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Gonadal transcriptome analysis of wild contaminated female European eels during artificial gonad maturation

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HIGHLIGHTS

- Strong differences were observed on the contamination levels of eels from the two sites.
- Eels from the reference site showed a higher gonad growth compared to contaminated fish.
- Genes associated to gonad growth were involved in cell division and gametogenesis.
- Genes associated to contaminants were involved in FOXO1 regulation and steroidogenesis.

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ABSTRACT

Since the early 1980s, the population of European eels (*Anguilla anguilla*) has dramatically declined. Nowadays, the European eel is listed on the red list of threatened species (IUCN Red List) and is considered as critically endangered of extinction. Pollution is one of the putative causes for the collapse of this species. Among their possible effects, contaminants gradually accumulated in eels during their somatic growth phase (yellow eel stage) would be remobilized during their reproductive migration leading to potential toxic events in gonads. The aim of this study was to investigate the effects of organic and inorganic contaminants on the gonad development of wild female silver eels. Female silver eels from two sites with differing contamination levels were artificially matured. Transcriptomic analyses by means of a 1000 candidate gene cDNA microarray were performed on gonads after 11 weeks of maturation to get insight into the mechanisms of toxicity of contaminants. The transcription levels of several genes, that were associated to the gonadosomatic index (GSI), were involved in mitotic cell division but also in gametogenesis. Genes associated to contaminants were mainly involved in the mechanisms of protection against oxidative stress, in DNA repair, in the purinergic signaling pathway and in steroidogenesis, suggesting an impairment of gonad development in eels from the polluted site. This was in agreement with the fact that eels from the reference site showed a higher gonad growth in comparison to contaminated fish.

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1. Introduction

Historically abundant and widespread in Europe, European eels (*Anguilla anguilla*) have suffered a sharp decline and the species' recruitment now represents only one tenth of what it was in the early 1980s (Stone, 2003; ICES report 2013). It is currently considered as critically endangered of extinction by the International Union for Conservation of Nature (IUCN). European eel is a catadromous fish species with a complex life cycle including marine (larval phase, sexual maturation and spawning) and continental (feeding and somatic growth) environments. The unusual life cycle

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of European eels makes them particularly vulnerable to pollution. Reproduction of the species takes place in the Sargasso Sea. Eel larvae drift back to the European coasts following oceanic currents. After metamorphosis of the larvae into glass eels, the organisms reach the juvenile growth phase stage (yellow eel) in continental habitats. During this stage, for up to 20 years, eels feed in order to grow and accumulate fat reserves. Then, yellow eels undergo a second metamorphosis called silvering which prepares the future genitors (silver eels) for their transoceanic reproductive migration (Tesch, 2003; Van Ginneken and Maes, 2005). However, when silver eels leave the European coasts, their gonads are still immature and maturation is blocked at a prepubertal stage (Vidal et al., 2004). This implies that gonad development must occur during the 5500 km transoceanic migration. During the migration, silver eels no longer feed and use their lipid reserves accumulated during the growth phase (yellow eel stage) for swimming and gonad maturation. Finally, this life cycle ends with the death of genitors after reproduction. During the growth phase, yellow eels will accumulate lipids but also metal and organic contaminants present in continental environments (Durrieu et al., 2005; Tapie et al., 2011). The lipid reserves will be remobilized but contaminants could be also remobilized toward gonads during their reproductive migration (Robinet and Feunteun, 2002; Palstra et al., 2006; Pierron et al., 2008). Consequently, a particular emphasis was placed in recent years on the hypothesis that spawner quality might play an essential role in the decline of the European eel. However, despite the increasing awareness that pollution might impair the reproductive success of silver eels, the potential effects of pollution on the eels' gonad development remain poorly known. Investigating the effects of contaminants on developing gonads in future spawners is highly relevant since effects observed at the individual level have implications at the population level.

The aim of this study was to investigate the possible impacts of contaminants on gonad growth as well as the toxic mechanisms of contaminants on gonads during artificial maturation of European female silver eels by means of a transcriptomic approach using a DNA microarray comprising 1000 candidate gene targets (Baillon et al., 2015).

