Genome-wide DNA methylation of the liver reveals delayed effects of early-life exposure to 17-α-ethinylestradiol in the self-fertilizing mangrove rivulus

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Abstract

Organisms exposed to endocrine disruptors in early life can show altered phenotype later in adulthood. Although the mechanisms underlying these long-term effects remain poorly understood, an increasing body of evidence points toward the potential role of epigenetic processes. In the present study, we exposed hatchlings of an isogenic lineage of the self-fertilizing fish mangrove rivulus for 28 days to 4 and 120 ng/L of 17-α-ethinylestradiol. After a recovery period of 140 days, reduced representation bisulfite sequencing (RRBS) was performed on the liver in order to assess the hepatic genome-wide methylation landscape. Across all treatment comparisons, a total of 146 differentially methylated fragments (DMFs) were reported, mostly for the group exposed to 4 ng/L, suggesting a non-monotonic effect of EE2 exposure. Gene ontology analysis revealed networks involved in lipid metabolism, cellular processes, connective tissue function, molecular transport and inflammation. The highest effect was reported for nipped-B-like protein B (NIPBL) promoter region after exposure to 4 ng/L EE2 (+ 21.9%), suggesting that NIPBL could be an important regulator for long-term effects of EE2. Our results also suggest a significant role of DNA methylation in intergenic regions and potentially in transposable elements. These results support the ability of early exposure to endocrine disruptors of inducing epigenetic alterations during adulthood, providing plausible mechanistic explanations for long-term phenotypic alteration. Additionally, this work demonstrates the usefulness of isogenic lineages of the self-fertilizing mangrove rivulus to better understand the biological significance of long-term alterations of DNA methylation by diminishing the confounding factor of genetic variability.

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Abstract

Organisms exposed to endocrine disruptors in early life can show altered phenotype later in adulthood. Although the mechanisms underlying these long-term effects remain poorly understood, an increasing body of evidence points toward the potential role of epigenetic processes. In the present study, we exposed hatchlings of an isogenic lineage of the self-fertilizing fish mangrove rivulus for 28 days to 4 and 120 ng/L of 17-α-ethinylestradiol. After a recovery period of 140 days, reduced representation bisulfite sequencing (RRBS) was performed on the liver in order to assess the hepatic genome-wide methylation landscape. Across all treatment comparisons, a total of 146 differentially methylated fragments (DMFs) were reported, mostly for the group exposed to 4 ng/L, suggesting a non-monotonic effect of EE2 exposure. Gene ontology analysis revealed networks involved in lipid metabolism, cellular processes, connective tissue function, molecular transport and inflammation. The highest effect was reported for *nipped-B-like protein B* (NIPBL) promoter region after exposure to 4 ng/L EE2 (+ 21.9%), suggesting that NIPBL could be an important regulator for long-term effects of EE2. Our results also suggest a significant role of DNA methylation in intergenic regions and potentially in transposable elements. These results support the ability of early exposure to endocrine disruptors of inducing epigenetic alterations during adulthood, providing plausible mechanistic explanations for long-term phenotypic alteration. Additionally, this work demonstrates the usefulness of isogenic lineages of the self-fertilizing mangrove rivulus to better understand the biological significance of long-term alterations of DNA methylation by diminishing the confounding factor of genetic variability.

Keywords : mangrove rivulus, 17-α-ethinylestradiol, delayed effects, DNA methylation, RRBS, nipped-B-like protein B, Transposable Elements

List of abbreviations : DMF, differentially methylated fragment; EDC, endocrine disrupting chemical; EE2, 17-α-ethinylestradiol; NIPBL, nipped-B-like protein B; RRBS, reduced representation bisulfite sequencing; RR genome; reduced representative genome; TE, Transposable element; WGBS, whole genome bisulfite sequencing

Introduction

Early-life exposure to environmental stressors, encountered during the sensitive period of embryogenesis or in juveniles, can be critical in shaping the long-term control of tissue physiology and homeostasis. Although this paradigm, referred to as the Developmental Origins of Health and Disease (DOHaD) (Barker, 2004), is now widely recognized, the molecular mechanisms by which early exposures influence the propensity of disease and phenotype later in life remain elusive. Identifying these mechanisms has become extremely important to understand the long-term effects of toxicants in human and wildlife and ensure the proper risk assessment of xenobiotic exposure (Brockmeier et al., 2017).

Nowadays, endocrine disrupting chemicals (EDCs) – xenobiotics able to interfere with the proper functioning of the endocrine system – are ubiquitous in the environment and our everyday life. The timely release and tightly regulated concentrations of hormones in early-life is crucial for the proper development of the organism, including the reproductive, nervous, and immune systems. Therefore, early-life exposure to EDCs can have dramatic consequences on homeostasis and physiology. In fact, mounting epidemiological evidence link exposures to EDCs to the increased incidence of metabolic diseases, immune diseases, neurological disorders, cancer and alteration of fertility in humans (Braun, 2017; Heindel, Newbold, & Schug, 2015; Kajta & Wójtowicz, 2013; Kuo et al., 2012). Of the many EDCs present in the aquatic environment, 17-α-ethinylestradiol (EE2) - a synthetic derivative of estradiol used in oral contraceptives - is of particular concern due to its high potency and resistance to degradation (Clouzot et al., 2008). In treated seawage, EE2 is often the major compound with estrogenic activity and its impact on aquatic wildlife has been deeply investigated (reviewed in (Matthiessen, Wheeler, & Weltje, 2018). In aquatic species, field studies and laboratory experiments show that exposure to EE2 affects fecundity, fertility, reproductive behavior and induces intersex, endangering natural populations (Aris, Shamsuddin, & Praveena, 2014).

Potential long-term and persistent effects of early-life EDC exposure involve the stable alteration of gene expression. This is possible through epigenetic regulation, involving histone modifications, non-coding RNAs, and DNA methylation. The latter refers to the transfer of a methyl group from a methyl-donor, S-adenosylmethionine (SAM), to the fifth position of cytosines in the context of CpG dinucleotides, forming a 5-methylcytosine (5mC) (Feng et al., 2010, #41462; Law & Jacobsen, 2010; Lister & Ecker, 2009). This process is catalyzed by two families of DNA methyltransferases (DNMTs): whereas DNMT3 enzymes are responsible for *de novo* methylation during development and differentiation, the DNMT1 family is maintaining methylation through each cell division by copying hemimethylated DNA, making DNA methylation a stable and heritable modification (de Mendoza, Lister, & Bogdanovic, 2020; Moore, Le, & Fan, 2013). DNA methylation regulates gene expression by altering chromatin state and accessibility to CpG sites. For instance, methylation occurring at promoter regions can prevent the binding of transcription factors, resulting in gene silencing. Yet, the relationship between methylation and gene expression is more complex, as methylation also occurs at intragenic sites, enhancers or suppressor elements (Edwards et al., 2017; Moore et al., 2013). DNA methylation is dynamically regulated throughout life: during gametogenesis and embryogenesis, two waves of demethylation/remethylation deeply reprogram the methylome to produce a totipotent zygote (Edwards et al., 2017; Guo et al., 2014; Smith et al., 2014). Then, DNA methylation plays an important role throughout development as it directs cellular differentiation after reprogramming. During early development and juvenile stages, DNAme is thus thought to be particularly sensitive to environmental factors (Dorts et al., 2016).

An increasing number of studies highlight the potential roles of DNA methylation in mediating long-term effects of sub-toxic developmental exposure to xenobiotics and roles in the etiology of diseases including cancer, obesity, cardiovascular disease, diabetes, hypertension, and neurodegenerative disorders (Eid & Zawia, 2016; Gore et al., 2011; Goyal, Limesand, & Goyal, 2019; Stel & Legler, 2015; Wadhwa et al., 2009). Indeed, DNA methylation could act as a long-term memory of past exposures mediating stable changes in gene expression (Barouki et al., 2018; Mirbahai & Chipman, 2014). In ecotoxicology, the role of epigenomics is receiving more and more attention to explain the long-term delayed and potential transgenerational effects of xenobiotics (Brander, Biales, & Connon, 2017; Vandegehuchte & Janssen, 2011; Vandegehuchte & Janssen, 2014). In particular, accumulating evidence indicates that hormones and endocrine disrupting compounds can alter the epigenome (Stel & Legler, 2015; Walker, 2016; Zhang & Ho, 2011). For instance, the regulation of DNMT transcription by ESR1 (an estrogen receptor that acts as a transcription factor) may represent one of the possible mechanisms by which hormones influence methylation (Shi et al., 2012). Other potential mechanisms may involve the reduction of SAM availability (Lee, Jacobs, & Porta, 2009), the alteration of histone activity as a result of membrane receptor estrogenic signaling (Anderson et al., 2012; Casati et al., 2015), the expression of miRNAs, or direct interactions between estrogen receptors and enzymes involved in the methylation machinery, such as thymine-DNA glycosylase (TDG) (Liu et al., 2016).

So far, relatively few studies have examined the effects of endocrine disruptors on DNA methylation in aquatic species. Using bisulfite conversion and pyrosequencing, Strömqvist et al. (Strömqvist, Tooke, & Brunström, 2010) have shown decreased methylation levels of three CpG sites located in the 5' flanking region of the *vitellogenin I*gene, a known biomarker of estrogenic exposure, in the liver of male and female zebrafish, following a 14-day exposure to 100 ng/L EE2. Using Methylated DNA Immunoprecipitation (MeDIP) and high-throughput sequencing, followed by validation using bisulfite sequencing PCR (BSP) and RT-PCR, Mirbahai et al. (Mirbahai et al., 2011) investigated the whole-genome methylation and gene expression in tumors in the liver of the Common dab (*Limanda limanda*), sampled in various sites in English rivers. Genes involved in pathways related to cancer, including apoptosis, wnt/β-catenin signaling and genomic and non-genomic estrogen responses, were altered both in methylation and transcription. In that case the exact mixture of environmental contaminants causing the liver tumors was not identified but the molecular responses point towards estrogenic disruption. On zebrafish early life stages, Falisse et al. (Falisse et al., 2018) showed that exposure to the antibacterial agent and EDC triclosan modified the methylome after 7 days exposure, as well as the expression of related genes, using Reduced Representation Bisulfite Sequencing (RRBS).

The identification of environmentally-induced alterations in the epigenome ideally requires the use of individuals with no existing or very low genetic variability to rule out the confounding factors of the genotype on the observed phenotype (Heard & Martienssen, 2014). Such a model species is provided by the mangrove rivulus, *Kryptolebias marmoratus* , one of the only known self-fertilizing vertebrates (the other one being its sister species, *K. hermaphroditus*). Although males and hermaphrodites are present in natural populations, most reproduction occurs by self-fertilization of hermaphrodites. Exclusive selfing of hermaphrodites during several generations results in highly homozygous and virtually "clonal" lineages that can occur naturally or be bred in a laboratory setting (Avise & Tatarenkov, 2015; Mackiewicz et al., 2006). A naturally clonal and sexually-reproducing vertebrate species allows the study of epigenetic mechanisms ruling out the confounding factor of genetic variability, which is especially useful in ecotoxicology experiments in the sublethal range and/or dealing with delayed effects where non-dramatic molecular changes are expected. Little is known about the epigenetic mechanisms in *K. marmoratus*but it has been reported that the sex-ratio can be modulated by temperature-sensitive DNA methylation (Ellison et al., 2015). Recent studies described the DNA methylation reprogramming event during embryogenesis (Fellous et al., 2018), as well as the time course expression of enzymes involved in epigenetics during its development (Fellous, Earley, & Silvestre, 2019a; Fellous, Earley, & Silvestre, 2019b). Another study has shown that parasite load can modify DNA methylation (Berbel-Filho et al., 2019a), while these authors also found a higher differentiation of the methylation landscape between different genotypes than between environments (Berbel-Filho et al., 2019b).

