

Impact of Endocrine Disrupting Chemicals [EDCs] on Hypothalamic-Pituitary-Gonad-Liver [HPGL] Axis in Fish

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Abstract: The aquatic ecosystem is one of the most exposed environments to pollutants. A lot of xenobiotics are known to disrupt the reproductive endocrine system. Fish are one of the primary risk organisms for Endocrine Disrupting Chemicals [EDCs]. In common with all vertebrates, reproduction in fish is controlled by the Hypothalamic-Pituitary-Gonad-Liver [HPGL] axis. The multitude of hormones controlling this axis and the complexity of hormones regulation, make from the HPGL axis a major target of EDCs. Variation of hormones synthesis levels, disturbance of genes expression, alteration of gonads structure and disruption of liver functions were noted. HPGL axis represents so one of the most important endpoints in the risk assessment of EDCs. In this review, we seek to extract from recent literature concepts regarding the effects of EDCs on the endocrine reproductive system in fish that might be of interest to explain some alterations in the wide populations of fish.

Key words: Endocrine Disrupting Chemicals % Hypothalamic-Pituitary-Gonad-Liver axis % Fish % Reproductive system % Gene expression

INTRODUCTION

The aquatic ecosystem is one of the most exposed environments to pollutants since it represents the ultimate destination for most anthropogenic contaminants. In fact all chemicals, whether initially released on land, in the atmosphere or directly in rivers will eventually find themselves in the rivers and oceans at the end. Therefore, aquatic systems contain a wide range of pollutants from sewage effluents, factory wastes, agrochemicals, pesticides, etc. Many of these chemicals are suspected or known to interfere with the endocrine system of models organisms such as mammals, fishes etc. and can lead to a disturbance of hormone metabolism, hormone-regulated cellular or physiological processes [1,2]. A disruption of the normal hormonal function modifies the organization and the functioning of the reproductive system. In fact, wild fish may be affected by drugs and hormones used in the manipulation of growth and reproductive cycles in

human and hormonal treatments. Similar effects might be expected in fish exposed to effluents from pharmaceutical industries, or to sewage effluents which can contain high levels of human estrogens or androgens [3] or industrial chemicals [4]. Such xenobiotics are known to disrupt the reproductive endocrine system and to affect gamete development and viability either by their cytotoxicity or by altering the hormonal environment during gamete development. They also may affect sexual differentiation of the gonad, timing of sexual maturation, gonadosomatic index, reproductive tract and gonad morphology [5].

Fish are considered as one of the primary risk organisms for Endocrine Disrupting Chemicals [EDCs] [6]. Not only they are directly exposed to a wide variety of EDCs, but also sex determination in fish is known to be very labile and can be disturbed or even reversed by exogenous hormone exposure at critical developmental stages [7].

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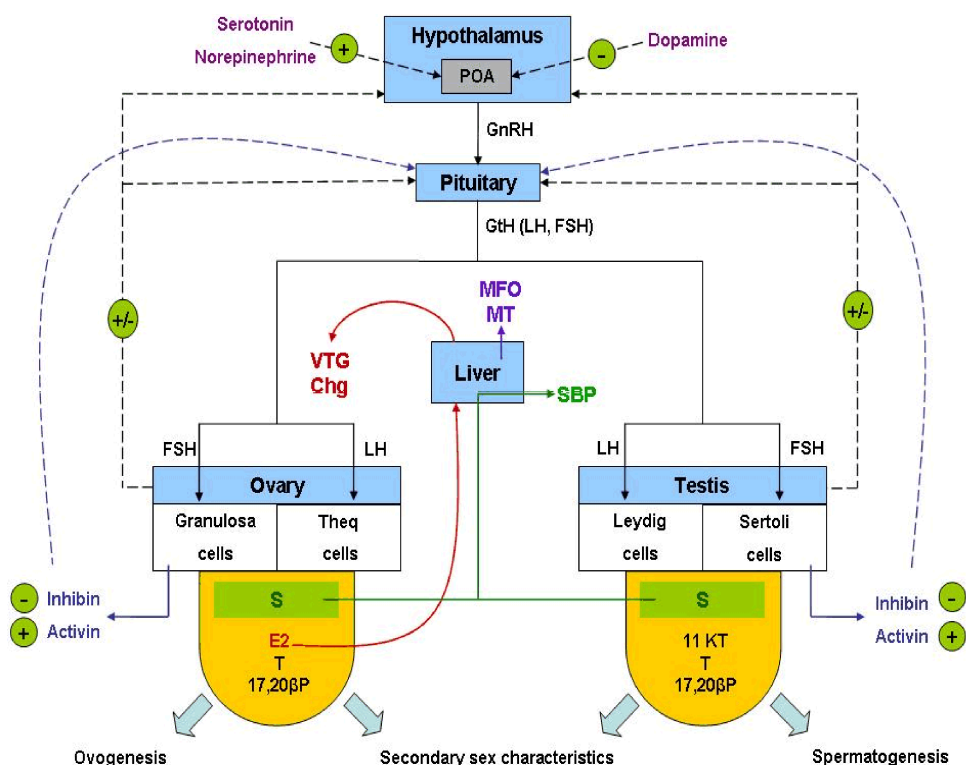


Fig. 1: Schematic presentation of the Hypothalamic-Pituitary-Gonad-Liver axis in fish.

