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Abnormal Ovarian DNA Methylation Programming during Gonad Maturation in Wild Contaminated Fish

Fabien Pierron,*[,†,‡] Sarah Bureau du Colombier,[§] Audrey Moffett,[∥] Antoine Caron,[∥] Laurent Peluhet,[†,‡] Guillemine Daffe,[†,‡] Patrick Lambert,[§] Pierre Elie,[§] Pierre Labadie,[†,‡] Hélène Budzinski,[†,‡] Sylvie Dufour,[⊥] Patrice Couture,[∥] and Magalie Baudrimont[†,‡]

[†]University of Bordeaux, EPOC, UMR 5805, F-33400 Talence, France

[‡]CNRS, EPOC, UMR 5805, F-33400 Talence, France

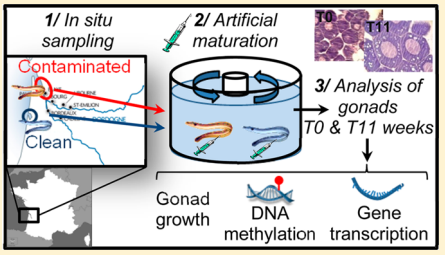
[§]Irstea, UR EABX, 50 avenue de Verdun, 33612 Cestas, France

[∥]Institut National de la Recherche Scientifique, Centre Eau Terre Environnement, 490 de la Couronne, Québec (Québec) G1K 9A9 Canada

[⊥]Muséum National d'Histoire Naturelle, UMR BOREA UPMC, CNRS 7208, IRD 207, UCBN, 7 rue Cuvier CP 32, F-75231 Paris, France

Supporting Information

ABSTRACT: There is increasing evidence that pollutants may cause diseases via epigenetic modifications. Epigenetic mechanisms such as DNA methylation participate in the regulation of gene transcription. Surprisingly, epigenetics research is still limited in ecotoxicology. In this study, we investigated whether chronic exposure to contaminants experienced by wild female fish (*Anguilla anguilla*) throughout their juvenile phase can affect the DNA methylation status of their oocytes during gonad maturation. Thus, fish were sampled in two locations presenting a low or a high contamination level. Then, fish were transferred to the laboratory and artificially matured. Before hormonal treatment, the DNA methylation levels of the genes encoding for the aromatase and the receptor of the follicle stimulating hormone were higher in contaminated fish than in fish from the clean site. For the hormone receptor, this hypermethylation was positively correlated with the contamination level of fish and was associated with a decrease in its transcription level. In addition, whereas gonad growth was associated with an increase in DNA methylation in fish from the clean site, no changes were observed in contaminated fish in response to hormonal treatment. Finally, a higher gonad growth was observed in fish from the reference site in comparison to contaminated fish.



INTRODUCTION

Epigenetics is the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA code.[1−4] In eukaryotic cells, DNA is packaged into chromatin. Covalent modifications upon histones, the primary protein component of chromatin, or upon the DNA molecule itself, can influence the transcription level of neighboring genes and by extension the phenotype of organisms. One of the most studied epigenetic mechanisms is DNA methylation. DNA methylation, which refers to the addition of a methyl group to cytosine to form the 5-methylcytosine base, is involved in fundamental biological functions such as development, cell differentiation and silencing of transposable elements.[2] There is increasing evidence that environmental factors such as nutrition, maternal care, temperature or exposure to pollutants can trigger defects or unintended changes in the DNA methylation status of cells.[2,5,6] Epigenetic modifications that occur in response to environmental factors have a special significance in germ cells. Indeed, such epigenetic errors can not only have a direct impact on how genes are expressed in oocytes but also later in embryos.[2,4,7,8] These epigenetic modifications can lead to negative health outcomes such as embryonic lethality, cancer, diabetes, and infertility.[2,5,6,9] Nowadays, most of the current knowledge relating to such epigenetic effects of contaminants comes from rodent models and experimental investigations during which exposure to contaminants was carried out during embryogenesis. Such data are currently lacking for nonmammalian species and very limited in ecotoxicology.[1,3,4]

Therefore, the aim of the present study was to test whether environmental pollution can influence the DNA methylation status of oocytes in an environmentally relevant aquatic species, the European eel (*Anguilla anguilla*), during gonad maturation. The European eel is currently considered critically endangered of extinction by the International Union for Conservation of Nature. Reproduction of the European eel takes place in the Sargasso Sea from where larvae drift back toward the European coasts following oceanic currents. After metamorphosis of the

