Gene transcription profiling in wild and laboratory-exposed eels: Effect of captivity and in situ chronic exposure to pollution

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HIGHLIGHTS

• Need of improved method to predict and discriminate in situ effects of pollutants
• Fish were caught in the wild or laboratory-exposed to different abiotic factors.
• Hepatic transcriptome profiles of wild and laboratory-exposed fish were compared.
• Captivity markedly altered the profiles, especially genes involved in histone marks.
• Genes affected by pollution were involved in cell differentiation and development.

ABSTRACT

Aquatic ecosystems are subjected to a variety of man-induced stressors but also vary spatially and temporally due to variation in natural factors. In such complex environments, it remains difficult to detect, dissociate and evaluate the effects of contaminants in wild organisms. In this context, the aim of this study was to test whether the hepatic transcriptome profile of fish may be used to detect in situ exposure to a particular contaminant. Transcriptomic profiles from laboratory-exposed and wild eels sampled along a contamination gradient were compared. During laboratory experiments, fish were exposed during 45 days to different pollutants (Hg, PCBs, OCPs or Cd) or natural factors (temperature, salinity or low food supply) at levels close to those found in the sampling sites. A strong difference was observed between the transcriptomic profiles obtained from wild and laboratory-exposed animals (whatever the sites or experimental conditions), suggesting a general stress induced by captivity in the laboratory. Among the biological functions that were up-regulated in laboratory eels in comparison to wild eels, histone modification was the most represented. This finding suggests that laboratory conditions could affect the epigenome of fish and thus modulate the transcriptional responses developed by fish in response to pollutant exposure. Among experimental conditions, only the transcription profiles of laboratory animals exposed to cold temperature were correlated with those obtained from wild fish, and more significantly with fish from contaminated sites. Common regulated genes were mainly involved in cell differentiation and liver...
1. Introduction

Human activities have dramatically increased the concentration of metallic and organic pollutants in aquatic environments (Thrush et al., 2009). The goal of ecotoxicology is to assess and predict the impact of these contaminants on organisms, populations and ecosystems. Towards this aim, experimental approaches were developed with aquatic model animals to understand and assess the impacts of contaminants in natural ecosystems. However, it remains difficult to extrapolate the results obtained in the laboratory to a realistic effect in natural environments due to the presence of other natural factors (temperature, salinity, dissolved oxygen concentration, parasitism...) as well as variations of these factors from one sampling site to another. Indeed, aquatic ecosystems are complex and dynamic entities in structure, composition and functioning change on a daily and seasonal basis in response to variations in natural factors. A huge challenge for ecotoxicologists is thus to distinguish the effects triggered by contaminants from those triggered by natural factors. It is even more difficult to detect and assess the contribution of individual contaminants in a multi-pollutant field context (Denslow et al., 2007).

In this context, gene transcription profiling has received increasing attention in recent years. By allowing the simultaneous measurement of the transcription level of a large number of genes belonging to various metabolic pathways, gene expression profiling analysis has been used to identify and provide mechanistic insights into pollutant toxicity as well as to provide chemical signatures of toxicity (Denslow et al., 2007; Pierron et al. 2011; Bougas et al. 2013; Baillon et al. 2015a, 2015b). Such signatures are constituted by a set of genes that respond to a particular factor and may be used to discern the effects of chemicals in pollutant mixtures (Poynton et al., 2008a, b). The emergence of analytical tools allowing high-throughput sequencing of mRNA molecules has allowed ecotoxicologists to develop such approaches in wild and non-model organisms. In a previous work, we took advantage of RNA-Seq to discover without any a priori method statistically confident “candidate genes” for which transcription levels were more likely related to contaminant exposure than to natural stressors in wild Atlantic eels, i.e. in European (Anguilla Anguilla) and American (Anguilla rostrata) eels (Baillon et al. 2015a). A total of 1000 candidate genes were retained and used to construct a DNA microarray (Baillon et al. 2015b). Historically abundant and widespread in Europe and North America, populations of Atlantic eels have suffered a sharp decline since the 1980’s. The European species (Anguilla Anguilla) is currently considered as critically endangered of extinction by the International Union for Conservation of Nature. The Committee on the status of Endangered Wildlife in Canada (COSEWIC) has revised the status of the American eel from “special concern” in 2006, to “Threatened” in 2012. Among hypotheses advanced to explain these declines, the possible contribution of pollution has received considerable attention in recent years. The unusual life cycle of Atlantic eels makes them particularly vulnerable to pollution (Belpaire and Goemans 2007; Geeraerts and Belpaire 2010). Spawning takes place in the Sargasso Sea and leptcephali larvae are transported by ocean currents until they reach the European or American coasts. After metamorphosis of the larvae into glass eels, the organisms reach the juvenile growth phase stage (yellow eel) in continental habitats. During this stage, eels adopt a more sedentary lifestyle and accumulate substantial energy reserves in the form of lipids. This stage can last from several years to 20 years, depending on the hydrosystem, and ends with a second metamorphosis called silvering which prepares the future genitors (silver eels) for their transoceanic reproductive migration without feeding (Tesch 2003; van Ginneken and Maes 2005).