Abbreviations: Chg = Choriogenin, E₂ = 17β-Estradiol, FSH = Follicle Stimulating Hormone, LH = Luteinizing Hormone, MFO = Mixed Function Oxydase, MT = Metallothionein, S = Steroides, SBP = Steroid Binding Proteins, T = Testosterone, VTG = Vitellogenin, 11KT = 11-Ketotestosterone, 17,20βP = 17",20β-dihydroxy-4-pregnen-3-one

Even if the binding affinity of most EDCs to the estrogen or androgen receptors [ER/AR] is low compared to endogenous steroid hormones, exposure to even low concentrations of xenoestrogens/xenoandrogens can disrupt normal developmental and reproductive processes. This is evident for exposure to anthropogenic chemicals continuously detected at low concentrations in the environment. A well-known example is ethinylestradiol [EE₂] used in contraceptive pills and finally discharged into the aquatic environment. EE₂ is found in the lower ng/L range in aquatic water, but it is enough to cause sustainable impacts on reproduction and population evolution in fish [8].

There is no doubt now that EDCs can have long-term effects on reproduction and subsequent population development in natural fish populations [9-12]. An ecotoxicological approach is suitable to evaluate the impact of EDCs on fish. In addition, investigations based on the evaluation of reproductive assessments are still costly, time-consuming and laborious, what can explain why these endpoints are not routinely integrated into most chemical safety assessments. However, since

endocrine disruption can be linked to molecular interactions, the variation of gene expression of appropriate biomarkers could be used as predictor for reproductive disruption [13] and would be a pertinent tool for the evaluation of reproductive assessments.

Metals (copper, mercury, lead, cadmium), polycyclic aromatic hydrocarbons polyhalogenated compounds and chemical mixtures induced different alterations such as antioxydant defense mechanisms, lysosomal system and metallothionein secretion. A large number of bioindicators was even identified such as *Trematomus bernacchii* [14-17]. However, the potential endocrine effects are still not enough investigated, although similar properties have been demonstrated for several chemicals. That makes it pertinent to look over previous work in order to determine perspectives for future research. In this review, we seek to extract from recent literature concepts regarding the effects of EDCs on the endocrine reproductive system in fish that might be of interest to explain some alterations in the wide populations of fish. For this, we will review the

basics of reproductive system in fish, then we will define EDCs and their different modes of action. Then we will explore the effects of EDCs along the Hypothalamic-Pituitary-Gonad-Liver [HPGL] axis in fish at different levels which will constitute a true mirror of the reproduction processes. Finally we will summarize some effects of pollutants on the reproductive systems in fish and conclude with a few considerations of future research directions.

Basics of Reproductive System in Fish: In common with all vertebrates, reproduction in fish is controlled by the Hypothalamic-Pituitary-Gonadal [HPG] axis. Signals from the brain control the hypothalamic secretion of gonadotropin-releasing hormone [GnRH] which stimulates the adenohypophysis to release gonadotropins: luteinizing hormone [LH] and follicle stimulating hormone [FSH] (Fig 1).

In the ovary, granulosa contains specific receptors to FSH [FSHR]. The role of this hormone consists on stimulating follicular maturation and androgen conversion to estrogen by aromatization. In the theca cells, LH binds to its receptor [LHR] and induces androgen production and ovulation.

In the testis, FSHR are localized to Sertoli cells. FSH would stimulate Sertoli cell proliferation and spermatogenesis but their exact role remains unclear. LHR are located on Leydig cells. The LH-receptor complex triggers androgen synthesis. Then these pituitary hormones induce and regulate steroidogenic enzymes [18].

Gonadotropins stimulate gonadal development and production of steroid hormones: 17 β -estradiol [E₂] in females, testosterone [T] and predominantly 11-ketotestosterone [11KT] in males, as well as maturation-inducing hormones [MIHs] mainly 17 α ,20 β -dihydroxyprogesterone [17,20 β P] in both sexes. The main difference between fishes and mammals is that in fish the major testicular androgen is 11KT [19] and the principal ovarian estrogen is the estradiol which acts differently than in mammals. It stimulates the liver, its target tissue, to produce the yolk protein vitellogenin [VTG] which is incorporated into the oocyte under the influence of the gonadotropin FSH [20].