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larvae into glass eels, the organisms reach the juvenile growth phase stage (yellow eel) in continental habitats. This stage can last from several years to more than 20 years, depending on the sex and hydrosystems, and ends with a second metamorphosis called silvering which prepares the future genitors (silver eels) for their transoceanic reproductive migration.[10,11] However, when silver eels leave the European coasts, their gonads are still immature and maturation is blocked at a prepubertal stage.[12] This implies that gonad development must occur during their 5500 km migration, that is, a 5−6 months period, marked by swimming activity and fasting that ends with the death of genitors after reproduction. Such a peculiar life cycle makes them particularly vulnerable to pollution. Indeed, during their long somatic growth phase, fish must store large lipid reserves to achieve their reproductive migration at the silver stage. Concomitantly, fish accumulate large quantities of persistent contaminants.[13,14] Thereafter, since silver eels do not eat during their transoceanic migration, contaminants previously accumulated can be massively remobilized and redistributed to their gonads.[15,16] Gonads of female European eels could be then especially prone to epigenetic modifications. As oocyte growth and maturation were found to be associated with de novo DNA methylation establishment in mammals and zebrafish,[2,8,17,18] pollutants could impair the DNA methylation reprogramming that occurs during gonad development.

In this view, migrating female silver eels were sampled in two different locations on the basis of their known gradient of contamination by metallic and organic pollutants. Then, in order to mimic the reproductive migration of European eels, fish were transferred to the laboratory where they were forced to swim in uncontaminated seawater and artificially matured. Although artificial reproduction of the European eel still remains a challenge, full gonadal development of females can be induced by long-term hormonal treatment with injections of carp pituitary extract (CPE).[19] In order to test whether pollutants could impair the DNA methylation reprogramming event that occurs during early gametogenesis, before and after 11 weeks of hormonal treatment, the methylation level of genes involved in both silvering (i.e., the second metamorphosis of the European eel) and early gonad development was investigated by means of methylation-dependent enzymatic restriction coupled with quantitative PCR analysis. We measured the methylation level of the gene encoding for (i) the receptor of the follicle stimulation hormone (*fshr*), a pituitary hormone involved in oocyte growth and differentiation,[20] (ii) the aromatase (*aro*), the enzyme that converts androgens into estrogens,[21] and (iii) the corticosteroid 11-beta-dehydrogenase isozyme 2 (*11β-hsd*), a steroidogenic enzyme involved in the synthesis of 11-ketotestosterone, an hormone that was recently shown to induce silvering and the early stages of oocyte development in Japanese eels.[22] In this view, we must note that unlike other teleosts, which possess duplicated genes for aromatase, cyp19a1a and cyp19a1b, expressed in the gonads and in the brain, respectively, eel species possess a single cyp19a1.[23] In parallel, the contamination levels of eel gonads for 19 organic pollutants and 10 metals was determined. Finally, the analysis was completed by the determination of the gene transcription level of *fshr*, *aro*, and *11β-hsd*.

MATERIALS AND METHODS

1.1. Experimental Design. All procedures used in this experiment were approved by the Aquitaine fish-birds ethic committee. Female silver eels (*Anguillla Anguilla*) were captured in the southwest of France in the Domaine de Certes salt marshes (located in the Arcachon Bay; 44°41′18″N 1°1′39″W) and the Gironde estuary (45°12′06.62″N 0°43′34.72″O) during their continental downstream migration in winter 2012−2013. The animals were transferred to the laboratory (Irstea experimental station, Saint-Seurin-sur-l'Isle), individually marked with pit-tags and randomly distributed in two separate swim tanks (salinity 30, natural seawater) thermostated at 15 °C. After at least one month of acclimation, five eels per sampling site were removed and dissected (T0 samples).

Remaining eels were then submitted to a water current forcing them to swim at a speed around 16 $cm.s^{-1}$.[24] To induce gonad maturation, eels received one perivisceral injection per week of CPE without anesthesia at a dose equivalent to 20 mg pituitary powder/kg body weight, according to a method previously described.[19] Sixteen animals, eight fish per sampling site, were removed for analysis after 11 weekly CPE injections (T11 samples).