In previous studies, we successfully used the mangrove rivulus to assess the delayed effects of BMAA (beta-N-Methylamino-L-alanine) (Carion et al., 2020) and 17-α-ethinylestradiol (EE2) (Voisin et al., 2016; Voisin, Kültz, & Silvestre, 2018) in adults after an early-life stage exposure. Hatchlings exposed to 4 and 120 ng/L EE2 for 28 days, and allowed to recover for 140 additional days in clean water, reported delayed and persistent effects of EE2 on growth, reproduction and steroid levels as well as liver, ovotestis and brain molecular phenotypes assessed by label-free quantitative proteomics. Effects of EE2 were tissue and dose-dependent, with most effects occurring at the environmentally relevant concentration (4 ng/L) in brain and liver, and at the higher concentration in the gonads. Among the three studied organs, the liver showed the most sensitive response to EE2. In this organ, EE2 affected known estrogen-responsive pathways such as lipid, fatty acid and steroid metabolism, apolipoproteins, innate immune system and inflammation. Interestingly, several proteins involved in SAM metabolism were affected in the liver, providing further indications of the potential effect of EE2 on DNA methylation. The liver serves an essential and conserved role for metabolic homeostasis in all vertebrates, and reproduction in the case of oviparous species. Several studies have shown that the liver is an important target of endocrine disruption in mammals (Foulds et al., 2017) and fish (De Wit et al., 2008; Feswick, Munkittrick, & Martyniuk, 2017; Humble et al., 2013; Mirbahai et al., 2011). Using a RRBS approach, the present study aimed at characterising the hepatic methylation landscape in adults and investigating the genome-wide changes in DNA methylation in the liver of 168 dph adults that were exposed to EE2 as hatchlings to test the hypothesis of the potential involvement of DNA methylation in the delayed effects of EDCs.

Materials and methods

2.1. Experimental fish, EE2 exposure and sample collection

Individuals used in this study were from the same experiment reported in Voisin et al. (Voisin et al., 2016), which also described the generation of breeding stock population and collection of eggs. Upon hatching, mangrove rivulus were transferred to 300 mL glass jars filled with 100 mlL of 25 ppt reconstituted salt water (Instant Ocean sea salt), and the corresponding treatment: vehicle control (0.000012 % ethanol), 4 ng/L and 120 ng/L 17-α-ethinylestradiol (EE2) (Sigma-Aldrich E4876-1G). Fish were raised at a temperature of 26°C and a 12:12 h photoperiod. Detailed preparation of EE2 solutions and the measurement of actual exposure concentrations by ELISA are reported in Voisin et al. (Voisin et al., 2016). Average measured concentration of the nominal 120 ng/L exposure solution was 132.3 ± 14.7 ng/L. Since 4 ng/L is situated below the detection limit (20 ng/L), we refer to the measure of the stock solution, which was 10.15 ± 0.15 µg/L for the nominal 10 µg/L stock solution. Throughout the paper, we will refer to the nominal concentrations of 4 and 120

ng/L. Fish were exposed for a period of 28 days with a 100 % water renewal 3 times a week. At 28 dph, fish were transferred to individual 1.2 L plastic containers filled with 400 mL of 25 ppt clean salt water and reared until 168 dph. Rivulus were fed 1 mL of concentrated newly hatched brine shrimp (*Artemia* nauplii) until 56 dph, 2 mL starting at 56 dph and 4 mL starting at 91 dph. At 168 dph, fish were sacrificed by a rapid transfer to 4°C 25 ppt salt water and sectioning the spinal cord. For each experimental condition, livers from five individuals were dissected out at 168 dph, snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. All rivulus husbandry and experimental procedures were performed in accordance with the Belgian animal protection standards and were approved by the University of Namur Local Research Ethics Committee (UN KE14/230). The agreement number of the laboratory for fish experiments is the LA1900048.

2.2. DNA extraction, RRBS library preparation and post-processing

Genomic DNA from individual livers was extracted using the NucleoSpin Tissue XS kit (Macherey-Nagel, Germany) following the manufacturer's protocol. DNA concentration and quality were assessed using the NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA) and 1% agarose gel electrophoresis. A total of 15 RRBS libraries (5 for each experimental condition) were prepared following established protocols (Chatterjee et al., 2013; Chatterjee et al., 2014). In brief, genomic DNA was digested with MSPI (New England Biolabs, Ipswich, MA) followed by end repair, addition of 3' A overhangs and addition of methylated adapters (Illumina, San Diego, CA) to the digested fragments. Following adapter ligation, size selection was performed by cutting 40–220 bp DNA fragments (pre-ligation size) from a 3% (w/v) NuSieve GTG agarose gel (Lonza, Basel, Switzerland). Subsequently, libraries were bisulfite converted using the EZ DNA methylation kit (Zymo Research, Irvine, CA) with an extended incubation time of 18–20 h. Bisulfite converted libraries were amplified by PCR and sequenced (100-bp single ended reads) by New Zealand Genomics Limited (University of Otago, New Zealand) on an Illumina HiSeq2000 sequencer. Sequences were obtained in FASTQ format.

The quality of sequenced reads was performed using the FastQC application (Babraham Institute, Cambridge, UK). Adaptor sequences were removed from the reads using the *cleanadaptors* program of the DMAP package (Chatterjee et al., 2012b). Single-ended bisulfite reads were aligned against the *Kryptolebias marmoratus* assembly (GCF_001649575.1_ASM164957v1) using the Bismark software (Krueger & Andrews, 2011). Proximal genes and genomic positions of the fragments were identified using the *identgeneloc* program from the DMAP package (Stockwell et al., 2014). Fragments were linked to the nearest protein-coding gene and the genomic position was attributed as follows: internal (within the gene body), promoter (0-5 kb upstream of the transcription start site), upstream (5-10 kb upstream of the TSS) and intergenic (>10 kb). The annotation of intergenic fragments or fragments located upstream of genes (5-10 kb) and in intergenic regions (>10 kb) is provided as an indication and should be considered with caution as methylation of these regions may not have any influence on the corresponding gene expression. Fragments with a methylation level [?] 80% are qualified as highly methylated, while those with a methylation level [?] 20% are qualified as lowly methylated.

2.3. Analysis of differential DNA methylation

Base resolution differences in DNA methylation following developmental exposure to EE2 were assessed based on MSPI fragments (40-220 bp range) as a unit of analysis. To filter the fragments suitable for differential methylation analysis, we selected the fragments with [?] 2 CpG sites, [?]10 reads per fragment (CpG10) and present in at least 3 of 5 individuals for each experimental condition. These fragments are referred to as "analyzed fragments". The 102,018 analyzed fragments form our reduced representative genome (RR genome) corresponds to a total of 14,679 unique RefSeq identifiers, as more than one fragment often maps to a same gene. Differential methylation analyzes were performed as previously described using the diffmeth program from DMAP (Chatterjee et al., 2012; 2017a; 2017b; Stockwell et al., 2014) between each experimental group, resulting in three group comparisons: Ctl versus 4 ng/L EE2 exposed individuals, Ctl versus 120 ng/L and 4 versus 120 ng/L. Determination of significantly differentially methylated fragments (DMFs) was based on [?] 10% differential methylation between the groups and P-value <0.01, and are qualified as hyper- or hypomethylated fragments.

2.4. Network analysis

RefSeq identifiers were retrieved using BLAST for all analyzed DMFs. To establish a reference gene list for the network analysis, a list including all DMFs was filtered to include only gene body and promoter genomic locations and contained 68,762 fragments mapped to rivulus RefSeq and human RefSeq identifiers. As more than one fragment often mapped to a same gene, this list corresponded to 10,440 unique human RefSeq identifiers. The list, containing P-values and methylation difference for each of the three treatment comparisons was uploaded in the Ingenuity Pathway Analysis software (IPA, QIAGEN, www.qiagen.com/ingenuity). To gain an overall view of the pathways and functions associated with promoter and gene body DMFs, as well as the connections between differentially abundant proteins, we performed a network analysis for each group comparison.

3. Results 3.1. Characterization of the hepatic DNA methylation landscape in*K. marmoratus*

Using RRBS, we generated 15 *K. marmoratus* liver methylomes with an average of 29.5 million sequenced reads (between 19.7 and 40 million reads) (Table 1, Supplementary Table 1). Unique alignment efficiency of these libraries ranged from 40.3 % to 46.8 % (except the library 23-4 with 28.5%, which was discarded in further analyses), with an average of 44.4 %. This corresponds to a total of 102 018 fragments suited for the analysis after MSPI digestion of genomic DNA and 40–220 bp size-selection (Figure 1A). The total size of the RR genome was 9.37 Mb, which accounted for 1.4% of the published full genome (GCF_001649575.1_-ASM164957v1) (Rhee et al., 2017). Within this reduced genome, we counted 701 862 CpG dinucleotides, representing 6.1% of the 11.5 million CpG in the reference genome. Consequently, the enrichment factor in CpGs of the RR genome was 4.3-fold. Of all the analyzed fragments, the majority was located within gene bodies (47%), followed by intergenic regions (30%), promoter (16%) and upstream regions (7%) (Figure 1B). The distribution of CpGs followed exactly the same pattern as the fragments (data not shown). We consider this dataset of about 6% of all genomic CpGs, albeit not uniformly distributed across the genome, as a representative sample that permits the investigation of changes in DNA methylation in liver due to exposure to toxicants. Global CpG methylation levels in liver tissue ranged from 63.5% to 66.4% in all samples. On the contrary, methylation level of non-CpG cytosines remained low between 1.7 and 2.4%.

For the following description, we took only the control group into consideration, in order to avoid any possible effect of the EE2 treatment. The average DNA methylation level of all MSPI fragments in mangrove rivulus followed a bimodal distribution with most fragments below 20% of methylation (20% of all fragments - considered as lowly methylated) or above 80% (67% of all fragments - considered as highly methylated) (Figure 1C). The level of DNA methylation was the lowest in fragments located in promoter regions (mean=42.3%; median=12.8%) compared to upstream (mean=66.2%; median=88.4%), intergenic (mean=72.6%; median=89.1%) and gene body (mean=78.0%; median=91.5%). Figure 1D depicts the distribution and spread (25-75 quantiles) of DNA methylation level for fragments within each region. We can observe that the methylation range was the highest for fragments located in the promoter region, showing that even if the majority of these fragments were lowly methylated (52%), a considerable part was highly methylated (38%). Despite the fact that fragments in promoter regions represented only 16% of the total analyzed fragments, they accounted for 42% of all lowly methylated fragments (Figure 2A), compared to fragments located in gene bodies (27%), intergenic (23%) and upstream (8%) regions. In contrast, only 9% of all highly methylated fragments were located in the promoter regions, compared to 53% in gene bodies, 31% in intergenic regions and 7% in upstream regions (Figure 2B). Supplementary figure 1 depicts the coefficient of variability of the methylation level within each genomic region, for lowly methylated fragments on the one hand, and for highly methylated fragments on the other hand. Highly methylated fragments presented a low level of variability ($< 3\%$), in opposition to lowly methylated fragments for which the coefficient of variability was higher than 33%. This pattern was the same for all the fragments, independently of the genomic region.