Although studied in mammals, very little information is available in fish on the specific roles of FSH and LH in regulating androgen production by the testis and the precise function of each gonadotropin in teleosts is scarce [21].

In fish, FSHR is expressed in Sertoli cells as it was shown in mammals [22,23], but further studies on Japanese

eel (*Anguilla japonica*, Temminck and Schlegel, 1846) and African catfish (*Clarias gariepinus*, Burchell, 1822) have demonstrated FSHR presence in Leydig cells in [24,25].

Gonadotropin functions were more explored in the salmonid model. Their transcripts and plasmatic concentrations showed that FSH is linked to the initiation of gametogenesis through the production of E₂ and 11KT. LH stimulates the production of MIHs, progestins 17,20 β P and 17 α ,20 β ,21-trihydroxy-4-pregnen-3-one [20 β S]. It plays so a crucial role in final steps of oocyte and sperm maturation [e.g., 26 and references therein].

Steroids initiate changes in secondary sex characteristics, behaviour as well as development and maturation of gametes [27,28]. Their biosynthesis has an acute (on the order of minutes) and a chronic (on the order of hours) hormonal regulation. Chronic regulation implies the transcription/translation of the genes encoding steroidogenic enzymes involved in the long term steroid synthesis, while the acute regulation of steroidogenesis depends on the mobilization of precursor cholesterol in the mitochondria [29]. In mammals, it was shown that this transport is mediated by the steroidogenic acute regulatory [StAR] protein [29]. Complementary DNA-encoding proteins with high homology to StAR of mammals were cloned from zebrafish (*Danio rerio*, Hamilton, 1822), rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792), cod (*Gadus morhua*, Linnaeus, 1758) and stingray (*Potamotrygon hystrix*, Müller and Henle, 1841) [e.g., 30 and references therein].

In fact, synthesis of steroids needs the contribution of several oxidative enzymes responsible of cholesterol conversion into steroids. For example, the cytochrome P450 11 β -hydroxylase and the cytochrome P450 aromatase are involved in the final steps of the synthesis of 11KT and the conversion of T to E₂ respectively [30]. Final plasma concentration of steroids depends on their level of synthesis and on the rate of deactivation by the liver. For example, estradiol acts on the liver which induces VTG synthesis, whereas it is catabolised in the liver [6].

Gametogenesis is controlled by the interference of systemic and intragonadal systems. Each system is less or more solicited depending on the stage of gametogenesis.

Gonadal recrudescence in both sexes requires several months, until viable gametes can be produced. E₂ and 11KT secretions then stop and the biosynthetic pathway turns to progestogen secretion which induces the final maturation of oocytes or testis. In most fish this progestogen is 17,20 β P, while in others 11-deoxycortisol or other progestogens may be involved [19].

The HPG axis plays a major role in maintaining normal reproduction and synchronization of the seasonal reproductive cycles [31-33]. In fact like most vertebrates, teleosts also exhibit distinct cyclic reproductive patterns during which, levels of GnRH, GtH and sex steroids vary according to the reproductive phase [32,34-36]. In mammals, peak levels of plasmatic E₂ and low progesterone levels precede preovulatory LH surge [37,38]. In fishes, decreased plasma E₂ levels and increased 17,20\$P levels precede spawning [39,40].

In addition to hormonal factors, external factors, such as light and water temperature, act on brain to control the timing of gonad development and maturation in fishes. Any irregularity in the GnRH, GtH or sex steroid levels in a particular phase could lead to a reproductive functions disturbance and to reduced fertility or even sterility [41]. Another system which modulates the functioning of the HPG axis is the monoaminergic system. Its neuromediators like dopamine could inhibit the release of LH while others like norepinephrine and serotonin stimulate LH release not only directly but also via the GnRH axis. Other factors such as seasonal changes are also able to induce variations in hypothalamic and pituitary monoamines [e.g., 42 and references therein].

Depending on the physiological stage, sex steroids exert negative and positive feedback effects at the pituitary and hypothalamus levels to modulate the synthesis and release of GnRH and GtH [43,44]. The gonads can also signal back their status to the pituitary and hypothalamus by other messengers than steroids, such as inhibins secreted by Sertoli cells [6].

The multitude of hormones controlling HPGL axis and the complexity of their regulation by control and feedback control signals make from the HPGL axis a major target of EDCs.

EDCs: There is an increasing public concern over the adverse effects of anthropogenic contaminants on the health and well-being of both humans and aquatic wildlife [45]. In fact it is crucial to preserve fish populations not only because they provide to humans an essential food source, but also because they are an important component of ecosystems and they provides their balanced functioning. To date, a wide variety of both developmental and reproductive disorders observed in wildlife species have clearly been linked to the exposure to environmental contaminants which act as EDCs [45-47]. EDCs are “exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body

responsible for the maintenance of homeostasis and the regulation of developmental processes” [2]. The most frequently EDCs are described as agents that could alter the hypothalamic-pituitary-thyroidal or gonadal axis functioning inducing disturbances in reproduction or development [48].

