebrafish (*Danio rerio*)	Kwok et al., 2005
not detected*	brain** #	Atlantic cod (*Gadhus morua*)	Maugars and Schmitz, 2006
brain (female and male)	brain (female and male)	Atlantic cod (*Gadhus morua*)	Mittelholzer et al., 2009
not detected*	brain and pituitary #	Seabass (*Dicentrarchus labrax*)	Rocha et al., 2007
pituitary and brain (female and male)	pituitary and brain (female and male)	Atlantic halibut (*Hippoglossus hippoglossus*)	Kobayashi et al., 2008
pituitary and brain (female and male)	brain (female and male)	Atlantic salmon (*Salmo salar*)	Andersson et al., 2009
not detected*	-	Protogynous grouper (*Epinephelus merra*)	Alam et al., 2010
brain (female and male)	brain (female and male)	Rare minnow (*Gobiocypris rarus*)	Qin et al., 2014
-	brain and pituitary (female)***	Orange-spotted grouper (*Epinephelus coioides*)	Peng et al., 2018

- not analyzed
the sex was not specified
* in neither brain and pituitary
** not detected in the pituitary
*** l*hr1* and *lhr2*

Thicklip grey mullet is sensitive to the effects of xenoestrogens (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Ortiz-Zarragoitia et al., 2014; Ros et al., 2015). One of the effects of xenoestrogen exposure in mullets is the development of the intersex condition in males, with testes showing oocytes (Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). Transcript levels of genes controlling gamete development, such as *cyp19a1a*, 5SrRNA and associated proteins in intersex testes showed to be similar to ovarian levels (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Rojo-Bartolomé et al., 2017). In addition, *fshr* is down-regulated in intersex testes (Chapter 2), suggesting that the main molecular mechanisms leading to the development of the intersex condition occur at gonad level.

Studies dealing with effects mediated by EDCs on teleost BPG-axis related genes are abundant, but effects on the transcription levels of such genes outside their principal tissue of expression are scarcely studied. In the present work, we have quantified the transcription levels of BPG-axis genes outside their common organ in order to assess their potential implication in the development of the intersex condition in mullets. Gonadotropin receptors *fshr* and *lhr,* were thus studied in the pituitary and *kiss2, gpr54, gnrh1, gthα, fshβ* and *lhβ* in the gonads of female and male mullets at different stages of the gametogenic cycle, and also in intersex mullets.

2. Material and methods

2.1. Study area, fish sampling and tissue collection

The inner part of the Pasaia harbor, located in the SE Bay of Biscay (43°19'18'' N; 1°55'53'' W), was selected as sampling area. Adult (>20 cm) thicklip grey mullets (*Chelon labrosus*) were captured by fishing-rod. Fish included in these analyses were captured between September 2010 and September 2011, and between October 2016 and January 2017. Pituitaries were only collected during the 2016/17 sampling period. Fish were anaesthetized upon capture in a saturated ethyl-4-aminobenzoate water bath. All fish handling and procedures were approved by the UPV/EHU Ethical Committee for Experimental Animals and by the regional authorities. Dissections were immediately performed *in situ*. Pituitary and a portion of the gonad of each fish were collected in RNAlater solution (Ambion; Life Technologies, Carlsbad, USA) and then frozen in liquid nitrogen until arrival at the laboratory where they were immediately stored at -80°C until processing. Another portion of the gonad of each fish was fixed in 4% neutral buffered formalin for histological analysis.

2.2. Histological analysis of the gonad

Gonad samples were fixed for 24 h in 4% neutral buffered formalin, then dehydrated in a graded ethanol series (70%, 90%, and 96%), and embedded in paraffin. Sections of 5 μm thickness were cut in a Leica RM 2125 RT manual microtome (Leica Microsystems, Nussloch, Germany) and then stained with hematoxylin/eosin (Gamble and Wilson, 2002) using the Leica Autostainer XL and mounted with the aid of the Leica CV 5030 workstation. Three sections per individual were microscopically analyzed with an Olympus BX61 light microscope (Tokyo, Japan). Sex and gametogenic stage of each individual were determined using the criteria published by McDonough et al. (2005). The Intersex Severity Index described by Jobling et al. (2006) was used to classify intersex males.

Individuals belonging to each sex and gametogenic stage were selected for the transcription analysis of target genes in pituitary and gonads. The four gametogenic groups stablished for each sex were; previtellogenic, cortical alveoli, vitellogenic and regressing stage for females and early, mid and late spermatogenesis and regressing stage for males. Mullets were classified as immature, if the gonad could not be identified as ovary or as testis. Analyses of transcription levels were also carried out in all the intersex individuals identified during the study.

2.3. Gene transcription analysis

2.3.1. Extraction of total RNA

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

2.3.2. cDNA synthesis and quantification

For each sample, first strand cDNA synthesis was performed from 2 μg of RNA using the Affinity Script Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, La Jolla, USA) with random primers, following manufacturer's protocol and using a 2720 Applied Biosystems Thermal Cycler (Life Technologies). Quant-iT OliGreen Kit (Life Technologies)

was used to quantify ssDNA concentration in the pituitary and gonad cDNA samples, following manufacturer's instructions. 96 well plates (Corning Incorporated, Corning, New York, USA) were used and fluorescence was measured in a Synergy HT Multi-Made Microplate Reader (Biotek, Winoosky, USA).

2.3.3. Real-time qPCR

Real time qPCR analyses were performed in a 7300 Real-Time PCR system thermocycler (Life Technologies). *kiss2*, *gpr54*, *gnrh1*, *gthα, lhβ, fshβ* were analyzed in the gonads, *fshr* and *lhr* in the pituitary. Specific primers are shown in Table 5. Each sample was analyzed in triplicates in a total volume of 20 µL containing 7.88 µL of water, 10 µL of SYBR Green fluorescent dye master mix (Roche Diagnostics, Indianapolis, USA) and 0.12 µL of 12.5 pmol primer pair. A control without template was run (also in triplicate) in each plate using the same reaction conditions. The qPCR conditions were as follows: an initial step at 50°C for 2 min and 95°C for 10 min, 40 cycles of a denaturing step at 95°C for 15 s and an annealing step at Tm (Table 5) for 1 min. Finally, a dissociation stage was included at 95°C for 15 s, followed by 1 min at 60°C for and 15 s at 95°C. The reaction efficiency of each plate was calculated using a standard curve consisting in serial dilutions of pooled cDNA. The specificity of the reaction was determined confirming the presence of a single peak in the dissociation curve, and observing that no primer dimmers were formed. For a more detailed description of the procedure, see Appendix. cDNA concentration obtained by fluorescent quantification method was used for normalization in, pituitary, ovary and testis, as performed by Rojo-Bartolomé et al. (2016).

Table 5
List of target genes with their specific forward and reverse primers, used melting temperature and fragment size.

Gene	Forward (5'-3') Reverse (5'-3')	Fragment size (bp)	Tm (°C)	Accession number
kiss2	ATTGGATTCCGCACAAAGGACA CTCAGGGAGAAGCACAGGT	111	58	KT248850
gpr54	ACCGCTGTTATGTGACAGTCTA TACATCAAAATCGGGGTGGACA	124	58	KT248849
gnrh1	GAGGGAAGAGGGAACTGGA TGGCGAAAGGCGTGTCCT	114	58	KT248847
gthα	GATTCATACCCCAACATTGACTTG TGGAGAAGCAGCAGCCACT	118	58	KT248848
fshβ	CGCCAACACCAGCATCAC CACCAATGAAGGAGAAATCCCT	104	59	KX758589
lhβ	TTCCTGGCTGTCTGCGG TTCAAAGGTGCAGTCGGACG	103	59	MH251322
fshr	CCTTGCTCATCTTCACCGAC CAGGACCAGGAGGACTTTAG	116	60	MH251323
lhr	GTTAATCCGACTGGGAACAACA GCAGGCCCATAAGCAGAGTT	112	58	KX171008

2.4. Statistical analysis

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

3. Results

3.1. Identification of the intersex condition

A total of 13 intersex individuals were detected. Intersex males were at different spermatogenic stages, ranging from immature or early spermatogenic stage to late spermatogenesis. All intersex individuals showed oocytes at previtellogenic stage with a very low intersex severity index that was in all cases regarded to be 1 in a scale of 7 (Jobling et al., 2006). No intersex gonad was identified in the individuals specifically captured for pituitary dissection in the sampling 2016/17.

3.2. Transcription levels of *kiss2, gpr54, gnrh1, gthα, fshβ* and *lhβ* in the gonads

Amplification product for *gnrh1* in the gonads was detected, but the specificity of the reaction could not be determined due to the several peaks observed in the qPCR dissociation curve.

In the case of *kiss2*, immature gonads showed levels of transcription similar to those observed in mature ovaries and testes, with significantly higher transcription levels than in regressing ovaries and testes (Figure 1). Ovaries showed a slight decrease in transcript levels, although not statistically significant, from previtellogenesis to the other gametogenic stages (Figure 1). In testes, a moderate upregulation was observed from mid to late spermatogenesis, with a decrease at the regressing stage (Figure 1). Although not statistically significant, intersex testes showed the lowest transcription levels for *kiss2*, compared to ovaries and to mature testes (Figure 1).

Immature gonads showed significantly higher transcription levels of *gpr54* in comparison to ovaries at any oogenic stage and to testes at mid and late spermatogenesis (Figure 1). In ovaries, uniform transcription levels were quantified along the whole oogenesis (Figure 1). On the other hand, testes at early spermatogenesis and at regressing stage showed significantly higher transcription levels than testes at mid and late spermatogenesis (Figure

1). Intersex testes showed very similar transcription levels to testes of males at mid and late spermatogenesis (Figure 1).

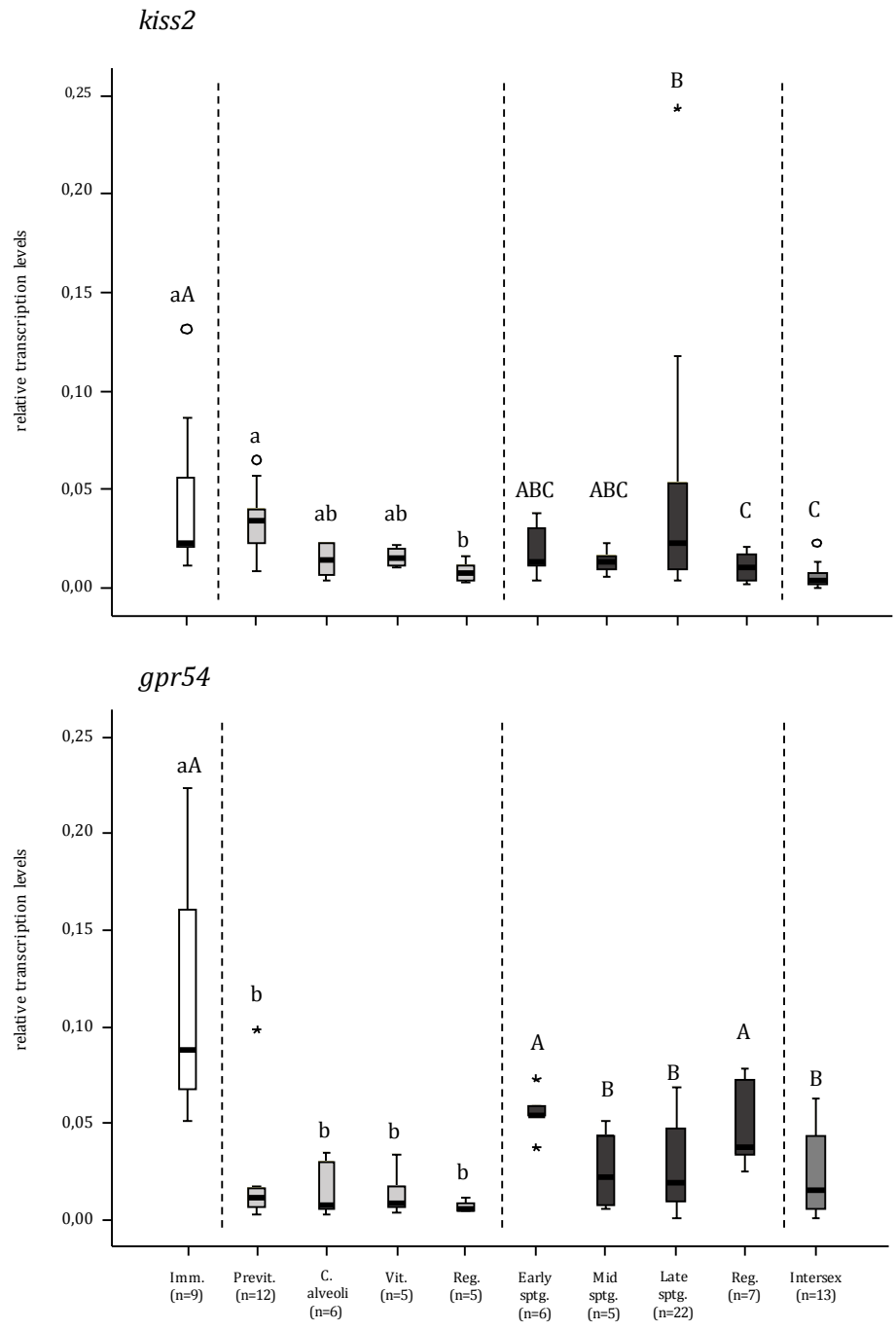

Figure 1
Gene transcription levels of *kiss2* and *gpr54* in gonads of immature (white), female (grey), male (black) and intersex male (dark grey) thicklip grey mullets. Different gametogenic stages are shown for females (previtellogenic, cortical alveoli, vitellogenic and regressing) and males (early, mid and late spermatogenesis, and regressing). Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Different lower case letters denote statistical differences between means ($p \leq 0.05$, after Kruskal-Wallis test followed by Dunn's post hoc test) among females, including immature individuals, while different upper case letters denote statistical differences between means ($p \leq 0.05$) for male, intersex and immature individuals. Number between brackets indicates n for each group.

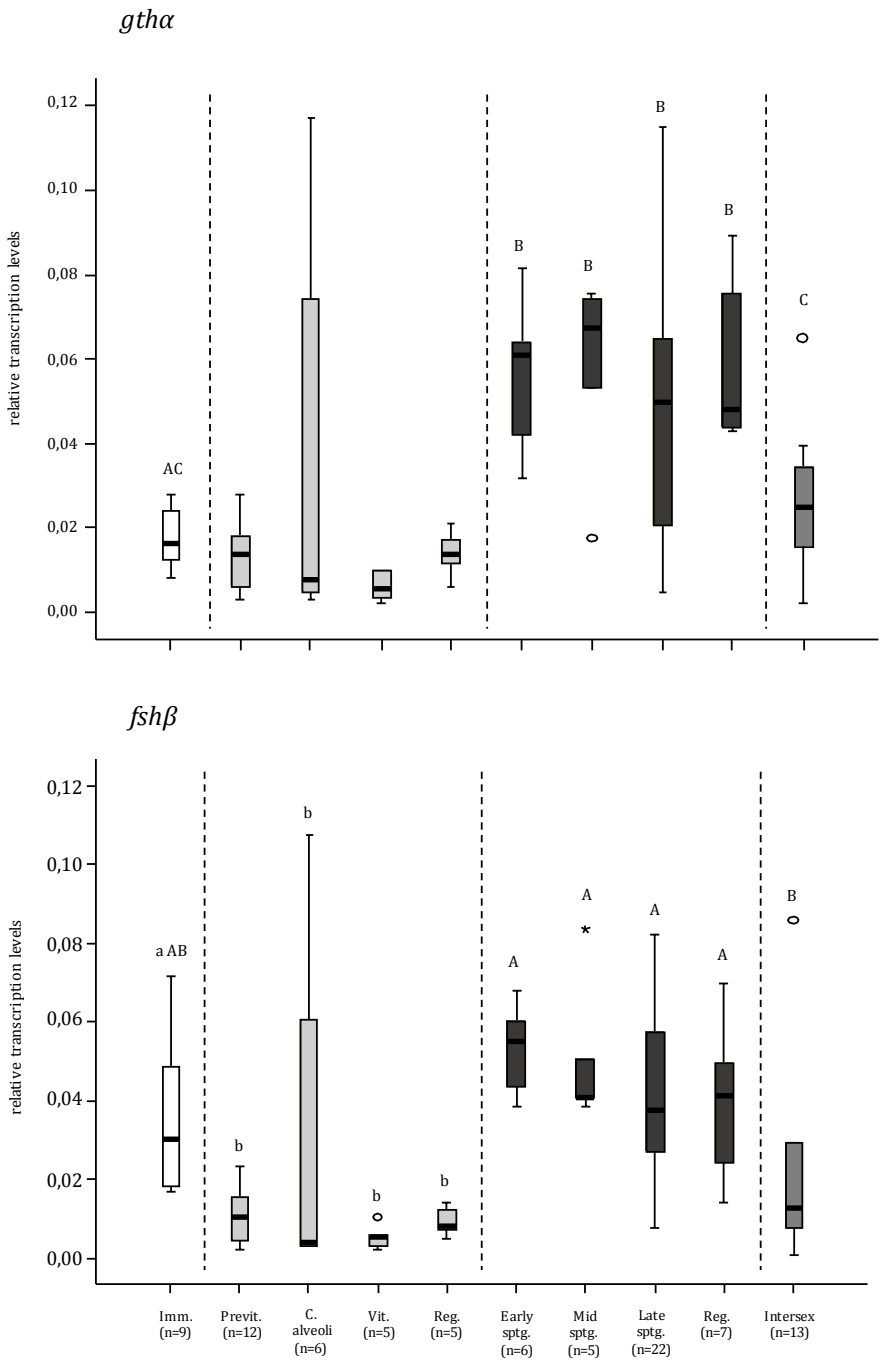

Figure 2
Gene transcription levels of *gthα* and *fshβ* in gonads of immature (white), female (grey), male (black) and intersex male (dark grey) thicklip grey mullets. Different gametogenic stages are shown for females (previtellogenic, cortical alveoli, vitellogenic and regressing) and males (early, mid and late spermatogenesis, and regressing). Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Different lower case letters denote statistical differences between means ($p \leq 0.05$, after Kruskal-Wallis test followed by Dunn's post hoc test) among females, including immature individuals, while different upper case letters denote statistical differences between means ($p \leq 0.05$) for male, intersex and immature individuals. Number between brackets indicates n for each group.

All gonad samples showed detectable *gthα* transcripts. Immature mullets showed similar transcription levels to those observed in ovaries but lower than those observed in testes (Figure 2). Ovaries showed constant transcription levels during the different stages of oogenesis, with slight upregulation at cortical alveoli stage compared to other stages (Figure 2). Testes also showed uniform *gthα* transcription levels at all spermatogenesis stages but higher than those observed in ovaries (Figure 2). Intersex testes showed lower transcription levels, and very similar to immature gonads and ovaries at cortical alveoli stage (Figure 2). *lhβ* transcripts were not detected in any of the analyzed gonads. Immature gonads showed higher transcription levels of *fshβ* than of *gthα* (Figure 2), which was not the case for ovaries and testes, being *fshβ* transcription levels very similar to those observed for *gthα.* In addition, intersex testes showed lower transcription levels of *fshβ* than normal testes, and similar to those shown by immature gonads and ovaries at cortical alveoli stage (Figure 2).

3.3. Transcription levels of *fshr* and *lhr* in the pituitary

Transcription of *fshr* and *lhr* were detectable in pituitaries of immature and mature females and males. Differences for both transcripts between immature and mature mullets were absent (Figure 3). No differences were observed when comparing the pituitaries from individuals at different developmental stages (Figure 3). In addition, both sexes showed very similar transcription levels for *fshr* and *lhr* (Figure 3).

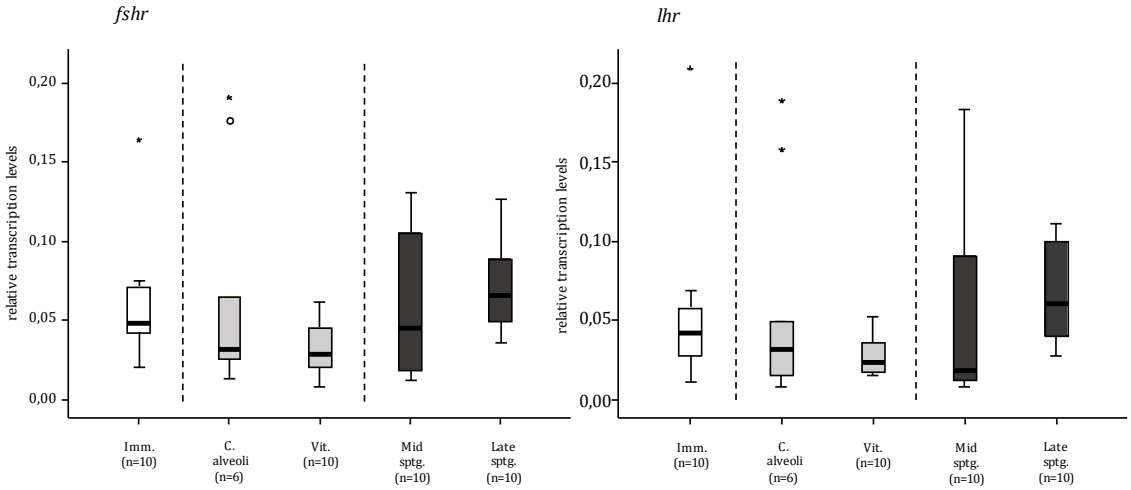

Figure 3
Gene transcription levels of *fshr* and *lhr* in the pituitaries of immatures (white), females (grey) and males (black) thicklip grey mullets. Different gametogenic stages are shown for females (cortical alveoli and vitellogenic) and males (mid and late spermatogenesis). Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Dots and asterisks denote outlayers. Number between brackets indicates n for each group.