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 needed for histology and genetic analyses were collected according to a standardized method, in the middle part of the ovaries. Samples for genetic analyses were stored in RNA later at −20 °C until further processing. For both organic and metal analyses, samples were stored at −80 °C.

During the two steps (i.e., acclimation and hormonal treatment), fish were not fed as eels undergo a natural period of fasting at the silver stage.

1.2. Histology. Ovary samples were immediately fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, cut in sections (thickness 4 μm), rehydrated, and stained with the HES method (hematotxylin-eosin-saffron). Oocytes were staged according to the method described in Takashima and Hibiya.[25] To determine the frequency of different oocytes types, oocytes at each stage were counted manually in 10 fields at 20× magnification. The relative abundance of germ cell stages in the ovary was then calculated as follow: [(number of oocytes at stage II or III)/(total number of oocytes)] × 100.

1.3. Metal analyses. Gonad samples were freeze-dried in PFA vials (Savillex) and kept in these vials during the whole digestion process. Subsamples of 50 mg were processed in a mercury analyzer (Milestone DMA-80) and MESS-3 certified reference material (National Research Council Canada) was used, with a recovery rate of 98.8 ± 2.5%. The rest of the samples was digested overnight at room temperature in pure trace metal grade nitric acid (HNO_3). Sample digestion was completed by autoclave (All American 25X-1) for 2 h at 15 PSI. Samples were then diluted in Milli-Q water to obtain a final concentration of 10% HNO_3. Metal concentrations for Ag, As, Cd, Cr, Cu, Ni, Pb, and Zn were measured by inductively coupled plasma−mass spectrometry (Thermo Scientific XSeries 2), inductively coupled plasma−atomic emission spectrometry (Varian Vista AX) or both methods. Blanks and standards (TORT-2 and DOLT-4, National Research Council Canada) were submitted to the same digestion protocol as gonad samples to monitor the efficiency of the procedure. Recovery rates were: Ag = 93.4 ± 10.3%; As = 99.8 ± 6.1%; Cd = 105.1 ± 5.8%; Cr = 76.6 ± 16.9%; Cu = 98.7 ± 2.6%; Ni = 97.6 ± 3.8%; Pb = 92.1 ± 13.1%; Se = 98.9 ± 6.2; Zn = 105.0 ± 2.2% (mean ± SD, n = 10).

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Table 1. Mean Concentrations of Organic Pollutants (Expressed as ng·g^{-1}, dw) and Metals (Expressed as μg·g^{-1}, dw) in Gonads of Female Silver Eels from Certes and Gironde before Hormonal Treatment (T0)[a]

		origin	
		certes	gironde
organic pollutants	PCB 50 + 28	2.42 ± 0.13 [a]	16.15 ± 4.18 [b]
	PCB 52	8.8 ± 0.22 [a]	55.9 ± 12.82 [b]
	PCB 101	8.53 ± 0.63 [a]	74.54 ± 16.00 [b]
	PCB 118	14.26 ± 1.64 [a]	102.45 ± 24.82 [b]
	PCB 153	41.66 ± 9.17 [a]	517.31 ± 125.49 [b]
	PCB 138	29.05 ± 6.47 [a]	385.94 ± 102.72 [b]
	PCB 180	18.33 ± 6.14 [a]	347.9 ± 98.37 [b]
	Sum PCB	123.1 ± 23.4 [a]	1500.2 ± 366.0 [b]
	HCB	1.64 ± 0.16 [a]	2.53 ± 0.32 [b]
	Lindane	1.26 ± 0.04	1.07 ± 0.09
	2,4′DDE	1.17 ± 0.08 [a]	6.16 ± 1.59 [b]
	4,4′DDE	7.87 ± 3.43 [a]	143.92 ± 40.29 [b]
	2,4′DDD	ND	1.96 ± 0.37
	4,4′DDD	5.63 ± 3.76 [a]	43.77 ± 6.52 [b]
	4,4′DDT	1.26 ± 0.8 [a]	9.31 ± 1.65 [b]
	Sum OCP	18.83 ± 5.97 [a]	208.74 ± 42.68 [b]
	PBDE 47	ND	11.49 ± 2.71
	PBDE 49	ND	0.68 ± 0.14
	PBDE 100	ND	5.02 ± 1.31
	PBDE 153	ND	0.31 ± 0.01
	PBDE 154	ND	0.51 ± 0.08
	∑PBDE	ND	17.85 ± 4.19
metals	Cr	0.52 ± 0.03	0.61 ± 0.05
	Ni	0.36 ± 0.05	0.52 ± 0.06
	Cu	1.35 ± 0.12	1.53 ± 0.07
	Zn	186.1 ± 19.5	169.0 ± 27.7
	As	1.51 ± 0.15	1.4 ± 0.41
	Se	1.23 ± 0.1 [a]	2.33 ± 0.35 [b]
	Ag	0.007 ± 0.002 [a]	0.061 ± 0.047 [b]
	Cd	0.013 ± 0.005 [a]	0.094 ± 0.020 [b]
	Pb	0.012 ± 0.002	0.012 ± 0.002
	Hg	0.009 ± 0.001 [a]	0.026 ± 0.004 [b]