2. Materials and methods

2.1. Experimental design

All procedures used in this experiment were approved by the Aquitaine fish-birds ethics committee. Migrating female silver eels (*Anguillla anguilla*) were captured during their continental downstream migration in winter 2012–2013, in the Arcachon Bay in the Domaine de Certes salt marshes (southwest of France, 44°41′18″N 1°1′39″W) a site considered as "clean", and in the Gironde estuary (45°12′06.62″N 0°43′34.72″O), considered as highly contaminated (Baillon et al., 2015). The animals were transferred to the laboratory (Irstea experimental station), individually marked with pit-tags and randomly mixed in two separate swim tanks (salinity 30‰, natural seawater) thermostated at 15 °C. After at least one month of acclimation period, 5 eels from Gironde and 8 from Certes were removed and dissected.

Remaining eels were then submitted to water current to force them to swim at a speed around 16 cm s^{-1} (Davidsen et al., 2011). To induce gonad maturation, eels received one perivisceral injection per week of CPE without anesthesia at a dose equivalent to 20 mg of pituitary powder/kg body weight (Durif et al., 2006). Sixteen animals, 8 fish per sampling site, were removed for analysis after 11 CPE injections.

At each sampling time, the total length and weight were recorded for each fish in order to estimate the Fulton condition factor (K): (weight (g) $\times 10^5$)/(length (mm)^3). Fish ovaries were weighed to calculate the gonadosomatic index (GSI expressed as a percentage: (gonad weight/total body weight) $\times$ 100). Samples for genetic analyses were stored in RNA later at −20 °C until needed. For both organic and metal analyses, samples were stored at −80 °C.

During the experiment, fish were not fed as eels undergo a natural period of fasting at the silver stage.

2.2. Metal and organic contaminant analyses

Metal analyses and organic contaminants analyses were carried out as previously described in Baillon et al. (2015). Metal concentrations were measured by inductively coupled plasma–mass spectrometry (Thermo Scientific XSeries 2), inductively coupled plasma–atomic emission spectrometry (Varian Vista AX) or both methods. Extraction of the seven indicator PCBs (CB50 + 28, CB52, CB101, CB118, CB138, CB153, and CB180), 14 OCPs (hexachlorobenzene or HCB, lindane or γ-HCH, dieldrin, heptachlor, heptaclorepoxide, cis-chlordane, trans-nonachlor, mirex, and DDTs), and 4 PBDEs (BDE47, BDE99, BDE119, and BDE153) was performed using microwave assisted extraction and analyses were carried out on an HP 5890 series II gas chromatograph coupled to a ^{63}Ni electron capture detector. For PCBs, LoQs were comprised between 0.2 ng/g dw and 2 ng/g dw; for OCPs they were comprised between 0.1 ng/g dw and 0.4 ng/g dw; for PBDEs they were comprised between 0.1 ng/g dw and 0.2 ng/g dw.

2.3. RNA extraction, labeling, and cDNA hybridization

Total RNA was extracted from the gonads of 8 individuals per condition. RNA was extracted from 20 to 25 mg of tissue using the SV total RNA isolation system (Promega) with minor modifications. Briefly, for the first step of reverse-transcription (RT), 15 μg of total RNA were used. After RT reaction, cDNA purification was made with Qiagen PCR purification kit following the manufacturer's protocol. Purified cDNA were then labeled with CyDye™ Post-Labeling Reactive Dye Pack (Cyanine 3 for sample and Cyanine 5 for reference). In order to normalize microarray data, we used a common reference design. The reference was composed by pooling total RNA from liver of 30 wild eels from the clean site; i.e. Certes (15 fish collected in year 2011 and 15 in year 2012). This reference was combined in equal amounts with each sample before to be hybridized on the microarray slide (15 h at 55 °C). A total of 16 microarrays were used, 8 microarrays were performed for each sampling site (i.e. Certes and Gironde). Each sample has been hybridized once on DNA microarray. Data acquisition was carried out by means of the Innoscan 710 microarray scanner (Innopsys) using Mapix software. More details on sample preparation and microarray processing are available in NCBI/Gene Expression Omnibus (GEO) under the accession number GPL19017.

2.4. Statistical analysis

For the contaminant analyses, comparisons among fish groups were performed by analysis of variance (ANOVA), after checking assumptions of normality and homoscedasticity of the error terms. When the assumptions were not met as deduced graphically and from ad-hoc tests, we used box-cox data transformations or the non-parametric Kruskal–Wallis test. If significant effects were detected, the Least Square Deviation (LSD) or U-Mann Whitney tests were used to determine whether means between pairs of samples were significantly different from one another. Computations were performed using STATISTICA version 6.1 software (StatSoft, USA).