4. Discussion

Transcription of brain-pituitary-gonad axis (BGP axis) genes can occur in organs different to their common source. In mammals, the local expression of kisspeptin system genes in ovaries and testes has been suggested to regulate several gonadal processes (Castellano et al., 2006; Gaytán et al., 2009; Saadeldin et al., 2012; Tariq et al., 2013). Transcription of *kiss1* and *kiss2* and their receptors, *gpr54-1* (or *kiss1-r*) and *gpr54-2* (or *kiss2-r*) in tissues other than the brain including the gonads, has been reported in some fishes (Oakley et al., 2009). Sex dimorphic transcription for *kiss1* or *kiss2* was found in both testis and ovary of zebrafish (*Danio rerio*), seabass (*Dicentrarchus labrax*), medaka (*Oryzias latipes*) (Felip et al., 2009), sablefish (*Anoplopoma fimbria*) (Fairgrieve et al., 2016) and rohu (*Labeo rohita*) (Saha et al., 2016). However, *kiss1* could only be detected in the testes but not ovaries of goldfish (*Carassius auratus*) (Li et al., 2009). In chub mackerel (*Scomber japonicus*) none of those kisspeptin genes was detected in the gonads (Selvaraj et al., 2010). Gonadal transcription of *gpr54* was reported in several teleost species such as tilapia (*Oreochromis niloticus*) (Parhar et al., 2004), zebrafish (Biran et al., 2008), fathead minnow (*Pimephales promelas*) (Filby et al., 2008), goldfish (Li et al., 2009), Senegalese sole (*Solea senegalensis*) (Mechaly et al., 2009), grass puffer (*Takifugu niphobles*) (Shahjahan et al., 2010), orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010), sablefish (*Anoplopoma fimbria*) (Fairgrieve et al., 2016) or golden mahseer (*Tor putitora*) (Shahi et al., 2017). Nevertheless, only a few studies have gone deeper in the characterization of the gene transcription pattern along gametogenesis. Species-specific transcription patterns can be noticed, which could be due to the broad range of reproductive and spawning strategies, timing of sexual maturation or gametogenic cycle duration (Saha et al., 2016).

Transcription of the kisspeptin-system genes *kiss2* and *gpr54* was detectable in the ovaries and testes of thicklip grey mullets. *kiss2* transcription was present in ovaries of mullets in all the analyzed gametogenic stages. Transcription levels decreased from previtellogenesis to regressing stage. On the contrary, ovarian *gpr54* transcription levels did not vary during oogenesis. In golden mahseer, high kisspeptin and *gpr54* transcription levels detected in the ovaries of vitellogenic females suggested a potential role in sex steroid secretion and final oocyte maturation (Saha et al., 2016; Shahi et al., 2017). Therefore, the absence of significant variation in ovarian *kiss2* and *gpr54* transcription levels during mullet oogenesis, suggests that these genes are not directly involved in the regulation of estrogen synthesis in female mullets. Nevertheless, the presence of kisspeptins in gonads of rohu was related with a role in the control of hormonal secretion during gametogenesis (Saha et al., 2016). Accordingly, interactions between the kisspeptin system in the gonads and synthesis of steroid

hormones were demonstrated in mammals (Colledge, 2009), suggesting the possible role of gonad kisspeptins in steroid activation. Therefore, we can hypothesize that a sustained transcription of the kisspeptin system genes in ovaries of mullets could be required to support oogenesis and that this system could be essential for the preparation and recruitment of a new gametogenic cycle, demonstrated by the up-regulation of *kiss2* and *gpr54* detected at immature stage. Nocillado et al. (2007) reported *gpr54* transcription in the ovaries of female flathead mullets (*Mugil cephalus*) suggesting a role in the regulation of the pubertal process, a function well known for the kisspeptins in the hypothalamus. They showed that transcription of *gpr54* in the ovary increased as puberty advanced. In sablefish, transcription levels in the gonads of juveniles were shown to have higher *gpr54-1* transcription levels than ovaries from adult females (Fairgrieve et al., 2016).

In testes, like in ovaries, the transcription of *kiss2* and *gpr54* was uniform during spermatogenesis, with a slight increase at late spermatogenesis followed by a drop at regressing testes. A possible local role of the kisspeptin system in the regulation of the reproductive functions at the spawning period was suggested for grass puffer and sablefish (Shahjahan et al., 2010; Fairgrieve et al., 2016). In the case of golden mahseer, *kiss1r* transcription in testes declined at maturation stage, rising again at spermiation stage, suggesting the involvement in the control of steroidogeneisis in testis (Shahi et al., 2017). As mentioned for mullet ovaries, a sustained transcription of *kiss2* and *gpr54* during gametogenesis could be required for an optimal spermatogenesis progression. Furthermore, high transcription levels of those genes at immature stage gonads of adult mullet, suggests a potential role in testis recruitment. Further works could help understanding the role of kisspeptin system in both ovaries and testes of mullets.

Gonads of intersex males showed transcription levels of *kiss2* and *gpr54* similar to testes at the corresponding spermatogenesis stage. Thus, implication of gonadal kisspeptin system in the development of intersex condition in mullets could not be established. Nevertheless, we cannot discard the possible attenuating effects of xenoestrogens over studied endpoints. Presence of xenoestrogens in the mullet population from Pasaia has been previously described and up-regulation of vitellogenin, together with the presence of intersex condition, are signs of endocrine disruption effects (Bizarro et al., 2014; Ros et al., 2015).

Transcription of GnRHs was described in peripheral tissues in several fish species, but their possible role in gametogenesis has not been elucidated yet. However, autocrine role in gametogenesis is suggested. Nocillado et al. (2007) described detectable transcript levels of *ghnr1* in the ovaries of *Mugil cephalus* mullets, which increased at advanced puberty stage. The authors highlighted the possibility that, as seen in mammals, GPR54 would mediate

GnRH release in mullet ovaries (Nocillado et al., 2008), as it occurs in the brain. Unfortunately, the primers designed for *C. labrosus gnrh1*, and that worked properly in the brain (Chapter 2), resulted in amplification of multiple bands in the gonads.

Transcription of gonadotropin subunits outside the brain and pituitary in teleost are poorly described. So et al. (2005) suggested that the extrapituitary expression of gonadotropins could be extrapolated to all teleost. In this work, mRNA transcription of *gthα* and *fshβ* was detected in both ovaries and testes with no signs of *lhβ* transcription. Wong and Zohar (2004) identified the three gonadotropin subunits in the ovary of gilthead seabream (*Sparus aurata*). The same was observed for zebrafish (So et al., 2005), with the three subunits detected in ovary, but only the *fshβ* and *lhβ* subunit in testis. In rare minnow (*Gobiocypris rarus*) Qin et al. (2014) identified *fshβ* and *lhβ* transcripts in various extrapituitarian tissues including ovary and testis, which showed the highest transcription levels, but less than brain. In mullets, transcription of *gthα and fshβ* were unaltered along gametogenesis with transcription levels being higher in testes than in ovaries, except at cortical alveoli stage. Transcription of *fshr* in gonads followed this same sex dependent pattern, as described in Chapter 2. In addition, transcription levels of *fshβ* resembles *fshr* in immature mullet gonads. These data could be indicative of the local synthesis of functional FSH in mullet gonads. The concerted transcription of both *gthα* and *fshβ* along gametogenesis may indicate local synthesis of active FSH. A constant synthesis of FSH in mullet gonads might indicate a local role during the whole gametogenic cycle in mullets. Wong and Zohar (2004) suggested a gonadal GnRH-gonadotropin axis with the expression of ovarian gonadotropins mediating the actions of intraovarian factors for in gilthead seabream. Although we lack data on gonadal *gnrh1* transcription in mullets, a possible interaction between GnRH and gonadotropin subunits via steroid hormones can be suggested. In intersex testes, downregulation of both *gthα* and *fshβ* was observed in comparison to normal testes. In addition, the transcription levels in intersex gonads were similar to those shown in ovaries at cortical alveoli stage, which could be related with the presence of previtellogenic oocytes in the testes of intersex mullets. Furthermore, the transcription of *fshβ* in intersex testes can be also related with the low *fshr* transcription levels observed in the same samples in Chapter 2. For both *fshβ* and *fshr* transcripts, intersex testes showed significantly lower transcription than non-intersex testes. Thus, as pointed above, *fshr* downregulation in intersex gonads could be a consequence of the testis feminization process.

Pituitary gonadotropins are transported to the gonads and bind to their specific receptors, FSHR and LHR. The transcription pattern of such receptors usually matches the transcription of the gonadotropins, therefore, levels of FSHR are higher at early stages of

gametogenesis and LHR peaks at gamete maturation stage (Rocha et al., 2009; Levavi-Sivan et al., 2010). Teleost show extragonadal expression of the gonadotropin receptors, mainly in brain and pituitary, but also in other tissues (e.g. Kobayashi et al., 2008; Andersson et al., 2009). Hereby, we have detected uniform transcription of *fshr* and *lhr* in the pituitaries of immature, female and male mullets. This could suggest a regulation unrelated to the cyclic reproductive events occurring during gametogenesis. Pituitary transcription of *lhr* was reported in seabass (Rocha et al., 2007) and orange-spotted grouper (*Epinephelus coioides*) (Peng et al., 2018); in Atlantic salmon for *fshr* (*Salmo salar*) (Andersson et al., 2009) and both *lhr* and *fshr* in Atlantic halibut (*Hippoglossus hippoglossus*) (Kobayashi et al., 2008). Transcription of *lhr* and *fshr* was also detected in the brain of several other fish species. *lhr* transcription was found in the brain of Amago salmon (*Oncorhynchus rhodurus*) (Oba et al., 1999), Atlantic cod (*Gadhus morua*) (Maugars and Schmitz, 2006; Mittelholzer et al., 2009), rare minnow (Qin et al., 2014) and African catfish (*Clarias gariepinus*) (Vischer and Bogerd, 2003). However, no transcription was detected in the Channel catfish (*Ictalurus punctatus*) (Kumar et al., 2001). On the other hand, *fshr* transcription was detected in the brain of Atlantic cod (Mittelholzer et al., 2009), Atlantic halibut (Kobayashi et al., 2008), rare minnow (Qin et al., 2014) and Atlantic salmon (Andersson et al., 2009). However, the transcription pattern of *fshr* and *lhr* in brain and pituitary at different gametogenic stages has never been studied in teleost. Although the exact physiological significance of gonadotropin receptor transcription in pituitary and brain has not been determined, a probable role in the activation of the steroid synthesis pathway in the brain can be suggested, which would resemble their role in the gonads. Nevertheless, taking into consideration the transcription pattern of brain aromatase (*cyp19a1b*) during the gametogenic cycle of thicklip grey mullets (Bizarro, 2015), a clear link between the transcription of gonadotropin receptors in the pituitary and *cyp19a1b* in the brain cannot be stablished. The high levels of transcription for *cyp19a1b* described in male and previtellogenic stage female mullets, do not match the transcription observed in the pituitary for the gonadotropin receptors in the present work. Due to the absence of pituitary samples from intersex mullets, we cannot present results for the possible role of gonadotropin receptors in intersex mullets.

5. Conclusions

To summarize, the presence of transcripts belonging to the kisspeptin and the gonadotropin systems outside their common tissue of expression, suggests a function for those genes at other levels of the BPG-axis. The transcription of *kiss2*, *gpr54*, *gthα* and *fshβ* in mullet gonads can be indicative of a local role in testis and ovary activities. The high *gpr54* transcription

levels detected in immature mullets could suggest its participation in the activation of the gametogenic cycle events, as it would occur during puberty. The similar transcription of both *gthα* and *fshβ* might reflect active FSH synthesis in the gonads of mullets and a possible paracrine role in the control of gametogenesis. The constant transcription levels of the gonadotropin receptors *fhsr* and *lhr* in the pituitary, suggests basal transcription unrelated to the reproductive cycle, probably linked to other neuronal activities. Regarding intersex mullets, low transcription levels of *gthα* and *fshβ* in gonads compared with normal testes might suggest disruption due to gonad feminization. In future works special attention should be driven to understand the interactions between the studied genes at the gonad level, in order to better understand paracrine processes occurring in the development of intersex condition in mullets. This preliminary evidence suggests a local alteration of the transcription of reproduction related genes in intersex gonads.

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Chapter 4

Evolution and trends of the incidence of the intersex condition in thicklip grey mullets exposed to environmental xenoestrogens in Gernika and Pasaia (SE Bay of Biscay)

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Abstract

Thicklip grey mullet (*Chelon labrosus*) is a sentinel species of exposure to endocrine disrupting chemicals (EDC) in the Basque Coast (South East Bay of Biscay). Mullets have been studied in several estuaries and harbors of the area. The presence of xenoestrogenic EDCs has been confirmed by chemical analysis in water and sediment of the sampled areas as well as in bile of the captured mullets, showing the presence, among others, of alkylphenols, phthalates, musk fragrances and pesticides. Especially high concentrations of alkylphenols were detected in the bile of mullets sampled in the harbor area of Pasaia and in the estuary of Gernika. The most striking biological effect identified in some of the sampled populations was the presence of males with intersex testes, this meaning that oocytes are formed within testis as a consequence of exposure to xenoestrogens. Samplings have been performed in Gernika since 2007, and intersex males have been consistently identified in all circumstances. The highest intersex prevalence can be reported for February 2014, with 83% of males showing intersex testes, while the average prevalence has been maintained in between 20-50% until 2018. In the case of Pasaia, the situation has changed in the last years and the prevalence of intersex males has decreased significantly, as since 2016 the highest intersex prevalence recorded was 20% in July 2018, and no intersex individuals were detected in October 2016 and January 2017. Previously, intersex prevalences of up to 56% were reported in Pasaia in October 2010, with levels above 20% in 6 out of 12 samplings carried out in the years 2010 and 2011. In relation to the severity of the intersex condition that takes into account the coverage of oocytes in the microscopically analyzed testes sections, in Pasaia most of the cases of intersex were described as low with an intersex severity score of 1 in a scale of 7. Instead, in Gernika, that was always showing the same low score, the situation seems to have changed after 2014 with higher severity scores becoming apparent. In this scenario intersex severity indexes of 3 to 6 have been identified in 21 individuals out of the 31 intersex males found in the 8 samplings conducted from February 2014 up to July 2018. The point sampled in Gernika receives the effluent of an old WWTP, which combined with the low water volume during low tide in the area contribute to a hot spot of EDC pollution. Chemical analyses up to now do not show any evident deterioration of the situation in the area, although plans to close the old WWTP and to connect Gernika and upstream localities to the new WWTP of Bermeo, allow thinking on a lack of investment in the present day facility. Continuous monitoring of the area would allow to analyze the evolution of the intersex condition in Gernika, as the new WWTP enters into full operational activity collecting the waters of the whole river basin.

Laburpena

Korrokoia (*Chelon labrosus*) disruptore endokrinoen (DE) esposaketako espezie zentinela da Euskal kostaldean (Bizkaiko golkoko hego ekialdea), eta bertako hainbat tokitan aztertu izan dira. Lagindutako puntuetako ur eta sedimentuen zein korrokoien behazunaren analisi kimikoek DE xenoestrogenikoen presentzia konfirmatu dute, beste batzuen artean alkilfenolen, ftalatoen, fragantzien eta pestiziden presentzia erakutsiz. Bereziki, behazunean alkilfenol kontzentrazio altuak neurtu ziren Pasaiako portuan eta Gernikako estuarioan lagindutako korrokoietan. Populazio batzuetan aurkitu den efektu biologikorik ikusgarriena intersex arren presentzia izan da, hau da, xenoestrogenoen esposizioaren ondorioz testikuloan gertatzen den obozitoen eraketa. 2007. urteaz geroztik laginketak egin dira Gernikan eta intersex presentzia kasu guztietan aurkitu da. Prebalentziarik altuena 2014ko otsailean ikusi zen, arren %83 intersexak zirelarik. Prebalentziaren batezbestekoa 20-50% tartean mantendu da 2018 arte. Pasaiako portuaren kasuan, azken urteetan egoera aldatu da, intersex arren prebalentziaren beherakada esanguratsua eman delarik. Izan ere, 2016tik jasotako prebalentziarik altuena 2018ko uztailean eman zen, %20, eta 2016ko urrian eta 2017ko urtarrilean ez zen intersexik aurkitu. Aurretik, %56-ko intersex prebalentzia ikusi zen 2010eko urrian, eta 2010-2011 urteetan egindako 12 laginketetatik 6etan %20-ko prebalentzia baino altuagoa ikusi zen. Intersex egoeraren larritasun indizeari dagokionez, mikroskopikoki analizatutako gonada zatian aurkitutako obozito kopurua kontutan hartzen duena, Pasaiako kasu gehienetan baxu bezala deskribatu zen, 7-ko eskala batean 1 larritasunekoak kalifikatu zirelarik. Gernikan aldiz, aurretik larritasun maila bera aurkeztu bazuten ere, 2014. urtetik hona egoera aldatu egin da, 5 eta 6 larritasun indizeak arruntago bilakatzen ari direlarik. Egoera honetan, 2014ko otsailetik 2018ko uztailera egindako 8 laginketetan aurkitutako 31 intersex arren artean, 21 indibiduok 3 eta 6 arteko intersex larritasun mailak aurkeztu zituzten. Gernikan lagintzen den puntura araztegiko isurkinak iristen dira. Marea beheran estuarioak duen ur emari baxuarekin konbitauz, ingurua ED kutsaduraren *hot spot* bilakatu da. Orain arteko analisi kimikoak ez dira tokiaren hondatzearen adierazgarri, hala ere, araztegiaren itxieraren eta Gernika zein ur gora dauden herrien Bermeoko araztegi berriari konektatzeko planek, gaur egungo instalazioan inbertsio falta eman dela aditzera ematen dute. Gunearen etengabeko jarraipena egiteak Gernikako intersex egoeraren analisia egitea baimenduko du, ibai arro osoko urak bilduz araztegi berria martxan jartzen denean.

1. Introduction

Endocrine disrupting chemicals (EDCs) are substances that can disrupt the endocrine system. Altering the endocrine system can result in effects for the organism and its progeny (OECD, 1996), which in addition could affect the population dynamics (Bernanke and Kohler, 2009; WHO/UNEP, 2013). EDCs include a wide range of substances with different chemical structure and from different sources. Among them, we can find manufactured chemicals such as alkylphenol surfactants, phthalate plasticizers, polychlorinated biphenyls (PCBs), polybrominated ethers, polycyclic aromatic hydrocarbons (PAHs), bisphenol-A (BPA), pharmaceuticals, fungicides, pesticides and their degradation products, as well as natural (17β-estradiol, estrone) and synthetic (17α-ethinlylestradiol) steroid hormones (e.g. Tyler et al., 1998; López de Alda and Barceló, 2001; Langston et al., 2005; Porte et al., 2006). These compounds reach the aquatic environment in the form of complex chemical mixtures through agricultural runoff waters, wastewater treatment plant discharges and urban and industrial effluents. Thus, it is difficult to identify the specific chemical (or chemicals) causing any observed adverse effects on the biota (Tyler and Routledge, 1998). In fact, some estuaries in the United Kingdom, have biologically described as estrogenic (Allen et al., 1999), although the chemicals causing such hormone-linked alterations could not be identified or singled out (Sumpter and Jobling, 2013).

Fish exposed to EDCs exhibit a variety of pathologies related with reproduction, ranging from reduced fecundity and altered courtship behavior to abnormal gamete production, which can affect the viability of a population (Jobling et al., 1998; Mills and Chichester, 2005; Vajda et al., 2008). Due to their ability to mimic estrogens, some EDCs classified as xenoestrogens, are of special concern. Such chemicals bind to estrogen receptors in vertebrates and are able to exert feminizing effects at different levels of biological organization, from the molecular up to the population level (Brander, 2013; WHO/UNEP, 2013). Alkylphenols, BPA, some phthalates and pesticides in addition to synthetic estrogens have been reported to be xenostrogenic. As an example, the alkylphenol nonylphenol was proved to be estrogenic to fish in the 90s (Jobling and Sumpter, 1993; Jobling et al., 1996; Routledge and Sumpter, 1996). Some studies also reported estrogenic activity of the sewage treatment plant effluents that was linked to the presence of natural and synthetic hormones (Desbrow et al., 1998). Among them, 17α-ethinlyletsradiol has received special attention, due to its potent estrogenicity (Lange et al., 2001).

Table 1
Cases of intersex condition in wild fish populations, ordered alphabetically by species. The intersex prevalence is shown as the percentage of intersex individuals out of total males, except for the cases marked by an asterisk (*) where the prevalence of intersex males is calculated as the percentage of total fish. Ns: not specified. WWTP: wastewater treatment plant. PAH: polycyclic aromatic hydrocarbon. PCB: polychlorinated byphenil. EDC: endocrine disrupting chemical.