[a]All data are expressed as means ± SE (n = 5 at T0). Means designated with different letters are significantly different (LSD test, $P < 0.05$). ND = non-detectable.

1.4. Organic Contaminant Analyses. Analysis 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) were performed on gonad samples following the procedures described by Tapie et al.[26,27] Lipids were determined by gravimetry after filtration and evaporation of an aliquot of the DCM extract. Persistent organic pollutants (POPs) were extracted 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 all the analyses, POPs were quantified relative to internal standards. Quality control consisted of the analysis of solvent procedural blanks, reproducibility and repeatability tests, injection of standard solutions as unknowns and analysis of certified reference material SRM 2974a (National Institute of Standards and Technology) for PCBs, OCPs, and PBDEs. The recoveries for five replicates on this SRM were between 70% and 110% with reproducibilities ranging from 10% to 20% depending on the compounds. As described in Labadie et al.,[28] POPs levels were blank corrected and the method detection limit (LoD) was derived from the blank value variability. 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.

1.5. Quantitative PCR Analysis of DNA Methylation. To design specific primer pairs, coding sequence (cds) from *Anguilla japonica* were obtained from Genbank for cytochrome P450 aromatase (*aro*; AY540622.1), follicle-stimulating hormone receptor (*fshr*; AB360713.1), Corticosteroid 11-beta-dehydrogenase isozyme 2 (*11β-hsd*; AB061225) and non-LTR retrotransposon UnaL2 (*Unal2*, AB179624). These sequences were compared and aligned to the genome of *Anguilla anguilla*[29] using Blast algorithm and BioEdit program. As the de novo DNA methylation event that occurs in mammals during oocyte growth is predominately associated with CpG rich regions,[7,8] genomic sequences from *Anguilla anguilla* were screened for CpG islands by means of the CpG island searcher program.[30] Finally, specific primer pairs were designed in sequences presenting high content of CpG using Primer3Plus software (see Supporting Information (SI), Table S1 and Figure S1). All primers used in this study were confirmed to amplify a single product of appropriate size.

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Genomic DNA from gonad tissue was isolated by overnight digestion with proteinase K, followed by standard phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The pellet was resuspended in 100 μL of water and treated with RNase A (2 mg.mL^{-1}) at 37 °C during 1 h. DNA was de novo treated with phenol/chloroform and precipitated with absolute ethanol. The pellet was resuspended in 100 μL of water. DNA methylation was monitored by qPCR analysis of untreated DNA and McrBC-digested DNA using a method previously described.[30] Genomic DNA (2 μg) was digested overnight at 37 °C with 20 U (2 μL) of McrBC endonuclease (New England Biolabs) in 100 μL total volume including 10 μL of NEB buffer 2 10×, 1 μL of BSA 100× (final concentration 1 μg.ml^{-1}), 1 μL of GTP 100× (final concentration 1 mM) and 66 μL of water. Reactions were stopped by heat treatment (65 °C, 25 min). To avoid variation in DNA recovery, no further purification was performed prior to qPCR. PCR reactions were then performed in an MX3000P (Stratagene; 95 °C for 10 min, followed by 40 cycles of 95 °C for 1 min and 60 °C for 30 s and 72 °C for 1 min). Each 25 μL reaction contained 12.5 μL of GoTaq qPCR master mix (Promega), 5 μL template (100 ng of DNA) and the specific primer pairs at a final concentration of 250 nM each. Digestion by McrBC was quality controlled by qPCR analysis of retrotransposon *UnaL2*. Retransposon-like sequences are known to be highly methylated even during gametogenesis to maintain them in a silenced state.[7] All samples displayed a delta Ct value for Unal2 superior to 7 (>99% depletion of the amplified region in the McrBC-digested portion relative to untreated DNA). For each sample and each amplicon, a delta Ct value corresponding to $Ct_{McrBC} - Ct_{untreated\ DNA}$ was calculated. A proxy of DNA methylation level was calculated as follow: $[1-1/2^{(Ct\ McrBC - Ct\ untreated\ DNA)}] \times 100$ as previously described in Ordway et al.[31]