3.2. Changes in DNA methylation following developmental exposure to EE2

Global DNA methylation levels of rivulus liver averaged 65.1 +- 1.0% in Ctl samples, 66.0 +- 0.2% in 4

ng/L and 65.5 +- 0.6% in 120 ng/L exposed individuals and were not significantly different (Anova on arcsin squareroot transformed values). Compared to the Ctl group, 58 DMFs were reported for the low EE2 concentration of 4 ng/L, 33 for the higher concentration of 120 ng/L, while 62 DMFs were significant between 120 and 4 ng/L groups. The majority of DMFs had a higher methylation level at 4 ng/L compared to Ctl and 120 ng/L groups, and at 120 ng/L compared to Ctl (Table 2, Figure 3A). Figure 3B depicts the DMF location within the RR genome and showed a similar pattern as the general distribution of the analyzed fragments (Figure 1B) with the DMFs located in the order gene body > intergenic > promoter > upstream regions. None of the significant DMF was common to all three group comparisons (Figure 3C). However, 2 DMFs were significant at both EE2 treatments compared to Ctl: one located in an intergenic region, in proximity of the *liprin-alpha-4* (PPFIA4) gene, and one located in the gene body of the *ATP-binding cassette sub-family A member 1* gene (ABCA1) (Figure 3D). In addition, there were 3 common DMFs to 4 ng/L vs Ctl and 4 ng/L vs 120 ng/L comparisons, of which two were found within gene bodies:*calcium/calmodulin-dependent protein kinase type 1D* (CAMK1D),*testis-specific serine/threonine-protein kinase* (TSSK1B) and one in the intergenic region in proximity of the *lymphoid-restricted membrane protein-like* gene (LRMP). Finally, one fragment was significantly differentially methylated in both 120 vs Ctl and 120 vs 4 ng/L, located in the intergenic region in proximity to the PTK2B (*protein-tyrosine kinase 2*) gene. Tables 3, 4 and 5 report the DMFs for the three comparisons: 4 ng/L vs Ctl, 120 ng/L vs Ctl, and 4 ng/L vs 120 ng/L, respectively. Among all group comparisons, the maximum effect size (difference in mean methylation) observed for hypermethylation was 21.9 % (promoter region of *nipped-B-like protein B* , NIPBL) and -19.0% for hypomethylation (intergenic region,*eukaryotic translation initiation factor 4 gamma 3* EIF4G3). Both DMFs were significant between 4 ng/L and Ctl groups. It is also important to report the fact that very few DMFs were classified as hypomethylated fragments. When comparing exposed groups to Ctl, only the NIPBL was hypomethylated in Ctl at 19.6% and increased to 41.5% after exposure to 4 ng/L EE2.

3.3. Networks associated with gene body and promoter DMFs

A network analysis was performed with the IPA software using genes corresponding to DMFs located in gene body and promoter regions. Only networks with the highest number of associated DMFs are reported (Table 6). The highest network scores were found in the 4 ng/L vs Ctl comparison: the first network involved 16 molecules, with functions related to organismal abnormalities, cell morphology and nervous system function and is represented in Figure 4. The second network was composed of 15 molecules and was related to gene expression, drug metabolism and lipid metabolism. In 120 ng/L exposed individuals compared to Ctl, only one network with more than 9 molecules was established in IPA, related to lipid metabolism, molecular transport and small molecule biochemistry. Finally, four networks were created when comparing 120 ng/L to 4 ng/L exposed individuals, endorsing the differences in the response at these two concentrations. The first network was related to antigen presenting, cell-to-cell signalling and the inflammatory response. The 2^{nd} and 3^{rd} networks were mostly associated with cellular processes (e.g. cellular organization and morphology, cell-to-cell signalling) and the 4^{th} network was linked to cellular death and survival and connective tissue function.

4. Discussion

4.1. Characterization of the mangrove rivulus RR genome

Cytosine methylation is a predominant epigenetic regulation of gene expression. It occurs mainly on CpG dinucleotides and is classically associated with gene silencing when it occurs at CpG rich promoter regions (Bock et al., 2012). Whole genome bisulfite sequencing (WGBS) provides global genome coverage of DNA methylation at single-base resolution, and is consequently referred as the 'gold standard' method. Despite its benefits, including a high coverage of CpGs (>90% in human) and an unbiased representation, it remains expensive and difficult to use with large number of samples, mostly because the detection of changes in methylation between samples demands high sequencing depth (Wreczycka et al., 2017). In the present study, we opted for reduced representation bisulfite sequencing (RRBS), an approach that also resolves single-base DNA methylation, combining the use of restriction enzymes and bisulfite sequencing. Unlike WGBS, RRBS can only sequence rich-CpG regions, and has limited coverage of the genome (Meissner et

al., 2005). However, the fact that it enriches the analyzed fragments with a high density of CpGs makes this technique cost-effective and allows to work on a higher number of samples with a high sequencing depth (Chatterjee et al., 2012a). Here we reported the DNA methylation pattern of mangrove rivulus liver using RRBS. This species shows a very peculiar reproduction strategy as it is the only vertebrate which alternates between cross-fertilization and selfing, the latter being the most common. It results in several lineages of isogenic individuals, and epigenetic mechanisms have been proposed to explain the occurrence of phenotypic variability despite a low level of genetic diversity (Fellous et al., 2018). We reported a RR genome representing 1.4% of the whole genome, which was comparable to what was obtained in mouse (1.4%) or in zebrafish (2.2%) brain (Chatterjee et al., 2013; Smith et al., 2009). The percentage of CpG covered in the RR genome reached 6.1% of the total number of genomic CpGs, which was similar to mouse and zebrafish (7.0% and 5.3%, respectively, (Chatterjee et al., 2013)), but higher than what other studies reported for diverse fish species such as stickleback [1% (Metzger & Schulte, 2017)], Atlantic salmon [2.75% (Uren Webster et al., 2018)], rainbow trout [<1% (Baerwald et al., 2016)] and guppies [1.5-2% (Hu et al., 2018)]. In mangrove rivulus brain, Berbel-Filho et al. (Berbel-Filho et al., 2019b) also reported a lower CpG coverage at 1.2%. This is surprising considering that different tissues of the same species usually show the same methylation profile (Zhang, Hoshida, & Sadler, 2016), and methodological aspects can not be rejected to explain this discrepancy. Our data indicated a 4.3-fold enrichment in CpG sites, which is higher than the enrichment obtained in zebrafish brain (2.4-fold) and a bit lower to the one obtained in mouse brain (5-fold).

The distribution of the analyzed fragments across the RR genome showed 16% located in promoter region. The analyzed fragments in this region are also low in zebrafish liver at 12%, and even lower in Atlantic salmon (4%) (Uren Webster et al., 2018). On the contrary, this percentage is higher in mammal species such as mouse liver (52%) (Zhang et al., 2016). This proportion of analyzed fragments located in promoter region can explain the observed difference of the mean global methylation level reported in fish species and in mammals, the latter having a lower value (28% in mouse vs 65% in the mangrove rivulus, 70% in zebrafish, 75% in Atlantic salmon). The promoter regions being less methylated on average than other regions in both fish and mammals, a higher proportion of fragments from this region decreases the average global methylation as observed in mouse RR genome. These differences between mammals and fish can also be reflected in the proportion of highly and lowly methylated fragments. If the distribution of methylation is bimodal in all reported vertebrates (fragments being either highly or lowly methylated), a higher proportion of fragments present a methylation level higher than 80% in fish than in mammals (67% of fragments in our study) (Zhang et al., 2016).

Regarding the mangrove rivulus, even if the majority of promoter fragments were lowly methylated, a significant proportion were highly methylated (38%), which indicates an elevated range of the methylation level in promoter regions, and consequently a regulatory activity of gene expression. In contrast, other genomic regions presented a narrower range in methylation levels, with the majority of fragments being highly methylated. When compared to zebrafish, an interesting difference appeared regarding the proportion of analyzed fragments located in gene bodies. While it reached 47% in mangrove rivulus, zebrafish has only 25% of CpGs in introns and exons. Conversely, the proportion of analyzed intergenic fragments is higher in zebrafish (63%) than in mangrove rivulus (37% including intergenic and upstream regions). A possible explanation could reside in the higher proportion of transposable elements (TEs) in zebrafish genome. There is increasing evidence that TEs are important targets of DNA methylation to silence their expression and consequently show a high level of CpG methylation. The zebrafish genome is rich in TEs as 52% of its genomic sequence is composed of the different classes of TEs (Chalopin et al., 2015; Shao, Han, & Peng, 2019), compared to 27.3% in the mangrove rivulus (Rhee et al., 2017). This difference could explain the relatively lower proportion of analyzed fragments located in intergenic regions in the mangrove rivulus compared to zebrafish. When considering other fish species, the part of TEs in genomes is very variable and values as low as 5% have been reported in *Tetraodon nigroviridis* (Shao et al., 2019). This variability of TE amount is explained by the large range of genome size among fish, and Chalopin et al. (Chalopin et al., 2015) reported a positive correlation between TE content and genome size in teleosts species, which also applies to the mangrove rivulus. Contrary to what was advanced by Zhang et al. (Zhang et al., 2016), it

is therefore unlikely that the pattern of high global methylation observed in fish RR genomes is the result of an enrichment in TEs. Nevertheless, the role of DNA methylation in TEs should be further investigated, including a precise look at the different types of TEs. For example, the rolling-circle transposons, known as Helitrons, are particularly abundant in the mangrove rivulus genome (0.65%) compared to other fish species including its phylogenetic relatives [Japanese medaka (0.03%) and Turquoise killifish (0.06%)]. From all studied fish species, only zebrafish showed a higher level of Helitrons at 1.5% (Shao et al., 2019). It is known that TEs in general, and the Helitron subfamily in particular, are mediator of host genes regulation and contribute to genome evolution and adaptation (Volff, 2005). It has been suggested that the mobilization of TEs and the changes in epigenetic pattern could be important in the process of organism's rapid adaptation to environmental changes (Lerat et al., 2019). The exact role of these elements and their methylation level in the mangrove rivulus is a promising perspective to investigate how this species adapt and evolve using a mixed-mating reproduction system and consequently a low genetic diversity within lineages.