Previous transcriptomic studies carried out on silver European eels chronically exposed to pollutants in their natural environment have reported an altered pattern of transcription of genes involved in detoxification and a down-regulation of genes involved in oxidative phosphorylation in eels inhabiting a highly polluted site (contaminated notably by polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and brominated flame retardants) in comparison to eels inhabiting clean sites, suggesting that pollutants may have a significant effect on energy metabolism in these fish (Pujolar et al. 2012, 2013). However, many biotic and abiotic factors can influence the transcription of fish in nature, leading to potential ambiguities in their interpretation. Here, we first established specific transcriptomic profiles for different anthropogenic (mercury (Hg), cadmium (Cd), PCBs, or OCPs) and natural (temperature, salinity or low food supply) factors under controlled laboratory conditions. The aim was to obtain a “gene transcription signature” elicited by each stressor (Ramaswamy et al. 2001; Denslow et al., 2007) by means of a DNA microarray. The second objective was to test whether these gene transcription signatures could help to detect and separate the effects of each stressor in wild fish chronically exposed to multiple stressors.

2. Material and methods

2.1. Wild eel sample collection

Seven sampling sites were selected in Québec (Canada; St.-Jean, Sud-Ouest, St. François, St. Pierre) and in France (Dordogne, Garonne, Gironde) on the basis of their known gradients of contamination by metallic and organic pollutants as previously described in Baillon et al. (2015a). European eels (Anguilla Anguilla) and American eels (Anguilla rostrata) were collected between May 24 and June 24 of 2012, using a trawl, a fyke net or by electrical fishing. For each sampling site, a total of five immature and sexually undifferentiated eels (mean ocular index 3.32 ± 0.1) were used for subsequent analyses. Fish were dissected as soon as possible at the proximity of the sampling sites and organs (liver and muscle) were divided into several samples. Samples for gene transcription analyses were stored in RNA later at −20 °C until needed. For both organic and metal analyses, samples were stored at −80 °C.

2.2. Experimental design

For laboratory experiments, European yellow eels were exposed to only one factor at a time. While environmental exposures rarely contain a single stressor, these single physicochemical studies are important because they set the stage against which responses to more complex exposures can be compared (Denslow et al., 2007). During laboratory experiments, fish were exposed to different pollutants or natural factors at levels close to those found in the sampling sites Table 1 and Table 2A and B). Pollutants selected for laboratory exposures were those found in high concentration levels in eels sampled in St. Lawrence and Gironde hydrosystems such as PCBs, Cd, Hg and OCPs (Baillon et al. 2015a). Finally, immature eels were exposed to natural stressors that showed the strongest variations among the natural sites, i.e. salinity and temperature. Eels were also submitted to food restriction, a factor known to influence fish metabolism (Dave et al. 1975). Experimental conditions are summarized in Table 1.
All procedures used in this experiment were approved by the Aquitaine fish-birds ethics committee. Immature and sexually undifferentiated yellow eels (*Anguilla anguilla*), averaging 37.7 cm in length and 75 g in weight (37.71 ± 5.05 cm and 75.00 ± 3.84 g, respectively, mean ± SE, n = 80) were captured from a cleaner site (*Cerises, Baillon et al. 2015a, 2015b, 2016*) in May 2012. The animals were transferred to the laboratory (Marine Station of Arcachon) and kept in running aerated tap water thermostated at 23 °C over a 1-month maintenance period, to simulate natural conditions of the Gironde estuary (unpublished data). For Hg exposure, the food was enriched with a mix of OCP solution to reach a concentration of 0.09 ± 0.01 μg·L⁻¹, 0.42 ± 0.04 μg·L⁻¹ and 4.00 ± 0.18 μg·L⁻¹ (mean ± SE, n = 18) in control and contaminated tanks, respectively. For our experiment, control animals were maintained in uncontaminated brackish water and fed with uncontaminated artificial food. For natural factor exposures, animals were maintained in the same conditions as controls. However, for the cold temperature exposure, animals were maintained in water thermostated at 13.4 ± 0.3 °C (mean ± SE, n = 34). For the high salinity exposure, the salinity was increased to 17.7 ± 0.2‰, (mean ± SE, n = 36, natural seawater dilution with aerated tap water). For the food restriction condition, animals were fed at only 2% wet weight animal/day.