1.6. Quantitative PCR Analysis of Gene Transcription Level. For each gene, specific primers previously designed to amplify exonic DNA regions were used. Total RNA was extracted from 20 mg of gonad and treated with DNaseI using the SV total RNA Total RNA Isolation System (Promega), according to the manufacturer's instructions. For each sample, RNA quality was evaluated by electrophoresis on a 1% agarose gel and RNA concentrations as well as purity were determined by spectrophotometry (Take3, Epoch, Biotek). First-strand cDNA was then synthesized from 2.5 μg of total RNA using the GoScript Reverse Transcription System (Promega), according to the manufacturer's instructions. Following the reverse transcriptase reaction, cDNA was diluted 10-fold. Real-time PCR reactions were then performed as described above. Amplification efficiencies for all primer sets were checked by making serial dilutions of cDNA; all values proved to be sufficient to allow direct comparison of amplification plots. In addition the reaction specificity was determined for each reaction from the dissociation curve of the PCR product. Relative quantification of gene expression was achieved by concurrent amplification of the *β-actin* endogenous control. Hence, during our experiment, total RNAs were quantified and 2.5 μg was used to be reverse-transcribed. During the subsequent qPCR amplifications, the output cycle corresponding to the *β-actin* was examined.

1.7. Statistical 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 nonparametric 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). Numerical results are given as means ± SE.

RESULTS AND DISCUSSION

As shown in Table 1, at the beginning of the experiment (T0), gonads of female silver eels from the Gironde estuary were generally more contaminated in both metals and organic pollutants than those of animals from Certes. Concerning organic pollutants, with exception of lindane for which no significant difference was observed between the two sampling sites, concentrations of contaminants were significantly higher in fish from Gironde in comparison to fish from Certes. Concerning metals, concentrations in Se, Ag, Cd, and Hg were significantly higher in fish from Gironde in comparison to fish from Certes. For the other metals, i.e. Cr, Ni, Cu, Zn, As, and Pb, no significant difference were observed between fish from Gironde and Certes.

Concerning gonad growth, the hormonal treatment was more effective to induce gonad development in fish from Certes, that is, fish from the cleanest site, in comparison to fish from Gironde (Figure 1A). Although there was no difference in fish GSI

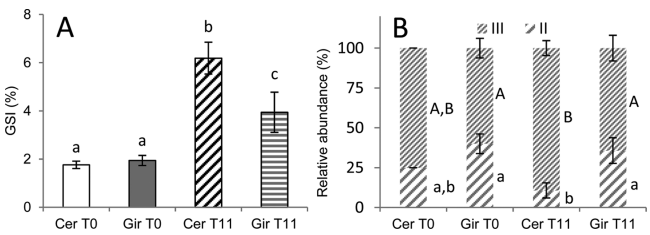

Figure 1. Mean GSI (A) and relative abundance of germ cell stages in the ovary (B) of female silver eels from Certes (Cer, clean site) and Gironde (Gir, contaminated site) before (T0) and after (T11) hormonal treatment. Stage II: previtellogenic oocyte, oocyte with chromatin nucleolus with strongly basophilic cytoplasm and no or few oil droplets; stage III: cortical alveolus stage, oocyte with oil droplets in the cytoplasm but not strongly basophilic system. Values are represented as mean percentages ± SE, n = 5 at T0 and n = 8 at T11. Means designated with different letters are significantly different, Anova and Krukal-Wallis tests, $P < 0.05$.