4.2. Effects of EE2 on the liver methylome

Growing evidence suggest that EDCs can modify DNA methylation and consequently gene expression pattern. Recently, experiments on zebrafish embryos clearly established a link between exposure to bisphenol A, DNA methylation and behavioral impairments (Olsvik et al., 2019). Data also showed that EE2 can modify the methylation status of target genes (Stromqvist et al., 2010). In a previous study, we showed that the liver proteome of adult mangrove rivulus was impaired by exposure to EE2 during early life stages (ELS) (Voisin et al., 2018). It has been hypothesized that exposure to EE2 during ELS can impair DNA methylation and induce long term consequences later in life as observed on the proteome and on the phenotype (Voisin et al., 2016). To test this hypothesis, we aimed at detecting potential long-term changes in the hepatic DNA methylation of 168 dph adults following a 28-day early-life exposure of mangrove rivulus hatchlings. We obtained a total of 146 DMFs among all group comparisons, from which a majority were observed between the two EE2 treatments and between 4 ng/L exposed individuals and Ctl. The larger observed impacts of the lowest EE2 concentration on DNA methylation is in accordance with a non-monotonic dose-response relationship often reported for exposure to endocrine disruptors. Hormones are effective at very low concentration, having a high affinity for their receptors, and so are the endocrine disrupting chemicals (Vandenberg et al., 2012). The present results are reinforced by a similar response detected in brain and liver protein expression profiles of the mangrove rivulus exposed in the same conditions to EE2 (Voisin et al., 2018). To our knowledge, only one other study investigated the long-term effects of early-life exposure to chemicals on fish methylome (Kamstra et al., 2017). This study investigated the effects of two compounds, mono(2-ethylhexyl) phthalate (MEHP, 30 µM) and 5-azacytidine (5AC, 10 µM, an inhibitor of DNMT1) in zebrafish. They found that most methylation changes occurred at conserved non-genic regions with cis-regulatory functions (i.e. enhancers, silencers, transcription factor binding sites).

Among the differentially methylated fragments (DMFs) found in adult mangrove rivulus liver following early-life exposure to EE2, none was significantly affected in all three group comparisons (Ctl vs 4 ng/L; Ctl vs 120 ng/L; 4 vs 120 ng/L). Six DMFs were significant in two out of three comparisons, of which three were located in gene bodies. A DMF corresponding to *ATP-binding cassette sub-family A member 1* (ABCA1) was hypermethylated at both 4 ng/L and 120 ng/L compared to Ctl. ABCA1 is a known estrogen-responsive gene (Srivastava, 2002). It was included in networks in both comparisons and appeared to play a central role in the response to EE2, showing interactions with the estrogen receptor and several other molecules (Figure 4). It is a major regulator of cholesterol and phospholipid homeostasis by regulating efflux of cholesterol and phospholipids outside of the cell, that are then taken up by apolipoproteins A1 and E to form high density lipoprotein (HDL) molecules. Namely, estradiol exposure of smooth muscle cells has been shown to enhance cholesterol efflux to APOA1 and HDL, which was associated to ABCA1 overexpression via ESR2 and liver X receptor (LXR) activation (Wang et al., 2014). We suggest that the alteration of methylation level of ABCA1 gene might be related to the altered abundances of liver apolipoproteins and other downstream proteins involved in lipid metabolism previously reported (Voisin et al., 2018). The fact that both concentrations of EE2 impacted this gene methylation level indicates that this mechanism might be a general mode of action of EE2, and could induce effects at environmental concentrations as low as 4

ng/L.

Two DMFs were significantly hypermethylated at 4 ng/L in comparison to both Ctl and 120 ng/L treatments: the *calcium/calmodulin-dependent protein kinase type 1D* (CAMK1D), and the *testis-specific serine/threonine-protein kinase* (TSSK1B). Both DMFs were included in the first network of 4 ng/L vs Ctl and 120 vs 4 ng/L comparisons. CAMK1D is a protein kinase that participates in the calcium-regulated calmodulin-dependent kinase cascade, regulating calcium-mediated granulocyte function and respiratory burst and activating the transcription factor CREB1. According to Figure 4, CAMK1D interacts not only with calmodulin, but also with the amyloid precursor APP, which can be induced by estrogens through extracellular-regulated kinase 1 and 2 (ERK1/2) phosphorylation (Zhang et al., 2005). Finally, TSSK1B is essential for spermatid development and spermatogenesis. While TSSK1B is likely not expressed in the liver, it can be possible that an epigenetic alteration of this gene could be found in the ovotestis following EE2 exposure, with potential consequences on reproduction. Although no study to date has linked EE2 and TSSK1B, a study in rats has shown that bisphenol A exposure decreases the expression of TSSK1B mRNA (Marmugi et al., 2012).

We must nevertheless be careful when interpreting the changes of methylation in these 3 DMFs. First, the observed differences in methylation, if significant, ranged between 10.3 and 14.6%. Further studies should determine whether these differences have a biological impact on gene expression. However, the individuals used in the present study being issued from the same isogenic lineage, the confounding factor of genetic variability is reduced, which may help to reveal more subtle difference in DNA methylation level, and stress the usefulness of this species in epigenetic studies. Moreover, these 3 DMFs occurred in gene bodies, as did the majority of DMFs in this study (and MSPI fragments). While methylation of DNA at promoters is well known to suppress transcription, the role of DNA methylation in gene bodies is more difficult to interpret (Maunakea et al., 2010). Gene body methylation is conserved among plants and animals, with a general preference for exons (Feng et al., 2010). Potential roles include the stimulation or inhibition of transcription elongation (Yang et al., 2014) and the regulation of splicing (Jones, 2012). In mammals, most genes possess alternative transcription start sites that can be located within the gene bodies, complicating the link between methylation and expression (Jones, 2012). Nevertheless, it is now recognized that gene body methylation is positively correlated with transcriptional activity in most species (de Mendoza et al., 2020) and we should not disregard the possible implication of these observed changes in methylation after exposure to EE2.

Beside significant changes of DNA methylation within gene bodies, we also reported several DMFs belonging to intergenic regions. Interpreting alterations in DNA methylation in these regions is challenging as the gene annotation may not directly apply. Intergenic regions are typically highly methylated in all vertebrates and bear a conserved role of maintaining genome stability, by preventing the transposition of repetitive TEs and silencing cryptic promoters and cryptic splice sites (Zemach et al., 2010). Moreover, TEs possess strong promoters that may be able to transcribe neighboring genes (transcriptional interference). Finally, a compact chromatin state in these regions can prevent recombination. Most (>90%) methylcytosines occur in transposons, and low methylation of these regions could reduce genomic stability (Baccarelli & Bollati, 2009). As above mentioned, the diversity and composition of TEs in the mangrove rivulus could contribute to the genetic recombination and evolutionary adaptation in this self-fertilizing species (Rhee et al., 2017). To gain further insights into the role of intergenic methylation, future research should investigate the overlapping of DMFs with potential TEs.

In addition, we pointed out the very low proportion of DMFs classified as lowly methylated fragment (< 20% methylation). The*nipped-B-like protein B* (NIPBL) is the exception as it showed 19.6% methylation in Ctl but increased to 41.5% in fish exposed to 4 ng/L EE2. It is remarkable that this fragment is also the one showing the highest effect size of our study (+ 21.9%) and is located in the promoter region. Few more lowly methylated fragments were significant when comparing the 2 treatments, but all others were either highly methylated or showed an intermediary methylation level. Two explanations can be advanced. First, the lowly methylated fragments were about 1/3 less numerous than the highly methylated ones in the RR genome as reported in Figure 2. This is related to the proportionally low occurrence of fragments located in the promoter

region, which have the lowest methylation level. Second, we also showed that lowly methylated fragments have a 10-fold higher coefficient of variability (33% vs 3% in highly methylated fragments). It results that the statistical power to detect changes of lowly methylated fragments is lower and that we can consequently report only DMFs with a high effect size, such as NIPBL. To overstep this limitation, one should include a higher number of replicates in future RRBS analyses. It is even more important for this kind of studies for which effects are searched long after the end of the exposure, which decrease the probability to observe high effect sizes. Nevertheless, our study is the first to report the effect of an endocrine disruptor exposure on the methylation level of NIPBL promoter region. This effect, which was significant while the exposure had stopped 140 days earlier, could have serious long-term consequences on adult fish. NIPBL is associated to cohesin in order to mediate chromatin cohesion, important for mitosis. This complex plays an important role in histone acetylation, chromosome architecture and therefore in regulation of gene expression (Gao et al., 2019; Zhu & Wang, 2019). In human, a mutation of NIPBL is associated to the Cornelia de Lange syndrome, a developmental disorder (Newkirk et al., 2017). In zebrafish, the zygotic genome activation is facilitated by cohesin and was a key for the embryogenesis (Meier et al., 2018). Intriguingly, the cohesin complex has been proposed to modulate estrogen-dependent gene transcription and a link has been established with breast cancer (Antony et al., 2015). Our results suggest that NIPBL hypermethylation could be a mode of action of EE2 exposure and could potentially modify gene regulation. Further studies should investigate the long-term links between the change in NIPBL methylation level and the overall changes in gene expression in adults, long after the exposure to EE2.

Little correspondence was found between the proteome previously published (Voisin et al., 2018) and the epigenome (Supplementary Figure 2). Although the number of identified genes was about 10 x greater in the methylome analysis, only about half of the total identified proteins in the liver was found in the methylome analysis. Similarly, we could retrieve the methylation level of fragments belonging to approximately half (52) of the differentially abundant proteins. None of these differentially abundant proteins could be related to a change in DNA methylation level of the promotor region. Conversely, only five of the significantly DMFs were also identified, but not significant, in the proteomic analysis in liver: *G1/S-specific cyclin-D2-like* (CCND2, promoter region, significant between 4 ng/L and Ctl),*teneurin-1-like* (TENM1, gene body, significant between 120 ng/L and Ctl), *protein NDRG1-like* (NDRG1, promoter, significant between 120 ng/L and Ctl), *fibrinogen alpha chain* (NGA, intergenic, significant between 120 ng/L and Ctl), and*death-associated protein 1* (DAP, gene body, significant between 4 and 120 ng/L). Comparison between DNA methylation and protein expression pattern is rare but is fully justified. The proteome is the main functional product of gene expression and is closely related to the molecular phenotype (Silvestre, Gillardin, & Dorts, 2012). As such, the proteomic level can generate new hypothesis on the modes of action of xenobiotics and on the adaptive responses of organisms (Diz, Martínez-Fernández, & Rolán-Alvarez, 2012). Epigenetics being at the interface between the genotype and the environment, a more systematic link with the proteome would improve the understanding of the adverse outcome pathways (AOPs) and the outcomes in terms of adaptation to the environment. However, it is challenging to correlate these two levels, because of technical variability, and because differentially abundant proteins are not necessarily regulated by differential methylation, but rather the end product of upstream regulators, themselves differentially methylated. Also, protein abundance results not only from transcription, which can be influenced by DNA methylation, but also from mRNA stability, alternative splicing, translation, protein turnover and post-translational modifications. Nevertheless, comparison of proteome and methylation data can be eased by the analysis of gene ontology. Some biological processes were identified in both datasets, such as lipid metabolism, inflammation, connective tissue development and molecular transport, which makes their long-term impairment plausible mechanisms of EE2 toxicity.