Concerning microarrays, the filtering of raw data was done for both green and red channels. The median foreground and median background were used. If the ratio Fmedian/Bmedian was lower than 1.5, data were removed from the analysis. In addition, data from bad replicates spots (three spots for each probe) were excluded from the data set. For each spot, the background was removed of the foreground and the mean of triplicates was then calculated. Finally, both green and red channels were used for data normalization. Normalization and statistical analysis were done by using the BRB-arrayTools version 4.4.0 software package (http://linus.nci.nih.gov/BRB-ArrayTools.html). Each array was median-normalized over entire arrays and genes were excluded if 50% of values were missing in the complete set of arrays. Microarray data have been deposited on the NCBI/Gene Expression Omnibus (GEO) platform under accession number GSE64278. In order to detect differences in transcription profiles among the conditions, data were analyzed by means of the class comparison test available in the BRB software.

Principal Component Analysis (PCA) and Spearman's Rank Correlation Analysis (SRCA) were used to help identifying a set of contaminants or morphometric traits that best described the transcription pattern of the genes that were differentially transcribed between fish from Certes and Gironde. PCA was used here to identify contaminants and morphometric traits from the initial list that were highly correlated with the first two components. Variables that were not strongly correlated with the first few components were excluded from further analysis.

3. Results and discussion

3.1. GSI and pollutant bioaccumulation

Despite no significant difference was observed at T11 between the GSI of fish from Certes and Gironde, the GSI of eels from Certes increased 3.5-fold, compared to 2.4-fold for eels from Gironde between the beginning of the experiment (T0) and after 11 weeks (T11) of artificial maturation. This increase was statistically significant for Certes but not for Gironde (Table 1). This suggests that pollution could have a negative impact on gonad development in female eels, i.e. pollution could impair or delay gonad maturation. However, since Certes and Gironde are two distinct aquatic ecosystems, we cannot rule out an effect of other abiotic or biotic factors. Yet, it is noteworthy that fish from Certes and Gironde did not differ significantly at T0 in term of length, weight or condition index (Table 1), i.e. factors that are known to influence the maturation response of female silver eels (Durif et al., 2006). After 11 weeks of hormonal treatment, only the gonad weight of eels from Certes significantly increased. However, the total weight of eels from the two sites significantly differed at T11. Furthermore, a high variability in GSI for eels from Gironde was observed after 11 weeks of CPE treatment, suggesting a high variability of the response to hormonal treatment in this group. This high variation among individuals from Gironde reflects that some fish had well-developed gonads while others showed no development at all.

Concerning the bioaccumulation levels of contaminants at T11 in ovaries, results are given in term of contaminant content (ng/whole tissue) in order to avoid a weight dilution phenomenon and to compare fish between the two sampling sites (Table 2). However, tissue concentrations, expressed in ng g^{-1} and µg g^{-1} (dry weight) for organic compounds and metals respectively, are given in the Supporting Information (Table S1).

For metals, except for chrome (Cr), nickel (Ni) and arsenic (As), bioaccumulation levels measured in gonads showed significant differences between animals from Certes and Gironde. Non-essential metals such as silver (Ag), cadmium (Cd) and mercury (Hg) showed significantly higher levels in gonads of eels from Gironde compared to fish from Certes. This is in agreement with the fact that the Gironde Estuary is a historically Cd-contaminated ecosystem (Durrieu et al., 2005). Moreover, recent studies have reported high levels of Ag in surface water and suspended particulate matter along the Gironde Estuary (Lanceleur et al., 2013). In contrast to non-essential metals, essential metals such as copper (Cu), selenium (Se) and zinc (Zn) showed significant lower levels in gonads from Gironde compared to Certes. Regarding Cu and Zn, these two metals are known to accumulate in gonads of fish during oocyte growth and maturation (Riggio et al., 2003). Thus, the lower Zn and Cu bioaccumulation levels in eel gonads for Gironde could be a consequence of the lower growth of their gonads in response to

Table 2
Mean persistent organic contaminants (POPs, expressed as ng, dw) and metals (expressed as µg, dw) contents in gonads of female European silver eels sampled in 2 sites (Certes and Gironde) after 11 weeks of hormonal treatment. All data are expressed as means ± SE (n = 8 per site). For each variable, means designated with different letters (a, b) are significantly different (ANOVA, $P < 0.05$). ND = Non-Detectable.

