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# Early-life exposure to methylmercury induces reversible behavioral impairments and gene expression modifications in one isogenic lineage of mangrove rivulus fish *Kryptolebias marmoratus*

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Methylmercury (MeHg) is a ubiquitous bioaccumulative neurotoxicant present in aquatic ecosystems. It is known to alter behaviors, sensory functions and learning abilities in fish and other vertebrates. Developmental and early-life stages exposure to MeHg can lead to brain damage with immediate consequences on larvae behavior, but may also induce long term effects in adults after a detoxification period. Epigenetic mechanisms are possible explanation for such delayed disruption. However, very little is known about developmental origin of behavioral impairment in adults due to early exposure to MeHg. The aim of this study is to assess whether early-life MeHg exposure induces immediate and/or delayed effects on behaviors, related genes expression and DNA methylation (one of epigenetic mechanisms). To reach this goal, newly hatched larvae of mangrove rivulus fish, *Kryptolebias marmoratus*, were exposed to two sub-lethal concentrations of MeHg (90 µg/L and 135 µg/L) for 7 days, and immediate and delayed effects were assessed respectively in 7 dph (days post-hatching) and 90 dph fish. This species naturally produces isogenic lineages due to its self-fertilizing reproduction system, which is unique among vertebrates. It allows to study how environment stressors can influence organism's phenotype while minimizing genetic variability. As results, both MeHg exposures are associated with a decreased foraging efficiency and thigmotaxis, and a dose-dependent reduction in larvae locomotor activity. Regarding molecular analysis in larvae whole bodies, both MeHg exposures induced significant decreased expression of DNMT3a, MAOA, MeCP2 and NIPBL, and significant increase of GSS, but none of those genes underwent methylation changes in targeted CpGs. None of significant behavioral and molecular impairments observed in 7-dph larvae were found in 90-dph adults, which highlight

a distinction between immediate and delayed effects of developmental MeHg exposure. Our results suggest implications of aminergic system and its neurotransmitters, redox/methylation trade-off and possibly other epigenetic mechanisms in MeHg neurotoxicity underlying behavioral alterations in rivulus.

Key words: methylmercury, delayed effects, behaviors, DNA methylation, mangrove rivulus

## **1. Introduction**

Methylmercury (MeHg) is a neurotoxic contaminant generated through methylation of heavy metal mercury by anaerobic bacteria in aquatic environments. It enters the aquatic food web through the consumption of these bacteria by zooplankton, and undergoes bioaccumulation and biomagnification resulting in high concentrations in large predatory fish and other top predators including humans (Baeyens et al., 2003). It is established that MeHg crosses the blood-brain barrier and can induce brain damages, impaired neurological development and behaviors. In fish, MeHg exposure causes altered swimming activity and prey capture success (Mora-Zamorano et al., 2017; Samson, 2001; Xiaojuan Xu, 2012; Zhu et al., 2020), visual deficit (Carvan et al., 2017; Weber et al., 2008), learning and memory impairments (Smith et al., 2010; Xu et al., 2016). Several cellular mechanisms have been proposed for MeHg-induced neurotoxicity including oxidative stress (Carvan et al., 2017; Farina and Aschner, 2019), alterations of neuronal differentiation (Tamm et al., 2008), and disruption of glutamatergic and dopaminergic neurotransmitter systems (Faro et al., 2002).

Most recent studies of MeHg neurotoxicity have focused on adverse outcome pathways and behavioral effects of developmental and early-life stages exposure. MeHg exposure during these stages can cause delayed effects that can be observed later in life, but also transgenerational effects (Carvan et al., 2017; Xu et al., 2016). It has been suggested that delayed and transgenerational MeHg-altered phenotypes result from gene expression modification without changes to the underlying nucleotide sequence of DNA (i.e., epigenetic mechanism) (Carvan et al., 2017; Skinner, 2011). Generally, MeHg alterations of epigenome include downregulation of microRNA expression, reduced histone acetylation and increased histone methylation, and global DNA hypomethylation in brain (reviewed in Culbreth and Aschner, 2019). As such, epigenetic modifications have become an attractive mechanistic target for the impacts of environment on phenotypic variation. Even with this extensive compilation of cellular

and molecular data, it is still challenging to link MeHg-induced behavioral impairments to specific molecular alterations, and to understand the underlying mechanisms of delayed behavioral effects of early-life stages exposure.