4.3. Conclusions

In conclusion, our study provides further evidence on the capacity of EDCs to alter the methylome and shows that these changes can be apparent several months after the exposure, supporting the hypothesis of possible long-term modulation of gene expression through epigenetics. It also confirms the non-monotonic response to EDCs as most significant effects were observed at the environmental concentration of 4 ng/L. The DMFs belonging to promoter and gene bodies revealed networks involved in several biological functi-

ons potentially regulated by estrogens, including lipid metabolism, cellular processes (death and survival), connective tissue function, molecular transport and inflammation, many of which were also identified in a previous proteomic analysis. Importantly, we reported that methylation of *nipped-B-like protein B* (NIPBL) might be an important mode of action of EE2 and, as involved in chromosome organization and gene expression, could have long term impact on gene regulation and on organism's general functions. To gain a more comprehensive view of the significance of DMFs in promoter, gene body, upstream and intergenic regions, future studies should investigate the overlapping of the DMFs with potential enhancers, insulators, transcription start sites, TEs, estrogen-responsive elements and other transcription factor binding sites that may interact with estrogen receptors. Among them, TEs, and more particularly helitrons, are potentially important targets of environmental stressors that could be involved in the response of organisms to environmental changes. Despite its limitations, RRBS has proved to be an efficient method to investigate effects of EE2 on DNA methylation in the mangrove rivulus. This self-fertilizing fish should be further considered as a model species to investigate the interplay between environmental stressors, epigenetics and adaptation. The possibility to naturally discard the confounding factor of genetic variability makes this species a top model to better understand the roles of epigenetics in the long-term response to environmental xenobiotics and in the developmental origin of health and diseases.

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6. References

Anderson, A. M., Carter, K. W., Anderson, D., & Wise, M. J. (2012). Coexpression of nuclear receptors and histone methylation modifying genes in the testis: implications for endocrine disruptor modes of action. *PLoS One* , *7* (4), e34158. https://doi.org/10.1371/journal.pone.0034158

Antony, J., Dasgupta, T., Rhodes, J. M., McEwan, M. V., Print, C. G., O'Sullivan, J. M., & Horsfield, J. A. (2015). Cohesin modulates transcription of estrogen-responsive genes. *Biochim Biophys Acta* ,*1849* (3), 257-269. https://doi.org/10.1016/j.bbagrm.2014.12.011

Aris, A. Z., Shamsuddin, A. S., & Praveena, S. M. (2014). Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environ Int* , *69* , 104-119. https://doi.org/10.1016/j.envint.2014.04.011

Avise, J. C., & Tatarenkov, A. (2015). Population genetics and evolution of the mangrove rivulus Kryptolebias marmoratus, the world's only self-fertilizing hermaphroditic vertebrate. *J Fish Biol* ,*87* (3), 519-538. https://doi.org/10.1111/jfb.12741

Baccarelli, A., & Bollati, V. (2009). Epigenetics and environmental chemicals. *Curr Opin Pediatr* , *21* (2), 243-251. https://doi.org/10.1097/mop.0b013e32832925cc

Baerwald, M. R., Meek, M. H., Stephens, M. R., Nagarajan, R. P., Goodbla, A. M., Tomalty, K. M. H., Thorgaard, G. H., May, B., & Nichols, K. M. (2016). Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. *Mol Ecol* , *25* (8), 1785-1800. https://doi.org/10.1111/mec.13231

Barker, D. J. (2004). The developmental origins of adult disease.*J Am Coll Nutr* , *23* (6 Suppl), 588S-595S. https://doi.org/10.1080/07315724.2004.10719428

Barouki, R., Melén, E., Herceg, Z., Beckers, J., Chen, J., Karagas, M., Puga, A., Xia, Y., Chadwick, L., Yan, W., Audouze, K., Slama, R., Heindel, J., Grandjean, P., Kawamoto, T., & Nohara, K. (2018). Epigenetics

as a mechanism linking developmental exposures to long-term toxicity. *Environment International* , *114* (January), 77-86. https://doi.org/10.1016/j.envint.2018.02.014

Berbel-Filho, W. M., de Leaniz, C. G., Moran, P., Cable, J., Lima, S. M. Q., & Consuegra, S. (2019a). Local parasite pressures and host genotype modulate epigenetic diversity in a mixed - mating fish. *Ecology and Evolution* , *9* (July), 8736-8748. https://doi.org/10.1002/ece3.5426

Berbel-Filho, W. M., Rodriguez-Barreto, D., Berry, N., Garcia De Leaniz, C., & Consuegra, S. (2019b). Contrasting DNA methylation responses of inbred fish lines to different rearing environments. *Epigenetics* ,*14* (10), 939-948. https://doi.org/10.1080/15592294.2019.1625674

Bock, C., Beerman, I., Lien, W. H., Smith, Z. D., Gu, H., Boyle, P., Gnirke, A., Fuchs, E., Rossi, D. J., & Meissner, A. (2012). DNA methylation dynamics during in vivo differentiation of blood and skin stem cells. *Mol Cell* , *47* (4), 633-647. https://doi.org/10.1016/j.molcel.2012.06.019

Brander, S. M., Biales, A. D., & Connon, R. E. (2017). The Role of Epigenomics in Aquatic Toxicology. *Environ Toxicol Chem* ,*36* (10), 2565-2573. https://doi.org/10.1002/etc.3930

Braun, J. M. (2017). Early-life exposure to EDCs: role in childhood obesity and neurodevelopment. *Nature Reviews Endocrinology* ,*13* (3), 161.

Brockmeier, E. K., Hodges, G., Hutchinson, T. H., Butler, E., Hecker, M., Tollefsen, K. E., Garcia-Reyero, N., Kille, P., Becker, D., Chipman, K., Colbourne, J., Collette, T. W., Cossins, A., Cronin, M., Graystock, P., Gutsell, S., Knapen, D., Katsiadaki, I., Lange, A., . . . Falciani, F. (2017). The Role of Omics in the Application of Adverse Outcome Pathways for Chemical Risk Assessment. *Toxicol Sci* ,*158* (2), 252-262. https://doi.org/10.1093/toxsci/kfx097

Carion, A., Markey, A., Hetru, J., Carpentier, C., Suarez-Ulloa, V., Denoel, M., Earley, R. L., & Silvestre, F. (2020). Behavior and gene expression in the brain of adult self-fertilizing mangrove rivulus fish (Kryptolebias marmoratus) after early life exposure to the neurotoxin β-N-methylamino-L-alanine (BMAA). *Neurotoxicology* . https://doi.org/10.1016/j.neuro.2020.04.007

Casati, L., Sendra, R., Sibilia, V., & Celotti, F. (2015). Endocrine disrupters: the new players able to affect the epigenome. *Front Cell Dev Biol* , *3* , 37. https://doi.org/10.3389/fcell.2015.00037

Chalopin, D., Naville, M., Plard, F., Galiana, D., & Volff, J. N. (2015). Comparative analysis of transposable elements highlights mobilome diversity and evolution in vertebrates. *Genome Biol Evol* , *7* (2), 567-580. https://doi.org/10.1093/gbe/evv005

Chatterjee, A., Ozaki, Y., Stockwell, P. A., Horsfield, J. A., Morison, I. M., & Nakagawa, S. (2013). Mapping the zebrafish brain methylome using reduced representation bisulfite sequencing. *Epigenetics* ,*8* (9), 979-989. https://doi.org/10.4161/epi.25797

Chatterjee, A., Rodger, E. J., Stockwell, P. A., Weeks, R. J., & Morison, I. M. (2012a). Technical considerations for reduced representation bisulfite sequencing with multiplexed libraries. *J Biomed Biotechnol* , *2012* , 741542. https://doi.org/10.1155/2012/741542

Chatterjee, A., Stockwell, P. A., Horsfield, J. A., Morison, I. M., & Nakagawa, S. (2014). Base-resolution DNA methylation landscape of zebrafish brain and liver. *Genom Data* , *2* , 342-344. https://doi.org/10.1016/j.gdata.2014.10.008

Chatterjee, A., Stockwell, P. A., Rodger, E. J., & Morison, I. M. (2012b). Comparison of alignment software for genome-wide bisulphite sequence data. *Nucleic Acids Res* , *40* (10), e79. https://doi.org/10.1093/nar/gks150

Clouzot, L., Marrot, B., Doumenq, P., & Roche, N. (2008). 17α-Ethinylestradiol: An endocrine disrupter of great concern. Analytical methods and removal processes applied to water purification. A review. *Environmental Progress* , *27* (3), 383-396. https://doi.org/10.1002/ep.10291

de Mendoza, A., Lister, R., & Bogdanovic, O. (2020). Evolution of DNA Methylome Diversity in Eukaryotes. *J Mol Biol* , *432* , 1687-1705. https://doi.org/10.1016/j.jmb.2019.11.003

De Wit, M., Keil, D., Remmerie, N., van der Ven, K., van den Brandhof, E. J., Knapen, D., Witters, E., & De Coen, W. (2008). Molecular targets of TBBPA in zebrafish analysed through integration of genomic and proteomic approaches. *Chemosphere* , *74* (1), 96-105. https://doi.org/10.1016/j.chemosphere.2008.09.030

Diz, A. P., Martínez-Fernández, M., & Rolán-Alvarez, E. (2012). Proteomics in evolutionary ecology: linking the genotype with the phenotype. *Mol Ecol* , *21* (5), 1060-1080. https://doi.org/10.1111/j.1365-294X.2011.05426.x

Dorts, J., Falisse, E., Schoofs, E., Flamion, E., Kestemont, P., & Silvestre, F. (2016). DNA methyltransferases and stress-related genes expression in zebrafish larvae after exposure to heat and copper during reprogramming of DNA methylation. *Scientific Reports* ,*6* (October), 34254. https://doi.org/10.1038/srep34254

Edwards, J. R., Yarychkivska, O., Boulard, M., & Bestor, T. H. (2017). DNA methylation and DNA methyltransferases. *Epigenetics & Chromatin* , *10* (1). https://doi.org/10.1186/s13072-017-0130-8

Eid, A., & Zawia, N. (2016). Consequences of lead exposure, and it's emerging role as an epigenetic modifier in the aging brain.*NeuroToxicology* , *56* (2015), 254-261. https://doi.org/10.1016/j.neuro.2016.04.006

Ellison, A., Rodríguez López, C. M., Moran, P., Breen, J., Swain, M., Megias, M., Hegarty, M., Wilkinson, M., Pawluk, R., & Consuegra, S. (2015). Epigenetic regulation of sex ratios may explain natural variation in self-fertilization rates. *Proc Biol Sci* ,*282* (1819). https://doi.org/10.1098/rspb.2015.1900

Falisse, E., Ducos, B., Stockwell, P. A., Morison, I. M., Chatterjee, A., & Silvestre, F. (2018). DNA methylation and gene expression alterations in zebrafish early-life stages exposed to the antibacterial agent triclosan. *Environ Pollut* , *243* (Pt B), 1867-1877. https://doi.org/10.1016/j.envpol.2018.10.004

Fellous, A., Earley, R. L., & Silvestre, F. (2019a). Identification and expression of mangrove rivulus (Kryptolebias marmoratus) histone deacetylase (HDAC) and lysine acetyltransferase (KAT) genes.*Gene* , *691* (December 2018), 56-69. https://doi.org/10.1016/j.gene.2018.12.057