	Certes	Gironde
Metals		
Cr	10.32 ± 0.75[a]	6.94 ± 0.81[a]
Ni	37.79 ± 2.25[a]	34.38 ± 4.48[a]
Cu	41.5 ± 2.56[a]	22.77 ± 2.14[b]
Zn	2424 ± 135[a]	1565 ± 129[b]
As	11.92 ± 0.55[a]	7.93 ± 0.48[a]
Se	33.08 ± 2.35[a]	31.26 ± 2.98[a]
Ag	0.18 ± 0.01[a]	0.44 ± 0.06[b]
Cd	0.17 ± 0.02[a]	0.83 ± 0.11[b]
Pb	0.40 ± 0.05[a]	0.11 ± 0.01[b]
Hg	0.55 ± 0.06[a]	0.76 ± 0.07[b]
POPs		
HCB	34.73 ± 2.82[a]	34.60 ± 4.34[a]
Lindane	30.50 ± 2.61[a]	14.76 ± 1.91[b]
2,4′DDE	14.16 ± 2.39[a]	118.09 ± 16.59[b]
4,4′DDE + dieldrin	78.00 ± 9.42[a]	1140.64 ± 117.41[b]
2,4′DDD	ND	27.38 ± 2.19
4,4′DDD	49.31 ± 6.98[a]	490.02 ± 51.88[b]
2,4′DDT	ND	ND
4,4′DDT	16.57 ± 3.58[a]	114.34 ± 16.46[b]
OCP_sum	239.3 ± 24.4[a]	1657.4 ± 231.9[b]
PBDE_sum	ND	209.69 ± 21.61
PCB_sum	2065 ± 231[a]	15,529 ± 1433[b]

Table 1
Morphometric parameters of female silver eels from Certes and Gironde before (T0) and after (T11) hormonal treatment. All data are expressed as means ± SE (n = 8, except for Gironde T0 where n = 5). For each variable, means designated with different letters (a, b, c) are significantly different (LSD test, $P < 0.05$).

	Certes T0	Gironde T0	Certes T11	Gironde T11
Weight (g)	279.8 ± 20.0[a,b]	284.3 ± 18.4[a,b]	315.9 ± 31.9[a]	237.4 ± 22.8[b]
Length (mm)	561.0 ± 13.8[a]	558.8 ± 10.8[a]	574.3 ± 20.3[a]	531.6 ± 22.8[a]
Fulton condition factor	0.157 ± 0.004[a]	0.162 ± 0.007[a]	0.163 ± 0.003[a]	0.154 ± 0.007[a]
Gonad weight (g)	4.8 ± 0.4[a]	5.6 ± 0.8[a,b]	20.6 ± 3.9[c]	12.2 ± 3.0[b]
GSI (%)	1.74 ± 0.09[a]	1.94 ± 0.21[a,b]	6.19 ± 0.66[c]	4.67 ± 0.1.08[b,c]

Table 3
List of the genes that were differentially transcribed between eels from Certes and Gironde after 11 weeks of hormonal treatment. Results are presented as fold change as compared to eels from Certes. Analyses were carried out by means of the class comparison tool available in the BRB array tools software ($P < 0.01$).

Fold-change	Gene name	Symbol	Biological function
1.85	Forkhead box protein O1	*foxo1*	Tissue development; regulation of cell proliferation; positive regulation of gluconeogenesis
1.61	Ethanolamine-phosphate cytidylyltransferase	*pcyt2*	Biosynthetic process
1.59	COP9 signalosome complex subunit 3	*cops3*	In utero embryonic development; signal transduction
1.52	Phosphoserine aminotransferase	*psat1*	L-serine biosynthetic process
1.49	Ectonucleoside triphosphate diphosphohydrolase 8	*entpd8*	Nucleoside monophosphate biosynthetic process
1.49	Heterogeneous nuclear ribonucleoprotein L-like	*hnrnpll*	Positive regulation of RNA splicing
1.47	Ephrin-A4	*efNA4*	Ephrin receptor signaling pathway
1.47	Adrenodoxin, mitochondrial	*fdx1*	Steroid biosynthetic process; cholesterol metabolic process; hormone biosynthetic process
1.45	GTP-binding protein 1	*gtpbp1*	GTP catabolic process
0.69	Kinectin	*ktn1*	Microtubule-based movement; protein transport
0.61	Golgi resident protein GCP60	*acbd3*	Steroid biosynthetic process, protein transport
0.59	Protein furry homolog	*fry*	Microtubule organization
0.58	Prefoldin subunit 6	*pfdn6*	Protein folding; chaperone-mediated protein complex assembly
0.56	Zinc finger CCCH domain-containing protein 13	*zc3h13*	/
0.55	Ubiquitin carboxyl-terminal hydrolase CYLD	*cyld*	Protein K63-linked deubiquitination; negative regulation of canonical Wnt receptor signaling pathway
0.54	Polyadenylate-binding protein 1	*pabpc1*	Gene silencing by RNA; positive regulation of nuclear-transcribed mRNA catabolic process
0.38	Nardilysin	*nrd1*	Proteolysis