EDCs can have severe impact on the environment, since hormones control many essential physiological processes such as growth and development, stress response and reproduction.

EDCs were shown to affect an organism through receptor mediated actions, but also receptor independent pathways (involving estrogens androgens and thyroid hormones) [49], altered hormone synthesis or degradation and binding to plasma proteins [50,51]. For example, it is known that exposure to E₂ induces the synthesis of specific proteins required for reproduction. Corresponding genes encode for ER, VTG and choriogenins necessary for the development of the egg membrane [49]. Other mechanisms including circulating steroid concentrations, steroidogenesis and hepatic steroid metabolism are susceptible to induce alterations and these effects are species and dose dependent [5].

The chemicals present in the environment that can disrupt reproductive system in fish are classified into four categories: estrogenic, anti-estrogenic androgenic and anti-androgenic compounds. However, other chemicals, able to interfere with the synthesis and binding of releasing and trophic hormones or the control of final MIH, could have similar effects and are also considered as EDCs [13]. Among the long list of EDCs we can mention: non ionic surfactants, such as nonylphenol and octylphenol [52-54], the fungicide vinclozolin [55-57], the metabolite 1,1-dichloro-2, 2-bis (*p*chlorophenyl) ethylene (*p,p*9-DDE) of the insecticide DDT [56-58], the industrial compound bisphenol A [59-61], effluent from sewage treatment plants [62,63], EE2 used in oral contraceptives [64] and run-off from animal farms [65]. Arcand-Hoy and Benson [66] provide a review of additional contaminants.

In recent years, trace metals such as mercury, cadmium, lead and copper have also been identified as EDCs able to interfere with the synthesis, transport and/or degradation of endogenous hormones. There is a wealth of literature available on the effects of trace metals on many different hormones and hormone functions [67]. For example cadmium [Cd], was indicated to have estrogenic effects through different molecular and cellular mechanisms [68,69]. Several studies in mammals and fish suggest that Cd has a high affinity to ER [68-69]. Estrogen receptor cadmium-binding sites have even been identified

[70]. Cd can also induce other endocrine disturbances such as development reproductive alterations [21,71]. Cadmium has the potential to bioaccumulate in the liver and is sequestered in eggs after waterborne exposures [72,73].

Besides the variety and the large presence of EDCs on the environment, it seems to be judicious to focus on the interactions they could have with the endocrine system of organisms and on the repercussion they could induce on their reproductive life.

IMPACT OF EDCs ON HPGL AXIS: Different kinds of hormones could be targets of environmental chemicals: reproductive hormones such as estrogens and androgens, thyroidal hormones, corticosteroids, growth hormone. As we have seen above, HPGL axis controls many important processes which are crucial for population viability. Therefore several research projects have focused on the interference of EDCs with the reproductive hormones, especially as they are of vital importance during the early critical stages of embryonic development and sex differentiation. In fact, HPGL axis represents one of the most important endpoints in the risk assessment of EDCs since it includes all aspects of reproductive development from sex differentiation to puberty.

Endocrinology: Gametogenesis, secondary sexual characters and reproductive cycles are controlled by signals emitted by the neuroendocrine system. In fact, the neuroendocrine system changes endogenous and exogenous signals on internal signals controlling different reproductive pathways. Changes in steroid levels can disturb the normal functioning of the neuroendocrine system in two manners, directly through agonist and antagonist effects or indirectly involving feedback signals [5,74].

The steroidogenic pathway appears to be affected by numerous pharmaceutical, agricultural and industrial contaminants. Exposure to various EDCs can result in decreased androgen production (demasculinization) or increased estrogen production (feminization) in males. Depressed plasma androgens and/or elevated E_2 are often characteristic of antiandrogenic or estrogenic xenobiotics exposition. Summer flounder (*Paralichthys dentatus*, Linnaeus, 1766) experimentally exposed to E_2 showed reduced plasma T and elevated plasma E_2 [96]. Similarly, flounder treated with o, p'-DDT and octylphenol showed a decreased plasma T concentration. The octylphenol treated flounders also had an initial increase in plasma E_2 concentration [75].

In white sturgeon (*Acipenser transmontanus*, Richardson, 1836) from the Columbia River, plasma T and 11KT concentrations were negatively correlated with p,p0-DDE levels found in the liver [76]. Male largemouth bass (*Micropterus salmoides*, Lacépède, 1802) taken from a contaminated site (Escambia River, exposed to two identified sources of pollution) had a plasma testosterone concentration lower than in males from a reference site [77].