between the two sampling sites at the beginning of the experiment (T0), the GSI of fish from Certes was significantly higher than in fish from Gironde at the end of the experiment (T11). This assumption is further supported by histological investigations (Figure 1B). Whereas no differences were observed in the relative abundance of oocytes at stage II and III at T0 between fish from Certes and Gironde, after CPE treatment, oocytes of fish from Certes were more mature with a significantly higher proportion of Stage III oocytes and a lower proportion of Stage II oocytes in comparison to fish from Gironde. This could indicate a detrimental effect of pollution on gonad development. Note that fish from Certes and Gironde did not differ at T0 and at T11 in term of length, weight, condition index or lipid content in muscle (Table 2), that is, factors that are known to influence the maturation response of female silver eels.[11,19] Only the gonad weight of eels from Certes significantly increased between T0 and T11. Obviously, as Certes and Gironde are two distinct aquatic ecosystems, an effect of other abiotic or biotic factors cannot be ruled out.

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Table 2. Morphometric Parameters of Female Silver Eels from Certes and Gironde Before (T0) and After (T11) Hormonal Treatment[a]

	T0		T11	
	certes	gironde	certes	gironde
weight (g)	290.7 ± 21.1 [a]	284.3 ± 18.4 [a]	315.9 ± 31.9 [a]	269.9 ± 46.2 [a]
length (mm)	575.0 ± 14.2 [a]	558.8 ± 10.8 [a]	574.3 ± 20.3 [a]	548.5 ± 24.9 [a]
gonad weight (g)	5.0 ± 0.4 [a]	5.6 ± 0.8 [a]	20.6 ± 3.9 [b]	13.5 ± 4.0 [a,b]
fulton condition factor	0.152 ± 0.003 [a]	0.162 ± 0.007 [a]	0.163 ± 0.003 [a]	0.154 ± 0.007 [a]
lipid percentage in muscle	57.2 ± 1.6 [a]	58.7 ± 2.1 [a]	57.3 ± 1.1 [a]	60.8 ± 1.7 [a]

[a]All data are expressed as means ± SE (n = 5 at T0 and n = 8 at T11). Means designated with different letters are significantly different (LSD test, P < 0.05).

Concerning the DNA methylation level of genes involved in the early development of gonads in eels, the methylation level of the exon 1 of the *fshr* gene and the promoter of the *aro* gene was found to be significantly higher in fish from Gironde in comparison to fish from Certes (1.7- and 1.4-fold, respectively) at the beginning of the experiment (T0, Figure 2). No differences

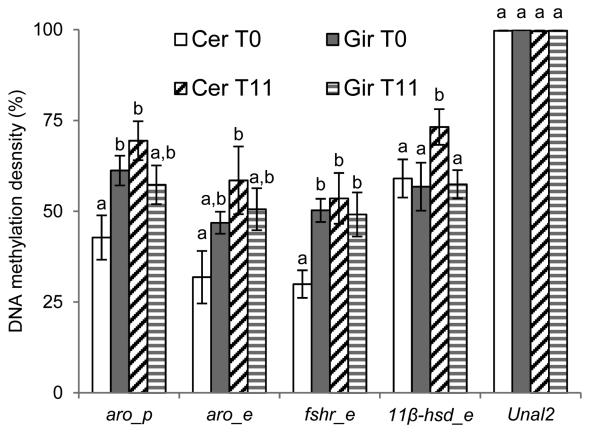

Figure 2. Means of DNA methylation densities of *aro*, *fshr*, *11β-hsd* genes and *Unal2* transposon in gonads of female silver eels from Certes (Cer, clean site) and Gironde (Gir, contaminated site) before (T0) and after (T11) hormonal treatment. p refers to promoter region and e to exonic region. Values are represented as mean percentages ± SE, n = 5 at T0 and n = 8 at T11. Means designated with different letters are significantly different, LSD test, P < 0.05.

were observed for the exonic regions of the *aro* and the *11β-hsd* genes. Moreover, highly significant correlations were observed between the DNA methylation level of the *fshr* gene and the pollutant content of gonads in fish sampled at T0, thus suggesting an effect of pollution on the DNA methylation level of *fshr*. The highest correlations (Spearman's rank correlation coefficient (ρ) > 0.9, P < 0.001) were observed with a metabolite of DDT, that is, 4,4′DDE, Hg, PCB 118 and Cd. An example of correlation is presented in Figure 3. As pollutants are correlated among them, it is not possible to discriminate the pollutant that could be mostly responsible for the observed pattern. Moreover, previous studies dealing with the effect of environmental contaminants on DNA methylation in fish suggest that both hypomethylation and hypermethylation are generalized responses.[1,32−35] In addition, these results must be viewed with caution because any differences in other factors between sites are likely to show relationship through a confounding effect. For example, the correlations between the DNA methylation level of the *fshr* gene and the Hg content of gonads in fish sampled at T0 (Figure 3) did not appear significant if the analyses were carried