Fellous, A., Earley, R. L., & Silvestre, F. (2019b). The Kdm/Kmt gene families in the self-fertilizing mangrove rivulus fish, Kryptolebias marmoratus, suggest involvement of histone methylation machinery in development and reproduction. *Gene* , *687* (November 2018), 173-187. https://doi.org/10.1016/j.gene.2018.11.046

Fellous, A., Voisin, A.-S., Labed-veydert, T., Locrel, M., Earley, R. L., & Silvestre, F. (2018). DNA methylation in adults and during development of the self- fertilizing mangrove rivulus, Kryptolebias marmoratus. *Ecology and Evolution* , *8* (June), 6016-6033. https://doi.org/10.1002/ece3.4141

Feng, S., Cokus, S. J., Zhang, X., Chen, P. Y., Bostick, M., Goll, M. G., Hetzel, J., Jain, J., Strauss, S. H., Halpern, M. E., Ukomadu, C., Sadler, K. C., Pradhan, S., Pellegrini, M., & Jacobsen, S. E. (2010). Conservation and divergence of methylation patterning in plants and animals. *Proc Natl Acad Sci U S A* , *107* (19), 8689-8694. https://doi.org/10.1073/pnas.1002720107

Feswick, A., Munkittrick, K. R., & Martyniuk, C. J. (2017). Estrogen-responsive gene networks in the teleost liver: What are the key molecular indicators. *Environ Toxicol Pharmacol* , *56* , 366-374. https://doi.org/10.1016/j.etap.2017.10.012

Foulds, C. E., Treviño, L. S., York, B., & Walker, C. L. (2017). Endocrine-disrupting chemicals and fatty liver disease. *Nature Reviews Endocrinology* , *13* (8), 445-457. https://doi.org/10.1038/nrendo.2017.42

Gao, D., Zhu, B., Cao, X., Zhang, M., & Wang, X. (2019). Roles of NIPBL in maintenance of genome stability. *Semin Cell Dev Biol* ,*90* , 181-186. https://doi.org/10.1016/j.semcdb.2018.08.005

Gore, A. C., Walker, D. M., Zama, A. M., Armenti, A. E., & Uzumcu, M. (2011). Early life exposure to endocrine-disrupting chemicals causes lifelong molecular reprogramming of the hypothalamus and premature reproductive aging. *Mol Endocrinol* , *25* (12), 2157-2168. https://doi.org/10.1210/me.2011-1210

Goyal, D., Limesand, S. W., & Goyal, R. (2019). Epigenetic responses and the developmental origins of health and disease. *The Journal of endocrinology* , *242* (1), T105-T119. https://doi.org/10.1530/JOE-19-0009

Guo, H., Zhu, P., Yan, L., Li, R., Hu, B., Lian, Y., Yan, J., Ren, X., Lin, S., Li, J., Jin, X., Shi, X., Liu, P., Wang, X., Wang, W., Wei, Y., Li, X., Guo, F., Wu, X., . . . Qiao, J. (2014). The DNA methylation landscape of human early embryos. *Nature* , *511* (7511), 606-610. https://doi.org/10.1038/nature13544

Heard, E., & Martienssen, R. A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* , *157* (1), 95-109. https://doi.org/10.1016/j.cell.2014.02.045

Heindel, J. J., Newbold, R., & Schug, T. T. (2015). Endocrine disruptors and obesity. *Nat Rev Endocrinol* , *11* , 653-661. https://doi.org/10.1038/nrendo.2015.163

Hu, J., Pérez-Jvostov, F., Blondel, L., & Barrett, R. D. H. (2018). Genome-wide DNA methylation signatures of infection status in Trinidadian guppies (Poecilia reticulata). *Mol Ecol* ,*27* (15), 3087-3102. https://doi.org/10.1111/mec.14771

Humble, J. L., Hands, E., Saaristo, M., Lindström, K., Lehtonen, K. K., Diaz de Cerio, O., Cancio, I., Wilson, G., & Craft, J. A. (2013). Characterisation of genes transcriptionally upregulated in the liver of sand goby (Pomatoschistus minutus) by 17α-ethinyloestradiol: identification of distinct vitellogenin and zona radiata protein transcripts. *Chemosphere* , *90* (11), 2722-2729. https://doi.org/10.1016/j.chemosphere.2012.11.053

Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* , *13* (7), 484-492. https://doi.org/10.1038/nrg3230

Kajta, M., & Wójtowicz, A. K. (2013). Impact of endocrine-disrupting chemicals on neural development and the onset of neurological disorders.*Pharmacol Rep* , *65* (6), 1632-1639. https://doi.org/10.1016/s1734-1140(13)71524-x

Kamstra, J. H., Sales, L. B., Aleström, P., & Legler, J. (2017). Differential DNA methylation at conserved non-genic elements and evidence for transgenerational inheritance following developmental exposure to mono(2-ethylhexyl) phthalate and 5-azacytidine in zebrafish.*Epigenetics Chromatin* , *10* , 20. https://doi.org/10.1186/s13072-017-0126-4

Krueger, F., & Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications.*Bioinformatics* , *27* (11), 1571-1572. https://doi.org/10.1093/bioinformatics/btr167

Kuo, C. H., Yang, S. N., Kuo, P. L., & Hung, C. H. (2012). Immunomodulatory effects of environmental endocrine disrupting chemicals. *Kaohsiung J Med Sci* , *28* (7 Suppl), S37-42. https://doi.org/10.1016/j.kjms.2012.05.008

Law, J. A., & Jacobsen, S. E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat Rev Genet* , *11* (3), 204-220. https://doi.org/10.1038/nrg2719

Lee, D. H., Jacobs, D. R., & Porta, M. (2009). Hypothesis: a unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. *Environ Health Perspect* , *117* (12), 1799-1802. https://doi.org/10.1289/ehp.0900741

Lerat, E., Casacuberta, J., Chaparro, C., & Vieira, C. (2019). On the Importance to Acknowledge Transposable Elements in Epigenomic Analyses.*Genes (Basel)* , *10* (4). https://doi.org/10.3390/genes10040258

Lister, R., & Ecker, J. R. (2009). Finding the fifth base: genome-wide sequencing of cytosine methylation. *Genome Res* , *19* (6), 959-966. https://doi.org/10.1101/gr.083451.108

Liu, Y., Duong, W., Krawczyk, C., Bretschneider, N., Borbély, G., Varshney, M., Zinser, C., Schär, P., & Rüegg, J. (2016). Oestrogen receptor β regulates epigenetic patterns at specific genomic loci through interaction with thymine DNA glycosylase. *Epigenetics Chromatin* , *9* , 7. https://doi.org/10.1186/s13072-016-0055-7

Mackiewicz, M., Tatarenkov, A., Perry, A., Martin, J. R., Elder, J. F., Bechler, D. L., & Avise, J. C. (2006). Microsatellite documentation of male-mediated outcrossing between inbred laboratory strains of the self-fertilizing mangrove killifish (Kryptolebias Marmoratus). *J Hered* , *97* (5), 508-513. https://doi.org/10.1093/jhered/esl017

Marmugi, A., Ducheix, S., Lasserre, F., Polizzi, A., Paris, A., Priymenko, N., Bertrand-Michel, J., Pineau, T., Guillou, H., Martin, P. G., & Mselli-Lakhal, L. (2012). Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver. *Hepatology* , *55* (2), 395-407. https://doi.org/10.1002/hep.24685

Matthiessen, P., Wheeler, J. R., & Weltje, L. (2018). A review of the evidence for endocrine disrupting effects of current-use chemicals on wildlife populations. *Critical Reviews in Toxicology* ,*48* (3), 195-216. https://doi.org/10.1080/10408444.2017.1397099

Maunakea, A. K., Nagarajan, R. P., Bilenky, M., Ballinger, T. J., D'Souza, C., Fouse, S. D., Johnson, B. E., Hong, C., Nielsen, C., Zhao, Y., Turecki, G., Delaney, A., Varhol, R., Thiessen, N., Shchors, K., Heine, V. M., Rowitch, D. H., Xing, X., Fiore, C., . . . Costello, J. F. (2010). Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* , *466* (7303), 253-257. https://doi.org/10.1038/nature09165

Meier, M., Grant, J., Dowdle, A., Thomas, A., Gerton, J., Collas, P., O'Sullivan, J. M., & Horsfield, J. A. (2018). Cohesin facilitates zygotic genome activation in zebrafish. *Development* ,*145* (1). https://doi.org/10.1242/dev.156521

Meissner, A., Gnirke, A., Bell, G. W., Ramsahoye, B., Lander, E. S., & Jaenisch, R. (2005). Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. *Nucleic Acids Res* , *33* (18), 5868-5877. https://doi.org/10.1093/nar/gki901

Metzger, D. C. H., & Schulte, P. M. (2017). Persistent and plastic effects of temperature on DNA methylation across the genome of threespine stickleback (Gasterosteus aculeatus). *Proc Biol Sci* ,*284* (1864). https://doi.org/10.1098/rspb.2017.1667

Mirbahai, L., & Chipman, J. K. (2014). Epigenetic memory of environmental organisms: a reflection of lifetime stressor exposures.*Mutat Res Genet Toxicol Environ Mutagen* , *764-765* , 10-17. https://doi.org/10.1016/j.mrgentox.2013.10.003

Mirbahai, L., Yin, G., Bignell, J. P., Li, N., Williams, T. D., & Chipman, J. K. (2011). DNA methylation in liver tumorigenesis in fish from the environment. *Epigenetics* , *6* (11), 1319-1333. https://doi.org/10.4161/epi.6.11.17890

Moore, L. D., Le, T., & Fan, G. (2013). DNA Methylation and Its Basic Function. *Neuropsychopharmacology* , *38* (1), 23-38. https://doi.org/10.1038/npp.2012.112

Newkirk, D. A., Chen, Y. Y., Chien, R., Zeng, W., Biesinger, J., Flowers, E., Kawauchi, S., Santos, R., Calof, A. L., Lander, A. D., Xie, X., & Yokomori, K. (2017). The effect of Nipped-B-like (Nipbl) haploinsufficiency on genome-wide cohesin binding and target gene expression: modeling Cornelia de Lange syndrome. *Clin Epigenetics* , *9* , 89. https://doi.org/10.1186/s13148-017-0391-x

Olsvik, P. A., Whatmore, P., Penglase, S. J., Skjærven, K. H., Anglès d'Auriac, M., & Ellingsen, S. (2019). Associations Between Behavioral Effects of Bisphenol A and DNA Methylation in Zebrafish Embryos.*Front Genet* , *10* , 184. https://doi.org/10.3389/fgene.2019.00184

Rhee, J. S., Choi, B. S., Kim, J., Kim, B. M., Lee, Y. M., Kim, I. C., Kanamori, A., Choi, I. Y., Schartl, M., & Lee, J. S. (2017). Diversity, distribution, and significance of transposable elements in the genome of the only selfing hermaphroditic vertebrate Kryptolebias marmoratus.*Sci Rep* , *7* , 40121. https://doi.org/10.1038/srep40121