Species	Common name	Intersex prevalence		Contaminants or source of contaminants	Habitat	Country	Reference
Abramis brama	Bream	0-6%		wwtp effluent	River	Germany	Hecker et al., 2002
		0-43%		wwtp effluent	River, coast, harbor	Netherlands	Vethaak et al., 2002
Astyanax fasciatus	Lambari	0-29%	ns	agricuture, industry and sewage effluents	River	Brazil	Prado et al., 2011
Barbatula barbatula	Stoneloach	11%		wwtp effluent, industry, agriculture	River	Belgium	Douxfils et al., 2007
Barbus plebejus	Barbell	50%	*	untreated sewage discharge, trace metals; PAHs, PCBs, pesticides	River	Italy	Viganò et al., 2001
Catostomus commersoni	White sucker	0-100%		wwtp effluent	River	USA	Woodling et al., 2006
		0-22%	*	wwtp effluent	River	USA	Vajda et al., 2008
Chelon haematocheilus	Red lip mullet	0-28%	*	environmental estrogens	Coastal area	China	Soyano et al., 2010
Chelon labrosus	Thicklip grey mullet	0-83%		wwtp, industry	Estuaries and harbors	Spain	Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014
Clarias gariepinus	Sharptooth catfish	14-25%	*	wwtp effluent, industry, agriculture,	River	South Africa	Barnhoorn et al., 2004
		14,81%	*	wwtp effluent, industry, agriculture,	River	South Africa	Pieterse et al., 2010
Coregonus clupeaformis	Lake whitefish	1,2%	*	wwtp effluent	River	Canada	Mikaelian et al., 2002
Cyprinus carpio	Carp	18-40%		wwtp effluent	River	Spain	Sole et al., 2003
		0-16,6%		wwtp effluent, agriculture, heavy industrialization	river	Spain	Lavado et al., 2004
		10%		PCBs, PAH, organochlorine pesticides in the sediment	River	USA	Baldigo et al., 2006
		0,1%		PCBs, metals	river	USA	Hinck et al., 2009
Escox lucius	Pike	0-26%	*	wwtp effluent	Rivers and canals	United Kingdom	Vine et al., 2005
Etheostoma blennioides	Geenside darter	0-60%		wwtp effluent	River	Canada	Tetreault et al., 2011
Etheostoma caeruleum	Rainbow darter	0-75%		wwtp effluent	River	Canada	Tetreault et al., 2011
Euthynnus alletteratus	Little tunny	2/449		(not specified)	Sea	Mediterranean Sea (Italy and Spain)	Macias et al., 2014
Gasterosteus aculeatus	Three spined stickleback	0-12,5%		(not specified)	River	Germany	Gercken and Sordyl, 2002
Gobio gobio	Gudgeon	5-20%		wwtp effluent, industry, agriculture	River	Belgium	Douxfils et al., 2007
		(1/3)		wwtp effluent	River	France	Minier et al., 2000
		0-15%	*	wwtp effluent	River	United Kingdom	van Aerle et al., 2001
Hypophthalmichthys molitrix	Silver carp	8%		(not specified)	River	USA	Papoulias et al., 2006
Hypophthalmichthys nobilis	Bighead carp	7%		(not specified)	River	USA	Papoulias et al., 2006
Ictalurus punctatus	Channel catfish	13-33%		PCBs, metals	river	USA	Hinck et al., 2009
Leuciscus cephalus	Chub	50%		PCBs, PAHs, organochlorine pesticides, Hg, As	River	Czech Republic	Randak et al., 2009
		0-3,6%		wwtp effluent	River	France	Minier et al., 2000
Limanda limanda	Dab	2/14		EDCs (not specified)	Sea	North Sea (United Kingdom)	Stentiford and Feist, 2005
Liza ramada	Thinlip mullet	0-24%	*	wwtp effluent	River	Italy	Tancioni et al., 2015
Micropterus cataractae	Shoal bass	67-100% (total fish)		wwtp effluent	River	USA	Ingram et al., 2011
Micropterus dolomieu	Smallmouth bass	25-50%		PCBs, PAH, organochlorine pesticides in the sediment	River	USA	Baldigo et al., 2006
		0-100%		agriculture	river	USA	Blazer et al., 2007
		14-73%		PCBs, organochlorine pesticides, mercury	river	USA	Hinck et al., 2009

		0-100%		wwtp effluent; PCBs, PAHs, pesticides	river	USA	Iwanowicz et al., 2009
Micropterus salmoides	Largemouth bass	20-50% (total fish)		Pesticides (tissue)	River	USA	Schmitt et al., 2005
		20-40%		PCBs, PAH, organochlorine pesticides in the sediment	River	USA	Baldigo et al., 2006
		8-91%		PCBs, organochlorine pesticides, mercury	river	USA	Hinck et al., 2009
Morone americana	White perch	22-83%		wwtp effluent	Lake	Canada	Kavanagh et al., 2004
Mugil cephalus	Flathead grey mullet	0-41%	*	environmental estrogens	Coastal area	Japan	Soyano et al., 2010; Aoki et al., 2010
		21%		PCBs, organochlorine pesticides	Estuary	Portugal	Ferreira et al., 2004
		0-15,7%	*	environmental estrogens	Coastal area	South Korea	Soyano et al., 2010; Aoki et al., 2010
		14%		(not specified)	Coastal area	India	Dhanasekar et al., 2018
Mullua barbatus	Red mullet	0-50% (1/2)		wwtp effluent	River	France	Martin-Skilton et al., 2006
Notropis hudsonius	Spottail shiner	0-27%		wwtp effluent	River	Canada	Aravindakshan et al., 2004
Oreochromis mossambicus	Java tilapia	33-39%		Pesticides	River	South Africa	Barnhoorn et al., 2010
Oreochromis spp.	Tilapia	50%		PCBs, NP, phthalates	River	Taiwan	Sun and Tsai, 2009
Pangasius nasutus	Pangasiid catfish	1 specimen		possible pesticide contamination	Pond	Indonesia	Rodriguez et al., 2012
Perca fluviatilis	Perch	0-33%		(not specified)	River	Germany	Gercken and Sordyl, 2002
Platichthys flesus	Flounder	0-8,3%		Industrial impact	Estuary	United Kingdom	Stentiford et al., 2003
		20%		wwtp effluent	Estuary, offshore	United Kingdom	Allen et al., 1999
Pristipomoides multidens	Goldband snapper	<1% (2/206)		(not specified)	Offshore	Australia	Hassell et al., 2018
Pseudorhombus arsius	Large-toothed flounder	1/12		Industrial and sewage effluents	Bay	Kuwait	Stentiford et al., 2014
Rutilus rutilus	Roach	9-25%		wwtp effluent	River	France	Minier et al., 2000
		4-100%		wwtp effluent	River	United Kingdom	Jobling et al., 1998
		4,5-26,5%		wwtp effluent	River and lake	Denmark	Bjerregaard et al., 2006
		20-50%		anthropogenic impact	River	France	Geraudie et al., 2017
Salmo trutta fario	Brown trout	4,5-26,7%	*	wwtp effluent	River	Switzerland	Korner et al., 2005
Sander vitreus vitreus	Walleye	7% males, 9% females		Bleached kraft pulp and paper mill	River	Canada	Pollock et al., 2010
		<1%		EDC contamination	River	USA	Miller et al., 2012
Sarotherodon malenotheron	Tilapia	71,5-80,7%	*	untreated waste waters from different origin	Dam	Nigeria	Adeogun et al., 2016
Scaphirhynchus platyorynchus	Shovelnose sturgeon	29%		organochlorines	River	USA	Harshbarger et al., 2000
		4,7%		(not specified)	River	USA	Colombo et al., 2007
		7,5%		(not specified)	River	USA	Amberg et al., 2010
Synaptura orientalis	Oriental sole	1/28		Industrial and sewage effluents	Bay	Kuwait	Stentiford et al., 2014
Tilapia guineensis	Tilapia	19,3-28,5%	*	untreated waste waters from different origin	Dam	Nigeria	Adeogun et al., 2016
Xiphias gladius	Mediterranean swordfish	17-27%		suspected estrogen-mimicking exposure	Sea	Several countries	De Metrio et al., 2003
Zoarces viviparus	Eelpout or viviparous blenny	0-27,8%		(not specified)	Coastal area	Germany	Gercken and Sordyl, 2002
		0-25%		Industrial impact	Estuary	United Kingdom	Stentiford et al., 2003

One of the most remarkable and evident effects of exposure to estrogenic compounds in fish is the development of ovotestis, or intersex condition, in males. Intersex condition is characterized by the development of oocytes within the testis of any gonochoristic species, thus, resulting in the simultaneous presence of ovarian and testicular tissues in the gonad (Tyler and Jobling, 2008). Many wild freshwater and marine fish species worldwide show intersex testes related to contamination by EDCs (Table 1).

Several studies have pointed out that mugilid species such as flathead mullet *Mugil cephalus* and thicklip grey mullet *Chelon labrosus* could be employed as sentinels of EDC pollution in monitoring programs in estuarine and coastal areas (Ferreira et al., 2004; Whitfield et al., 2012; Ortiz-Zarragoitia et al., 2014). Intersex condition has been described in *M. cephalus* from South Korea, Japan, India and Portugal (Ferreira et al., 2004; Aoki et al., 2010; Soyano et al., 2010; Dhanasekar et al., 2018); *Liza ramada* from Turkey and Italy (Bayhan et al., 2006; Tancioni et al., 2015) and *Chelon haematochelius* from China (Soyano et al., 2010). *Chelon labrosus* is commonly found in the coastal waters and estuaries of the Southern Bay of Biscay and is an adequate pollution sentinel species, due to its widespread distribution, its ability to bio-accumulate pollutants and its resilience and responsiveness to pollution (Ortiz-Zarragoitia et al., 2014). The study of mullets in the Basque coast dates back to the 90s ,but it was only after 2005 that attention to endocrine disruption effects was paid, describing feminizing effects and intersex condition in male mullets from different estuaries (Raingeard et al., 2009; Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Sardi et al., 2015; Rojo-Bartolomé et al., 2017; Valencia et al., 2017). The presence of EDCs was confirmed by analytical methods in water, sediment and mullet bile samples (Puy-Azurmendi et al., 2010; 2013; Bizarro et al., 2014; Ros et al., 2015). High concentration of PAHs, alkylphenolic substances, BPA and 17β-estradiol were measured in the bile of fish captured in Gernika and Pasaia (Puy-Azurmendi et al., 2010; 2013; Bizarro et al., 2014; Ros et al., 2015). These mullets showed alterations in the transcription levels of genes related to steroidogenesis and gamete development. In addition, intersex condition was detected in male mullets from the Bilbao estuary, the harbors of Ondarroa and Pasaia and in the Urdaibai estuary, near the WWTP of Gernika (Diaz de Cerio et al., 2012; 2018; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). The highest intersex condition prevalence correspond to the populations of Pasaia and Gernika, which showed sustained intersex presence over the last 7 to 10 years. Thus, the objective of this work was to analyze the temporal evolution of the intersex condition in mullets form Gernika and Pasaia.

The town of Gernika (43º19'01''N, 2º40'36''W) is located in the Biosphere Reserve of Urdaibai. Although protected, the area receives significant anthropogenic impacts from surrounding agriculture, industrial and urban activities. According to the quality environmental levels established by the European Union Water Framework Directive (2000/60/EC) the estuary is under medium environmental pressure (Borja et al., 2006). In addition, the WWTP of Gernika showed to be obsolete, which is reflected by the deterioration suffered in water physico-chemical parameters during the 2002-2014 period (Borja et al., 2018).

The Pasaia harbor is located in the Oiartzun estuary (43º19'35''N, 1º55'9''W) and it is the second most important harbor in the Basque Country. The activities negatively impacting the estuary's environmental quality include maritime traffic, extensive dredging activities and urbanization, diverse industrial and pollutant discharges (Cearreta et al., 2004; Borja et al., 2006; 2009; Montero et al., 2013). According the EU WFD the estuary is classified as a site under very high pressure (Borja et al., 2006). In the last annual campaign for the analysis of the ecological status of the transitional and coastal waters of the Basque Country, both Urdaibai and Oiartzun estuary water masses were classified with a status of *worse than good*, as the inner part of Urdaibai was classified as *deficient* and the ecological potential of the Oiartzun water body as *moderate* (Borja et al., 2018).

2. Materials and methods

2.1. Sampling site and sample collection

We selected the inner part of the Pasaia harbor and the river channel downstream the town of Gernika. These two places were previously extensively studied for the detection of feminizing effects and intersex condition in native mullet populations. Data from Pasaia included September 2010 to September 2011 (monthly) and June 2012 (Bizarro et al, 2014; Bizarro, 2015). New samplings in the contest of the present work were carried out in this population from October 2016 to January 2017 (monthly) and in July 2018. The analysis in Gernika included published data from April and October 2007, April 2008, July 2010, June 2012 and 2013 and February 2014 (Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). In addition, new samples in Gernika were obtained from the Biscay Bay Environmental Biospecimen Bank (BBEBB). These samples were collected every June or July from 2014 to 2018 and in December 2015 and 2017.

All analyzed thicklip grey mullets (*Chelon labrosus*) were adults (>20 cm), individually collected by fishing rod. The procedure for gonad tissue and bile collection was identical to that mentioned in previous chapters. Briefly, after anaesthetizing fish in a saturated ethyl-

4-aminobenzoate water bath, dissection was immediately performed. For histological analysis, a portion from the middle part of the gonad from each fish was fixed in 4% neutral buffered formalin. A portion of each gonad and the whole bile sac were independently frozen in liquid nitrogen and then stored at -80°C until processing for molecular biology analysis and analytical chemistry determination of EDCs. All fish handling procedures were approved by the UPV/EHU Ethical Committee for Experimental Animals and by the regional authorities.

2.3. Histological analysis of the gonad

After fixing the gonad samples for 24 hours in 4% neutral buffered formalin, they were dehydrated in a graded ethanol series (70%, 90% and 96%) and embedded in paraffin. Then, sections of 5 μm thickness were cut in a Leica RM 2125 RT manual microtome (Leica Microsystems, Nussloch, Germany) and placed on 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 six sections per individual.

The criteria by McDonough et al. (2005) for flathead grey mullet *Mugil cephalus*, was 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 or cortical alveoli stage oocytes and testes during early, mid and late spermatogenesis. When an intersex testis was identified, the prevalence of intersex males out of the total number of males was calculated. The severity of the intersex condition was assigned according to the Intersex Severity Index described by Jobling et al. (2006); severity increases as the number of oocytes present in the testis increases. The intersex index ranges from 0 to 7. Normal male testis scores 0; presence of up to 5 oocytes scattered through the testis scores 1; testis showing 6 to 20 clustered oocytes = 2; presence of 21-50 oocytes = 3; 50-100 oocytes = 4; more than 100 oocytes = 5; more than 50% of the gonad is formed by ovary clearly separated from the testicular tissue = 6; and a completely feminized and ovarian-like gonad scores 7.

2.4. Chemical analysis of fish bile

The determination of alkylphenols (4-*tert*-octylphenol, 4*n*-octylphenol, nonylphenol isomer mixture), bisphenol A and estrogenic natural and artificial hormones (17β-estradiol, 17α-ethynilestradiol, diethylstilbestrol) from bile samples of mullets from Pasaia in 2010 (September and November), 2011 (January, March, May, July and September), 2012 (July),

2016 (November) and 2017 (January) was performed according to Gibson et al. (2005), with some modifications published in Ros et al. (2015). Briefly, an enzymatic hydrolysis of 100 μL of the bile sample was done first. followed by an extraction step using Bond Elut Plexa (200 mg, Agilent Technologies, Avondale, PA, USA). Then a clean-up step with solid phase extraction cartridges LC-Florisil (1 g, SigmaeAldrich, Steinheim, Germany) was carried out. LC-Florisil cartridge elution produced two fractions. In the first one, alkylphenols were eluted and a direct analysis by means of gas chromatography-mass spectrometry (GC-MS) was performed. In the second one, hormones were submitted to a derivatization step prior to their analysis by GC-MS.

3. Results

3.1. Histology of the gonads

In the June-July samplings performed in Gernika and Pasaia males showed immature testes, with cysts composed only of spermatogonia. In the samplings carried out in winter, males showing testes at mid and late spermatogenesis were the most abundant, although some males showed immature testes.

Records of intersex individuals detected in Gernika and Pasaia showed that the intersex condition developed regardless of the spermatogenic stage of the testis and in most cases showing previtellogenic oocytes (Figure 2A-D). Oocytes could be inside (Figure 1 B, C, D) or outside (Figure 1A) the spermatic tubules. However, in some few cases intersex gonads showed mature oocytes. These intersex mullets showed testis at mid and late spermatogenesis with cortical alveoli or vitellogenic oocytes, as shown in February 2014 in Gernika and in December 2016 in Pasaia (Figures 1E and 2). Oocytes at advanced oogenic stages are more abundant in mid and late spermatogenic testis than in early or immature testis. Accordingly, high intersex severity index (4-6) were only detected in intersex individuals showing testis tissue at mid and late spermatogenesis.

Intersex condition was present in all sampling campaigns performed in Gernika from April 2007 to July 2018 (Figure 3A). Intersex prevalence ranged from 5% in April 2008 to 83,3% in February 2014. In the new samplings included in the present work, the lowest prevalence was detected in June 2014 (17%) and the highest in June 2017 (50%). During the whole study period the prevalence of intersex mullets in Gernika did not change significantly (Figure 3A). Most intersex mullets from Gernika showed a severity index of 1, but after February 2014 severity index values of 4 to 6 became predominant (Figure 4A). Unfortunately, data for severity index prior to 2013 is not available, but according to the

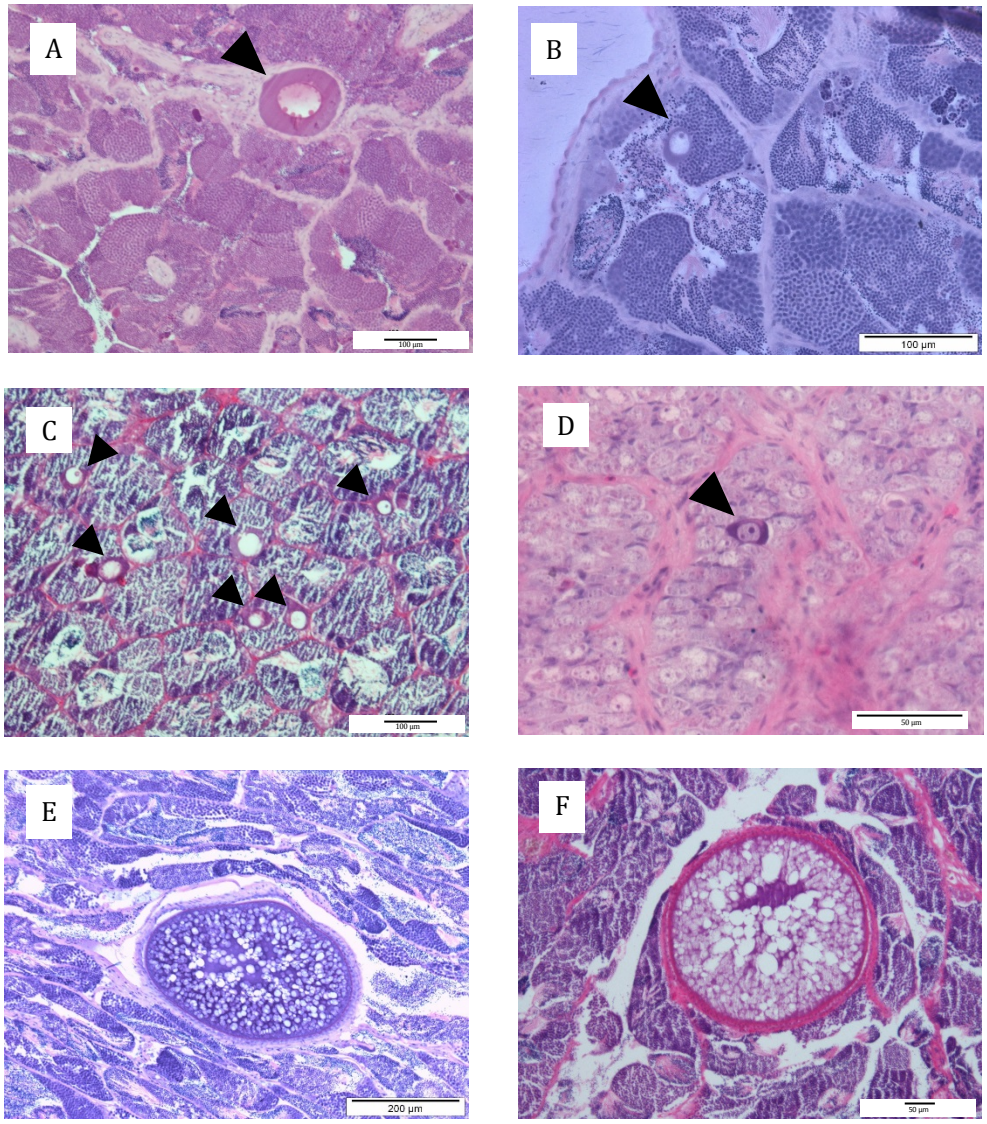

Figure 1
Micrographs of gonad sections of intersex thicklip grey mullet. A) Mature testis with solitary previtellogenic oocytes (arrowheads) surrounded by connective tissue. B) Mature testis with a solitary previtellogenic oocyte inside a spermatic cyst (arrowhead). C) Mature testis with dispersed previtellogenic oocytes (arrowhead) in different spermatogenic cysts. D) Immature testis with showing one previtellogenic oocyte (arrowhead). E and F) Mature testes containing a vitellogenic oocyte.

descriptions published in those years all cases showed scattered oocytes within testicular tissue. Thus, severity index values of 1 to 3 could be suggested in samples of 2007 to 2012. Recent samplings demonstrated almost complete feminization of the gonad of one individual in December 2015 (Figure 2). This mullet showed a section of ovary delimited by connective tissue within the testis. Other sections of the testis contained dispersed clusters of previtellogenic oocytes (Figure 2B). Intersex mullets collected in later samplings also showed high intersex severity indexes (Figure 4A). In June 2016 and 2017, individuals with

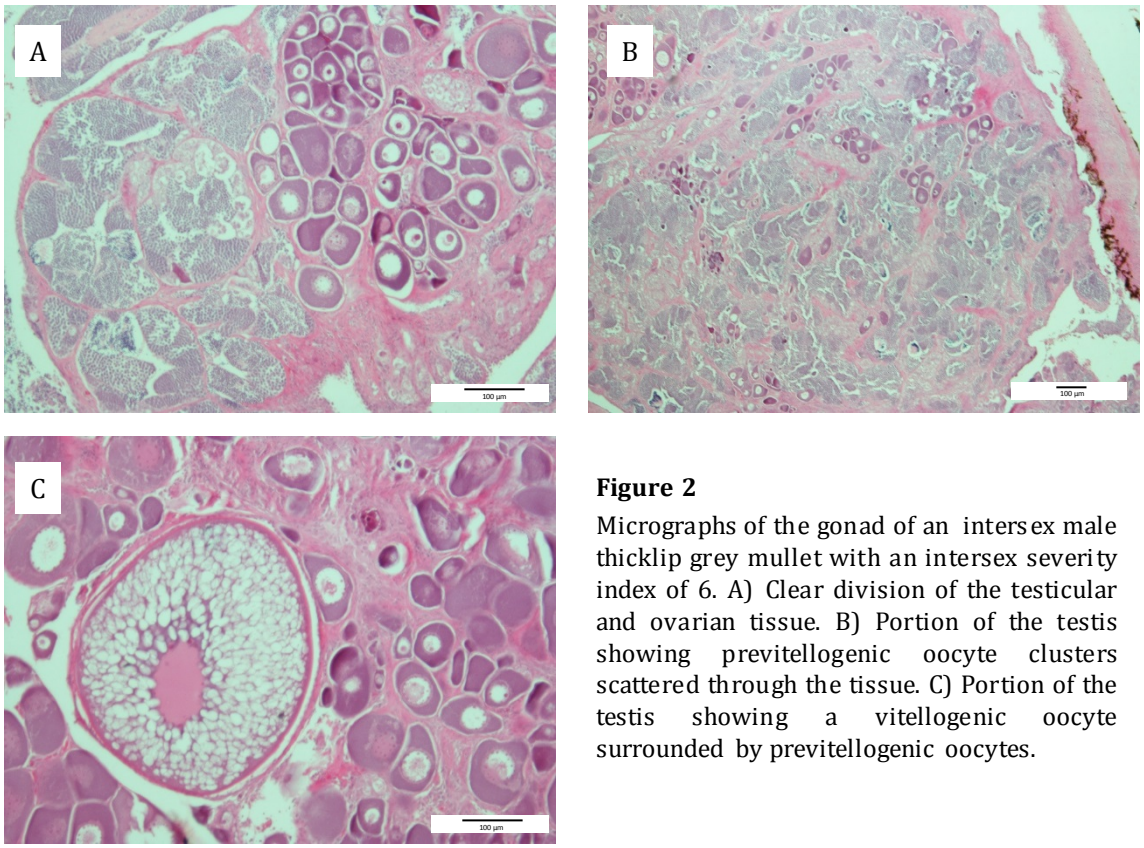

Figure 2

Micrographs of the gonad of an intersex male thicklip grey mullet with an intersex severity index of 6. A) Clear division of the testicular and ovarian tissue. B) Portion of the testis showing previtellogenic oocyte clusters scattered through the tissue. C) Portion of the testis showing a vitellogenic oocyte surrounded by previtellogenic oocytes.

intersex severity indexes ranging from 1 to 6 were observed, while in July 2018 the intersex severity indexes were between 3 and 5 (Figure 4A).