At the end of the exposure period (45 days), five eels per EU were removed and dissected. Samples of liver were immediately fixed in RNAlater solution and stored at −20 °C until needed for analyses. All experimental conditions are shown in Table 1.

### 2.3. RNA extraction, labelling, and cDNA hybridization

All procedures were carried out as previously described in *Baillon et al. (2015b)*. Microarrays contained one thousand oligonucleotide probes (60mers) in triplicates. These probes were designed from a set of transcripts comprising both an American and European eels transcriptome assembled from a previous RNA-Seq study (*Illumina Hiseq2000 technology, Baillon et al. 2015a*) from hepatic transcriptome of 48 wild eels, collected in the same sites as those described in the present manuscript. A total of 11,547 unique genes of known function were identified. In parallel, the contamination level of fish in 8 metals and 25 organic pollutants was determined and the temperature, salinity and dissolved oxygen concentration of each sampling sites were measured. Then, we carried out correlation analyses between the transcriptional level of a given gene and (i) contaminant concentrations in each individual fish, (ii) condition and morphometric indices, (iii) physico-chemical characteristics of sampling sites by means of the factor analysis for multiple testing (FAMT) method (*Friguet et al. 2009*). The FAMT model was used with a unique threshold of 0.003 in order to identify genes for which their expression levels were more likely correlated with a single natural or anthropogenic factor. In total, 61 genes were significantly correlated with Ag, 64 with As, 60 with Cd, 64 with DDT, 49 with HCB, 55 with Hg, 35 with PBDE-47, 51 with PCB-sum, 68 with trans-nonachlor, 115 with lindane, 51 with hepatosomatic index (HSI), 18 with the Fulton condition factor (K), 68 with spleen-somatic index (SSI), 91 with salinity and 84 with sampling site temperature. To reach a total of 1000 candidate genes 66 genes for which their transcription level was significantly correlated with two factors were retained. The probes were printed on Corning UltraGAPS TM Coated Slides with the Q-array Mini (Genetix) at the “Gênome et transcriptome” (GeT) microarray platform (Toulouse, France). SPM3 (Telechem) Microarray Printing Pins were used to deposit 2 nl per spot (100 µm/spot) onto the slide. The probes were then crosslinked with UV irradiation. Total RNA was extracted from liver of the 85 experimental and wild fish (5 individuals per condition or sampling site).
RNAs were extracted from 15 to 25 mg of tissue using the SV total RNA isolation system (Promega) with minor modifications (more details on sample preparation and microarray processing are available in NCBi/Gene Expression Omnibus (GEO) under the accession number GPL19017). Then, we used an indirect cDNA labelling method. During the first step 15 μg of total RNA was reverse transcribed with oligo-dT priming and amino-allyl-dUTP (Sigma). 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 (Cyamine 3 for cyanine and Cyamine 5 for reference). In order to normalize microarray data, we used a common reference design. The reference was composed by pooling total RNA from 30 wild eels from a clean site; i.e. Certes (15 fishes 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 (one night at 55 °C). A total of 85 eels and 85 microarrays (one microarray per individual) were used, five microarrays were performed for each experimental condition (see Table 1) and 5 for each sampling site (i.e. St. Jean, Sud-Ouest, St. Pierre, St. François, Dordogne, Gironde and Garonne). Thus, five biological replicates were performed per experimental condition or sampling site. Data acquisition was carried out by means of the Innoscan 710 microarray scanner (Innopsys) using Mapix software.

2.4. Metal and organic concentration analyses

For wild and experimental yellow eels, analysis of the seven indicator PCBs (CB50 + 28, CB32, CB101, CB118, CB138, CB153, and CB180), 14 OCPs (hexachlorobenzene or HCB, lindane or γ-HCH, dieldrin, heptachlor, heptachlorepoxide, cis-chlordane, trans-nonachlor, mirex, and DDTs), were performed on muscle samples following the procedures described in Baillon et al. 2015a. For PCBs LQs 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. Metal concentrations for Cd and Hg were measured from liver samples following the procedures described in Baillon et al. 2015a.

For laboratory Cd exposure, analyses were performed on liver and water samples following the procedures described by Pierron et al. 2007. For Hg analyses in experimental yellow eels, food and muscle samples were first dried at 45 °C for 48 h before analysis. Total Hg concentrations in samples were determined by flameless atomic absorption spectrometry. Analyses were carried out automatically after thermal decomposition at 800 °C under an oxygen flow (AMA 254, LECO France). Detection limit of the method was 0.1 ng·Hg with an average analytical variability of 5%. The analytical method was validated using reference materials TORT2 (National Research Council of Canada, lobster hepatopancreas) every ten samples. Recoveries of certified reference material were 111 ± 4%.