CPE treatment in comparison to fish from Certes. The content of organic contaminants in gonads was significantly higher in animals from Gironde than in Certes, except for lindane and 2,4′DDT, an organochlorinated pesticide and a metabolite of DDT. Nevertheless, total organo-chlorine pesticides (OCPs) and PCBs contents in the ovaries were significantly 6.9- and 7.5-times higher in fish from Gironde in comparison to fish from Certes, respectively (Table 2). Moreover, whereas polybrominated diphenyl ethers (PBDEs) were not detectable in gonads of fish from Certes, PBDEs content reached a mean of 209.69 ± 21.61 ng (dw) in fish from Gironde. As pollutants are correlated among them, it is not possible to discriminate the pollutant that could be mostly responsible for the observed pattern on gonad development. Moreover, previous experimental studies dealing with the impacts of contaminants on fish oocyte development and maturation suggest that both stimulatory and inhibitory are generalized responses (Pierron et al., 2008; Szczerbik et al., 2006; Thomas, 1989; Tilton et al., 2003). Nevertheless, contaminant levels in fish from Certes were much lower than in eels from Gironde, supporting the use of Certes as a relatively pristine site suitable as a reference for this study.

3.2. Gonads microarray analysis

Seventeen genes were found to be differentially transcribed between fish from Certes and Gironde ($P < 0.01$) (Table 3). The first two components (shown in Fig. 1A) represented 72.95% of total variability (52.41% and 20.54% for each it). PCA showed a clear division between genes correlated with GSI or contaminants. Genes associated with GSI were under-expressed in most of the animals from Gironde. These included *polyadenylate-binding protein* (*pabpc1*), *protein furry homolog* (*fry*), *prefoldin subunit* 6 (*pfdn6*), *Golgi resident protein GCP60* (*acbd3*), *ubiquitin carboxyl-terminal hydrolase CYLD* (*cyld*), *Zinc finger CCCH domain-containing protein 13* (*zc3h13*), *kinectin* (*ktn1*) and *nardilysin* (*ndr1*). In contrast, genes associated to contaminants (i.e. modulated by pollution) were over-expressed in animals from Gironde and were particularly associated to OCPs, PCBs, Hg, Cd, Ag and Se. These included *forkhead box protein O1* (*foxo1*), *ethanolamine-phosphate cytidylyltransferase* (*pcyt2*), *GTP-binding protein 1* (*gtpbp1*), *phosphoserine aminotransferase* (*psat1*), *heterogeneous nuclear ribonucleoprotein L-like* (*hnrpll*), *ephrin-A4* (*efNA4*), *ferredoxin mitochondrial* (*fdx1*) and *COP9 signalosome complex subunit 3* (*cops3*). Eels' distribution on these two axes showed two distinct groups including animals from Certes on one side (left side) and animals from Gironde on other side (right side) (Fig. 1B). By projection on the correlation circle (Fig. 1A), the Certes group seemed to be more associated to GSI, i.e. associated to gonad growth and the Gironde group seemed more associated to contaminants. However, two Gironde individuals, Gironde 715 and 713, were placed on the left side of the axis 2. Individual contamination (data not shown) indicated that the Gironde 713 animal contained low tissue concentrations of metals but similar concentrations of POPs in comparison to the other fish from the Gironde estuary. Concerning Gironde 715, this animal presented a high GSI (9.17%) in comparison to the other fish from the Gironde. These findings show that while pollution may be an explanation for some of the results obtained in the present study, it cannot be fully responsible for all of the observed variability and other factors are involved in the response of animals to hormonal treatment.