In Pasaia, intersex condition prevalence ranged from 5% in May 2010 and August 2011 to 56% in October 2012 (Figure 3B). Intersex mullets were not detected in the samplings of October 2016 and January 2017, probably due to the reduced number of males captured. Nevertheless, a decreasing trend in the intersex prevalence was observed during the studied period in the population of Pasaia (Figure 3B). Most intersex mullets from Pasaia (94%) showed severity index value of 1 (Figure 4B). Higher values were only detected in three intersex individuals showing levels of 2 to 5. Nevertheless, and opposite to what was observed in the population of Gernika, after December 2016 all intersex found in Pasaia showed severity index 1 (Figure 4).

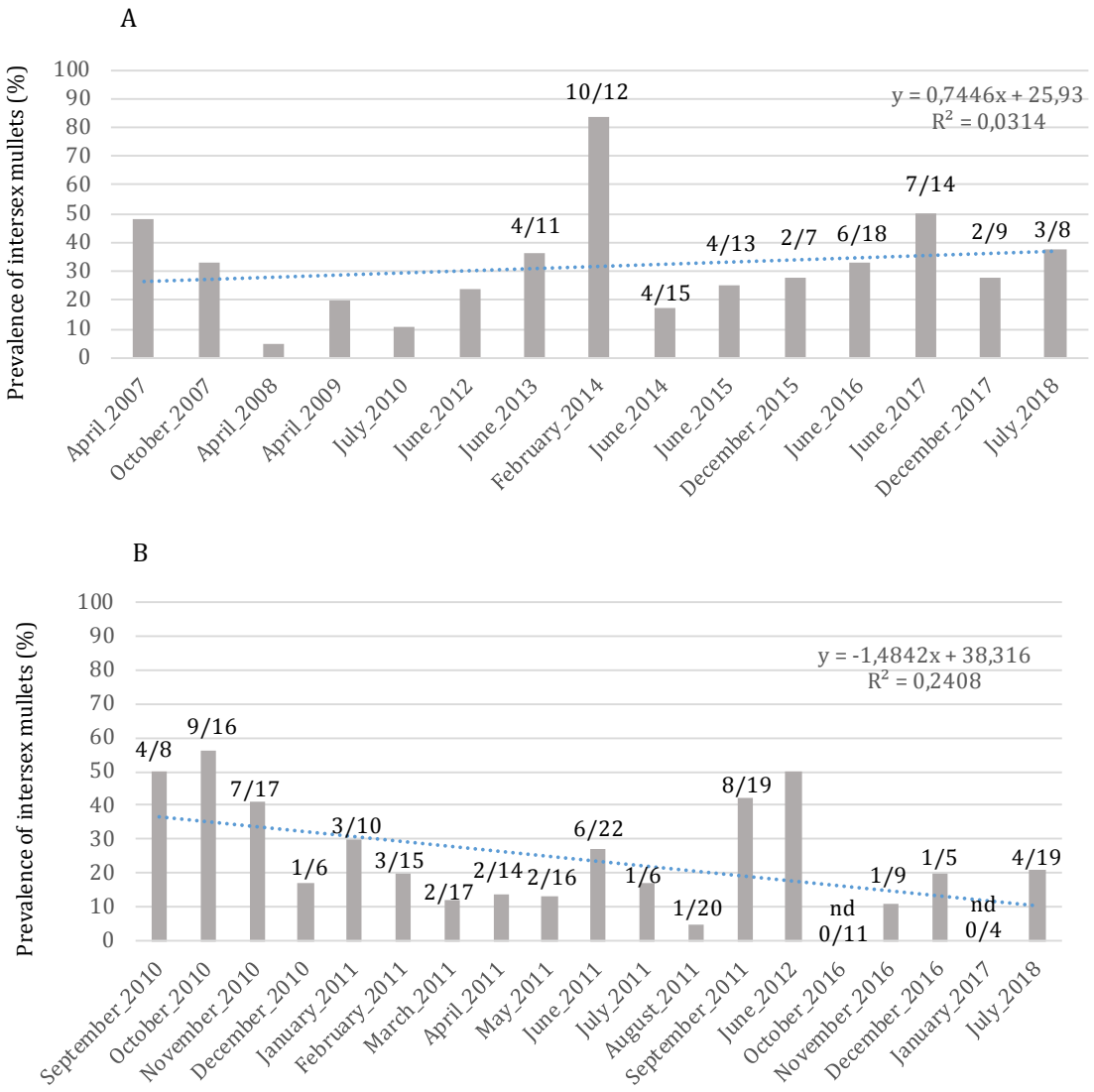

Figure 3
Prevalence of intersex mullets calculated as the percentage of intersex individuals out of the total amount of males per sampling (The ratio is showed when available). A) Prevalence of intersex mullets from Gernika at different samplings performed from April 2007 to June 2018. B) Prevalence of intersex mullets in Pasaia in the samplings performed from September 2010 to July 2018. The dotted lines represent the temporal trend of the intersex prevalence, their corresponding equations are represented in the upper right side of each graph. nd = no intersex.

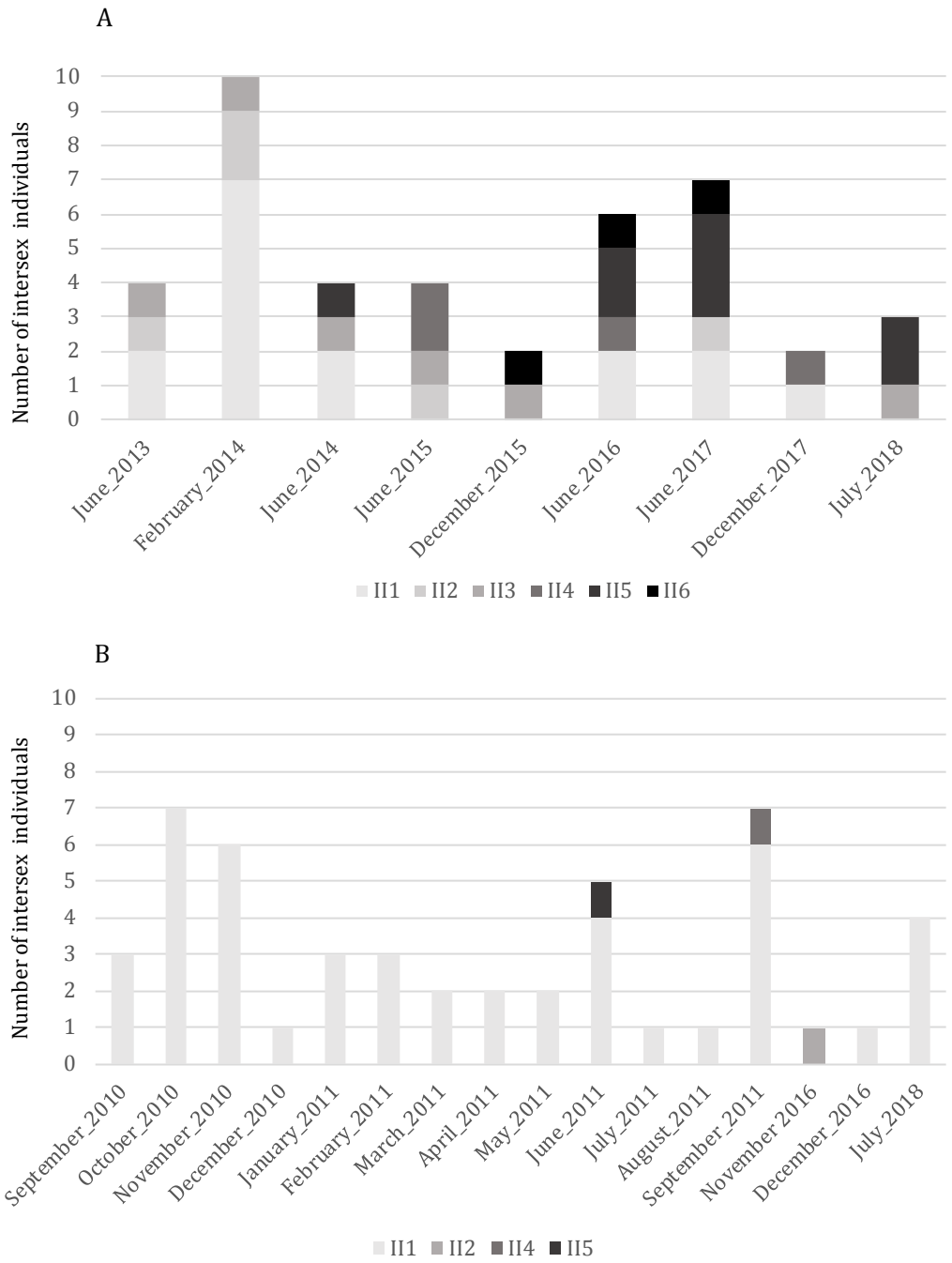

Figure 4
Number of intersex mullets (calculated as the number of intersex males out of the total number of males) captured at each sampling and Intersex Severity Index (II) associated to them in A) Gernika from June 2013 to July 2018 and B) Pasaia from September 2010 to July 2018.

3.2. Bile chemical analysis

Results of the chemical analysis of selected EDC analytes in the bile samples of mullets from Pasaia showed detectable levels of most of the analyzed chemicals (Table 2). 17α-ethinlyletsradiol (EE2) and diethylstilbestrol (DES) levels were not detected or were below their limit of detection. Levels of 17β-estradiol (E2) were high at all samplings, with very high levels detected in January 2011, November 2016 and January 2018. Concentrations of 4tOP and 4nOP alkylphenols were similar in all campaigns, being the range of 49-70 ng/g for 4tOP and 25-70 ng/g for 4nOP. NP mix showed a wider range of concentration variation between sampling campaigns. High NP mix concentrations (> 100 ng/g) were determined in the bile of mullets captured in January, March, July and September 2011. The lowest concentrations were measure in mullets from November 2010 and January 2017. BPA concentrations in the bile of mullets were high at all sampling times, without any seasonal distribution. Extremely high BPA levels were detected in bile from mullets collected in January 2017.

Table 2
Mean concentration ± standard deviation (ng/g) of selected endocrine disrupting chemicals quantified in the bile of mullets captured in Pasaia at several sampling campaigns.

Sampling point	Year	Analyte						
		Alkylphenols			BPA	E2	EE2	DES
		4tOP	4nOP	NP mix				
Pasaia								
	2010, September	70±9	44±5	64±9	922±220	28±5	nd	<LOD
	2010, November	46±2	25±2	25±6	291	24±4	nd	<LOD
	2011, January	65±4	41±4	137±33	373	226±5	nd	<LOD
	2011, March	54±1	33±2	140±23	459	31±4	nd	<LOD
	2011, May	47±2	32±1	64±3	397±40	40±4	nd	<LOD
	2011, July	49±1	57±5	272±49	452	42±8	nd	<LOD
	2011, September	49±2	32±1	115±16	472±29	55±8	nd	<LOD
	2012, July	55±0,2	35±10	87±2	849	30±4	nd	<LOD
	2016, November	55±1	44±1	40±6	521	108±13	nd	<LOD
	2017, January	52±4	70±4	28±7	1927	202±12	nd	<LOD

nd: not detected
LOD: limit of detection

4. Discussion

4.1. Intersex condition in Gernika and Pasaia

In the last decade, intersex mullets have been detected at several estuaries and littoral areas of the Basque coast (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017).

The intersex condition is an uncommon phenomenon in wild populations of mugilids and associated with the exposure to EDCs (Ortiz-Zarragoitia et al., 2014). The first evidence of intersex condition in mullets dated back to 1959 in one *Mugil cephalus* individual collected in the East coast of Florida (Stenger 1959). However, specific studies dealing with the development of intersex and its association with EDCs are scarce. Furthermore, a study in white sucker (*Catostomus commersonii*) and fathead minnow (*Pimephales promelas*) from Boulder Creek (Colorado, USA), suggested that the high prevalence of intersex condition is a recent phenomenon in the last 50 years associated to urban discharges and WWTP activities (Norris et al., 2018). In the Basque Country intersex condition in mullets was described for the first time in April 2007. In the Biosphere Reserve of Urdaibai 49% of analyzed males showed testes containing oocytes at previtellogenic stage and one individual showed vitellogenic oocytes (Puy-Azurmendi et al., 2013). Thereafter, analysis of mullets from other Basque estuary and coastal areas revealed that intersex condition was also present in populations of the harbors of Pasaia, Ondarroa and Arriluze (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Valencia et al., 2017). Furthermore, year-to-year analysis of mullets from Gernika showed that intersex severity in this population is increasing. Since June 2014, several intersex mullets in Gernika show severity index values between 4 and 6 (in a maximum scale of 7), which could indicate a worsening in the water quality due to EDC exposure in the area (Valencia et al., 2017 and present work results). Most of the chemicals accumulated in the bile of mullets from Gernika showed very high levels of alkylphenols, BPA and estrogenic hormones, signs of exposure to urban wastewaters. The WWTP of Gernika works with a primary physical treatment followed by an inefficient biological treatment, which contributes to the presence of EDC into the estuary (Ros et al., 2015).

The prevalence of intersex males in Gernika ranged from 5% to 83,3% (Puy-Azurmendi et al., 2013; Valencia et al., 2017) and in Pasaia from 5% to 56%, although not in all samplings in this area were intersex mullets detected. Similar prevalence were detected in other mugilids. For instance, Ferreira et al. (2004) reported 21% of intersex in *Mugil cephalus* from the Douro estuary in Portugal. In Korea and Japan prevalence of intersex ranged between 5% to a maximum of 41% in the Omuta River that receives discharges of urban and industrial origin in an area with 130000 inhabitants (Aoiki et al., 2010; Soyano et al., 2010). In a study performed with *Liza ramada* thinlip grey mullets from the Tiber river downstream de WWTP of the city of Rome, intersex male prevalence ranged from 12,5% to 24,4% of the total studied fish population (Tancioni et al., 2015).

Occurrence of intersex fish has been documented worldwide. In the United Kingdom, intersex individuals of wild roach *Rutilus rutilus* were found in all studied sites in the years

1995 and 1996. The minimum intersex prevalence was recorded at a low impacted site (4%) and the maximum (100%) at two populations downstream WWTPs (Jobling et al., 1998). High prevalence (100%) of intersex condition has been also reported in smallmouth bass *Micropterus dolomiteu* from the Potomac river (Blazer et al., 2007; Iwanowicz et al., 2009), in a site that receives agricultural runoff and industrial and domestic discharges (Blazer et al., 2007). Species differences in sensitiveness to pollutants and intersex condition development exist. Baldigo et al. (2006), reported intersex condition in bass (*Micropterus dolomieu* and *M. salmoides*) and carp (*Cyprinus carpio*) but not in bullhead (*Ameiurus nebulosus*), being all four species from same polluted locations. There are also studies that have not found any intersex incidence in fish living in highly polluted areas (Flammarion et al., 2000; Folmar et al., 1996). Chub (*Leuciscus cephalus*) from the Moselle River (France) showed high vitellogenin levels in plasma and necrotic sperm in testis, but no signs of intersex condition (Flammarion et al., 2000). Similarly, carps captured downstream a WWTP showed elevated levels of vitellogenin in plasma but no evidences of intersex condition (Folmar et al., 1996). Accordingly, in thicklip grey mullets from the Basque coast there is no a direct association between vitellogenin content in plasma or transcript up-regulation in the liver and the intersex condition (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Bizarro, 2015; Valencia et al., 2017). This suggest that development of intersex condition occurs by other molecular mechanisms different to those controlling vitellogenin synthesis. Lavado et al. (2004) for instance, found only one intersex carp downstream the WWTP of the city of Zaragoza, but plasma vitellogenin levels were detected in most of the male carps sampled in that site.

A full year intensive study, from September 2010 to September 2011, in the harbor of Pasaia demonstrated that intersex condition was present in mullets at all spermatogenic stages and all along the study period (Bizarro, 2015), suggesting that intersex condition in this species is not limited to a specific or more sensitive gametogenic stage. Similarly, absence of relation between intersex condition and testis spermatogenic stage were also described in smallmouth bass (Iwanowicz et al., 2019). However, seasonal dependent differences in the intersex prevalence were described in *Liza ramada*, as Tancioni et al. (2015) observed that the highest intersex prevalence were recorded in winter, corresponding to the period when testes can be found at late spermatogenesis. Smallmouth bass from the Potomac river showed seasonal changes in intersex prevalence, being intersex incidence higher in spring than in summer, coinciding with the species pre-spawning season (Blazer et al., 2007, Blazer et al., 2012). In the case of intersex mullets from Pasaia, the highest prevalence was observed in autumn months (September, October and November 2010 and September 2011) (Bizarro, 2015). In Gernika, although the highest intersex prevalence was detected in

winter (February 2014) (Valencia et al., 2017), in the remaining winter samplings (December 2015 and 2017), the prevalence was not higher than in summer samplings. Therefore, the election of a suitable sampling period should be considered when investigating the intersex condition in wild fish (Bahamonde et al., 2013). More developed gametogenic stages facilitate the identification of possible pathologies in gonad tissue including intersex (Barrett and Munkittrick, 2010).

As mentioned above the prevalence of intersex males with high severity indices has increased in Gernika since June 2014. In previous sampling campaigns, intersex mullets in Gernika showed dispersed oocytes through the testis, being their severity index between 1 and 3. The intersex individual with severity index 6 from December 2015 showed a clear division of the ovarian and the testis tissues, both separated by a layer of connective tissue. Furthermore, some of the oocytes were at advanced stages of maturation with male tubules at mid spermatogenesis stage. Although in most of the intersex cases reported the oocytes were at previtellogenic stage, some studies have reported the presence of vitellogenic oocytes within the testis, such as in the lake whitefish (*Coregonus clupeaformis*), salmonid fish (Mikaelian et al., 2012), flounder (*Platichthys flesus*) (Allen et al., 1999) or barbell (*Barbus plebejus*) (Vigano et al., 2001). In the lambari (*Astyanax fasciatus*), intersex individuals showed testis containing focal arrangement of previtellogenic and vitellogenic oocytes (Prado et al., 2011).

Most of the EDCs detected in water and sediment from Gernika and Pasaia are well known for their xenostrogenic activity. However, we cannot exclude the presence of androgenic or antiestrogenic compounds. Histological analysis and the structure of oocytes within spermatic tubules in studied mullets, suggest feminization rather than masculinization of an ovary. In this respect, van Aerle et al. (2001) reported the prevalence of intersex in gudgeon (*Gobio gobio*) captured downstream a WWTP as the percentage of the whole population studied, and not as the percentage of all males studied. They recalled the possibility of those intersex being feminized males, masculinized females or a combination of both, although they provided evidence in favor of feminization of males. Körner et al. (2005) described intersex in *Salmo trutta fario* brown trout, captured downstream of three different Swiss WWTPs, as phenotypically females with sperm nests. In a study with *Esox lucius* pikes in rivers from the United Kingdom (Vine et al., 2005), only one out of 16 intersex pikes was considered to be a male intersex, while the other 15 were described as female intersex. These individuals presented a gonad where the ovarian tissue was predominant with some dispersed male germ cells. A similar classification was reported for a carp population, where the only intersex found was described as an intersex female that

presented sperm in the ovary (Hinck et al., 2009). However, Gercken and Sordyl (2002) described intersex condition in eelpout (*Zoarces viviparous*) as males containing oocytes in testis. In any case, the evaluation of the intersex condition in mullets from Gernika and Pasaia showed evidences of the feminizing process occurring in males.