2.5. Data acquisition and analyses

For contaminant and morphometric data, comparisons among eel groups were performed by analysis of variance (ANOVA), after checking assumptions of normality and homoscedasticity of the error terms. The Least Square Deviation test (LSD) was used to determine whether means between pairs of samples were significantly different from one another. Computations were performed using the statistical software Sigma Stat. For all statistical results, a probability of \( P < 0.05 \) was considered significant. Results are given as mean ± SE.

Microarray analyses were conducted on the liver of animals from both the laboratory and field in order to test the potential usefulness of gene transcription signatures to predict and unravel the specific effects of contaminants in wild organisms. For this, the “signatures” obtained under laboratory conditions were compared with transcriptomic profiles obtained from wild Atlantic eels. Details about the raw data acquisition of microarrays are available on GEO (geo@ncbi.nlm.nih.gov).

Comparisons among fish groups were performed by using the class comparison test available in the BRB software (Simon et al. 2007). A hierarchical clustering analysis was performed on data obtained from both experimental and wild eels to assess the similarities and dissimilarities among the transcription profiles. For this, an average linkage hierarchical clustering was done with centered correlation and genes options using all arrays with BRB array tools software. Cluster reproducibility and the robustness were examined by the method of Mchane et al. 2002 based on 100 permutations. Finally, functional classification and assessment of significant differential representation of functional classes were performed with the Blast2go software (http://www.blast2go.com/b2ghome) using Gene Ontology annotation.

3. Results and discussion

3.1. Contamination levels

Results of physicochemical conditions at sampling sites indicated strong variations in temperature and salinity. Temperature ranged from 12 °C to 22 °C. For salinity, Sud-Ouest showed the highest value, reaching 16.5‰, compared with other French and Canadian sites that showed salinity comprised between 0 and 7.78‰. Concerning contaminants, the highest levels of contamination in both metallic and organic contaminants were observed in eels from France (Table 2A). Significant increases in pollutant concentrations were observed along the French gradient from Dordogne → Garonne → Gironde. For Québec, we observed an increasing contamination gradient from Sud-Ouest → St. Jean → St. François → St. Pierre. Eels from St. Pierre were the most contaminated eels, presenting contamination levels close to those determined in eels from Dordogne in France. Concerning metals, the highest variations were observed for Cd. Animals inhabiting the Gironde estuary were the most contaminated in Cd (8.39 ± 0.89 μg·g\(^{-1}\) dw) and were 17-fold more contaminated than eels from St. Jean, which was the cleanest site. Eels from Dordogne and St. Pierre were the second and third most contaminated sites by this metal with a mean concentration reaching 4.52 ± 0.92 μg·g\(^{-1}\) and 4.47 ± 1.64 μg·g\(^{-1}\) dw, respectively (Table 2A). For mercury, animals from St. Pierre were the most contaminated with a concentration averaging 1.8 ± 0.23 μg·g\(^{-1}\) dw.

Concerning organic pollutants, eels from Gironde and Garonne were the most contaminated compared to fish from all other sampling sites. The highest variations among sites were observed on muscle PCBs concentrations, which were 122-fold lower in eels from St. Jean than in fish from Gironde. In comparison, muscle OCP concentrations in eels from St. Jean were in mean 29-fold lower than in fish from Gironde.

Laboratory animals did not show significant differences among conditions in length or weight at the end of experiment. Concerning the hepatosomatic index (HSI), eels acclimated to cold temperature (13 °C compared to 23 °C for controls) showed a significantly higher HSI in comparison to other animals. For Hg and OCPs exposures that were carried out through the diet route (i.e. with food contaminated with Hg or OCPs), significantly higher bioaccumulation levels were observed in the muscle of exposed fish in comparison to controls (Table 2B). Concerning the PCBs condition, only fish that were exposed to the low PCBs condition presented significantly higher levels of contamination in comparison to controls. Indeed, even if eels exposed to PCB-contaminated food at 50 ng·g\(^{-1}\) (PCB_low) were fed with a food six times less contaminated than eels fed with PCB-contaminated food at 300 ng·g\(^{-1}\) (PCB_high), the concentration of PCBs in muscle of fish from the PCB low condition was 2.7-times higher than in eels from the PCB high condition (Table 2B). This is most likely explained by the low food intake rate of animals exposed to the high concentration of PCB. Indeed, for the PCB high condition, eels ate significantly less food in comparison to controls or fish of the PCB low condition (Table 2B).