Concerning genes that were positively correlated with GSI, previous studies carried out in male and female mice showed that the *pabpc1* gene is involved in gametogenesis (Ozturk et al., 2012, 2014). Stable mRNA during the multi-step differentiation from germ cells to gametes is particularly important, and *pabpc1* plays a role in both mRNA stability and longevity (Féral et al., 2001). The *fry* gene plays a role in cell division. The mechanism is not well understood, yet in mammals *fry* seems to regulate the activity of another gene that was down-regulated in eels from Gironde, *ndr1*, which is involved in cellular polarity. More precisely, *ndr1* encodes for a protein which primary role is the alignment of chromosomes on the mitotic spindle during cell division in mammalian cells (Chiba et al., 2009). The *pfdn6* gene is also involved in cell division and in some parts of gametogenesis, notably chromosomal migration to the most distal ends of the cell during gonad development (Lundin et al., 2008). The *abcd3* gene is known to be involved in steroidogenesis. Cholesterol, a building block for the synthesis of steroid hormones, must enter the mitochondria before a series of reactions which transform it into progesterone. The transfer of cholesterol into the mitochondrion is regulated by the protein encoded by the *abcd3* gene (Fan et al., 2010). This step allows the synthesis of a complex macromolecular signal which drives

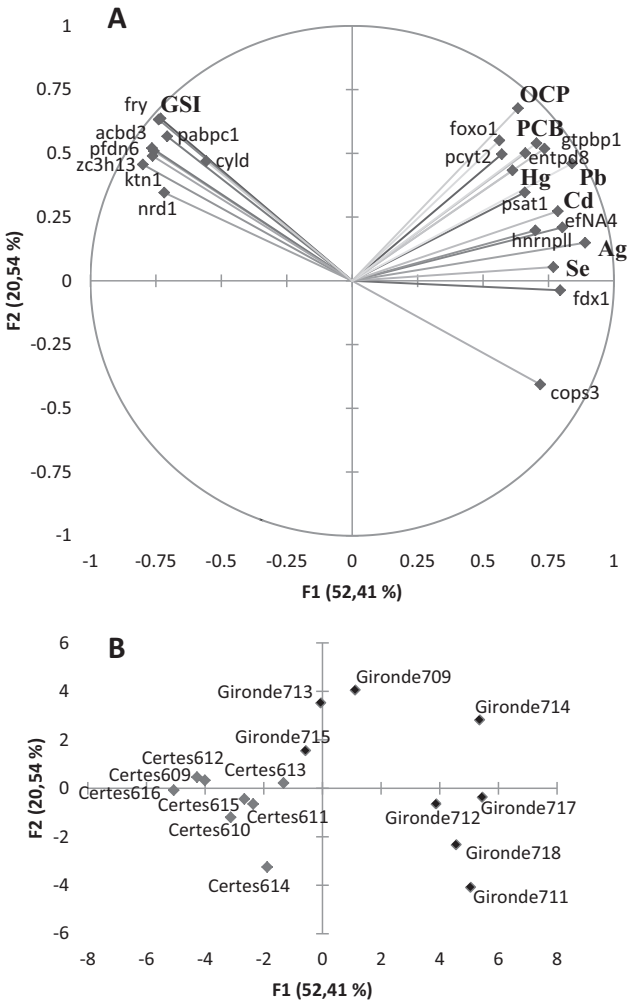

Fig. 1. Principal Component Analysis (PCA): (A) relationships between the transcription level of the 17 genes that were differentially transcribed between fish from Certes and Gironde and organic contaminant or metal contents or fish GSI. Scores for the principal components 1 and 2 were 52.41% and 20.54% respectively. (B) Screening of animals from Certes and Gironde on the principal components 1 and 2. See text and Table 3 for more details.

cholesterol metabolism. Translation of the *ubiquitin carboxyl-terminal hydrolase* (*cyld*) mRNA produces a protein known to regulate spermatogenesis and fertility in male mice (Wright et al., 2007). This gene was also found to be expressed in the ovaries of *Drosophila melanogaster* (Tsichritzis et al., 2007). The *kinectin* gene (*ktn1*) is involved in the transport of various cellular organelles and vesicles along the microtubules in the cytoplasm. Despite the paucity of research on the *zc3h13* gene, one article by Vizeacoumar et al. (2013) shows that it plays a key role in chromosome segregation during the cell cycle. Overall, the genes that appeared associated to GSI in this study are generally involved in mitotic cell division. This could be linked to a proliferation of follicle cells but also to a resumption of meiosis since some of the genes that were identified are involved in chromosome segregation, i.e. a mechanism that occurs during both mitosis and meiosis. The higher GSI of animals from Certes compared to Gironde corroborates this possibility.