In the present study we used the mangrove rivulus *Kryptolebias marmoratus* as model system to determine immediate and delayed behavioral impairments and associated molecular mechanisms following an early exposure to MeHg. Mangrove rivulus is a powerful emerging model vertebrate species in ecotoxicology due to its unique reproductive system: it is the only known vertebrate capable of self-fertilization, for which hermaphrodites can naturally produce highly homozygous and isogenic populations after a few generations (Costa et al., 2010). These characteristics allow scientists to drastically minimize genetic variation between individuals, and to focus on how epimutations and environment variation, such as pollutant exposure, influence phenotype. This is made possible thanks to the expanding repertoire of genetic and epigenetic data on mangrove rivulus (Kelley et al., 2016). Furthermore, rivulus is subject to MeHg exposure in its natural environment since mangroves are considered as potential MeHg hotspots due to their large amount of organic matter and their physico-chemical characteristics (Lei et al., 2019). Their location facilitates pollutant accumulation, as showed in Florida where Hg levels were elevated in mangrove transition zone compared to both the upstream canals and the open waters of Florida Bay (Rumbold et al., 2011). THg concentrations in freshwater ecosystems range from 0.3 ng/L to 450 µg/L, with higher levels found downstream of pollution sources including mines and industrial discharges (Kidd and Batchelar, 2011). There are fewer data available on the distribution of mercury in mangrove waters, and even less on MeHg. THg concentrations in mangroves water ranged from 0.04 to 110 ng/L, with large temporal and spatial variations. In waters collected from mangrove in south Florida, USA, Bergamaschi et al. (2012) recorded 26 ng/L MeHg. However, MeHg transfer from water into the base of the food web (bioconcentration) and subsequent biomagnification in the aquatic food web leads to most of the MeHg in higher trophic levels (Wu et al., 2019). Mangrove rivulus is a predator and can accumulate MeHg through contaminated prey consumption including bivalves that showed 100 to 940 ng/g dw Hg concentration (Saha et al., 2006) and polychaetes containing 50 to 280 ng/g dw Hg (Alam et al., 2010) in mangrove ecosystems. To our knowledge, there is no available data on THg and MeHg concentrations in mangroves rivulus.

Our objective is to assess whether an early exposure to sublethal MeHg of newly hatched rivulus larvae exposed from 0 to 7 dph induces immediate and delayed effects on behaviors including locomotor activity, thigmotaxis (used as index of anxiety) and foraging efficiency in 7 dph and 90 dph rivulus, and to unravel underlying mechanisms of these alterations by investigating expression and methylation of related genes. We choose a long detoxification period of 83 days after 7 days of exposure at high concentration of MeHg to highlight delayed and potentially irreversible effects, and to reveal underlying mechanisms that could be too subtle at lower MeHg concentrations. We hypothesize that MeHg exposure during early life can induce lasting epimutations that could impact gene expression, leading to behavioral impairments in rivulus larvae but also in adults. This is the first set of experiments that aimed to identify MeHg effects in mangrove rivulus, joining the restricted list of ecotoxicological studies on this fish species.

## **2. Materials and methods**

### *2.1. Experimental fish procurement and methylmercury exposure*

Mangrove rivulus used for this experiment are the 4<sup>th</sup> generation obtained from one single hermaphrodite to reduce genetic variation between individuals. This ancestor was sampled in 2019 in South Florida (Emerson Point Preserve, Palmetto, Florida, USA; N27°31'56.8", W82°37'46.4") by Kristy Marson, Ryan L. Earley, Frédéric Silvestre and Valentine Chapelle. It was transferred to the University of Namur where it generated a stock population raised in the laboratory of evolutionary and adaptive physiology (LEAP, University of Namur, Belgium), housed in  $12 \pm 1$  parts per thousand (ppt) saltwater (Instant Ocean™ sea salt), at  $25 \pm 1^\circ\text{C}$ , 12:12 light:dark cycle and fed every day *ad libitum* with living *Artemia salina*. These housing conditions are kept for the rest of the experiment as control condition.