Finally, we must note that, with exception of Hg, concentrations of pollutants in experimentally exposed eels were lower than those determined in wild eels from contaminated sites. These results showed
Table 2
Morphometric measures (mean ± SE, n = 5) and average concentrations of muscle organic pollutants (expressed as ng·g⁻¹, dw) and liver metal concentrations (expressed as µg·g⁻¹, dw) in Atlantic eels sampled in seven sites located in Canada and France (Table 2A) and eels exposed in laboratory (Table 2B). All data are expressed as means ± SE (n = 5 per site). For field and laboratory, means designated with different letters are significantly different (LSD test, P < 0.05). LQ: limit of quantification; ND: not determined.

### 2A

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<th>Field</th>
<th>St. Jean</th>
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<th>St. Pierre</th>
<th>Dordogne</th>
<th>Garonne</th>
<th>Gironde</th>
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### 2B

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### Metals

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### Organic pollutants

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<th>ND</th>
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<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pcb_sum</td>
<td>14.0 ± 4.5d</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89.4 ± 3.83a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Fig. 1. Hierarchical clustering of hepatic transcriptomic profiles of experimental and wild eels (see Table 1 for more details). In order to test an effect of the species, the Fig. 1A includes only wild eels from France (Dordogne, Garonne, Gironde) and all experimental eels. The Fig. 1B includes wild eels from France and Canada (St. Jean, Sud-Ouest, St. François, St. Pierre, Dordogne, Garonne and Gironde) and all experimental eels.
that a 45-day period of experimental exposure was not sufficient, in
term of bioaccumulation levels, to mimic several years of in situ chronic
exposure.

3.2. Microarray analyses

To test the potential usefulness of gene transcription signatures to predict and discriminate the specific effects of contaminants in wild or-
ganisms, the transcriptome profiles of wild and experimental eels were
analyzed. As many biotic and abiotic factors can influence the transcription
of fish in nature, rather than to perform comparisons among sam-
ping sites and experimental conditions (Table S1), a hierarchical
clustering analysis was performed on data obtained from both exper-
imental and wild eels. In addition, in order to test a potential effect of
the eel species, as both American and European eel species were used
in the present study, two analyses were performed. The first analysis in-
cluded all laboratory exposure conditions and only European wild eels
(i.e. the species used for experimental investigations (Fig. 1A)). The sec-
ond one included all laboratory and field conditions from both Canada
and France (Fig. 1B). For both analyses, a separation was observed be-
tween the gene transcription profiles obtained from laboratory and
wild animals. In addition, wild eels from the two species were clustered
together suggesting that the “laboratory” effect was more important
than the species effect (Fig. 1B). Thus, and as the second analysis allows
to cover a larger panel of abiotic conditions including eels with low con-
tamination levels (i.e. eels from Sud-Ouest and St. Jean), only the results
from the analysis that was carried out on the whole set of arrays will be
further discussed (i.e. Fig. 1B).