Shao, F., Han, M., & Peng, Z. (2019). Evolution and diversity of transposable elements in fish genomes. *Scientific Reports* ,*9* (1). https://doi.org/10.1038/s41598-019-51888-1

Shi, J. F., Li, X. J., Si, X. X., Li, A. D., Ding, H. J., Han, X., & Sun, Y. J. (2012). ERα positively regulated DNMT1 expression by binding to the gene promoter region in human breast cancer MCF-7 cells. *Biochem Biophys Res Commun* , *427* (1), 47-53. https://doi.org/10.1016/j.bbrc.2012.08.144

Silvestre, F., Gillardin, V., & Dorts, J. (2012). Proteomics to assess the role of phenotypic plasticity in aquatic organisms exposed to pollution and global warming. *Integr Comp Biol* , *52* (5), 681-694. https://doi.org/10.1093/icb/ics087

Smith, Z. D., Chan, M. M., Humm, K. C., Karnik, R., Mekhoubad, S., Regev, A., Eggan, K., & Meissner, A. (2014). DNA methylation dynamics of the human preimplantation embryo. *Nature* , *511* (7511), 611-615. https://doi.org/10.1038/nature13581

Smith, Z. D., Gu, H., Bock, C., Gnirke, A., & Meissner, A. (2009). High-throughput bisulfite sequencing in mammalian genomes. *Methods* , *48* (3), 226-232. https://doi.org/10.1016/j.ymeth.2009.05.003

Srivastava, R. A. (2002). Estrogen-induced regulation of the ATP-binding cassette transporter A1 (ABCA1) in mice: a possible mechanism of atheroprotection by estrogen. *Mol Cell Biochem* , *240* (1-2), 67-73. https://doi.org/10.1023/a:1020604610873

Stel, J., & Legler, J. (2015). The role of epigenetics in the latent effects of early life exposure to obesogenic endocrine disrupting chemicals. *Endocrinology* , *156* (10), 3466-3472. https://doi.org/10.1210/en.2015-1434

Stockwell, P. A., Chatterjee, A., Rodger, E. J., & Morison, I. M. (2014). DMAP: differential methylation analysis package for RRBS and WGBS data. *Bioinformatics* , *30* (13), 1814-1822. https://doi.org/10.1093/bioinformatics/btu126

Strömqvist, M., Tooke, N., & Brunström, B. (2010). DNA methylation levels in the 5' flanking region of the vitellogenin I gene in liver and brain of adult zebrafish (Danio rerio)–sex and tissue differences and effects of 17alpha-ethinylestradiol exposure. *Aquat Toxicol* ,*98* (3), 275-281. https://doi.org/10.1016/j.aquatox.2010.02.023

Uren Webster, T. M., Rodriguez-Barreto, D., Martin, S. A. M., Van Oosterhout, C., Orozco-terWengel, P., Cable, J., Hamilton, A., Garcia De Leaniz, C., & Consuegra, S. (2018). Contrasting effects of acute and chronic stress on the transcriptome, epigenome, and immune response of Atlantic salmon. *Epigenetics* , *13* (12), 1191-1207. https://doi.org/10.1080/15592294.2018.1554520

Vandegehuchte, M. B., & Janssen, C. R. (2011). Epigenetics and its implications for ecotoxicology. *Ecotoxicology* , *20* (3), 607-624. https://doi.org/10.1007/s10646-011-0634-0

Vandegehuchte, M. B., & Janssen, C. R. (2014). Epigenetics in an ecotoxicological context. *Mutat Res Genet Toxicol Environ Mutagen* , *764-765* , 36-45. https://doi.org/10.1016/j.mrgentox.2013.08.008

Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Lee, D. H., Shioda, T., Soto, A. M., vom Saal, F. S., Welshons, W. V., Zoeller, R. T., & Myers, J. P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev* , *33* (3), 378-455. https://doi.org/10.1210/er.2011-1050

Voisin, A.-S., Fellous, A., Earley, R. L., & Silvestre, F. (2016). Delayed impacts of developmental exposure to 17-α-ethinylestradiol in the self-fertilizing fish Kryptolebias marmoratus. *Aquatic Toxicology* , *180* , 247-257. https://doi.org/10.1016/j.aquatox.2016.10.003

Voisin, A.-S., Kültz, D., & Silvestre, F. (2018). Early-life exposure to the endocrine disruptor 17-α-ethinylestradiol induces delayed effects in adult brain, liver and ovotestis proteomes of a self-fertilizing fish. *Journal of Proteomics* , June), 1-13. https://doi.org/10.1016/j.jprot.2018.12.008

Volff, J. N. (2005). Genome evolution and biodiversity in teleost fish. *Heredity (Edinb)* , *94* (3), 280-294. https://doi.org/10.1038/sj.hdy.6800635

Wadhwa, P. D., Buss, C., Entringer, S., & Swanson, J. M. (2009). Developmental origins of health and disease: Brief history of the approach and current focus on epigenetic mechanisms. *Seminars in Reproductive Medicine* , *27* (5), 358-368. https://doi.org/10.1055/s-0029-1237424

Walker, C. L. (2016). Minireview: Epigenomic Plasticity and Vulnerability to EDC Exposures. *Mol Endocrinol* , *30* (8), 848-855. https://doi.org/10.1210/me.2016-1086

Wang, H., Liu, Y., Zhu, L., Wang, W., Wan, Z., Chen, F., Wu, Y., Zhou, J., & Yuan, Z. (2014). 17β-estradiol promotes cholesterol efflux from vascular smooth muscle cells through a liver X receptor α-dependent pathway. *Int J Mol Med* , *33* (3), 550-558. https://doi.org/10.3892/ijmm.2014.1619

Wreczycka, K., Gosdschan, A., Yusuf, D., Grüning, B., Assenov, Y., & Akalin, A. (2017). Strategies for analyzing bisulfite sequencing data.*J Biotechnol* , *261* , 105-115. https://doi.org/10.1016/j.jbiotec.2017.08.007

Yang, X., Han, H., De Carvalho, D. D., Lay, F. D., Jones, P. A., & Liang, G. (2014). Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell* , *26* (4), 577-590. https://doi.org/10.1016/j.ccr.2014.07.028

Zemach, A., McDaniel, I. E., Silva, P., & Zilberman, D. (2010). Genome-wide evolutionary analysis of eukaryotic DNA methylation.*Science* , *328* (5980), 916-919. https://doi.org/10.1126/science.1186366

Zhang, C., Hoshida, Y., & Sadler, K. C. (2016). Comparative Epigenomic Profiling of the DNA Methylome in Mouse and Zebrafish Uncovers High Interspecies Divergence. *Front Genet* , *7* , 110. https://doi.org/10.3389/fgene.2016.00110

Zhang, S., Huang, Y., Zhu, Y. C., & Yao, T. (2005). Estrogen stimulates release of secreted amyloid precursor protein from primary rat cortical neurons via protein kinase C pathway. *Acta Pharmacol Sin* ,*26* (2), 171-176. https://doi.org/10.1111/j.1745-7254.2005.00538.x

Zhang, X., & Ho, S. M. (2011). Epigenetics meets endocrinology. *J Mol Endocrinol* , *46* (1), R11-32. https://doi.org/10.1677/jme-10-0053

Zhu, Z., & Wang, X. (2019). Roles of cohesin in chromosome architecture and gene expression. *Semin Cell Dev Biol* , *90* , 187-193. https://doi.org/10.1016/j.semcdb.2018.08.004

7. Data Accessibility Statement

All the data generated by the RRBS workflow will be submitted to Dryad repository and will be publicly accessible. The BAM files after Bismark alignment and the excel sheet containing the values for all analyzed fragments have already been submitted and are available at this link:*https://datadryad.org/stash/share/k7Lq6mHbxBMF5BHdk8cOp1EFGSGVK3tfJoBx4tPYnzc.*

The DOI is: https://doi.org/10.5061/dryad.v41ns1rsv

The Fastq files will be submitted when a decision will have been given for the acceptance of the manuscript.

8. Author Contributions

Anne-Sophie Voisin: investigation, manuscript writing and editing, experimental design, data acquisition and analysis

Victoria Suarez Ulloa: data analysis, manuscript editing

Peter Stockwell: bioinformatic data analyses

Aniruddha Chatterjee: supervision, methodology, RRBS analyses, manuscript editing

Frédéric Silvestre: investigation, supervision, funding acquisition, manuscript writing and editing, experimental design, submission

9. Tables

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Table 2: Number of analyzed fragments, analyzed CpGs and number of DMFs in each group comparison.

	4 ng/L vs Ctl	120 ng/L vs Ctl	120 vs 4 ng/L
Fragments with P<0.01	1,674 (1.6%)	787 (0.75%)	1,471 (1.4%)
Fragments with [?] 10%	383 (0.36%)	311 (0.30%)	471 (0.46%)
Differentially methylated fragments (DMFs; P<0.01 and methylation difference [?] 10%)**	**58** (0.06%)(46—, 12—)	**33** (0.03%)(20—, 13—)	**62** (0.06%)(13—,49—)
Gene body DMFs	26 (22—, 4—)	12 (9—, 3—)	31 (6—, 25—)
Promoter (>5kb) DMFs	6 (4—, 2—)	4 (3—, 1—)	11(3—, 8—)
Upstream (5-10 kb) DMFs	3—	4 (2—, 2—)	3—
Intergenic (>10 kb) DMFs	23 (17—, 6—)	13 (6—, 7—)	17 (4—, 13—)

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10. Figure legends

Figure 1: Hepatic DNA methylation landscape in *K. marmoratus* . A: Frequency distribution of MSPI fragment size. Mean fragment size was 95 bp and median 88 bp; B: Overall distribution of analysed fragments in genomic elements; C: Frequency distribution of DNA methylation % for MSPI fragments. Distribution of MSPI fragments follows a bimodal distribution, which is expected given the binary character of DNA methylation in cells; D: Boxplot of DNA methylation (%) of fragments according to their location in genomic elements (including medians and quantile 25-75%).