4.2. Bile EDC content in mullets from Pasaia and Gernika

Chemical analysis in water and sediments from Basque estuaries have revealed the presence of a wide range of pollutants, many of them considered EDCs (Larreta et al., 2013; Ros et al., 2015; 2015b). The main contaminant sources in the study area are the discharges of wastewater treatment plants (WWTPs), which contain organochlorine pesticides, phthalates, PAHs, PCBs, BPA, alkylphenols and natural and synthetic hormones at the ng/L-µg/L level (Bizkarguenaga et al., 2012). Elevated levels of phthalates (up to 11223 $ng.L^{-1}$), BPA (up to 2615 $ng.L^{-1}$), 4tOP (47327 $ng.L^{-1}$) and NP (28917 $ng.L^{-1}$) were detected in the effluent of the WWTP of Gernika. In addition, concentrations of natural hormones 17β-estradiol, estriol and estrone were 54, 21 and 574 $ng.L^{-1}$ respectively. However, the synthetic hormone 17α-ethynylestradiol was below detection limit and diethylstilbestrol concentration was 7 $ng.L^{-1}$ (Bizkarguenaga et al., 2012). Similarly, high levels of EDCs were quantified in the bile of mullets inhabiting downstream the WWTP of Gernika (Puy-Azurmendi et al., 2010; 2013; Bizarro et al., 2014; Bizarro, 2015; Ros et al., 2015). Bile is a good biological matrix to study exposure and uptake of EDCs (Petterson et al., 2006). Puy-Azurmendi et al. (2013) suggested the association between alkylphenol levels in bile and intersex condition in mullets from Gernika. On the other hand, Bizarro et al. (2014) and Ros et al. (2015) did not observe such association, and the potential effect of the mixture was proposed to promote intersex in mullets.

In the present work, we determined the concentration in bile of several EDCs (alkylphenols 4nOP, 4tOP and NP, BPA, natural and synthetic hormones E2, EE2 and DES) in mullets from Pasaia. Unfortunately, we still do not have data for samples from Gernika. EE2 and DES were below the detection limits in mullets from Pasaia, similar to low levels reported by Ros et al. (2015). Low EE2 bile levels have also been reported in fish inhabiting waters downstream WWTP (Viganó et al., 2006; Fenlon et al., 2010; Harding et al., 2016). However, E2 levels were high in the bile of analyzed mullets, with extremely high levels in January 2011 and 2017 and November 2016. In previous studies in the same population, lower E2 levels were detected in bile (Bizarro et al., 2014; Ros et al., 2015). It should be considered, that in the present work bile of male and female mullets was pooled, while in the works of Bizarro et al. (2014) and Ros et al. (2015), bile of intersex and non-intersex males was

analyzed individually. Gametogenesis in mullets in the Basque coast develops during winter months, when higher plasma E2 levels were detected in females (Sardi et al., 2015). This could partially explain the high E2 levels detected in the bile samples of analyzed mullets. Nevertheless, bile concentrations of E2 in male roach (*Rutilus rutilus*) exposed to WWTP effluents were similar (158-332 $ng.mL^{-1}$) to the highest concentrations reported in the present study, and female roach showed even higher concentrations (549-2503 $ng.mL^{-1}$) (Fenlon et al., 2010). Thus, we could not discard that environmental E2 was also contributing to detected elevated E2 levels in mullets from Pasaia.

Very high BPA concentration was detected in the bile of mullets collected in Pasaia in January 2017. This is the highest concentration observed until now in the Basque coast (Bizarro et al., 2014; Ros et al., 2015), and ten times higher than BPA levels reported previously in mullets from Gernika. High BPA was also detected in the bile of roach (763-1951 $ng.mL^{-1}$) (Fenlon et al., 2010), juvenile rainbow trout (0,49 $\mu g.mL^{-1}$) (Rostkowski et al., 2011) and coho salmon (3900 $ng.mL^{-1}$) (Harding et al., 2016) exposed to WWTP effluents. On the other hand, levels of 4tOP and NP in mullets from Pasaia, were lower than those reported for mullets from Gernika (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Ros et al., 2015). 4tOP levels in bile of mullets from Gernika ranged from 21 up to 95 $ng.g^{-1}$, while NP levels were in the range of 2540-7483 $ng.g^{-1}$ (Puy-Azurmendi et al., 2013). However, mullets from Pasaia contained higher alkylphenol levels in bile than pouting (*Trisopterus luscus*) and four-spotted megrim (*Lepidorhombus boscii*) captured offshore in the Basque coast (Fernandes et al., 2008). Alkylphenols have been detected commonly in the bile of fish inhabiting downstream or nearby WWTPs and showing intersex condition. Red mullets (*Mullus barbatus*) collected close to the WWTP of the city of Marseille showed 28,31 $\mu g.g^{-1}$ of NP and 0,25 $\mu g.g^{-1}$ of 4tOP in their bile (Martin-Skilton et al., 2006). The bile of carps (*Cyprinus carpio*) downstream the WWTP of Zaragoza in the Ebro River (Spain), showed NP and OP average concentrations of 19,88 and 0,39 $\mu g.g^{-1}$ respectively (Lavado et al., 2006). In roach under the influence of WWTP effluents, bile NP levels were in the range of 5531-12678 $ng.mL^{-1}$ (Fenlon et al., 2010). All populations and species mentioned above showed intersex condition, similar to mullets analyzed in the present study. In addition to NP and 4tOP, there are more degradation products of alkylphenol polyethoxylates with xenoestrogenic effects, which could contribute to feminizing effects in impacted fish populations (Tyler and Routledge, 1998). Thus, the possibility that other alkylphenols or their derivatives are present in mullet bile cannot be discarded.

The presence of EDCs in mullet bile together with previous reports of EDCs in sediment and water from both Gernika and Pasaia are responsible for the development of the intersex condition in mullets. The chemical mixture from both areas comprises a wide range of chemical compounds that possibly act synergistically to produce the intersex condition (Tyler and Routledge, 1998). The increase in the severity of the intersex condition in mullets from Gernika during the last campaigns evidences that the exposure scenario in that area is getting worse. Determination of xenoestrogenic chemicals discussed above in the bile of mullets from Gernika, will help elucidating the contribution of those EDCs in the prevalence and severity of intersex condition. Furthermore, it is expected that the future connection of the wastewater system of the town of Gernika to the modern WWTP of Bermeo (scheduled for 2020), will improve the water environmental quality, which could also modulate the presence of endocrine disruption effects in local fish populations. In Pasaia, the lower intersex condition prevalence detected in the last years, could be related with the improvement observed in the water environmental quality. Nevertheless, the high contamination load still present in the sediments of the harbor combined with the dredging activities, suppose a risk to the mullet population of the area.

5. Conclusion

The exposure to EDCs evidenced by their accumulation in bile together with previous reports of EDCs in sediment and water from both areas is causing the development of the intersex condition in mullets from Gernika and Pasaia. The increase in prevalence and severity of the intersex condition observed in mullets in the last sampling campaigns in Gernika, evidences that the situation is becoming worse. In this respect, a new WWTP has been constructed in the area which has begun functioning downstream of the WWTP of Gernika, and the situation might be expected to improve when Gernika and the upstream localities are connected to the new facility. High concentrations of EDCs have been also observed in the harbor of Pasaia, although a decrease in the prevalence of the intersex condition has been notified, beginning in 2016. Although the water quality in Pasaia has improved in the last years, the high load of contaminants present in the sediments combined with dredging activities, can make chemicals available for the mullet population of the area. Altogether, the data suggests that the situation in Gernika and Pasaia will improve, this leading to a recovery of the impacted mullet populations that we shall continue analyzing during the annual sampling campaigns conducted by the Biscay Bay Environmental Specimen Bank.

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Chapter 5

Oocyte atresia and transcription levels of apoptosis and autophagocytosis marker genes in the gonads of females and intersex thicklip grey mullets (*Chelon labrosus*) downstream the wastewater treatment plant of Gernika

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Abstract

Atresia is a degenerative process through which ovarian follicles lose their integrity and are eliminated before completing maturation. Although it is a common feature of vertebrates, the mechanisms leading to atresia differ among species. This physiological process can be promoted by environmental stressors such as light/temperature regime changes, hypoxia, fasting, presence of biocidal agents and pollutants, and altered hormone levels. Endocrine disrupting chemicals (EDCs) can also trigger follicular atresia in fish. In previous studies with *Chelon labrosus* thicklip grey mullets high incidence of intersex condition due to EDC exposure has been reported downstream the wastewater treatment plant of Gernika and in the Pasaia harbor. In samples collected from July 2014 to June 2018, histopathological analysis of mullet gonads from Gernika showed high incidence of atresia in previtellogenic ovaries, together with high prevalence of intersex condition in males. In contrast, ovaries from Pasaia did not display atresia while intersex prevalence showed a decreasing trend from 2016, with no intersex found in some of the samplings. Atretic ovaries showed a high amount of strongly basophilic and shrunk oocytes, without connection with neighboring follicles and surrounding connective tissue. TUNEL analysis revealed no immunohistochemical signs of apoptosis. To molecularly better understand the ovarian atretic process, gene transcription analysis of apoptosis and autophagocytosis related genes was performed comparing atretic and non-atretic ovaries and intersex testes of mullets from Gernika, and ovaries of mullets from Pasaia. Transcriptional levels of *p53, mdm2*, *rpl5, caspase3, fshr* and *beclin1* were not different in atretic vs non-atretic ovaries from Gernika, but all ovaries from Gernika showed higher transcription levels than those from Pasaia. In general, intersex testes showed transcription levels similar to those quantified in ovaries from Gernika, with the exception of *fshr*, whose transcript levels in intersex testes were significantly higher than the transcription observed in all ovaries. These results show the upregulation of apoptotic/autophagocytic pathways in the ovaries of fish exposed to EDCs in Gernika but not in Pasaia, and point out to a possible molecular mechanism associated to the follicular atresia response detected in female mullets. Nevertheless, it remains unclear if these effects will have implications in population reproductive capacity.

Laburpena

Atresia endekapen prozesuaren bitartez, folikulu obarikoek beraien osotasuna galtzen dute eta heldutasunera iritsi aurretik deuseztatzen dira. Ornodunen ohiko ezaugarria bada ere, espezieen artean atresiara daramaten mekanismoen arteko desberdintasunak daude. Ingurune estresek prozesu fisiologiko hau sustatu dezakete, esaterako, argi eta tenperatura aldaketak, hipoxiak, baraualdiak, agente bioziden eta kutsatzaileen presentziak edo hormona mailen aldaketak. Disruptore endokrinoek (DE) ere atresia folikularra eragin dezakete arrainetan. Korrokoiekin (*Chelon labrosus*) egindako azken lanetan, DE-en eraginez sortutako intersex egoeraren gorakada nabarmendu da Gernikako araztegitik ur behera eta Pasaiako portuan. 2014ko ekainetik 2018ko uztailera jasotako laginetan, Gernikako korrokoien gonaden analisi histopatologikoak, atresia altua erakutsi zuen obario prebitelogenikoetan, arretan intersex egoeraren presentzia altuarekin batera. Pasaian aldiz, aztertutako obarioetan ez zen atresiarik antzeman eta intersex prebalentziaren beherakada ikusi zen 2016tik, kasu batzuetan intersexik aurkitu ez zelarik. Obario atretikoek oso basofilikoak ziren eta uzkurtutako obozito kopuru handia zuten, inguruko folikuloekin eta ehun konektiboarekin lotura galdu zutelarik. TUNEL analisiaren bitartez ez zen apoptosiaren zantzu immunohistokimikorik behatu. Obarioen atresia prozesua molekularki hobe ulertzeko asmoz, apoptosiarekin eta autofagozitosiarekin erlazionaturiko geneen transkribapen maila neurtu zen, Gernikako obario atretiko zein ez atretiko eta korrokoi intersexetan, Pasaiako korrokoien obarioekin konparatuz. *p53, mdm2*, *rpl5, caspase3, fshr* eta *beclin1* geneen transkribapen mailak berdinak ziren Gernikako obario atretiko zein ez atretikoetan, Pasaiakoak baino altuagoak ziren. Orokorrean, intersexen gonadek obarioen antzekoak ziren transkribapen mailak erakutsi zituzten, Gernikakoen antzekoagoak zirelarik, *fhsr*-ren salbuespenarekin, honen transkribapena intersexetan obario guztietan neurtutakoa baino nabarmenki altuagoa zelarik. Emaitzek ED-etara esposaturiko arrainen obarioetan apoptosi eta autofagozitosi bidezidorren gain erregulazioa erakusten dute Gernikan, baina ez Pasaian, atresia folikularrarekin erlazionaturiko mekanismo molekular batean pentsatzera eragiten dutelarik. Hala ere, argitu gabe geratzen da ea ikusitako efektuek populazioaren ugalkortasun gaitasunean eragina izango dezaketen.

1. Introduction

Ovarian atresia is a degenerative process through which ovarian follicles lose their integrity and are eliminated before they can complete their maturation process (Saidapur, 1978). Although follicular atresia is a common feature of vertebrate ovaries, the mechanisms undergoing atresia differ among species (Saidapur, 1978). In mammals, apoptotic cell death is the molecular mechanism responsible for ovarian follicular atresia and can occur in the oocyte or in the surrounding follicular granulosa or theca cells (Hughes and Gorospe, 1991; Tilly et al., 1991; Hsueh et al., 1994; Hussein 2005). Apoptosis is an evolutionarily conserved physiological and developmental process that occurs in several tissues and cell types (Steller, 1995), being the main process for selective cell deletion in order to prevent proliferation of damaged cells and tissue inflammation (Corcoran et al., 1994). The process is hormonally regulated (Hsueh et al., 1994). In this way, some substances are considered survival factors, among others, gonadotropins, estrogens, growth hormones and growth factors, as they are able to suppress directly or indirectly ovarian follicle apoptosis. While other molecules are considered apoptotic factors, for instance, androgens, some cytokines and gonadotropin releasing hormone-like peptides (Chakraborty et al., 2017). The balance between such survival and atretogenic factors will lead to follicular survival or to follicular atresia (Hussein, 2005). This physiological phenomenon can be increased by environmental stress factors such as light and temperature regime changes, hypoxia, fasting, presence of biocidal agents, confinement, inadequate hormone levels, infections and disease (Saidapur, 1978; Nagahama, 1983).

The follicular development in teleosts is different from that of mammals and less information is available regarding the mechanism underlying the ovarian atretic process. Atresia occurs as a normal physiological process during post-ovulatory follicle resorption in the ovary (e.g. Drummond et al., 2000; Santos et al., 2005; Luckenbach et al., 2008). Nevertheless, in earlier oogenic stages it would occur as a mechanism for the selection and recruitment of a pool of follicles that will proceed into vitellogenesis (Janz and Van Der Kraak, 1997). In addition, rates of follicular atresia can increase as consequence of environmental stressors such as thermal stress, reduced food intake or presence of pollutants (e.g. Bromley et al., 2000; Janz et al., 2001; Sato et al., 2005). Indeed, ovarian atresia in previtellogenic oocytes is an important indicator of pathology, related with exposure to environmental contaminants (reviewed by Blazer, 2002). Increased oocyte atresia has been observed in fish inhabiting waters rich in endocrine disrupting chemicals (EDCs), for instance, in white croaker (*Genyonemus lineatus*) (Cross and Hose, 1988) and in English sole (*Parophrys vetulus*) (Johnson et al., 1988). Induction of ovarian atresia has been

reported after laboratory exposure to EDCs in zebrafish (*Danio rerio*) (Van Den Belt et al., 2002), fathead minnow (*Pimephales promelas)* (Jensen et al., 2004) and common carp (*Cyprinus carpio*) (Altun et al., 2017).

Unlike in mammals, in teleosts the mechanisms of follicular atresia are not yet well understood and whether the process occurs by any of the three known types of programmed cell death, apoptosis, autophagocytosis or necrosis, is still unknown. Some studies have suggested that apoptosis is the main effector of ovarian atresia in postspawning teleost ovaries (Janz and Van Der Kraak, 1997; Drummond et al., 2000; Wood and Van Der Kraak, 2001; Santos et al., 2005; Thomé et al., 2006; Santos et al., 2008). In addition, apoptosis has been suggested to be the process by which the undifferentiated ovary-like zebrafish (*Danio rerio*) gonad is transformed into testis (Uchida et al., 2002). Related with that, the transcription pattern of tumor suppressor protein *p53* has been suggested to be involved in the germ cell apoptotic process that leads to testis development (Rodriguez-Marí et al., 2010). Moreover, the importance of *p53* and the related apoptotic pathways on the regulation of ovarian development have been recently described in the spotted knifejaw (*Oplegnathus punctatusthe*) (Du et al., 2017). In a study about the consequences of prolonged fasting, atresia in coho salmon (*Oncorhynchus kisutch*) was suggested to be mediated by apoptotic events (Yamamoto et al., 2011). The increase on the number of atretic follicles was accompanied by the elevation of the expression levels of genes associated with apoptotic pathways, such as *p53* and *caspase3* (Yamamoto et al., 2011). Nevertheless, the simultaneous activation of apoptosis and autophagocytosis has been observed during ovarian regression in some characiform species, by the detection of morphological features of both processes and the detection of coordinated activation of apoptotic, Caspase3 and autophagic Beclin1, proteins (Thomé et al., 2009; Morais et al., 2012). The elevated expression of both *p53* and *beclin1* genes after the induction of atresia has also been reported in the Japanese anchovy (*Engraulis japonicus*), showing that both pathways can be activated simultaneously (Chakraborty et al., 2017). This kind of interaction between different kinds of cell death mechanisms have been reported in mammalian cells, as molecules that participate in apoptosis can also mediate autophagy (reviewed in Lockshin and Zakeri, 2004; Gump and Thorburn, 2011). In addition, the pro-autophagic activity of beclin1 can be eliminated by cleavage by caspases, especially by caspase3 (Djavaheri-Mergny et al., 2010; Luo and Rubinsztein, 2010) and the contrary can also occur, as the depletion of beclin1 can lead to caspase-dependent apoptosis (Maiuri et al., 2007; Kang et al., 2011).

Regarding the hormonal regulation of atresia, similarities have been found between mammals and teleosts (reviewed by Habibi and Andreu-Vieyra, 2007). Wood and Van Der Kraak (2002) studied the effects of gonadotropins, 17β-estradiol and epidermal growth factor in vitellogenic trout ovarian follicles and concluded that, as in mammals, such factors can regulate the process, suggesting that pituitary gonadotropins (follicle stimulating hormone and luteinizing hormone) and estrogen have a role as potent follicle survival factors (Wood and Van Der Kraak, 2002). As described in mammals, gonadotropin releasing hormone, GnRH has shown to be able of promoting apoptosis in fish (Andreu-Vieyra and Habibi, 2000).

In previous studies with *Chelon labrosus* thicklip grey mullets from the Basque coast, the attention has been focused in the xenoestrogenic effects, especially prevalence of intersex males as a consequence of exposure to EDCs (Ortiz-Zarragoitia et al., 2014). Less attention has been payed to the possible xenoestrogenic effects on female mullets. In this work, we have focused in female mullets from the polluted area of Gernika with high prevalence of intersex condition. We have tried to elucidate how EDC pollution can trigger ovarian histopathological lesions such as atresia and the molecular mechanism underlying such effect.

2. Material and Methods

2.1. Sampling site and sample collection

The area surrounding the Wastewater Treatment Plant (WWTP) of Gernika (43°19'01"N, 2°40'36"W) which is located in the Oka river was selected for fish samplings. This WWTP receives urban waters from the town of Gernika and nearby settlements (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 with a first and a secondary biological treatment. Previous studies have shown xenoestrogenic effects in the local mullet population (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). Fish studied hereby were collected in February 2014 and at the annual sampling campaign of the Biscay Bay Biospecimen Bank in June 2013, 2014, 2015 and 2016. Mullets from the inner part of Pasaia (43°19’18’’ N; 1°55’53’’ W) harbor captured between September 2010 and September 2011, and between October 2016 and January 2017 were also analyzed. The sampling procedures were equal in both sites. Adult (>20 cm) thicklip grey mullets (*Chelon labrosus*) were captured by fishing-rod. Fish were anaesthetized upon capture in a saturated ethyl-4-aminobenzoatewater bath. All fish handling procedures were approved

by the UPV/EHU Ethical Committee for Experimental Animals and by the competent regional authorities. Dissections were performed immediately. A portion of the gonad of each fish was collected in RNAlater solution (Ambion; Life Technologies, Carlsbad, USA) and then frozen in liquid nitrogen until arrival at the laboratory where it was immediately stored at -80°C until processing. Another portion of each gonad was fixed in 4% neutral buffered formalin for histological analysis.

2.2. Histological analysis of the gonad

After fixing gonad samples for 24 hours in neutral buffered formalin, they were dehydrated in a graded ethanol series (70%, 90% and 96%), and then embedded in paraffin. Sections of 5 μm thickness were cut in a Leica RM 2125 RT manual microtome (Leica Microsystems, Nussloch, Germany) and stained with hematoxylin/eosin (Gamble and Wilson, 2002) using the Leica Autostainer XL and mounted with the aid of the Leica CV 5030 workstation. Three sections per individual from Pasaia and from June 2013 and February 2014 Gernika samplings were analyzed with an Olympus BX61 light microscope (Tokyo, Japan). The other samples from Gernika were microscopically analyzed using a slide scanner (Hamamatsu Photonics, France). Sex and gametogenic stage of each individual was determined using the criteria by McDonough et al. (2005). When intersex males were identified, the Intersex Severity Index described by Jobling et al. (2006) was applied.

For histological analysis and complementary downstream molecular analyses, females from Gernika that showed ovaries at previtellogenesis were selected. This was the predominant stage during June. Females were divided in two groups, those displaying atresia and those showing healthy oocytes. Intersex males from Gernika were also chosen. In Pasaia all previtellogenic females were chosen for the study and no signs of atresia were identified microscopically. Mullet ovaries were classified as atretic when high amounts of darkly stained oocytes were observed, with shrinked and deformed shape and loss of normal appearance.