Between the two main clusters identified in the second analysis
(Fig. 1B), one gathered the majority of experimental eels (with only
one wild eel from Dordogne) while the other one regrouped the great
majority of wild animals and only few experimental eels. These two
clusters showed high reproducibility indices reaching 0.862 and 0.976,
respectively (R-index = 0.937 and D-index = 3.19), which indicates
reasonably robust clusters (Mschane et al., 2002). A total of 11 experi-
mental animals (out of 50), including all five animals of the low temper-
ature condition, clustered with wild animals. Among them, 4 animals of
the PCB low condition and 4 animals of the OCP condition clustered
with wild animals. However, these experimental animals were mostly
associated with wild fish from clean sites (Fig. 1B). Such a discrepancy
between wild and laboratory tends to show that results obtained from
laboratory experiments do not follow the results found in the field. As
wild and laboratory animals of the different species were both exposed
to different pollutants and natural stressors and presented different
sizes and weights, the present results may best be explained by an effect
of captivity. For example, while eels from Dordogne (moderately con-
taminated French site, mean length = 363.8 mm), Garonne (highly con-
taminated French site, mean length = 417.6 mm) or Sud-Ouest (lowly
contaminated Canadian site, mean length = 302.8 mm) presented a
length comparable to those of experimental eels (European eels, mean
size = 375.0 mm), the transcriptome profile of wild eels and exper-
imental eels were separated in two different clusters. In addition, while
strong variations in temperature were observed among Canadian (12
to 22 °C) and French sites (21.3 to 22.2 °C) and despite the fact that
the temperature used during the experimental investigations (23 °C)
was close to the temperature measured in the French sites, the tran-
scriptome profiles of wild European eels were more closely associated
to the transcriptome profiles of wild Canadian eels, rather than with
the transcriptome profiles of experimental European eels. These results
could suggest that the effect of laboratory captivity on fish liver tran-
scriptome was more important than an effect of size or temperature
or even, species. As such an effect is rarely taken into account or often
underrated, we then investigated the effect of laboratory captivity. Sta-
tistical analyses were carried out to identify the genes that were differ-
entially regulated between laboratory (including cold-acclimated fish)
and wild fish. A total of 190 genes (%< 0.01; Table S2) were identified,
88 genes were up-regulated and 102 genes were down-regulated in lab-
oratory-exposed compared to wild eels. An enrichment analysis with
Fisher’s exact test was performed on the 190 differentially expressed
genes to highlight the most significant biological processes that differed
between wild and experimental animals. The three diagrams in Fig. 2
show the most specific biological functions that were significantly
enriched between wild and laboratory eels. The first diagram (Fig. 2A)
summarizes the principal functions represented among the 190 differ-
entially transcribed genes. Among the down-regulated functions in lab-
oratory eels (Fig. 2B), phagocytosis engulfment, protein targeting to
membrane (N-terminal protein myristoylation) and regulation of the
cell cycle (regulation of Cdc42 protein signal transduction and regula-
tion of G2/M transition of mitotic cell cycle) were the most represented.
Such results are in agreement with previous studies carried out on other
fishes where captivity and confinement were associated with lower
growth rate, lower phagocytic activity and significant changes in the
transcription level of genes involved in protein targeting and phospho-
lipid metabolism (Pickering 1993; Ortuno et al. 2001; Caldach-Giner
et al. 2010). Among the up-regulated functions in laboratory eels, macromolecule modification and cell differentiation were the most
represented. In line with the first diagram (Fig. 2A), genes included in
the functional class called histone modification were the same as
those included in the functional classes called macromolecule modifica-
tion, leukocyte differentiation, megakaryocyte differentiation and lung
epithelial cell differentiation in Fig. 2C. This may suggest that the major-
ity of up-regulated genes in laboratory animals were involved in histone
modification, i.e. in epigenetic mechanisms. Epigenetics is the study of
changes in gene function that are mitotically and/or meiotically herita-
ble and that do not entail a change in DNA code (Feil and Fraga 2012). In
eukaryotic cells, DNA is packaged into chromatin. Covalent modific-
ations upon histones, the primary protein component of chromatin, can
influence the condensation level of chromatin and the accessibility of
transcription factors to their binding sites, leading to changes in the
transcription level of genes. Such an up-regulation of genes encoding
for proteins involved in epigenetic mechanisms in response to laborato-
ry conditions could explain the discrepancy observed between wild and
laboratory animals. As such, even if a particular effort was made to en-
sure the welfare of animals (by using a flow-through system, low fish
densities and shelters, see material and methods), our results raise the
hypothesis that laboratory/captivity conditions could affect the epige-
nome of fish and thus modulate the responses developed by fish ex-
posed to pollutants or natural stressors under experimental conditions.