Regarding genes which transcription levels were correlated to the contaminants, many studies have shown that these genes encode for proteins involved in cell membrane repair, oxidative stress response, cell division and apoptosis (Essers et al., 2004). The *pcyt2* gene for example plays a role in membrane fusion and assembly of contractile rings which allow separation into two daughter cells via the biosynthesis of phosphatidyl ethanolamine (Pavlovic and Bakovic, 2013). The *hnrnpll* gene encodes for a protein known for its role in RNA splicing and telomere biogenesis, but more so for its role in DNA repair (Han et al., 2013). The expression of *psat1* activates biosynthesis of serine, an amino acid. A recent study showed that overexpression of this gene is a marker of ovarian cancer in humans (Toyama et al., 2012). Interestingly, strong and significant correlations (Spearman coefficient > 0.8; $P < 0.05$) were observed among the transcription levels of *entpd8*, *efNA4* and *gtpbp1*. This indicates that they might be co-regulated and implicated in a common biological process. The purinergic signaling pathway could be one of these processes (Fig. 2). ATP and ADP (adenosine tri- and diphosphate) are intermediates secreted into the extracellular space by a cell exposed to stimuli (pH, UV, reactive oxygen species; Burnstock, 2014), to communicate with adjacent cells. Here, these messages could be taken up by the enzyme ectonucleoside triphosphate diphosphohydrolase (ENTPDase) and bound to purine receptors in the membrane of a neighboring cell. Among ENTPDase, the gene *entpd8* was up-regulated in eels from Gironde. The *entpd8* gene encodes for a transmembrane protein that hydrolyzes ATP into ADP (Kojima et al., 2011). Both ATP and ADP could activate purinergic membrane receptors coupled to G proteins. The latter are implicated in numerous signaling processes, including the activation of the PI3K/Akt signal. Among the genes encoding for G proteins, the gene *gtpb1* was overexpressed in contaminated eels. G protein activation initiates an activation cascade of phosphatidylinositol-3 kinase (PI3K), phosphatidylinositol (3,4,5)-triphosphate (PIP3) and ultimately phosphorylation of a protein kinase, Akt (Roy et al., 2010). In its phosphorylated form, Akt protein adds a phosphate group to the transcription factor FOXO1 which is present in the nucleus. FOXO1 is then inactivated and a protein binds to it allowing its passage from the nucleus to the cytoplasm where it is degraded by the proteasome. In animal gonads, the primary functions of FOXO1 are to stop the cell cycle, initiate apoptosis, regulate glucose homeostasis and activate the production of manganese superoxide dismutase (SODMn), an important antioxidant defense (Essers et al., 2004). More recently, FOXO1 was shown to serve vital functions in gonads and to contribute to oocyte maintenance in mammals (Tarnawa et al., 2013). In addition, the gene ephrin-A4 (*efNA4*) was overexpressed in fish from Gironde. The protein product of this gene binds to a membrane receptor inducing two parallel processes both of which result in the inhibition of the transcription factor FOXO1. The activation of the ephrin receptor will simultaneously induce IRS-1 (insulin receptor substrate 1) and RAS (G protein) both of which activate PI3K and inhibit FOXO1. Thus, several genes involved in signaling pathways that lead to FOXO1 inactivation were up-regulated in fish from Gironde. In contrast, the gene encoding FOXO1 was significantly up-regulated in eels from Gironde. It is important to keep in mind that the processes presented above are described at the protein level while the present study was done on mRNA. Quantification of the FOXO1 protein would provide further insight into why increased gene expression is occurring at the same time as an increase in the transcription level of genes involved in pathways that are known to trigger FOXO1 inactivation. Moreover, an increase in the transcription level of a gene encoding for a protein that activates FOXO1 was also observed. The gene coding for the protein COPS3 was overexpressed. This protein acts on the PI3K/Akt signaling process by inhibiting Cullin2 which has among its roles to regulate the IRS1 protein by causing its ubiquitination and ultimately its degradation by the proteasome (Hartmann et al., 2013). The activation of this metabolic pathway can lead to activation of the FOXO1 transcription factor. We hypothesize that contamination disrupts many pathways implicated in the regulation of FOXO1 activity, and thus ultimately in defense mechanisms against oxidative stress, cell cycle arrest and energy metabolism regulation.