Exposure to the phytoestrogen genistein induces decreased testicular T production and a reduced plasma T concentration in medaka (*Oryzias latipes*, Temminck and Schlegel, 1846) [78]. Similarly, goldfish (*Carassius auratus auratus*, Linnaeus, 1758) exposed to bleached sulfite mill effluent showed a reduction in testicular T and 11KT synthesis [79].

In order to evaluate the effect of PCBs on the neuroendocrine system, male Atlantic croaker (*Micropogonias undulatus*, Linnaeus, 1766) was exposed through diet to Aroclor 1254 for 30 days during gonadal recrudescence. It was noted a decrease in serotonin and dopamine levels and an increase in their metabolites, the response to stimulation by a luteinizing hormone-releasing hormone analogue was also altered. Later it was demonstrated that Aroclor 1254 inhibits hypothalamic tryptophan hydroxylase [TPH] activity but not the one of monoamine oxidase [MAO] and that the gonadal growth disturbance that was noted is related to the disruption of the GtH synthesis through the decline of serotonin levels [80]. This study was followed by further experiments to ensure that these results were related to the decrease of GnRH release. When animals were treated by P-chlorophenylalanine (an irreversible TPH inhibitor) then an exogenous 5-hydroxytryptophan, serotonin has regained normal levels and none deleterious effects were observed. In addition, GnRH implants prevented the PCB-induced decline in GnRH receptors and restored the LH response to a GnRH analog. Then the decline of serotonin concentrations is really related to the PCB induced alteration of the stimulating effects of serotonin-GnRH controlling LH levels [81].

LH secretion is typically higher in sexually mature croaker compared to early recrudescing fish. Khan *et al.* [82] reported that during testicular maturation, GnRH content of the POA, pituitary GnRH receptor density and LH secretion were similar to those of recrudescing male croaker after Aroclor 1254 exposure. PCB seems to disrupt the GnRH-LH system through the decrease of GnRH and its receptors as well as neurotransmitters that control its synthesis like serotonin.

Concerning heavy metals, cadmium for example is known to act centrally to disrupt steroidogenesis at the pituitary level, but it can also act directly on the gonad or liver [83]. Cd activated ER^α in estrogen responsive breast cancer cells with similar binding affinity as E₂ and inhibited human and rainbow trout ER transcriptional activity in a recombinant yeast system by affecting DNA binding [84,85]. Cd also alters calcium homeostasis and disrupts signal transduction processes responsible to gonadal and pituitary hormone secretions [86-88]. Other studies have showed that Cd directly inhibits enzymes involved in gonadal steroidogenesis and hepatic steroid metabolism [89]. Cd was found to disrupt the HPGL axis in both males and females at multiple levels. Those parameters were even more sensitive to Cd than the reproductive or development alterations observed in some studies suggesting that they can be adequate indicators for EDCs exposure assessment. Changes in circulating steroid levels were not accompanied with significant effects on plasma VTG or hepatic ER. These results are consistent with a 2-week exposure of medaka hatchlings to the same Cd concentrations in which there were no changes in reproductive output or VTG and ER production [90]. Cd interacts directly with ER but also indirectly by affecting ER mediated processes, which could result in its cumulative effect on ER mediated proteins levels [91]. However, other studies have found Cd inhibits transcriptional activity of rainbow trout ER and to a lesser extent, human ER by affecting DNA binding activity [84]. It was also reported that Cd alters signal transduction and ER-mediated processes involving divalent cations, such as Ca²⁺ and Zn²⁺ which could affect signals along the HPGL axis and alter ER-mediated proteins such as VTG and ER [86].

Gonads Development and Gametogenesis: The measure of the GSI (gonad weight divided by the body weight) is a simple way to evaluate the gonadal dysfunction. Decreased GSI is indicative of decreased hypothalamic, pituitary or gonadal activity. To determine if a particular maturation stage is inhibited and to explore where the pollutant is precisely acting, it is interesting to complete this measure with gonadal histological examination. That can show a block in inducing vitellogenesis, development of spermatogonia, or growth and maturation of oocytes [6,92].

Typically, GSI is reduced after exposure to estrogenic or antiandrogenic contaminants. The decline of GSI could be linked to inhibited testicular development. Suppression of testicular development was shown in fathead minnows

(*Pimephales promelas*, Rafinesque, 1820) collected from a lake then treated with EE₂ [87], in adult fathead minnows exposed to bisphenol A [61] and European sea bass exposed to estrogens during gonadal differentiation [88].

Concerning gonadal histological alterations induced by EDCs, it was noted that mature fathead minnows exposed to environmentally relevant doses of E₂, 4-nonylphenol, or nonylphenol ethoxylate induced many histological lesions such as disturbances in the size and the number of Sertoli cells and germ cell syncytia, degenerative changes in Sertoli cells, necrotic aggregates of various stages of germ cells on males, presence of phagocytic cells in the lumina of seminiferous tubules. In females, in the ovaries follicles seems to be blocked in the primary stage of development and a decline in the secondary and graafian follicles were noted [93,94].