Eggs were collected and individually placed in plastic 24-wells microplates (Cellstar®) filled with 2 mL of  $12 \pm 1$  ppt saltwater. Plates with eggs were housed in an incubator at  $25 \pm 1^\circ\text{C}$ , 12:12 light:dark cycle. Water was changed every day until hatching. Newly hatched autonomous larvae were randomly assigned and individually exposed to

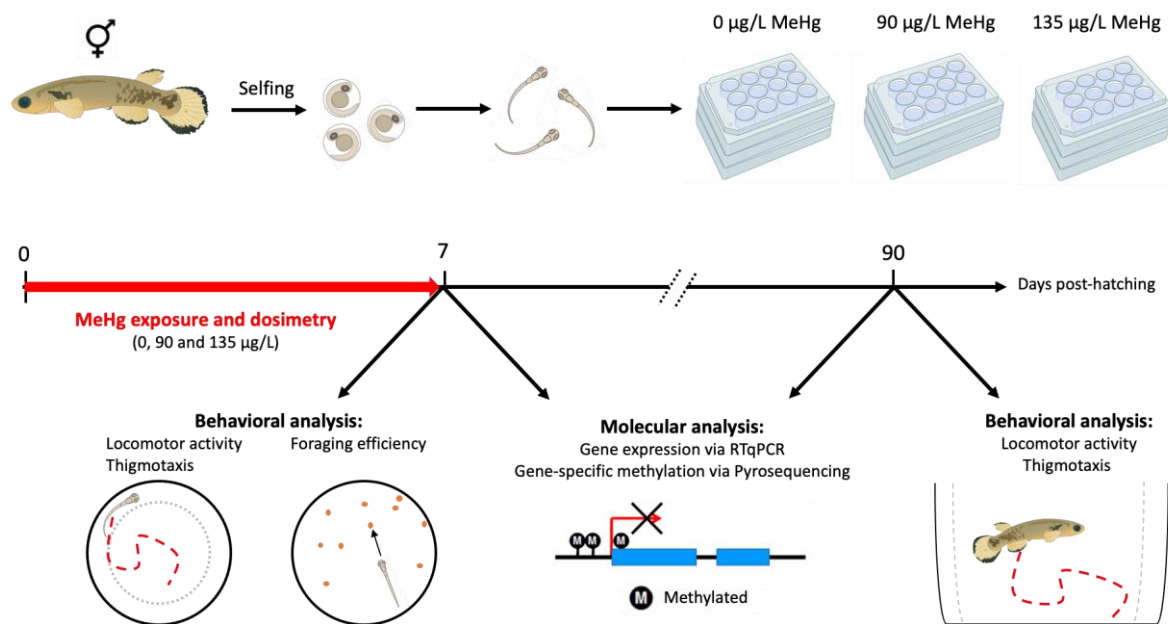
nominal methylmercury concentrations of 0 (control), 90, or 135 µg/L for 7 days in 12-wells microplates filled with 4 mL of exposure solution (Figure 1). Two of the twelve wells were dedicated to internal control. A total of 114 0-day post-hatching (dph) autonomous larvae were split into three exposure groups (Table 1).

Methylmercury solutions were obtained from lyophilized methylmercury chloride (CH<sub>3</sub>HgCl) purchased from Sigma-Aldrich® (ref: 442534). A stock solution of 135 mg/L MeHg was kept for the entire experiment at 4°C in the dark. From this stock solution, 90 and 135 µg/L exposure solutions were newly produced every day. These two MeHg concentrations were chosen based on pre-test results as they were the two lowest sublethal MeHg concentrations having behavioral effects among 7 other concentrations (no published data). Every day during the 7-days exposure, each larva was fed with one drop of their exposure solution containing *Artemia salina* and half of the solution was renewed to maintain stable MeHg concentrations. At the end of exposure, all larvae took the two behavioral tests described below. Then, they were separated in two different groups: 46 larvae were euthanized, measured, and conserved at -80°C for molecular analysis and 58 larvae were kept until 90 dph to study delayed effects of MeHg (Table 1). These 58 larvae were individually transferred into a « cleaning bath » filled with salt water (12 ± 1ppt at 25 ± 1 °C) and then to a glass jar filled with 200 mL of salt water and placed under the same physico-chemical conditions than control fish until 90 dph. Mature fish took the same behavioral tests they took at 7 dph, then were euthanized, measured and conserved at -80°C for molecular analysis.