2.3. In situ terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay

The selected females and intersex gonad samples from Gernika were used to perform the TUNEL assay. Paraffin sections of 3 μm thickness were placed on adhesion microscope slides (SuperFrost®Plus, Menzel-Glaser, Gerhald Menzel Gmbh, Braunschweig, Germany). TUNEL assay for the staining of apoptotic nuclei was performed using the DeadEnd™ Colorimetric TUNEL System Kit (Promega, Madison, USA) following the manufacturer's instructions. The principle of the reaction is based on the incorporation of a biotinylated nucleotide into the 3'-OH DNA ends in the cell. The reaction is performed by the

recombinant Terminal Deoxynucleotidyl Transferase (rTdT) enzyme. Peroxidase bound streptavidin is then added, which is able to bind to the biotinylated nucleotides. Peroxidase substrate hydrogen peroxide is used to precipitate the diaminobenzidine chromogen and provide the labeling. The assay was done in replicates, with one section of each sample used as negative control, with no rTdT enzyme added to the reaction.

2.4. Gene transcription analysis

2.4.1. Extraction of total RNA

Non-atretic previtellogenic females from Gernika (n=8) and Pasaia (n=11), atretic previtellogenic females from Gernika (n=5) and intersex males from Gernika (n=4) were selected for the transcription analysis of target genes in the gonads.

Total RNA was extracted from gonad (around 100 mg) samples using TRI Reagent Solution (Ambion; Life Technologies). The tissues were homogenized in 1 mL TRI Reagent Solution using zirconia/silica beads (Biospec, Bartlesville, USA) in a Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France). RNA concentration and quality were measured by spectrophotometry in a biophotometer (Eppendorf, Hamburg, Germany), all samples resulting in A260/A280 absorbance ratios between 1.8 and 2.2, were considered adequate for further analysis.

2.4.2. Capillary electrophoresis of gonad total RNA

RNA integrity and ribosomal rRNA concentration in gonads was determined in Agilent RNA 6000 Nano Kits (Agilent Technologies, Santa Clara, California, USA) using a Bioanalyzer 2100 (Agilent Technologies). Concentrations of 5S rRNA and 18S rRNA were obtained using the electropherograms provided for each sample and measuring the Time Corrected Area of the 5S and 18S rRNA peaks. Then, the ratio between both concentrations and its logarithm was calculated in order to obtain the 5S/18S RNA index per sample (Rojo-Bartolomé et al., 2016).

2.4.3. cDNA synthesis and quantification

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

2.4.4. Real-time qPCR

Specific primers were designed using the partial sequence available in GenBank for the genes *caspase3, p53, mdm2*, *beclin1, fshr* and the partial sequence obtained by Miseq Illumina RNASeq analysis of mullet gonads (unpublished data) for *rpl5* (Table 1). Primers were designed using IDT and Eurofins online tools. Real time qPCR analyses were performed in a 7300 Real-Time PCR system thermocycler (Life Technologies). Each sample was analyzed in triplicates in a total volume of 20 µL containing 7,88 µL of water, 10 µL of SYBR Green fluorescent dye master mix (Roche Diagnostics, Indianapolis, USA) and 0,12 µL 12,5 pmol primer pair. A control without template was run (also in triplicate) in each plate using the same reaction conditions. The qPCR conditions were as follows: an initial step at 50 °C for 2 minutes and 95 °C for 10 minutes, 40 cycles of a denaturing step at 95 °C for 15 seconds and annealing step at Tm (Table 1) for 1 minutes; finally a dissociation stage of 95 °C for 15 seconds, 60 °C for 1 minutes and again 95 °C for 15 seconds. The reaction efficiency of each plate was calculated using a standard curve consisting in serial dilutions of pooled cDNA. The specificity of the reaction was determined confirming the presence of a single peak in the dissociation curve, and observing that no primer dimmers were formed. cDNA concentration obtained by fluorescent quantification method was used for normalization in the gonad, as performed by Rojo-Bartolomé et al. (2016).

Table 1
List of target genes with their specific forward and reverse primers, melting temperature and fragment size. GeneBank accession number is provided.

Gene	Forward primer (5'-3') Reverse primer (5'-3')	Tm (ºC)	Fragment size (bp)	Accession number
p53	TCTTCAGAGTGGAGGGCAC CAGCAGGATGGTCGTCATTTC	59	136	DQ146943
mdm2	TCGACAGCTTCTCTGTTAAGAG CTGGCTGCTGCTTTGTGTTTG	59	127	KX758587
rpl5	ACTGACTACTTTGCTCGCAAGC TGTCACCCTCAATCTTGGCATAG	60	150	MK331988
caspase3	TCGTGGAACTGAAGTAC CTGTCCGTTTCAATCCCTGGA	60	130	JF732773
fshr	CCTTGCTCATCTTCACCGAC CAGGACCAGGAGGACTTTAG	60	116	MH251323
beclin1	CACAGAGGAGCTACAGTACCA GTACCAAACTGTCCGCTGTG	60	176	KY771085

2.5. Statistical analysis

Statistical analyses were performed with the aid of the SPSS.22 statistical package (SPSS Inc., Microsoft Co., Redmond, USA). Normality was assessed with Shapiro-Wilk test. Non-parametric Kruskal-Wallis test followed by Dunn's post hoc test was used for multiple comparisons. Significant differences were established at $p < 0,05$.

3. Results

3.1. Histological analysis of the gonads

Previtellogenic females were selected and classified as atretic or non-atretic. The prevalence of females showing atretic ovaries ranged from all females being non-atretic in June 2013 and February 2014, to a maximum of 100% showing signs of atresia in June 2018. In the other samplings, 83% of females showed atresia in June 2015 and 2016 and 20% and 23% in June 2017 and 2018, respectively. Females classified as atretic (Figure 1, C and D) showed the presence of a high amount of darkly stained oocytes within the ovary. Many of the oocytes present in the ovary had lost their structure and showed deformed shape with irregular surrounding follicular cell layer. Shrinkage of some of the oocytes was also observed. In some cases, a clear separation between the atretic oocytes and the surrounding connective tissue could be noticed. From all observed atretic females, five were selected for molecular and histochemical analysis, two from June 2014 and three from June 2016. Eight non-atretic females were selected for this study; two from June 2013, two from February 2014, one from June 2014 and three from June 2015. All previtellogenic females from Pasaia were classified as non-atretic.

Intersex males were also identified in the Gernika in a range from no males being intersex in the sampling from June 2018, up to 83% of males being intersex in February 2014 (see Chapter 4 for more information) Some of them showed high intersex severity index, with high amount of oocytes scattered through the testicular tissue. Four of them were selected for the present study; one from June 2015 and three from June 2016 with severity index 3, according to the classification by Jobling et al. (2006) (Figure 1E).

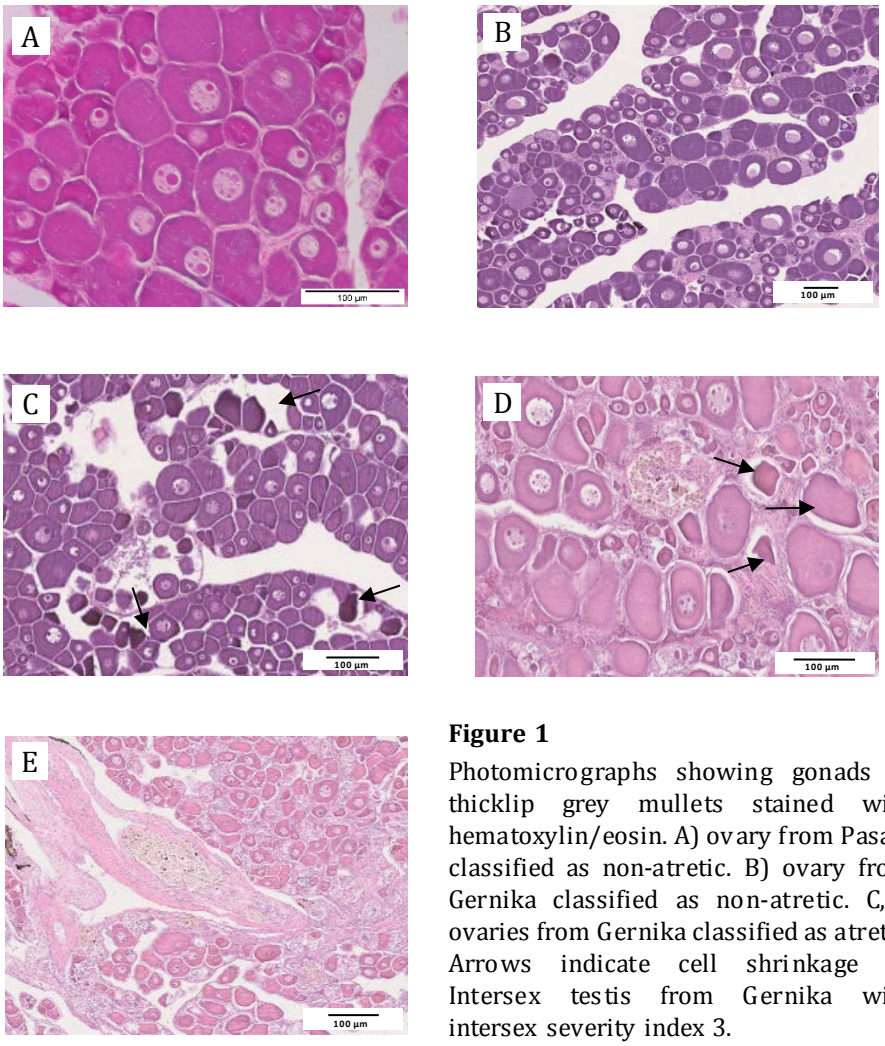

Figure 1

Photomicrographs showing gonads of thicklip grey mullets stained with hematoxylin/eosin. A) ovary from Pasaia classified as non-atretic. B) ovary from Gernika classified as non-atretic. C,D) ovaries from Gernika classified as atretic. Arrows indicate cell shrinkage E) Intersex testis from Gernika with intersex severity index 3.

3.2. In situ terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay

The TUNEL histochemical staining was performed in order to identify apoptotic cells in the ovaries histologically classified as atretic and non-atretic and in intersex testis samples from Gernika. No clear signs of apoptosis were detected (Figure 2) according to TUNEL. No clear Positive staining of the nuclei could not be detected. Non-atretic and atretic samples showed similar background immunolabelling (Figure 2, A2 and B2).

3.3. 5S/18S rRNA index

All studied ovaries at previtellogenic stage showed similar 5S/18S ribosomal RNA index levels, independent of the presence atresia (Figure 3). Intersex testes showed values lower than females but not significantly lower than those of non-atretic females from Gernika.

3.4. Transcription levels of target genes in the gonads

Transcription levels of genes related with atresia, were measured in gonads of studied fish (Figure 4). Overall, *p53*, *mdm2*, *rpl5*, *caspase3*, *fshr* and *beclin1* showed the same transcription profile, with ovaries from Pasaia showing lower transcription levels than ovaries (atretic or non-atretic) or intersex testes from Gernika. Only in the case of *p53* were these differences non-significant. In the same way, for *fshr*, ovaries of mullets from Pasaia did not differ from non-atretic ovaries from Gernika and in the case of *rpl5*, Pasaia ovaries were not different to intersex testes. Differences between atretic and non-atretic ovaries of females from Gernika were not detected. Intersex gonads showed higher *fshr* transcription levels than non-atretic ovaries and similar to atretic ovaries.

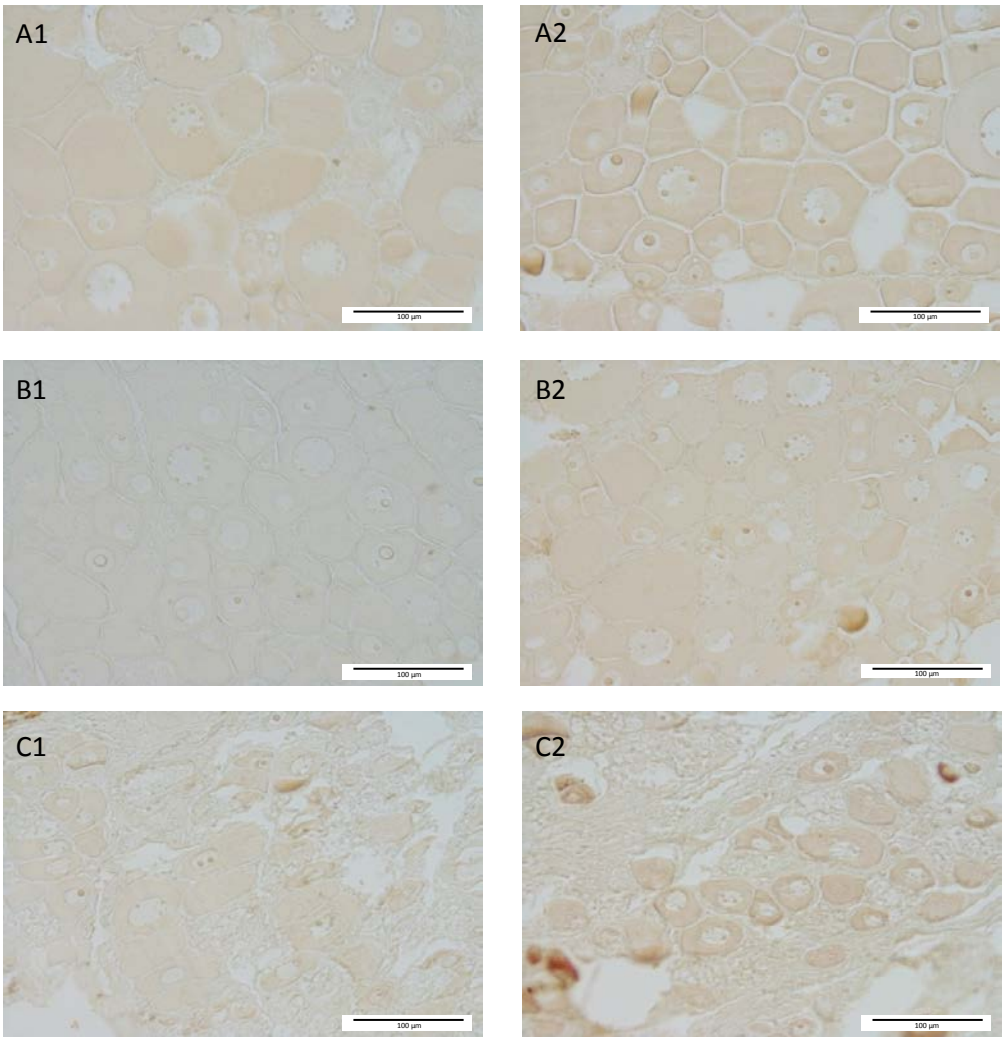

Figure 2
Representative TUNEL-immunostained histological sections of mullet gonads from Gernika. A) non-atretic ovary. Negative control (A1) and positive reaction (A2). B) atretic ovary. Negative control (B1) and positive reaction (B2). C) testis section of an intersex male showing a high intersex severity index. Negative control (C1) and positive reaction (C2).

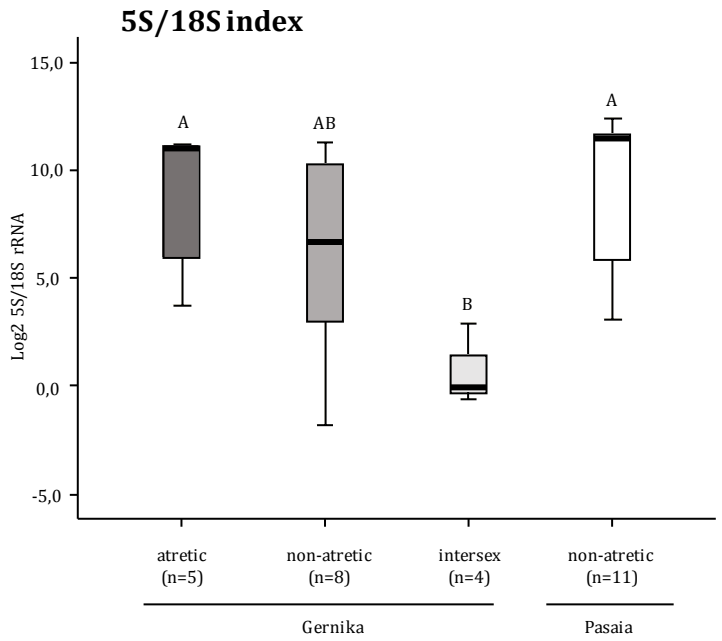

Figure 3
5S/18S rRNA index in the gonads of thicklip grey mullets. Atretic females (dark grey), non-atretic females (grey) and intersex (soft grey) from Gernika and non-atretic females (white) from Pasaia. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Upper case letters denote statistical differences between means after Kruskal-Wallis test followed by Dunn's post hoc test ($p \leq 0,05$).

4. Discussion

In this study, the histopathological analysis of female *Chelon labrosus* thicklip grey mullet ovaries from Gernika and Pasaia was performed. High incidence of ovarian atresia was observed in previtellogenic ovaries from mullets from Gernika. For a better understanding of the ovarian atretic process, immunohistochemical and gene transcription analyses concentrated in apoptosis and autophacytosis related genes were performed comparing atretic and non-atretic ovaries from Gernika, intersex testes from Gernika and non-atretic ovaries from Pasaia.

Ovaries classified as atretic showed a high amount of strongly basophilic oocytes, with high incidence of oocyte shrinkage. Oocyte follicles in many circumstances showed no connection with their neighboring follicles and the surrounding connective tissue, showing structural disorganization (Miranda et al., 1999; Thomé et al., 2010). In fish, the atretic process usually occurs at the end of the reproductive cycle, in preparation for the resorption of the non-spawned oocytes. Thus, when ovarian atresia occurs at post-ovulatory stage it is

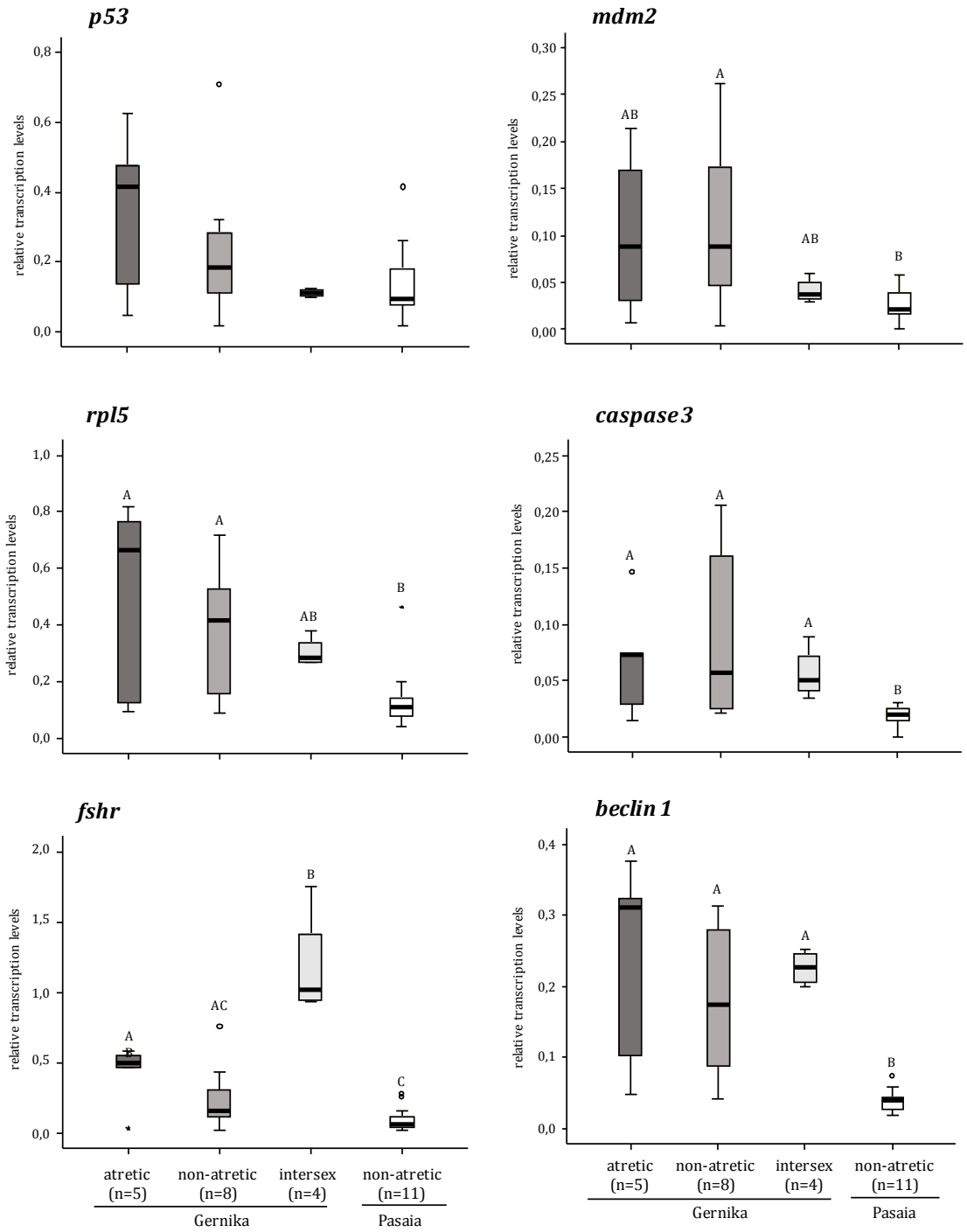

Figure 4
Transcription levels of target genes (*p53*, *mdm2*, *rpl5*, *caspase3*, *fshr* and *beclin1*) in gonads of thicklip grey mullets. Atretic females (dark grey), non-atretic females (grey) and intersex (soft grey) from Gernika and non-atretic females (white) from Pasaia. Box-plots show median value (line), 25%-75% quartiles (box) and standard deviation (whiskers). Circles show outlier values. Upper case letters denote statistical differences between means after Kruskal-Wallis test followed by Dunn's post hoc test ($p \leq 0,05$).

considered a physiological process for the recovery of the energy devoted in oocyte production and growth (reviwed by McBride et al., 2015). When the process occurs at previtellogenic stage, it is considered a pathological phenomenon or a phenomenon linked to adverse environmental conditions (Guraya, 1986). Nevertheless, selection of ovarian follicles has been suggested to be important in the recruitment of the best oocytes that will enter vitellogenesis (Janz and Van Der Kraak, 1997). In any case, the high incidence of atresia observed in the present study in previtellogenic ovaries from Gernika may indicate the incidence of external stress factors. Atresia was reported in English sole (*Parophys vetulus*) and white croaker (*Genyonemus lineatus*) from contaminated areas (Johnson et al., 1988; Cross and Hose, 1998). High prevalence of ovarian atresia was observed also by Jobling et al. (2002) in wild roach (*Rutilus rutilus*) from contaminated estuaries compared with roach from the reference estuaries. Baldigo et al. (2006) found high rates of ovarian atresia in largemouth bass (*Micropterus salmoides*) from a contaminated river, although without correlation with contaminant concentrations. In addition, they reported differences between fish species regarding atresia incidence, showing different interspecies susceptibility. In laboratory conditions, ovarian atresia has been observed in females from several species exposed to endocrine disrupting chemicals (EDCs). Medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) or sheepshead minnow (*Cyprinodon variegatus*) exposed to EDCs such as 17α-ethynylestradiol, nonlyphenol or the antiandrogen flutamine showed ovarian atresia (Zillioux et al., 2001; Van Den Belt et al., 2002; Weber et al., 2002; Jensen et al., 2004). The sampling point from Gernika receives discharge waters from the local wastewater treatment plant. In previous studies, high concentrations of EDCs were measured in mullet bile (Puy-Azurmendi et al., 2013; Bizarro et al., 2014). Males showing intersex testes as a consequence of xenoestrogenic exposure has also been reported (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Valencia et al., 2017). In addition, the classical xenoestrogen exposure biomarker vitellogenin was analyzed at the transcriptional level, showing hepatic transcription in males (Bizarro et al., 2014; Valencia et al., 2017). In addition to effects on males, responses in females from the same population should also be expected. Thereby, the high incidence of atresia in mullet ovaries recorded in the last samplings in Gernika could be indicative of exposure to EDCs.