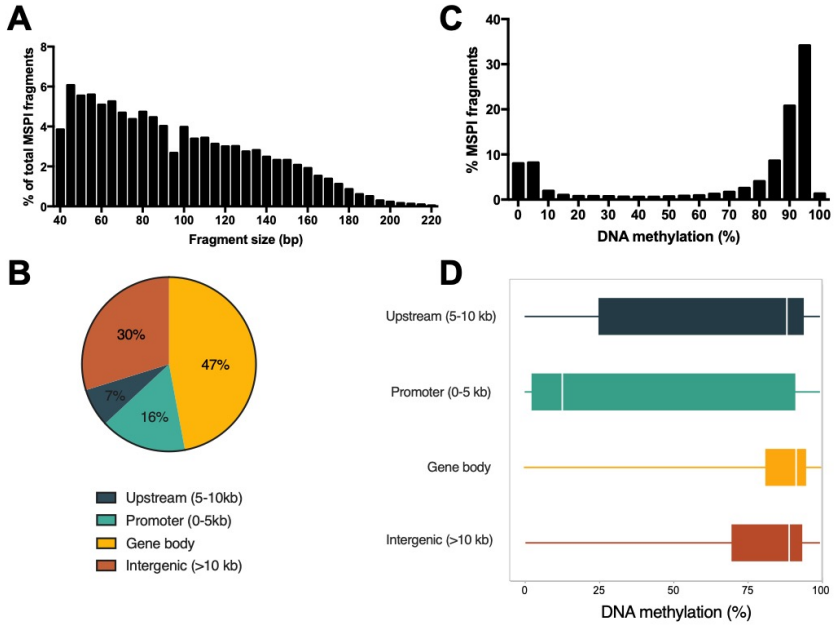
Figure 2: Distribution of lowly methylated (<20% methylation) (A) and highly methylated (> 80% methylation) (B) fragments among the genomic regions (gene body, promoter, upstream, intergenic) of the liver

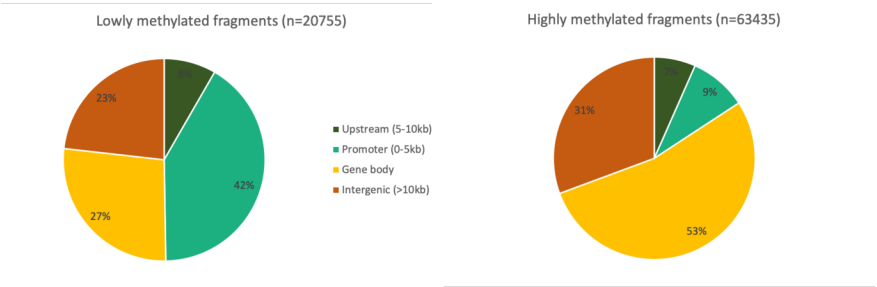
reduced representative genome of the mangrove rivulus.

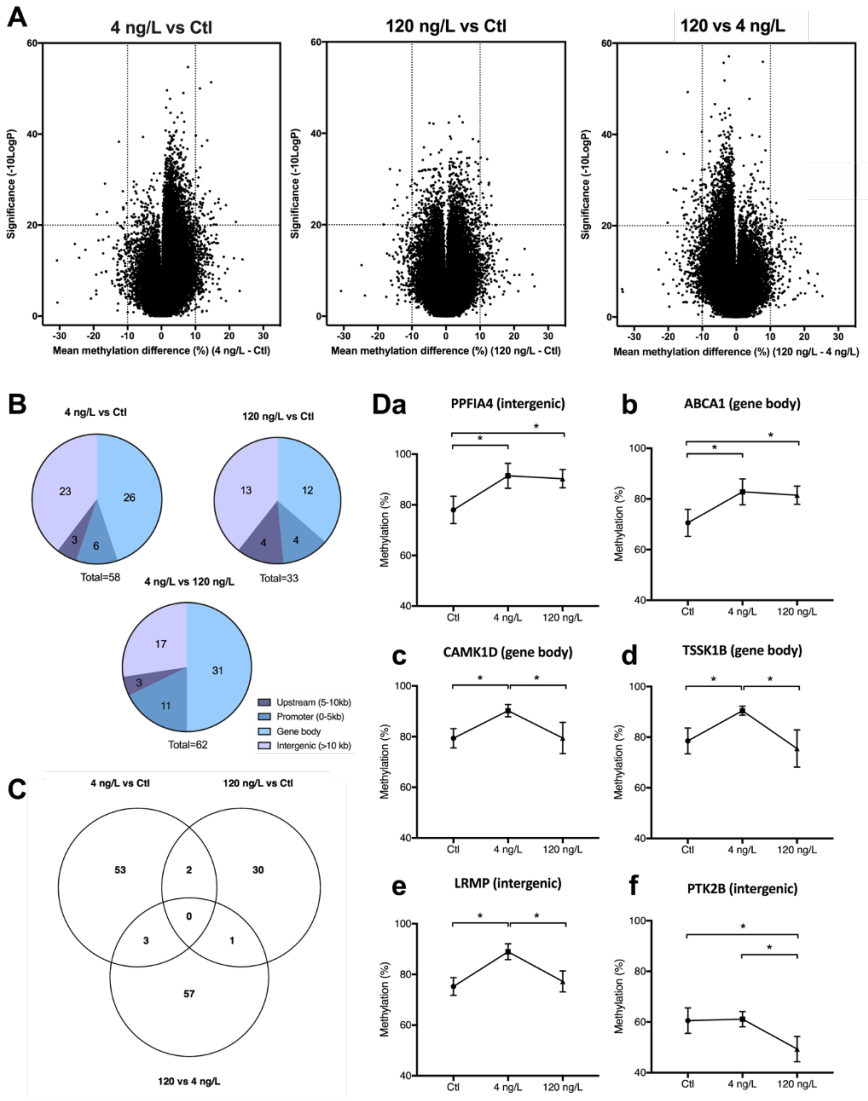
Figure 3: A: Volcano plot representations of significance and differential methylation in analysed fragments. Dotted lines represent thresholds of significance: -10LogP20, corresponding to P0.01 and mean methylation difference 10% A: 4 ng/L vs control; B:120 ng/L vs control; C: 4 ng/L vs 120 ng/L. B: Overall distribution of DMFs in genomic elements; C: Venn Diagram showing specific and common DMFs between group comparisons; D: Methylation levels (%) of common fragments between 4 vs Ctl and 120 vs Ctl comparisons (Da,Db), between 4 vs Ctl and 4 vs 120 comparisons (c,d,e) and between 120 vs Ctl and 4 vs 120 ng/L comparisons (f). For clearer representation of methylation differences, y axes start at 40 %.

Figure 4: Network of genes corresponding to significant DMFs (located in promoter region or in gene body) at 4 ng/L compared to control. Blue: hypermethylation at 4 ng/L compared to control, Yellow: hypomethylation at 4 ng/L compared to control.

11. Figures







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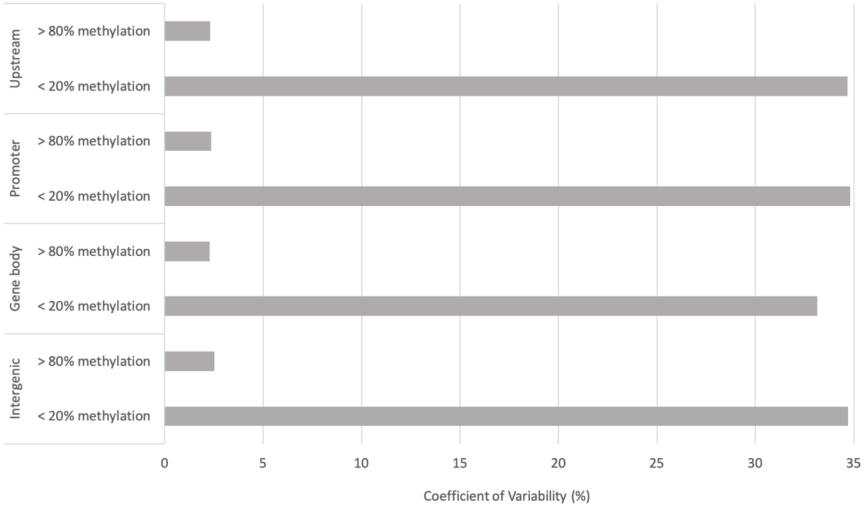
12. Supplemental data

Supplementary Table 1 : Sequencing, mapping outputs of RRBS librairies and corresponding global CpG and non-CpG methylation levels.

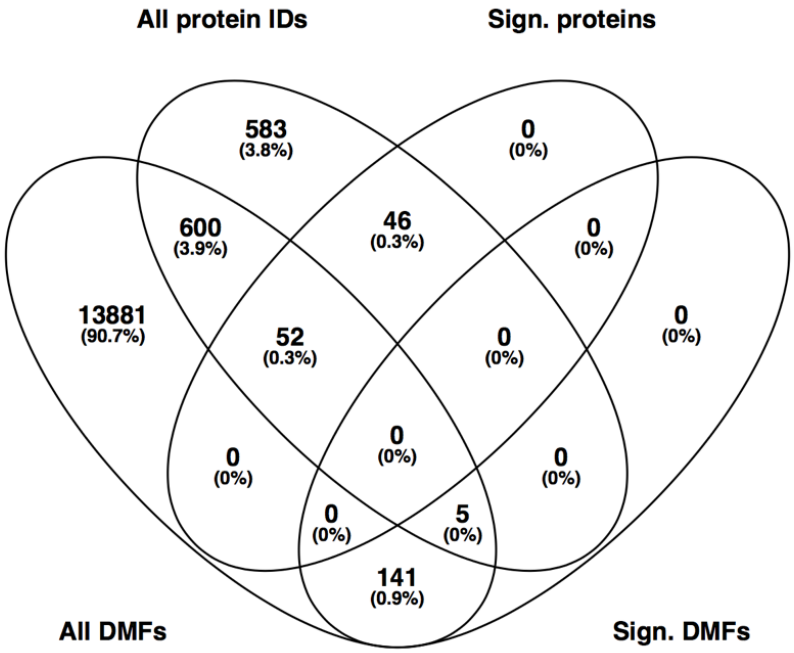
Sample ID	Sequenced reads (million)	Uniquely mapped reads (million)	Mapping efficiency (%)	CpG methylation (%
1-CTL	37.43	16.19	43.3%	65.6%
2-CTL	36.91	16.36	44.3%	65.5%
7-CTL	37.06	16.35	44.1%	65.6%
8-CTL	24.85	11.55	46.5%	65.2%
25-CTL	40.37	17.84	44.2%	63.5%

5-4	31.66	12.75	40.3%	65.8%
6-4	24.93	11.23	45.1%	65.8%
11-4	22.05	10.32	46.8%	66.4%
12-4	34.40	15.24	44.3%	66.0%
23-4	38.41	10.94	28.5%	65.9%
9-120	21.24	9.49	44.7%	65.9%
10-120	22.86	10.78	47.1%	64.9%
3-120	19.72	8.86	45.0%	66.0%
15-120	36.68	16.42	44.8%	65.8%
16-120	31.11	12.56	40.4%	64.8%

Supplementary Figure 1 : Coefficient of variability of hypomethylated (< 20% methylation) and hypermethylated (> 80%) fragments among the different regions of the liver reduced representative genome of the mangrove rivulus.



Supplementary Figure 2 : Venn diagram comparing the proteomic and methylome analyses of 168 dph adult livers. All protein IDs are all the proteins that were identified in the liver (with at least one unique peptide); Sign. Proteins are the differentially abundant proteins taking all three group comparisons into account (4 ng/L vs Ctl, 4 ng/L vs 120 ng/L and 120 ng/L vs Ctl); All DMFs represent all the identified genes corresponding to one or more MSPI fragment (fragments were either found in intergenic, upstream, promoter or gene body regions); Sign. DMFs are all the genes corresponding to significantly differentially methylated fragments across the three group comparisons. None of the differentially abundant proteins had significantly altered methylation. Five of the significant DMFs were identified in the proteomic analysis.


All protein IDs
Sign. proteins
583
(3.8%)
0
(0%)
600
(3.9%)
46
(0.3%)
0
(0%)
13881
(90.7%)
52
(0.3%)
0
(0%)
0
(0%)
0
(0%)
0
(0%)
0
(0%)
0
(0%)
5
(0%)
141
(0.9%)
All DMFs
Sign. DMFs