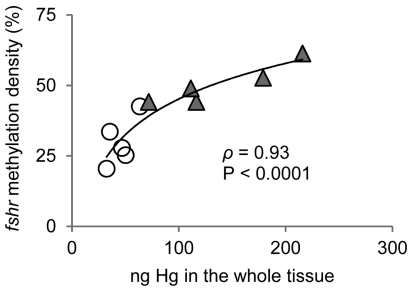

Figure 3. Relationships between the methylation density of the *fshr* gene and the Hg content of gonads of female silver eels from Certes (white circles) and Gironde (gray triangles) before hormonal treatment (T0). The Spearman coefficient (ρ) and level of significance (P) of the correlation are reported on the plots (n = 10).

out independently across individuals from each site (ρ = 0.6 with P = 0.284 and ρ = 0.82 with P = 0.088 in fish from Certes and Gironde, respectively). This could be also linked to the low number of biological replicates per site. Nevertheless, it is important to note that in our case such mechanisms occurred in juvenile organisms. European eel is a panmictic species that reproduces in the Sargasso Sea (~5500 km from Europe). Then, larvae drift back toward the European coasts before being randomly dispersed in coastal and continental habitats. Genetic studies failed to detect obvious spatial genetic differentiation of individuals across Europe.[11,36] Thus, our results support the hypothesis that chemical stressors can affect DNA imprinting in gonads whether during the somatic growth phase of eels (yellow eel stage) when sexual differentiation and contamination occur[37] or during the silvering process (i.e., the second metamorphosis) when a moderate development of gonads is observed (GSI increases from 0.5% to 2%).[19] This could imply that experiences in individual's life can have consequences on the reproductive capacities of animals via epigenetic modifications.

Thereafter, whereas hormonal treatment triggered a significant increase in the methylation level of all genes analyzed in fish from the cleanest site, that is, Certes, no effect of CPE treatment was observed in eels from Gironde. Thus, at the end of the experiment (T11), no significant differences were observed on the methylation levels of the *aro* and *fshr* genes between fish from Certes and Gironde. In addition, whereas no difference was observed at T0 on *11β-hsd*, this gene was significantly hypermethylated in fish from Certes in comparison to fish from Gironde at T11. In mammals, gametogenesis is characterized by extensive epigenetic reprogramming events. First, during early embryonic development, primordial germ cells (i.e., the precursors of gonads) undergo extensive demethylation. Thereafter, during oogenesis and more precisely during oocyte growth that takes place soon after birth, DNA methylation is

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reestablished. This de novo methylation coincides with the transition from primary oocyte (first meiosis arrested in prophase I) to the secondary follicle stage. The methylation level of oocytes progressively increases with the size of oocytes until the germinal vesicle stage when oocytes become transcriptionally silent.[7,8,18] Although such data are not available in fish, there is increasing evidence that while sperm or oocyte DNA of zebrafish is methylated (heavily methylated in the case of sperm), genomic DNA is substantially demethylated during early embryonic development before being methylated de novo during gametogenesis.[17,38,39] In eels, premigrating female silver eels are still at a prepubertal stage and far from sexual maturity.[40] Prepubertal blockage of European silver eels is due to a deficiency in pituitary gonadotropin secretion, resulting from both a deficiency in the gonadotropin-releasing hormone (GnRH) function of hypothalamic neurons and a strong dopaminergic inhibition exerted at the pituitary level, counteracting GnRH stimulated gonadotropin synthesis (including FSH) and release.[12] Thus, oocyte growth mainly occurs during the transatlantic spawning migration of wild eels or in response to CPE treatment in captive eels.[40] In our study, in eels from Certes, as in mammals or zebrafish, oocyte growth was associated with an increase in DNA methylation. In contrast, in contaminated eels, that is, eels from Gironde, such a pattern was not observed. This could suggest that contamination not only triggered an increase in the DNA methylation level of *aro* and *fshr* genes at T0 but also impaired de novo methylation during hormonal treatment.