The intersex males identified in Gernika during the 2014-2016 samplings, displayed high intersex severity indexes. This severity values were higher than the previously determined in Gernika and in other sampling sites within the Basque coast (Bizarro et al., 2014; Sardi et al., 2015; Rojo-Bartolomé et al., 2017; Valencia et al., 2017). All oocytes present in the intersex testes were in previtellogenic stage. In some of the intersex mullets, very slight signs of oocyte atresia were observed. Oocyte atresia has also been reported in other

intersex male fish such as European flounders (*Platichthys flesus*) from contaminated estuaries in the United Kingdom (Bateman et al., 2004). Authors suggested that intersex oocytes would develop as in normal ovaries and in the absence of spawning they would undergo atresia and resorption.

The TUNEL assay was used for the detection of DNA fragmentation in the cells and to identify whether the process of atresia occurs though apoptosis, and whether this occurs in oocytes themselves or/and in surrounding follicular cells. No positive staining was identified strongly suggesting that apoptosis is not the main process involved in thikclip grey mullet ovaries or intersex testes. Weber and Janz (2001), using TUNEL evaluation of histological channel catfish (*Ictalurus punctatus*) ovary also reported non-conclusive results. TUNEL positive staining, and apoptosis related atresia, has been identified instead in European hake (*Merluccius merluccius*) ovary, with both positively stained apoptotic nuclei in previtellogenesis oocytes and in follicular cells surrounding previtellogenic and vitellogenic oocytes (results not published). In studies done with postovulatory characiform fish induced to spawn, ovarian atresia was induced and suggested to be mediated by apoptosis, which was confirmed by TUNEL positive staining (Santos et al., 2005; Thomé et al., 2006; Santos et al., 2008). Ovarian atresia induced by exposure of fish to substances known to induce atresia such as EDCs, also resulted in TUNEL positive staining of follicular cells, as described in the medaka exposed to quercetin (Weber et al., 2002) or the cyprinid *Chalcanurnus tarichi* exposed to 17α-ethinylestradiol (Kaptaner and Unal 2011). In the first case, apoptosis was described as the pathway that is activated in the early stages of atresia in medaka (Weber et al., 2002). Zebrafish exposed to fadrozole showed TUNEL positive staining in the oocytes (Uchida et al., 2004). After exposure of goldfish to gonadotropin releasing hormone (GnRH), TUNEL positive staining was observed and thus, apoptosis was described as the leading molecular mechanism of GnRH mediated follicular atresia (Andreu-Vieyra et al., 2005). Hence, in several studies, apoptosis has been described as the mechanism underlying ovarian atresia.

The 5S/18S ribosomal RNA index was calculated for all groups. 5S rRNA is a valuable marker of oocyte presence (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2017) and the 5S/18S rRNA index changes along oogenesis. Previtellogenic oocytes do mainly produce 5S rRNA while vitellogenesis occurs together with an activation of 18S and 28S rRNA production (Rojo-Bartolomé et al., 2016; 2017). In the present study mullets display the 5S rRNA transcription levels common at early previtellogenesis (Rojo-Bartolomé et al., 2017), both in atretic and non-atretic ovaries. Intersex males also showed high values although

lower than in previtellogenic ovaries common to testes with intersex severity index 3 (Rojo-Bartolomé et al., 2017).

Genes related to the process of apoptosis and autophagocytosis were studied at the transcriptional level to decipher possible molecular mechanism related to the observed follicular atresia response. The same transcriptional trend was observed for all genes in the studied groups. Transcription levels were higher in ovaries of mullets from Gernika than in ovaries from Pasaia, independent of their classification as atretic or non-atretic. Intersex testes generally showed transcription levels similar to those in ovaries from Gernika, with the exception of *fshr*, that showed transcription levels higher in intersex testes than in any group of ovaries. No differences in transcription levels were observed between atretic and non-atretic females from Gernika for any of the measured genes. The results thus do not enable to establish a direct link related either to apoptosis or to autophagocytosis in the triggering of atresia in mullet ovaries from Gernika. In some fish such as rainbow trout (*Oncorhynchus mykiss*) and sole (*Solea senegalensis*), a constant transcription level of apoptotic-related genes has been observed, with no differences between atretic ovaries and ovaries at other stages (Von Schalburg et al., 2005; Cerdà et al., 2008; Tingau-Sequeira et al., 2009). This could suggest that the lack of difference between atretic and non-atretic ovaries may be due to the higher influence of the ovarian stage than of the atresia itself. However, it calls the attention on the upregulation of important genes in ovaries from Gernika in comparison to Pasaia, where no histological signs of follicle atresia were recorded. Measured marker genes would inform of exposure to apoptosis and autophagocytosis triggering chemicals in females inhabiting the polluted waters of Gernika, that eventually result in atresia in some individuals.

The tumor suppressor gene *p53* can mediate apoptosis in several tissues including the gonads. Its importance during the sex differentiation process from the bipotential gonad to the differentiated testis in zebrafish has been described (Rodriguez-Marí et al., 2010), related with its role in apoptosis (Uchida et al., 2002). In addition, it has also been related with ovarian germ cell proliferation, integrity maintenance and fertility in the spotted knifejaw (*Oplegnathus punctatus*) (Du et al., 2017). Under physiological or external stress the tumor suppressor gene *p53*, can be activated to trigger cellular responses such as apoptosis and autophagocytosis (Haupt et al., 2003; Gump and Thorburn, 2011). The presence of EDCs in Gernika (Puy-Azurmendi et al., 2013; Bizarro et al., 2014; Ros et al., 2015) could be linked to the recorded high ovarian *p53* transcription levels. Upregulation of *p53* transcription levels have been observed after exposure of zebrafish larvae and embryos to the EDCs acetochlor and petrilachlor respectively, showing the potential of such

substances as apoptosis inducers (Jiang et al., 2015; 2016). In the latter, upregulation of *p53* was accompanied with the upregulation of *mdm2* transcription levels (Jiang et al., 2016). P53 is under the regulation of Mdm2, which is considered an anti-apoptotic gene as under normal conditions binds to P53 for its ubiquitination and proteolytic degradation (Haupt et al., 2003; Bose and Gosh, 2007). In this work, only the levels of gene transcription have been measured, which does not allow to understand the effects at protein level or the post-translational regulation of P53, which involves Mdm2 in many cases (Harris and Levine, 2005). Interaction of P53 and Mdm2 with other molecules have also been described. The 5S ribosomal nucleoprotein (RNP) complex (Chakraborty et al., 2011; Sloan et al., 2013), formed by the 5S ribosomal rRNA and the ribosomal proteins RPL5 and RPL11, can bind Mdm2 under certain conditions, leading to the release and an activation of P53. The interaction between RNP complexes increases when ribosomal biogenesis is blocked (Sloan et al., 2013). It is known that in teleost oocytes the production of 5S rRNA is very high, to the point in which an electrophoresis of total RNA extracted from ovaries, specially during previtellogenesis, reveals 5S rRNA as the predominant band well above the typical 18S and 28S rRNA bands observed in any other eukaryotic cell (Diaz de Cerio et al.,2012; Rojo-Bartolomé et al., 2016; 2017). *rpl5* transcription levels were higher in ovaries from Gernika than in ovaries from Pasaia, suggesting a possible unbalance in the production of 5S RNP that if unable to incorporate into the nucleus, could bind to Mdm2 leading to high P53 activity.

Caspases are activated by P53, initiating a proteolytic cascade within the cells (Thornberry and Lazebnik, 1998). First, initiator caspases are activated which subsequently activate effector caspases, with Caspase3 as the main effector caspase (dos Santos et al., 2008). Some studies have described the presence of Caspase3 as a marker of apoptosis. In this way, in the ovarian atresia occurring after induction of spawning in the curimata-pacu (*Prochilodus argenteus*), Santos et al. (2008) observed Caspase3 immunostaining in the apoptotic ovaries. Similarly, Morais et al. (2012) detected Caspase3 immunostaining in regressing ovaries from the same species and other related characiforms, mainly at the late stages of atresia, confirming also in that case the execution of apoptosis. Results obtained in Gernika ovaries would suggest a *caspase3* activation that would enhance apoptosis even when histochemically no apoptotic nuclei could be identified. In female coho salmon (*Oncorhynchus kisutch*) exposed to prolonged fasting, ovarian *caspase3* transcription levels were elevated prior to any evidence of atresia (Yamamoto et al., 2011), which suggested the function of *caspase3* as early indicator of apoptosis. No differences in *caspase3* transcription levels were observed between ovaries and testes from Gernika. Higher transcription levels would have been expected in intersex, as in other species, higher Caspase3 activity or higher

mRNA and protein levels have been described in relation with testicular regression in natural sex reversal processes. It is the case for instance of gilthead seabream (*Sparus aurata*) and clownfish (*Amphiprion melanopus*) (Soverchia et al., 2007; Kim et al., 2013).

Gonadotropins are among the molecules described to have anti-apoptotic functions (Hsueh et al., 1996; Janz and Van Der Kraak, 1997; Wood and Van Der Kraak, 2002). In vitro culture of coho salmon follicles with follicle stimulating hormone, FSH, induced the transcription levels of anti-apoptotic genes, suggesting that FSH can protect ovaries from undergoing apoptosis (Luckenbach et al., 2011). In addition, exposure to endocrine disrupting chemicals can result in the downregulation of ovarian gonadotropin transcription, as described in common carp (*Cyprinus carpio*) exposed to chlorpyrifos (Altun et al., 2017). In the present study transcription levels of the FSH receptor *fshr* were measured. Atretic females from Gernika showed higher although non-significant transcription levels than non-atretic females and higher than those from Pasaia. These results are in contrast to what could be expected. In a normal situation, non-atretic females should be the ones showing higher transcription levels of the gonadotropin receptor and thus a higher steroid hormone synthesis ability that in turn would act as cell survival factor by inhibiting apoptosis. In this regard, in the normal atretic process of regressing ovaries in fathead minnows for instance, *fshr* transcription levels were reported to be decreased in comparison to other stages of oocyte development (Villeneuve et al., 2010). The same was observed in the atretic ovaries of largemouth bass (*Micropterus salmoides*) (Martyniuk et al., 2013). Also in atresia induced after fasting in coho salmon a declining trend in the transcription levels of *fshr* was observed (Yamamoto et al., 2011). This transcription pattern suggests that low transcription levels observed in non-atretic ovaries from Gernika are representative of the transcription levels that correspond to previtellogenic ovaries from thickip grey mullets. The slightly higher transcription levels observed in atretic ovaries could be related with the atretic process or with other activated pathways. In a study with white sucker (*Catostomus commersoni*) females exposed to paper mill effluent (Janz et al., 1997), increased transcription levels of antiapoptotic HSP70, heat shock protein 70, were observed in fish with high ovarian apoptosis levels. Nevertheless, it is likely that the elevated HSP70 levels were related to a general response to environmental stress. In addition, intersex males showed the highest *fshr* transcription levels. This result is surprising, as intersex testes from mullets collected in Pasaia showed the lowest *fshr* transcription levels, when compared to ovaries and normal testes (Chapter 2). The high transcription levels of *fshr* could be related to their high intersex severity index, as in Pasaia intersex index was normally 1 (Chapter 2). Furthermore, apoptosis inhibition in the testis has been described to be important for the homeostasis of spermatogenesis (Nadzialek et al., 2010). Higher transcription levels of the anti-apoptotic

bcl-2 compared with *caspase3* were measured in gudgeon (*Gobio gobio*) exposed to EDCs that had developed intersex gonads (Nadzialek et al., 2010). Thus, the observed transcription pattern in intersex from this study could be also related to the endeavor of the testicular tissue to maintain its condition.

Autophagy is a highly regulated catabolic process by which damaged or aged organelles are degraded (Debnath et al., 2005; Maiuri et al., 2007). It is an alternative pathway for cell death and is characterized by autophagosome synthesis and no nuclear affection (Maiuri et al., 2010; Liu et al., 2016). Under stressful situations autophagy can be enhanced (Maiuri et al., 2007). In this study, transcription levels of *beclin1*, a gene related with autophagy (Yoshimori, 2004; Kang et al., 2011) were similar to the other measured genes. Only intersex showed slightly higher transcription levels of *beclin1* compared with apoptotic genes. That both apoptosis and autophagy could occur at the same time should be considered as interactions between the apoptotic and autophagic pathway have been described. Interactions between Beclin1 and P53 or Caspase3, have been previously described in mammal cells (Gump and Thornburn, 2011; Kang et al., 2011), together with switching role of *beclin1* from autophagy to apoptosis (Maiuri et al., 2010; Gump and Thornburn, 2011; Kang et al., 2011). Caspase dependent cleavage of beclin1 can inhibit its autophagic activity and enhance apoptosis (Djavaheri-Mergny et al., 2010; Luo and Rubinsztein, 2010). This cannot be discarded in females from Gernika, where the transcription levels of *beclin1* and *caspase3* were in the same range. Both processes have been described to act in a coordinate manner during the process of ovarian atresia in some fish species (Santos et al., 2008b; Thomé et al., 2009; Morais et al., 2012; 2016; Chakraborty et al., 2017). In some characiform species, immunostaining showed double labeling of Caspase3 and Beclin1 during the late stages of follicular atresia occurring during ovarian regression (Morais et al., 2012), showing the coordination between both apoptotic and autophagic processes. After induction of ovarian atresia in Japanese anchovy (*Engraulis japonicus*), elevated expression of both *p53* and *beclin1* was observed. On the other hand, Gioacchini et al. (2013) showed that after probiotic feeding of zebrafish, although an interaction between both processes was likely to be happening, when upregulation of the autophagic *beclin1* was occurring, decrease of apoptotic genes *p53* and *caspase3* occurred. Such transcription pattern would be more similar to the transcription levels of *beclin1* and *caspase3* observed in intersex mullets, which showed more elevated transcription of *beclin1*.

In general, in studies where fish had been exposed to EDCs in the field or in laboratory conditions, apoptosis has been described as the ongoing process in ovarian atresia (e.g. Janz and Van Dr Kraak, 1997; Kaptaner and Unal, 2011; Luzio et al., 2016). Several factors are

able to suppress or induce apoptosis. In rainbow trout (*Oncorhynchus mykiss*) ovarian follicle culture, incubation with gonadotropins, epidermal growth factor and 17β-estradiol (E2) suppressed apoptotic DNA fragmentation (Janz and Van Der Kraak, 1997) acting as follicle survival factors. Instead, and in a field study, pre-spawning white sucker females (*Catostomus commersoni*) exposed to paper mill effluent with elevated E2 levels showed high apoptosis rates (Janz et al., 1997). Authors suggested that this response could have been due to the ovarian stage, as the physiological E2 levels of pre-spawning white suckers are normally low. Janz et al. (1997) observed high apoptosis rates in the ovaries of white sucker females from the same population and noted high concentrations of polycyclic aromatic hydrocarbons (PAHs). They described that apoptosis of follicular somatic cells could have been induced by effluent components that may affect steroidogenesis. They also suggested that apoptosis might have been stimulated via alterations in physiological hormone levels as a consequence of chemical exposure. Mullets from the present study showed ovaries at previtellogenesis stage, whose internal E2 levels have been described to be high (Sardi et al., 2015). Nevertheless, in previous studies in the mullet population from Gernika, suppressed transcription levels of ovarian aromatase were observed in previtellogenic females (Chapter 1 and Valencia et al., 2017). In zebrafish juveniles exposed to the aromatase inhibitor fadrozole, the observed oocyte apoptosis was thought to be directly or indirectly caused by the reduction in aromatase activity and depletion of estrogen synthesis (Uchida et al., 2004). Hormone imbalance and decrease in steroid hormone synthesis capacity as a consequence of exposure to EDCs were suggested to be involved in the induction of apoptosis in atretic ovaries of the cyprinid *Chalcarbornus tarichi* (Kaptaner and Unal, 2011).

5. Conclusion

To summarize, high ovarian atresia was observed in some female thicklip grey mullets from Gernika collected during the last 5 years. When compared with females from Pasaia, females from Gernika showed higher transcription levels of genes related with the apoptotic pathway, independently of their histological classification. These results suggest that the endocrine disruption chemical load present in Gernika is able to disrupt the normal ovarian development of mullets from that locality. Nevertheless, whether the observed ovarian atresia is being caused by the activation of apoptotic or autophagic pathways remains unclear. The studied intersex males showed similar transcription levels for all the analyzed genes except for *fhsr*. Further research is needed in order to understand if such upregulation is related with the anti-apoptotic function of the gene or with other molecular pathways.

6. References

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General Discussion

The effects of endocrine disrupting chemicals (EDCs), especially those of xenoestrogenic compounds have been extensively studied, many times by measuring the disruption of the ability to produce sex steroids and/or vitellogenin, or by measuring the transcription levels of genes related with reproduction processes. One of the most outstanding effects observed in fish exposed to xenoestrogens is the development of the intersex condition in testes, defined by the development of oocytes within the testicular tissue in gonochoristic fish (e.g. Tyler and Jobling, 2008; Bahamonde et al., 2013). Such condition has been described in several geographical locations and in many different fish species, always related with EDC contamination, the source of contaminants in many cases being the effluent of wastewater treatment plants (WWTPs) (Bahamonde et al., 2013; Abdel-Moneim et al., 2015). In the Basque coast (South East Bay of Biscay), *Chelon labrosus* thicklip grey mullets have been studied since 2007 in several estuaries and harbor areas, and intersex males have been detected in relation with exposure to xenoestrogens form industrial and agricultural origin (Diaz de Cerio et al., 2012; Puy-Azurmendi et al., 2010; 2013; Bizarro et al., 2014; Rojo-Bartolomé et al., 2017). Along the Basque coast, Gernika in the Oka estuary and the harbor of Pasaia in the Oiartzun estuary have been the most extensively studied localities. The presence of intersex mullets has been described in all the samplings performed in both locations until the year 2016. In most of the cases intersex males have shown a low to moderate affection with low intersex severity index, with a score of one. This means that at the most, five oocytes could be detected in each analyzed testis histological section, those gonads showing almost exclusively previtellogenic oocytes. In Gernika, intersex mullets with higher intersex severity index were observed in the last yearly samplings, especially since 2014, when scores of 4 and 5 in a scale of 7 were described. This means that more than 50 oocytes could be observed in the analyzed testes sections. In the samplings of the following years, until 2018, some of the identified intersex individuals have shown 50% of oocyte coverage in the gonad (intersex severity index 6). It has to be taken into consideration that the sampling site of Gernika is located in a site with low water level at low tide and where the effluent of the local wastewater treatment plant is discharged. Several xenoestrogens have been detected in such effluent, for instance, the natural estrogens 17α-estradiol (E2), estrone (E1) and estriol (E3), the synthetic estrogen 17β-ethynylestradiol (EE2), bisphenol-A (BPA), alkylphenols, and a wide range of pesticides and phthalates (Bizkarguenaga et al., 2012). In addition, the presence of such substances in the bile of mullets has also been described, confirming the bioavailability of the chemicals (Puy-Azurmendi et al., 2013; Ros et al., 2015). The mullet population from Gernika was compared with another population captured downstream the WWTP of Galindo, located in one of the tributaries of the Bilbao estuary. The xenoestrogens listed above have also been detected in

the effluent of the Galindo WWTP, but for some of them maximum concentrations are lower than the ones detected in Gernika´s WWTP effluent. This is for instance the case of alkylphenols (Bizkarguenaga et al., 2012). Although hepatic vitellogenin transcription was detected in males and intersex from both sites, which was indicative of the exposure to xenoestrogens in both populations, higher intersex prevalence was observed in Gernika (36% and 83%) than in Galindo (9%), in the two performed samplings. In addition, a more clear sexually dimorphic transcription pattern of genes related with sex differentiation, gonad formation and steroidogenesis, some of them being recently amplified, sequenced and annotated using degenerate primers as this information was not available for thicklip grey mullets, was observed in Galindo than in Gernika. This was the case of *cyp19a1a, foxl2, gtf3a* (ovaries)*, cyp11b*, *dmrt1, amh* and *sox9* (testes) in the gonads. However, in Gernika a clear sexual dimorphic transcription profile was only observed for *gtf3a, dmrt1* and *amh* in the two seasons in which samplings were performed (winter and late spring), and for *foxl2* in June and *cyp11b* in February. Surprisingly, in Gernika no *sox9* transcription was detected in none of the analyzed sexes in any of the seasons, probably due to the effect of xenoestrogen exposure. In the case of the transcription levels of *vasa*, no sexual dimorphic pattern was observed in neither of the localities and seasons. Regarding intersex individuals, they showed transcription levels more similar to those of females for most of such sexually dimorphic genes Altogether, the obtained results were considered to be indicative of a more marked impact on mullets collected downstream the WWTP from Gernika than from Galindo, with *dmrt1* and *amh* as the most promising markers of intersex condition in mullets.