No significant effects on the stages of follicular development were observed in females exposed to NP. In swordtails (*Xiphophorus helleri*, Heckel, 1848) treated with nonylphenol, bisphenol A and their mixture, an increase in apoptotic/necrotic cells in the testicular interstitial tissues and seminiferous tubules was observed [95].

Disruption of spermatogenesis may result in changes in proportions of sex cell types in the testis, blocked spermatogenesis in earlier stage, a decline of the number and the quality of sperm cells and decrease of the germinal cells number [5].

General decreases in spermatogenesis and ejaculated sperm counts have been observed in several species, including goldfish treated with E₂ [96], adult zebrafish after 24 days of laboratory exposure to 17-β-EE₂ [97], adult Japanese medaka exposed to 4-tert-octylphenol [98], swordtails exposed to nonylphenol [95] and adult and sexually developing juvenile guppies (*Poecilia reticulata*, Peters, 1859) exposed to vinclozolin and p,p'-DDE [56,57]. Van den Belt *et al.* [97] noted that those effects were reversible once fish were placed in clean water. In flounder (*Platichthys flesus*, Linnaeus, 1758) captured from contaminated waterways and in wild roach (*Rutilus rutilus*, Linnaeus, 1758) from rivers exposed to sewage effluent, it was observed a sharply reduction in spermatogenesis and an absence of running milt but that can be extracted by stripping [11,78,99,100]. Then an occlusion or a dysfunction of the spermatic ducts could be involved in this disturbance. Toft *et al.* [101] reported that male mosquitofish (*Gambusia affinis*, Baird and Girard, 1853) collected from Lake Apopka, FL (USA) had fewer sperm cells when compared to fish from less contaminated lakes nearby.

In adult fathead minnows exposed to bisphenol A, Sohoni *et al.* [61] observed changes in the proportions of sex cell types in the testis, suggesting an inhibition of spermatogenesis.

Four ppm of Cd significantly decrease ovarian and testicular GSI in the freshwater fish Bata (*Labeo bata*, Hamilton, 1822) whereas 25 ppb of Cd decrease GSI in winter flounder (*Pseudopleuronectes americanus*, Walbaum, 1792) [102,103]. While histopathological data is not available for these samples, histological analysis of testis from other chronic Cd exposures in fish exhibit degeneration of mature spermatocyte cells, absence of spermatids and spermatozoa and severe necrosis of Leydig cells [102].

Liver: The liver plays a key role in steroid, drug and xenobiotic biotransformation in all vertebrates that can explain why we can not exclude it from the HPG axis hence the relevance of the HPGL axis analysis (Fig 1). Hepatic biotransformation of steroids could be a relevant tool for the assessment of EDCs. Hepatic enzymes are known to have a large substrate specificity increasing then the number of xenobiotics which they can inactivate [5]. The liver also inactivates endogenous hormones, such as androgens and estrogens. Whereas hepatic biotransformation of steroids is regulated by endogenous sex steroid levels. It can be disrupted by EDCs. EDCs not only can disrupt the gonad development but also they can be involved in the biotransformation of steroids [104,105].

That results in changes in T, E₂ plasmatic concentrations inducing in turn disturbances in the normal functioning of reproductive system.

The enzyme complex cytochrome P450 enzymes is largely involved in the hepatic biotransformation of xenobiotics. It is divided in two families CYP 1 and CYP 2 and it induces oxygen atom to hydrophobic structures by transforming them into water-soluble metabolites (phase I). Then they will be conjugated (phase II) and excreted (phase III) [106].

Some of EDCs like phenobarbitol, 3-methylcolanthrine and β -naphthoflavone induce the cytochrome P450 complex but the families of enzymes involved differ depending on the model studied [e.g., 5 and references therein].

The HPGL axis reveals several potential modes of interaction for EDCs ranging from interference with hormone synthesis and secretion, binding to hormone receptors, or altering the availability of endogenous hormones. Eventually, endocrine disrupting effects are mediated via the available level of steroid hormones or its analogs.

Thus, biomarkers directly responsive to steroid hormone levels are the most widely used as molecular indicators of endocrine disruption. The synthesis of VTG for example is a widely parameter used for the detection of exposure to xenoestrogens *in vivo* and *in vitro* [107,108].

Apart from xenoestrogens, the effect of EDCs acting like androgens or anti-estrogens on VTG levels are not enough explored [109]. Methyltestosterone [MT] (an androgen) and fadrozole [F] (an aromatase inhibitor) were selected to study whether a VTG response can be measured also after exposure to compounds other than estrogens [110]. For this, juvenile fathead minnows were exposed to MT, to F and to a combination of both. The MT exposed fish showed *de novo* synthesis of VTG mRNA, but not the F exposed fish or the fish of the combination group. This result suggests that an endogenous aromatase converts MT to estrogen (methylestradiol). Accordingly, exposure to MT leads to a partial feminization of the exposed fathead minnows, while in combination with the aromatase-inhibitor, MT was able to exert its full androgenic potential [109]. In fact it was noted that all fishes showed premature secondary male sex characteristics (pigmented dorsal fin, tubercles, aggressive territory defence). The exposure to the combination of both MT and F was not followed by any increase in VTG levels, which proves even more that MT was aromatised to an estrogen.