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 21 367 KE). The agreement number of the laboratory for fish experiments is LA1900048.

**Table 1 : Total number of mangroves rivulus used per condition and analysis. MeHg immediate effects are measured on 7-days post-hatching (dph) larvae, while MeHg delayed effects are measured on 90 dph adults.**

Conditions	Larvae			Adults		
	Behaviors	Genes expression	Gene-specific methylation	Behaviors	Genes expression	Gene-specific methylation
Control	52	22	3	28	24	3
90 µg/L MeHg	31	12	3	16	13	3
135 µg/L MeHg	31	12	3	14	13	3
Total	114	46	9	58	50	9



**Figure 1: Experimental design: MeHg exposures, behavioral and molecular analysis timeline.** Eggs were produced by self-fertilization of hermaphrodite individuals from one single ancestor. Newly hatched autonomous larvae were randomly assigned and individually exposed to nominal methylmercury concentrations of 0, 90, or 135 µg/L for 7 days. At 7 dph, the locomotor activity and thigmotaxis were analyzed on all of the 114 larvae, directly followed by the assessment of foraging efficiency. 46 larvae were euthanized, measured, and conserved at -80°C for gene expression and DNA methylation analysis and 58 larvae were kept until 90 dph to study delayed effects of MeHg.

## 2.2. Methylmercury and inorganic mercury dosimetry by GC-ICP-MS

Methylmercury and inorganic mercury concentration were measured at different levels and time points. Firstly, newly prepared working solution of 90 and 135 µg/L of MeHg were sampled, stabilized by acidification (1% hydrochloric acid) and stored at -20°C to further assess the actual MeHg concentrations to which larvae were exposed. Inorganic mercury (iHg) concentration was also measured to evaluate MeHg demethylation rate. Secondly, 6 randomly selected larvae of 7 dph were euthanized at

the end of exposure to analyze MeHg and iHg concentration in their whole body (2 individuals per condition).

MeHg and iHg concentrations in working solutions and in larvae were determined using species-specific isotope dilution analysis and gas chromatography combined with inductively coupled plasma mass spectrometry (GC/ICP-MS). Limits of detection (LOD) are 0.005 pg for MeHg and 0.013 pg for iHg, and limits of quantification are 0.02 pg for MeHg and 0.04 pg for iHg (Cavalheiro et al., 2014). Those analysis were realized at IPREM (Institute of Analytical Sciences and Physico-Chemical for the Environment and materials) in Pau, France.

### 2.3. Behavioral tests

At the end of MeHg exposure, all of the 114 rivulus larvae were transferred from 12-wells to 6-wells microplates to evaluate locomotor activity and thigmotaxis (the tendency to remain close to the walls, used as index of anxiety), directly followed by the assessment of foraging efficiency. Locomotor activity and thigmotaxis were monitored and analyzed by using DanioVision (Noldus Information Technology, Wageningen, Netherlands). After 5 minutes of acclimation, total distance moved and cumulative duration spent in wells center were recorded for 10 minutes and calculated using EthoVision software version 15.0 (Noldus Information Technology). After this behavioral test, we directly evaluated foraging efficiency by introducing 10 *Artemia salina* nauplii per rivulus. They were allowed to feed for 10 min during which the number of capture successes and fails were manually counted based on video-records. Regarding 90-dph adult rivulus, similar tests were used to evaluate delayed effects of MeHg but in bigger tanks filled with one liter of 12 ppt saltwater.

### 2.4. Tissue samplings for molecular analysis

After the first run of behavioral tests, 46 of the 114 larvae were immersed in 4 °C water for euthanasia. Their standard length was measured, and whole larvae were snap-frozen in liquid nitrogen and stored at -80 °C for subsequent molecular analyses. Regarding 90 dph adults, they were immersed in 4 °C water for euthanasia directly



after the second run of behavioral tests. Their weight and standard length were measured. Death was ensured by decapitation. The brain was removed, snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for subsequent molecular analyses.