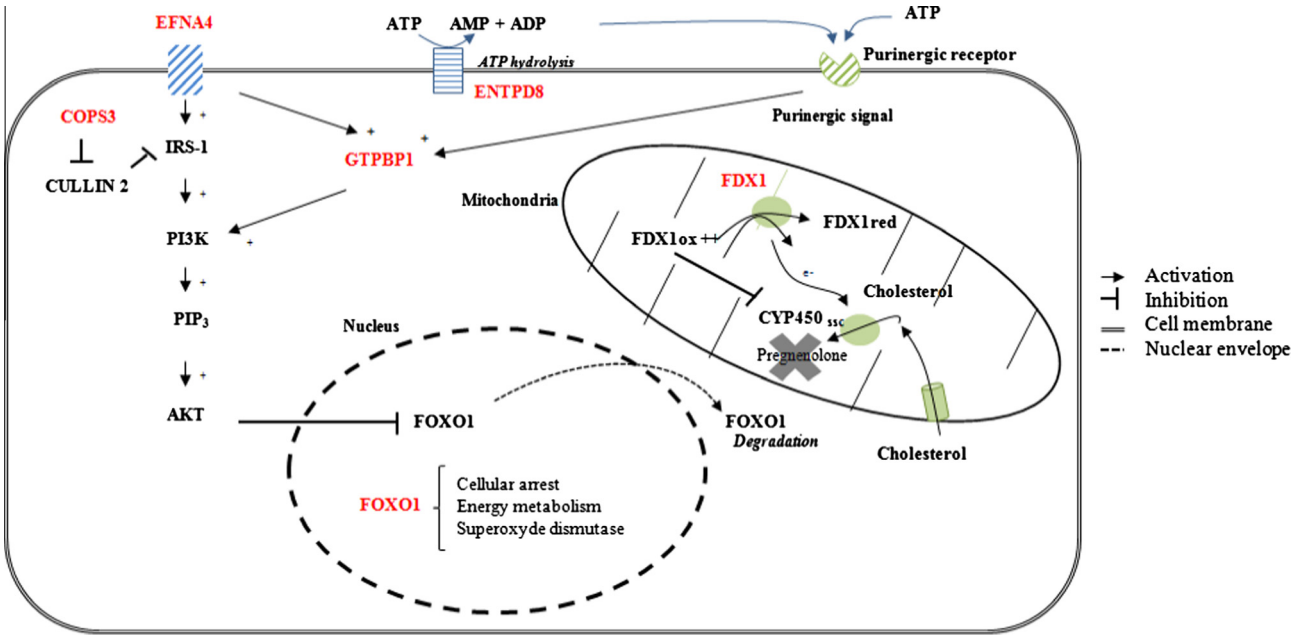

Fig. 2. Schematic diagram presenting the possible biological pathways affected by contaminants in gonads of female European silver eel. The genes that were down-regulated in eels from Gironde in comparison to fish from Certes are highlighted in red (efNA4: Ephrin-A4, entpd8: Ectonucleoside Triphosphate Diphosphohydrolase 8, P2X/P2Y: purinergic receptor, Cops3: signalosome complex subunit 3, gdpbp1: GTP Binding Protein 1, IRS-1: Insulin Receptor Substrate 1, PI3K: phosphatidylinositol-3 kinase, PIP3: Phosphatidylinositol (3,4,5)-trisphosphate, Akt: protein kinase B, foxo1: Forkhead box protein O1, fdx1: ferredoxin mitochondrial, fdx1, FDX1 red: reduced form of ferredoxin, FDX1ox: oxidized form of ferredoxin, CYP450scc: cytochrome P450scc). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Sexual maturation could also be disrupted by contamination causing interference with sex hormone synthesis. One step in steroid hormone biosynthesis takes place in the mitochondrion, by the enzyme cytochrome P450scc (also called CYP11A1). This enzyme (Fig. 2) converts cholesterol to pregnenolone which is rapidly transformed into progesterone (Tuckey et al., 2001), a precursor of estradiol. A gene involved in steroidogenesis and in the activity of cytochrome P450scc was overexpressed in contaminated eels, the *ferredoxin mitochondrial* gene (*fdx1*). While FDX1 is known for its role in steroid hormone biosynthesis by participating in the reduction of the mitochondrial cytochrome P450, an excess of it in mitochondria will inhibit the cytochrome P450scc activity. Tuckey et al. (2001) showed that in its oxidized form, *ferredoxin* inhibits the cytochrome P450scc through competitive inhibition with cholesterol.

4. Conclusion

Among genes that were differentially regulated between fish from Certes and Gironde, genes which transcription levels were more associated with contaminants were involved in the protection against oxidative stress, DNA repair, in the purinergic pathway but also in steroid hormones synthesis and in oocyte maintenance. An effect of contaminants on such pathways in eel gonads could impair their development and ultimately, the reproductive capacity of animals. This study, with previous studies carried out on female silver eels (Palstra et al., 2006; Pierron et al., 2008, 2009; see Geeraerts and Belpaire, 2009 for review), corroborate the fact that contamination could contribute to the decline of the European eel by impairing their reproduction.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.06.007.

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