In contrast to the other experimental conditions, all fish that were
experimentally exposed to low temperature clustered with wild ani-
mals, and more significantly so with fish from the most contaminated
sites (Fig. 1B). Indeed most of cold-acclimated fish were more closely
associated with fish from Dordogne, Garonne, Gironde and St Pierre
sites (Fig. 1B). Moreover, whereas the temperature applied during
cold-acclimation (13 °C) was closed to the temperature of the St Pierre
site (12 °C), the temperature in Dordogne, Garonne and Gironde sites
reached in mean 21.6 °C (Table 2A). In order to further explore such un-
expected results, statistical analyses were carried out to identify firstly
the genes and biological functions that were affected by cold tempera-
ture. A total of 112 genes (%< 0.05; Table S3) were differentially regulat-
ed between cold-acclimated fish and controls, 63 genes were found up-
regulated and 49 genes down-regulated. Then, an enrichment analysis
with Fisher’s exact test was performed on the 112 differentially tran-
scribed genes (Fig. 3). The first diagram (Fig. 3A) summarizes the prin-
cipal biological functions represented among the 112 differentially
expressed genes. Generally, the majority of the functions that were
up-regulated in response to cold acclimation were involved in the
response to growth factor stimulus, regulation of mitosis and mRNA
processing (Fig. 3C). This is quite surprising as the acclimation of fish
to low temperature leads to a decrease in their general metabolic activity in order to compensate the effect of cold temperature (Johnston and
Dunn 1987; Vergauwen et al. 2013). However, despite
a lower metabolic activity in cold-acclimated fish, many studies have reported an increase in mitochondrial density in liver and muscle in order to compensate for the lower activities of aerobic enzymes (Johnston and Dunn 1987, Sidell 1998, Gracey et al. 2004). In addition, an increase in the hepato-somatic index (HSI) have been reported in European eels acclimated to cold temperature at 10 °C compared to fish acclimated at 20 °C. This increase was associated with an increase in cell numbers (Johnston and Dunn 1987). Accordingly, eels exposed to cold water (13 °C) presented a significantly higher HSI than control eels (23 °C) in our study (Table 2B). Then the induction of growth factor signaling pathways, mitosis and mRNA processing agrees with previous studies carried out on liver of cold-acclimated eels and reflects a basic paradigm of cold acclimation: fish frequently compensate for the rate-depressing effects of cold temperature by synthesizing more enzymes (and by increasing the number of hepatic cells in the case of eels) to maintain biochemical performance (Gracey et al. 2004). Down-regulated functions (Fig. 3B) were vitamin A metabolism, regulation of kinase and cytokine activities and biosynthesis of monocarboxylic acids. First, our results showed a decrease in the biosynthetic process of monocarboxylic acids, i.e. fatty acids. This could suggest a decrease in energy storage in liver. This contrasts with a previous study where European eels acclimated to cold temperature at 10 °C (compared 13 °C in our study) did not show differences in liver triacylglycerol content compared to fish acclimated to 20 °C (23 °C in our study; Jankowsky et al. 1984). In contrast, a decrease of angiogenesis, i.e. a decrease in blood vessel growth, agrees with the reported decrease in the oxygen consumption rate of eels acclimated to cold (Jankowsky et al. 1984). Vitamin A (or retinol) is involved in several biological processes such as cell differentiation, immune system, hematopoiesis and lipid metabolism (Pierron et al. 2011). The down-regulation of vitamin A metabolism is consistent with the down-regulation of genes involved in lipid metabolism and angiogenesis, since hematopoiesis and angiogenesis are closely linked (Suda et al. 2000). Moreover, cytokine activity, involved in cell communication but also in the innate immune system (Calgani and Elenkov 2006), was down-regulated (Fig. 3B) in cold-acclimated animals, in agreement with the effects of cold acclimation in ectotherms. Interestingly, similar results were observed in the liver of wild yellow perch (Perca flavescens) chronically exposed to metal pollution. Using a transcriptome-wide approach (RNA-Seq), chronic metal exposure was found to be associated with a decrease in the transcription level of genes involved in the innate immunity and in the retinol and lipid metabolism (Pierron et al. 2011, Defo et al. 2014, Defo et al. 2015). Our results could suggest that wild eels chronically exposed to multi-pollutant mixture may adopt a somewhat similar strategy than cold acclimated animals. In order to deepen these findings, statistical analyses were carried out to identify the genes that were similarly regulated among animals exposed to low temperature and animals from the four contaminated sites. As a first step, we identified the genes
that were differentially transcribed ($P < 0.05$) between controls and animals from each sampling sites. These gene lists were then compared and 32 genes that were commonly regulated between fish from the four contaminated sites and fish acclimated to cold were identified. In addition, a gene list was also established comparing the low temperature exposure group and each lowly contaminated site, i.e., St. Jean,