The liver plays also a key role in the exposure to heavy metals by the production of metallothioneins [MTs] which are cysteine-rich metal binding proteins that play an essential role in the regulation of intracellular metal concentration [111].

MTs maintain the balance of the zinc which is indispensable for the gametes development. Heavy metals affect the metallothionein synthesis and produce serious damages in zinc homeostasis and *in fine* in gametogenesis [6,112].

The MTs levels are controlled by steroids [113]. MTs and another hepatic enzyme involved in steroids catabolism, the mixed function oxydase [MFO], are considered as good indicators of exposure to heavy metals and of alteration of reproduction [6]. But other stressors such as temperature changes or high rearing density are also able to increase the expression of gene encoding for MTs indicating that MTs are not a specific biomarker and that they can be a good tool to monitor fish stress conditions [114,115].

Sexual Differentiation: Sex steroids, especially androgens and estrogens play a main role in the regulation of the sexual differentiation process.

In fish, it is possible to induce sex reversal by treating fishes with estradiol or testosterone [116]. That has been demonstrated since the late of 1930s and with the expansion of fish culture in the past 25 years, it has been largely used in aquaculture for skewing natural mixed sex population [117,118]. Estrogen and its analogs have been used for generating females while T and androgen analogs, aromatase blockers have been used for generating males [119-122]. In fact, in fish one of the most important factors in sex differentiation is the steroidogenic enzyme cytochrome P450 aromatase, product of the *CYP19a* gene. The role of aromatase consists on converting androgens to estrogens in an irreversible manner and then it plays a main role in determining the final sex in fish. The expression level of *CYP19a* influences sex differentiation: when it is highly expressed, the sex seems directed to female differentiation, while it is directed to male differentiation when it is low expressed [123]. It was demonstrated that sex differentiation was influenced in a large number of teleost species exposed to EDCs that have an estrogenic effect [124]. Exposure of eggs or larvae to environmental xenohormones may lead to similar effects and thus, it has become relevant that reproduction and population dynamics in wildlife, can be influenced by EDCs. Therefore more and more studies focused on dysregulation of sexual development and reproduction and reported a widely deleterious effects such as intersex in medaka caused by the organochlorine (γ -BHC and nonylphenol [125,126] and in roach below sewage outfalls contaminated with estradiol [3,9], feminization of rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) exposed to sewage treatment plants [10,127,128], masculinizing effects of pulp and paper mill effluents in female mosquito fish from North-America and New Zealand [129-133]. Intersex or imposex result from disrupted gonadal differentiation and typically consist in the simultaneous presence of ovarian and testicular tissue in the same gonad. In roach intersex, a number of duct malformations associating a female-like reproductive duct and sperm ducts in the same gonad have been reported [11,134,135].

Dramatic induction of VTG and zona radiata proteins [ZRP] in the same or even higher average than those of reproductive females, were detected in males of Mediterranean swordfish (*Xiphias gladius*, Linnaeus, 1758) suggesting that this species may be exposed to xenoestrogens [136,137]. That was approved by De Metrio *et al.* [138] who revealed a high percentage of intersex in this species.

In fish populations exposed to environmental estrogens or antiandrogens, the sex ratio is typically modified in favor of females. This disturbance can either lead to the exposure of developing embryos to the EDC or to the parents exposure or even more to the juvenile fish exposure during the period of sex determination. In fact, it is clear now that the time of exposure to the xenobiotic plays a key role in the sex differentiation. Transgenic zebrafish assays in which the estrogen receptor was transactivated, have shown that ER activity is very high during sexual development and could explain why the period of gonadal differentiation may be especially susceptible to disruption by estrogenic compounds [139]. For example, exposure to E₂ during sexual differentiation increased the frequency of female differentiation in lumpfish (*Cyclopterus lumpus*, Linnaeus, 1758) [140], Argentinian silverside (*Odontesthes bonariensis*, Valenciennes, 1835) [141], Amur catfish (*Silurus asotus*, Linnaeus, 1758) [142], carp (*Cyprinus carpio*, Linnaeus, 1758) [143,144] and sheepshead minnow (*Cyprinodon variegatus*, Lacépède, 1803) [64]. Japanese medaka exposed during development to octylphenol [52] and bisphenol A [145] showed also a sex ratio in favor of females. Similar effects were noted in juvenile guppies following their exposure to xenobiotics that having an antiandrogenic action [57]. It has also been demonstrated that some xenoestrogens like 4-tert-pentylphenol induce intersex in carp gonads [143,144].