To strengthen the link between DNA methylation and gonad growth, we then measured the transcription level of the *aro*, *fshr* and *11β-hsd* genes (Figure 4) by RT-qPCR using *β-actin* as endogenous reference. Unfortunately, the transcript abundance of *β-actin* was significantly affected by the hormonal treatment (data not shown) which prevents the comparison between organisms sampled at T0 and organisms sampled at T11 and thus to investigate the effect of hormonal treatment on gene transcription levels. Such an effect has already been reported in various fish species, including eels, and is explained by the fact that gonadal growth is associated with an increase in the content of noncoding RNAs (i.e., mainly rRNA) and thus to lower ratio of mRNA to total RNAs.[20,41,42] Nevertheless, no difference was observed at each sampling time between fish from Certes and Gironde, thus allowing the comparison for each sampling time.

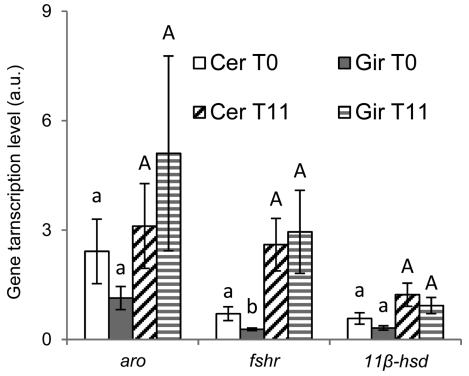

Figure 4. Changes in the transcription levels of *aro*, *fshr* and *11β-hsd* genes in gonads of female silver eels from Certes (Cer, clean site) and Gironde (Gir, contaminated site) before (T0) and after (T11) hormonal treatment. Values are represented as mean ± SE, $n = 5$ at T0 and $n = 8$ at T11. For each sampling time, means designated with different letters are significantly different, LSD test, $P < 0.05$.

In agreement with an increase in its methylation status at T0, the transcription level of the gene encoding for FSHr was found to be significantly down-regulated in fish from Gironde in comparison to fish from Certes at T0 (2.5-fold). Concerning the *aro* gene, although its transcription level was 2.1-times lower in fish from Gironde in comparison to fish from Certes, this down-regulation was not statistically significant at T0. In addition, no significant differences were observed on the transcription level of *fshr* and *aro* genes after CPE treatment (i.e., T11), which is in agreement with the absence of differences of their methylation status in the same animals. Concerning the *11β-hsd* gene, no significant differences were observed between animals from Certes and Gironde either at T0 or T11 and this, despite the fact that the *11β-hsd* gene was hypermethylated in fish from Certes at T11 in comparison to fish from Gironde.

Thus, as first described in cultured rat Sertoli cells,[43] our results emphasize a potential role of DNA methylation in the regulation of the transcription level of the *fshr* gene during early gonadal development in female silver eels. This could explain, at least in part, why CPE treatment was less effective to induce gonad development in contaminated eels. FSH is a pituitary-secreted hormone that plays a central role in reproductive biology by regulating steroidogenesis and gamete differentiation notably during early gonad development.[20] This hormone exerts its function by binding to its specific gonadal receptors. Thus, its ability to affect gonadal physiology depends greatly on the transcription level of its receptor in gonads.[20] In our case, CPE treatment contains a variety of pituitary hormones including FSH. Our results suggest that pollution could be responsible for an increase in the methylation level of gonadal genes involved in oocyte growth and differentiation. Thus, our results, obtained from wild contaminated organisms, support that the chronic pollution experienced by animals throughout their life can affect their reproductive capacities and possibly, their offspring.[7] Further studies are needed to investigate the potential transfer of such epigenetic marks to the next generation. In this sense, we must note that during our studies the genomic DNA from both oocytes and ovarian follicular cells was extracted and analyzed. Future studies, for instance on ovulated oocytes (i.e., after the rupture of the ovarian follicles), should help deciphering the potential impact of pollutants on the DNA methylome of the eel germ-line.

■ ASSOCIATED CONTENT

Supporting Information

Details of specific primer pairs and amplicons used in PCR analyses are presented in Table S1 and Figure S1, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +33556223927; fax: +33556549383; e-mail: f.pierron@epoc.u-bordeaux1.fr.

Notes

The authors declare no competing financial interest.

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