### Table 3

List of the genes that were similarly regulated between cold-acclimated fish and animals from wild sites St. Pierre, Dordogne, Garonne and Gironde and their respective fold changes compared to controls.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Biological process</th>
<th>Temperature</th>
<th>St. Pierre</th>
<th>Dordogne</th>
<th>Garonne</th>
<th>Gironde</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription factor E2-alpha</td>
<td>Cell differentiation</td>
<td>1.56</td>
<td>1.51</td>
<td>1.45</td>
<td>1.45</td>
<td>1.45</td>
</tr>
<tr>
<td>Arginine-glutamic acid dipeptide repeats protein</td>
<td>Regulation of histone deacetylation</td>
<td>1.89</td>
<td>1.84</td>
<td>1.7</td>
<td>1.77</td>
<td>2.09</td>
</tr>
<tr>
<td>Krueppel-like factor 12</td>
<td>Positive regulation of transcription from RNA polymerase II promoter</td>
<td>0.31</td>
<td>0.36</td>
<td>0.47</td>
<td>0.39</td>
<td>0.37</td>
</tr>
<tr>
<td>DNA damage-inducible transcript 4-like protein</td>
<td>Negative regulation of signal transduction</td>
<td>1.33</td>
<td>1.66</td>
<td>1.47</td>
<td>1.65</td>
<td>1.56</td>
</tr>
<tr>
<td>ATP-binding cassette sub-family D member 4</td>
<td>ATP catabolic process</td>
<td>1.75</td>
<td>1.64</td>
<td>1.8</td>
<td>1.8</td>
<td>1.86</td>
</tr>
<tr>
<td>Sortilin-related receptor</td>
<td>Transport</td>
<td>2.27</td>
<td>2.4</td>
<td>1.82</td>
<td>2.27</td>
<td>2.13</td>
</tr>
<tr>
<td>Integral membrane protein 2A</td>
<td>/</td>
<td>1.64</td>
<td>2.09</td>
<td>2.11</td>
<td>1.95</td>
<td>1.62</td>
</tr>
<tr>
<td>TATA-binding protein-associated factor 2N</td>
<td>Transcription initiation from RNA polymerase II promoter</td>
<td>1.89</td>
<td>1.79</td>
<td>1.96</td>
<td>2.51</td>
<td>2.14</td>
</tr>
<tr>
<td>Protein arginine N-methyltransferase 7</td>
<td>Regulation of transcription</td>
<td>1.56</td>
<td>1.85</td>
<td>1.65</td>
<td>1.7</td>
<td>1.61</td>
</tr>
<tr>
<td>Complement C3</td>
<td>Innate immunity</td>
<td>1.72</td>
<td>2.02</td>
<td>1.83</td>
<td>2.15</td>
<td>1.96</td>
</tr>
</tbody>
</table>
Sud-Ouest and St. François and 22 common genes were found. Comparing the two lists, 10 specific genes were identified between the low temperature condition and contaminated sites (i.e. St. Pierre, Dordogne, Garonne and Gironde; Table 3). These genes were all regulated in the same direction and only one gene was found down-regulated among all conditions. This down-regulated gene, *Krueppel-like factor 12* (*klf12*) confers strong transcriptional repression to the *tpaf2a* transcription factor. This gene encodes for the AP-2-α protein that is involved in cell differentiation, morphogenesis and development (Li et al. 2013). Among the up-regulated genes, the gene *integral membrane protein 2A* (*itm2a*) was found to be highly expressed in adipose tissue mesenchymal stem cells (ASC) and was shown to inhibit their differentiation into chondrogenic cells (Boeuf et al. 2009 36). The gene *transcription factor E2-alpha* (*tcf3*) encodes for a transcriptional regulator that is involved in cell fate specification and differentiation (Yoon et al. 2011). The gene *arginine-glutamic acid dipeptide repeats protein (rere)* encodes for the protein RERE that acts as transcriptional co-repressor during embryonic development and that plays a role in the control of cell survival (Zoltewicz et al., 2003). The association between RERE and a histone deacetylase alters the structure of chromatin and lead to transcriptional repression (Ng and Bird 2000). The gene *protein arginine N-methyltransferase 7* (*prmt7*) mediates the symmetric dimethylation of histone H4 and the recruitment of DNA methyltransferases at these sites (Miranda et al. 2004). PRMT7 was found to be associated with gene silencing. PRMT7 was notably found to negatively regulate the transcription of genes involved in DNA repair (Karkhanis et al. 2012). Thus, most of the genes that were similarly regulated between cold-acclimated and wild contaminated animals were involved in the regulation of cell differentiation, organism development and epigenetic mechanisms. In adults, differentiation of hepatic stem/progenitor cells contributes to liver development, homeostasis, repair/regeneration under pathological conditions and reprogramming. Cellular differentiation is regulated by epigenetic mechanisms and induces significant changes in the phenotype of cells, from their shape to their metabolic activity and their responsiveness to external and internal stimuli (Miyajima et al. 2014). As these effects were observed in wild eels that presented different patterns of contamination (Table 2A) and even in laboratory cold-acclimated fish, this response would not be specific to a particular contaminant or stress but would be rather a global and long-lasting response developed by fish in response to chronic exposure to multi-pollutant mixture.

4. Conclusions

Microarray analyses have first shown a clear separation between the gene transcription profiles of wild and laboratory-exposed fish, suggesting an important effect of captivity on gene transcription. This effect was more important than the species effect. The fact that captivity could induce epigenetic mechanisms could explain, at least in part, the differences observed between wild and laboratory-exposed fish. Further investigations are needed to investigate such a hypothesis. Although field studies are always challenging, involving numerous uncontrolled variables and multi-pollutant mixtures which often leads to results difficult to interpret, our results highlight the importance of in situ studies. Second, cold-acclimated animals were, among experimental animals, the closest to wild animals, and mostly to wild eels from contaminated sites (Gironde, Garonne, Dordogne and St. Pierre). The comparison between cold-acclimated and the most contaminated wild fish gave not a specific response to a particular contaminant but rather a global response to a chronic multi-pollutant exposure. The common genes were mainly involved in cell differentiation and organism development. Overall, our results provide a basis to explore at a higher biological level, a range of specific mechanistic hypotheses based on the involvement of hepatic progenitor cells in the adaptive response developed by fish in response to long-term, life-long and chronic exposure to water pollution.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.07.131.

References


Miranda, T.B., Miranda, M., Frankel, A., Clarke, S., 2004. PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. J. Biol. Chem. 279, 22902–22907.