The Pasaia harbor has been previously studied for the characterization of the transcription profile of genes related with sex differentiation and steroidogenesis in mullets (Diaz de Cerio et al., 2012; Bizarro et al., 2014; Sardi et al., 2015). In the monthly monitoring carried out from September 2010 to September 2011, intersex mullets were detected in all the samplings, with intersex prevalences depending on the month ranging from 5% to 56% (Bizarro, 2015). Intersex individuals displayed intersex severity scores higher than 1 only in two of the 12 samplings (Bizarro, 2015). Bile chemical analysis of mullets collected in June 2012 showed the presence of significant concentrations of some xenoestrogen chemicals such as BPA, E2, alkylphenols and pesticides, among others (Ros et al., 2015). Although in general the concentrations of such chemicals in the bile of mullets from Pasaia were quite similar to those observed in the mullets from Gernika, the mean concentrations of BPA and alkylphenols for instance, were lower (Ros et al., 2015). In the present PhD study, samplings were performed monthly from October 2016 to January 2017. The incidence of intersex mullets in Pasaia was lower than in previous monitoring campaigns. It

was also lower than in Gernika, as no intersex individuals were detected in October 2016 and January 2017 while the prevalences in the other months in which intersex individuals were identified ranged from 10% to a maximum of 20%. All except one of the observed intersex mullets showed an intersex severity index of 1. This data, compared with the results obtained from the samplings performed in Gernika since 2014, are indicative of a lower impact on the mullet population from Pasaia and a trend towards the improvement of the reproductive health status of the population. In contrast, the situation is becoming worse in Gernika with an increase in the intersex severity index, showing severities up to 6, in the last years. This situation is bound to change in the following years when the sewages of Gernika and the upstream towns and villages will be connected to the new WWTP constructed in Bermeo, which will discharge offshore instead of in the estuary.

In addition, we have identified that the EDC load coming from the WWTP effluent in Gernika is causing effects also in female mullets, as the histological analysis of the gonads of mullets collected since June 2014 have shown a high incidence of ovarian atresia. Such observations were not evident in previous monitorings in Gernika and have not been observed in any female from Pasaia. This histophatological effect was observed in previtellogenic ovaries, atresia occurring at early oogenic stages having been described to be related with exposure to environmental contaminants (Blazer, 2002). When comparing the gonad transcription levels of genes related directly or indirectly with apoptosis (*p53, mdm2, rpl5, caspase3* and *fshr*), and autophagocytosis (*beclin1* and *p53*) in order to understand if ovarian atresia in mullets is driven by one or both of the processes, differences were observed between the transcription levels in females from Gernika and Pasaia. Ovaries histologically classified as atretic, but also those classified as non-atretic in mullets from Gernika showed higher transcription levels than ovaries from Pasaia for all the measured genes, indicative of the activation of both the apoptotic and the autophagocytic pathways in Gernika.

Pasaia was chosen in this study to characterize the transcription pattern of genes involved in reproduction signaling in the brain-pituitary-gonad axis, during the gametogenic cycle of female and male mullets, previous to their analysis in intersex mullets. Most of those genes were previously amplified, sequenced and annotated using degenerate primers as this information was not available for thicklip grey mullets. The transcription levels of *kiss2* (kisspeptin), the *gpr54* (kisspeptin receptor), *gnrh1* (gonadotropin releasing hormone) were measured in the brain. The pattern observed was that females at cortical alveoli stage showed higher transcription than females at other oogenic stages, which could be related with the increase in the transcription of the genes coding for both gonadotropin subunits *fshβ* and *lhβ* observed in the pituitary from previtellogenesis to vitellogenesis. This would

show a functional interaction between the kisspetin system and gonadotropins in female thicklip grey mullets from Pasaia. A similar transcription pattern of the brain genes has been described at the cortical alveoli stage in chub mackerel (*Scomber japonicus*) for instance, and suggested to be important in the steps preceding vitellogenesis (Selvaraj et al., 2010). In male mullets instead, transcription levels in the brain of regressing males were higher than in the other stages of spermatogenesis for the three genes. This was also observed but only in the case of *gpr54* and *gnrh1* in mid spermatogenic males. No clear relationship between the transcription levels of brain target genes and the gonadotropin subunits in the pituitary was observed in males. In that sense, no spermatogenic stage dependent transcriptional differences were observed for the gonadotropin subunits in the pituitary. Transcription levels of the two genes coding for the gonadotropin receptors, *fshr* and *lhr*, were measured in the pituitary, but no clear differences were observed between the two analyzed gametogenic stages, in both females and males. In the gonads, the transcription levels of both gonadotropin receptors was also measured. In females, transcription levels of *fhsr* increased slightly during cortical alveoli and vitellogenesis stages, decreasing again at regressing stage. Such profile was similar to the pattern observed for the gonadotropin subunits in the pituitary, indicating a relationship between the expression of gonadotropin subunits in the pituitary and the transcription of their receptors in the gonads. *gthα* and *fshβ* were also found to be transcribed in mullet gonads, however, the transcription levels of both gonadotropin subunits in the ovaries did not show an oogenic stage dependent pattern and remained constant. In males, although not significant, an upregulation was observed for *fshr* from early to mid spermatogenesis, decreasing slightly again at late spermatogenesis. The opposite trend was observed for *lhr*, as early spermatogenic males showed higher transcription levels than males at more advanced stages. No differences in transcript levels were observed between males at mid and late spermatogenesis for the gonadotropin receptors, coinciding with the constant transcription pattern observed also for the gonadotropin subunits in the pituitary. A constant transcription pattern was also observed for *gthα* and *fshβ* in the testes. *kiss2* and *gpr54* transcript levels were also measured in the gonads of mullets, showing in general no differences among gametogenic stages. That the observed pattern of transcription of the genes involved in the BPG-axis is influenced by the presence of xenoestrogens affecting all individuals captured in Pasaia should be taken into consideration, as the BPG-axis is a potential target to endocrine disrupting chemicals (Hachfi et al., 2012). This could have modulated the normal signaling mechanisms in the studied mullets. In this sense though, contradictory examples can be found for xenoestrogen effects on gonadotropin gene transcription, as some studies describe suppression and others describe increase of the transcription levels of *fshβ* and *lhβ* under xenoestrogen

exposure (Mateos et al., 2002; Jeng et al., 2007; Zhang et al., 2008; Urbatzka et al., 2012; Liu et al., 2013; Qin et al., 2014; Alvarado et al., 2016; Chen et al., 2017).

The transcription levels of the BPG-axis related genes were also measured in the brain and gonads of intersex mullets from Pasaia. *kiss2*, *gpr54* and *gnrh1* in the brain of intersex mullets showed transcription levels similar to males at mid spermatogenesis and females at cortical alveoli stage, although the oocytes present in the testes of intersex mullets were all at previtellogenic stage. It is not clear if the observed transcription levels in intersex mullets could be the due to the exposure to xenoestrogens or the consequence of the development of the intersex condition and the development of oocytes in the testes. In some species, an increase in the transcription levels of kisspeptin genes after E2 exposure and an increase of transcription levels of *gnrh* after EE2 exposure have been described (Kanda et al., 2008; 2012; Zhang et al., 2008; Servili et al., 2011). In the intersex testis, the transcription levels of the same genes could be measured, showing transcription levels similar to normal testis and ovaries, as transcription was not modulated at all. Also in the intersex testis, transcription levels of *gthα* and *fshβ* were measured and resulted to be more similar to ovaries and lower than in normal testis. This was coincident with the pattern observed for *fshr*, as intersex testis showed lower transcription levels than normal testis. Instead, *lhr* transcription levels observed in intersex males were similar to those of normal testis. Thus, the main difference between intersex and non-intersex males was the downreagulation of *fshβ* and *fshr* in intersex testes. Taking these observations into account, and considering that the alterations in transcription levels occur mainly at the level of the gonad tissue, *fshr* could be suggested a suitable gene as a marker of the intersex condition in thicklip grey mullets from the Basque coast. In addition, in the work done with mullets from Gernika and Galindo, *dmrt1* and *amh*, which are male sex differentiation genes, were observed to be downregulated in intersex males in comparison to males. This allows us to suggest that *dmrt1* and *amh* can be suggested as useful biomarkers of the intersex condition for future xenoestrogen biomonitoring programs to be conducted in the Basque coast. In this context, the future connection of Gernika and upstream town sewage waters to the newly built WWTP of Bermeo, will offer an outstanding study-case for the monitoring of the predicable improvement of the intersex condition prevalence in the local mullet population.

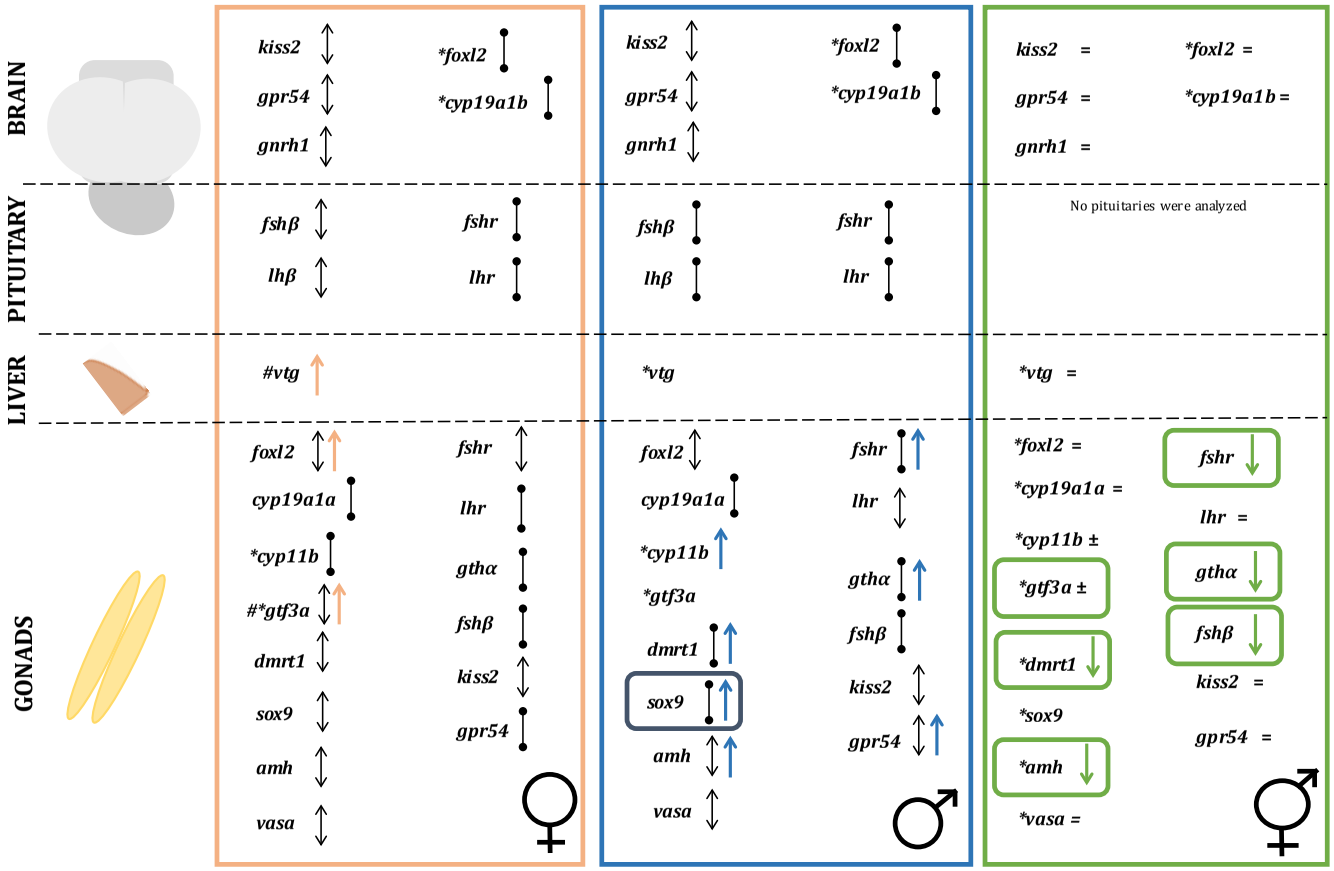

Figure 1
Schematic representation of the gene transcription trend in genes in the brain, pituitary, liver and gonads of female, male and intersex thicklip grey mullets. Data obtained from analyses conducted during the gametogenic cycle in mullets from Pasaia, except for the cases marked with an asterisk (*), meaning that data were obtained in mullets from Gernika. Hash (#) means that transcription levels of the gene were not analyzed in this work and information from previous studies has been used (Diaz de Cerio et al., 2012; Rojo-Bartolomé et al., 2017). For females and males, arrows with two arrowheads are indicative of fluctuating transcription levels along the gametogenic cycle and arrows with bold arrowheads are indicative of constant transcription levels along the gametogenic cycle. Arrows in color indicate if the transcription levels were higher for females (orange) or males (blue). For intersex, green arrows indicate that transcription levels were lower in intersex males than in normal males, "=" means that transcription levels were similar in intersex males and normal males, and ± means that transcription levels were in between females and males. Considering results from Pasaia and/or Gernika, genes whose transcription pattern was shown to be altered due to exposure to xenoestrogens are surrounded in dark blue and genes considered to be good indicators of the intersex condition are surrounded in green.

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Conclusions and Thesis

CONCLUSIONS

1. Intersex male mullets were found in Gernika and for the first time in Galindo in both studied seasons, late spring and winter, which, together with detectable hepatic transcription levels of vitellogenin in normal and intersex males suggested exposure to xenoestrogens under the influence of wastewater treatment plant effluents. In Gernika, the prevalence of intersex condition and its severity index were higher than in Galindo, the transcription profile of some of the analyzed genes showing alterations in males from Gernika, indicative of a more severe/prolonged exposure to xenoestrogens than in Galindo.
2. The transcription profile of genes related with sex differentiation, oogenesis and steroidogenesis in the brain and gonads in thicklip grey mullets in Galindo and Gernika revealed genes with sexual dimorphic transcription, with higher transcripton levels of *cyp11b, dmrt1* and *amh* in testes than in ovaries, and the other way around for *cyp19a1a*, *cyp19a1b, foxl2,* and *gtf3a*, indicating that exposure to xenoestrogens does not influence this sex dependent transcription pattern. On the contrary, transcription levels of *sox9* were not detectable in Gernika, in none of the sexes and seasons, indicative of the suppression of its transcription as a consequence of a strong xenoestrogenic or toxic impact.
3. Transcription levels of sex differentiation, oogenesis and steroidogenesis genes in the brain and gonads of thicklip grey mullets from polluted estuaries revealed only a significant downregulation of *dmrt1* and *amh* in testes of intersex males in comparison to normal males, suggesting their suitability as specific markers of the intersex condition in thicklip grey mullets from the Basque coast.
4. The transcription pattern of genes involved in development and reproduction signaling in the brain-pituitary-gonad axis of thicklip grey mullets showed no clear modulation during gametogenesis neither in females nor in males, which could be consequence of the presence of endocrine disrupting chemicals in the harbor of Pasaia but also possibly of an effect on the physiological capacity to regulate their transcription. Intersex males showed transcription patterns similar to those in normal males, except for *fshr* in the testis, whose levels were more similar to those in ovaries, suggesting that *fhsr* could be used as biomarker of intersex condition in thicklip grey mullets.
5. Transcription of genes related with development and reproduction signaling in the brain-pituitary-gonad axis was detectable outside their archetypal and functional tissue of expression. This suggested a possible feedback tissue-specific role in regulation of the gametogenic cycle in thicklip grey mullets although their

transcription profile was independent of the gametogenic cycle. Transcription of the gonadotropin subunits *gthα* and *fshβ* in gonads suggests a local production of the follicle stimulating hormone. Intersex mullets showed lower *gthα* and *fshβ* transcription levels in testes when compared to normal males, indicating a possible effect due to feminization of the gonads.

6. Intersex thicklip grey mullets have been identified in almost all the samplings performed in Gernika and Pasaia during the last decade. Such observations together with previously performed chemical analysis of mullet bile is indicative of the presence of a complex mixture of xenoestrogens in both sites that is readily accumulated with a feminizing effect on the population. An increase on the prevalence and the severity of the intersex condition detected in samplings after 2014 in mullets from Gernika are indicative of a deterioration of the situation in association to the defective performance of its wastewater treatment plant. On the contrary, intersex individuals have not been found in some of the samplings performed in Pasaia after 2016.
7. Ovarian atresia was observed microscopically in a considerable number of female thicklip grey mullets from Gernika, but not from Pasaia, during the last five years. Histochemical analysis resulted inconclusive and no signs of apoptosis were observed. Genes related with apoptosis and autophagocytosis showed higher transcription levels in the ovaries of mullets from Gernika when compared to mullets from Pasaia, independent of whether they presented microscopical signs of atresia. This suggests an activation of the genes involved in controlled cell death processes and could also be indicative of a possible effect of the exposure to xenoestrogens in female mullets from Gernika.

THESIS

The presence of *Chelon labrosus* thicklip grey mullets showing intersex testes is common in several estuaries in the Basque Coast due to the presence in the environment of xenostrogenic endocrine disrupting chemicals. In the locality of Gernika, the high incidence of intersex mullets and the increasing trend on its prevalence, together with the high prevalence of female mullets with atretic ovaries seen during the last years, is indicative of a deterioration in the area along the years. After the analysis of the transcription levels of genes related with gametogenesis and genes involved in the regulation of reproductive processes in the brain-pituitary-gonad axis in females, males and intersex individuals, gonadal *dmrt1, amh, fshr, gthα* and *fshβ* should be considered as suitable candidate genes, to be utilized as biomarkers of the intersex condition in thicklip grey mullets studied as sentinel organism in pollution biomonitoring campaigns in the Basque coast.

Appendix

Real Time qPCR method

For the calculation of the most suitable primer and sample concentration, serial dilutions done with a pool of cDNA were tested combined with primer concentrations of 12,5 pmol, 25 pmol and 50 pmol. The most suitable combinations of cDNA and primer concentration for each target gene were selected considering the lack of primer dimers and that the obtained Ct values were maintained in the range of 22-28. In all cases, the selected primer concentration was 12,5 pmol. For the sample concentration used for each gene see table 1.

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 primer pair. In each qPCR plate a standard curve made by serial dilutions prepared from a pool of samples, including the dilution used for each gene was added in the plate. The curve was run in triplicates, as the samples.

qPCR conditions: an initial step at 50ºC for 2 minutes and 95ºC for 10 minutes, 40 cycles of a denaturing step at 95ºC for 15 seconds and annealing step at Tm (Table 1) for 1 min, finally a dissociation stage of 95ºC for 15 seconds, 60ºC for 1 min and again 95ºC for 15 seconds

Normalization of the qPCR data:

1) All plates used for the quantification of each gene were compared assuring that the points of the standard curve had the same Ct value in each plate.
2) The mean Ct value of the triplicates of each sample and curve point was calculated.
3) The point of the curve corresponding with the dilution used for the samples was used as internal control and used to calculate the ΔCt of each sample.

$$\Delta Ct = Ct\ sample - Ct\ internal\ control$$

4) The efficiency (E) of the reaction was calculated using the slope (s) of the standard curve.

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

5) The cDNA concentration of each sample, which had been previously measured by fluorescence using the commercial Quant-iT™ OliGreen® ssDNA Kit (Life Technologies), was used to normalize the obtained results and to calculate the relative transcription level of the target gene for each sample.

$$RQ = (1+E)^{-\Delta Ct} / ng\ cDNA$$

Table 1

List of genes used in each chapter with their corresponding concentration for each target organ.

Gene	Sample dilution	Tm (ºC)	Target organ
vtgAa	1/40	59	liver
gtf3a	1/400	59	gonad
cyp19a1a	1/5	60	gonad
cyp19a1b		59	brain
dmrt1	1/20	59	gonad
foxl2	1/5	59	gonad
	1/10	59	brain
cyp11b	1/5	60	gonad
amh	1/5	58	gonad
sox9	1/2,5	58	gonad
vasa	1/20	58	gonad
kiss2	1/5	58	brain
	1/2,5	58	gonad
gpr54	1/5	58	brain
	1/5	58	gonad
gnrh1	1/5	58	brain
gthα	1/50	58	brain
	1/2,5	58	gonad
fshβ	1/20	59	pituitary
	1/2,5	59	gonad
lhβ	1/5	59	pituitary
fshr	1/20	60	gonad
	1/2,5	60	pituitary
lhr	1/5	58	gonad
	1/5	58	pituitary
p53	1/20	59	gonad
mdm2	1/5	59	gonad
beclin1	1/20	60	gonad
caspasa3	1/5	60	gonad
rpl5	1/20	60	gonad

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