Furthermore, it was reported that EDCs could induce other types of disturbances, such as altering the time of sexual maturation by speeding up or even delaying the timing of sexual maturation, what can severely affect reproductive processes, especially if maturation is completely delayed. In this context, Blazquez *et al.* [88] concluded that estrogens were able to delay sexual maturation in the European sea bass even if estrogen exposure happens after sex determination.

As we have seen the effects of EDCs are not limited to gonads, but it can affect different HPGL axis levels. During the critical period of sex reversal induced by exogenous steroid influence, POA-H and GnRH levels vary indicating an impact of sex steroids on GnRH-GtH synthesis [146]. That was approved by Smith and Jennes [147] who have demonstrated that E2 influenced the central monoaminergic system in POA-H regulating pulsatile secretion of GnRH in mammals. It was also shown that blockage of 5-HT by parachlorophenylalanine mimics estrogenic effect [148]. Heavy metals have also been described as affecting populations sex ratio. Matta *et al.* [149] have demonstrated in fact that mercury was able to induce feminization of the gonads in fish.

Gene Expressions Through the HPGL Axis: Studies concerning EDCs often focus on the involvement of steroid hormone receptors in the disturbances observed. Then other pathways such as disturbed steroidogenesis that can affect gene expression begin to be explored [e.g., 150 and references therein].

EDCs can alter normal patterns of gene expression either by direct (steroid hormone receptor-mediated pathways) or compensatory effects [151].

One mechanism for disrupting the steroidogenic pathway is to alter activity or expression of genes encoding for specific enzymes as the gene encoding for aromatase (*Cyp19* or *P450arom*). In male medaka, brain aromatase activity increased in a dose-dependent manner following E_2 exposure [152].

Another gene which expression could be severely be affected by EDCs is vtg. VTG is a female protein released by the liver and that's serves as a food reserve for embryos and larvae. The synthesis of VTG is hormone-dependent, mediated by the interaction between estradiol and ER, leading to induction of gene expression and transcription. In males activation of estrogen responsive genes is caused by many EDCs which can interfere with gonadotropin release, steroidogenesis and binding to nuclear ER [153-156]. VTG is normally present at high level only in females undergoing oogenesis. In males the vtg gene is normally suppressed. However, the expression of vtg can be induced in males by exposure to E_2 or estrogen mimics. In general the vtg gene is present, but only marginally expressed in males or juveniles [157]. In males and juveniles, abnormal changes in the VTG synthesis represent an indicator of an estrogenic contamination [158-161].

In addition to affecting the expression of vtg and ER, E_2 induces an array of genes, some by direct interaction with the ER and others by alternative pathways. The cascade of genes that are induced is tissue specific. For example, VTG is only produced in the liver, yet we know that E_2 targets other tissues besides the liver, such as the gonads and the brain. While the vtg gene is present in these other tissues, it is not induced by E_2 . Other hormones, such as androgens and thyroid hormones activate their own tissue specific cascades of genes. The identity of many of those genes is limited [49].

Along the HPGL axis many other genes could be affected by EDCs. The same chemical could generate over expression of some genes and under expression of others. Zhang *et al.* [150] have realized a striped view of concentration dependent response profile in EE_2 exposure of male Japanese medaka along the HPGL axis. The resulting map provides a lot of information concerning the genes affected on each level of the HPGL axis and their way to respond to EDCs.

CONCLUSION

Many EDCs were shown to disturb the reproductive system in fish by affecting the HPGL axis at different levels: pituitary, gonads, hormones, the liver catabolism of steroids, gametes quality... These disturbances are often considered as indicators of exposure to pollution, but they are far from specifying neither the corresponding physiological processes of pollutant nor its target components (tissues, genes, proteins). It seems important to determine which component is affected at first in order to know, if the EDC acts directly on the gametes or on a higher level on the HPGL axis (hypothalamus, pituitary, GnRH, GtH and steroid synthesis, the liver catabolism of steroids...) and to determine adequate molecular biomarkers.

It will be interesting for future research to focus on the development of molecular, physiological, or behavioural tools that will allow more extensive testing and analysis of fish response to EDCs [5]. The potential of molecular screening approaches has been recognized as a valuable tool in several applications and has contributed to the development of fish screening assays for EDCs [162-164].

The molecular screening assay, such as DNA and PCR-arrays allow studying the expression profiles of a considerable number of genes after exposure of organisms to xenobiotics. Specific expression profiles for each xenobiotic may be often described, which makes the screening assays important tools for monitoring the aquatic environment. Such tool will enable to the regulatory authorities to evaluate the real risk relied to the EDCs exposure of the aquatic ecosystems with easier and less expensive way [165].

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