## 2.5. *Genes of interest*

Expression and methylation of several genes of interest were analyzed to assess MeHg immediate and delayed effects at the molecular level. Genes coding for Toll interacting protein (Tollip), monoamine oxidase A (MAOA) and methyl CpG binding protein 2 (MeCP2) were chosen for their roles in neuronal functions and/or personality traits (Baronio et al., 2022; Newman et al., 2005; Oguro et al., 2011; Pietri et al., 2013; Sallinen et al., 2009). Genes coding for DNA methyltransferase 3a (DNMT3a) and Nipped-B-like protein (NipBL) were chosen for their roles in epigenetic mechanisms (Bell and Felsenfeld, 2000; Gao et al., 2019). Finally, we chose Glutathione synthetase (GSS) for its central role in glutathione biosynthesis pathway, and response to stressor such as heavy metals (Farina and Aschner, 2019).

## 2.6. *Simultaneous DNA and RNA extractions*

DNA and RNA were simultaneously extracted with the Quick-DNA/RNA<sup>TM</sup> Microprep Plus Kit from ZYMO research (ref: D7005) following the manufacturer's protocol. This purification allows us to obtain relative expression data and DNA methylation levels in the same individual. Briefly, whole larvae or adult brains were homogenized 2 minutes with bead beating, then incubated with proteinase K at  $55^{\circ}\text{C}$  for 30 minutes (brains) or 1 hour (larvae bodies). The supernatant was transferred to columns and washed multiple times to separately eluate approximately 20  $\mu\text{L}$  of DNA and RNA. DNA and RNA concentrations were measured using the Nanodrop (Thermo Fisher, Waltham, MA), and their integrity was evaluated on a 1% agarose gel. DNA and RNA samples were stored at  $-80^{\circ}\text{C}$  for subsequent molecular analyses, respectively gene-specific DNA methylation analysis via pyrosequencing and relative gene expression analysis via RT-qPCR.

## 2.7. *Gene expression by reverse transcription quantitative PCR (RT-qPCR)*

DNase treatment was performed on RNA samples with Invitrogen DNA-free™ DNA Removal Kit from Thermo Fisher Scientific (ref: AM1906) before converting 500 ng per RNA samples into cDNA with the RevertAid RT Reverse Transcription Kit from Thermo Scientific™ (ref: K1691). Samples were stored at -20 °C during primer design and tests.

Primers were designed with AmplifX 2.1.1 and Primer blast (NCBI genome: ASM164957v2) using the exon-spanning method. Primers efficiencies were tested on cDNA pool of samples made from all MeHg conditions. 2.5 µL of 50x diluted cDNA was added to 2.5 µL of primers mix and 5 µL of SYBR green (Bio Rad®). Three technical replicates were established per dilution. SYBR green quantitative PCR was conducted on a StepOnePlus Real-Time PCR System® (Applied Biosystems). A melting curve and an end-point agarose gel electrophoresis followed by SYBR safe (Thermo Fisher) staining were used to check for accurate amplification of the target amplicon. Primer efficiencies were calculated according to the MIQE guidelines and accepted between 90 and 110% (see RTqPCR primers in Table A.1).

Relative expression of genes of interest was normalized with mean expression of two house-keeping genes: ubiquitin-conjugating enzyme E2 A (ube2a) and selenoprotein 15 (SEP15). These genes were selected after testing their overall stability value and their intragroup and intergroup variation. The relative gene expression (RGE) is the gene of interest expression level in comparison to the mean of housekeeping genes expression.

## 2.8. *Gene-specific DNA methylation by pyrosequencing*

Pyrosequencing relies on bisulfite treatment of the genomic DNA, converting unmethylated cytosine to uracil (thymine after PCR amplification) while methylated cytosines are refractory to the treatment. Bisulfite treatment was performed on DNA samples with EZ DNA Methylation kit from Zymo Research (ref: D5001). For optimized bisulfite conversion, we used 500 ng of genomic DNA and followed the manufacturer's

protocol excepted for the last step of elution. We performed two elutions of 10  $\mu$ L M-Elution Buffer instead of a single 10  $\mu$ L elution to double our final elution volume.

PCR amplification was performed using PyroMark PCR Kit from Qiagen (ref: 978703) in 25  $\mu$ L reaction volumes containing 1  $\mu$ L of DNA template, 6  $\mu$ L nuclease-free H<sub>2</sub>O, 12.5  $\mu$ L of 2x PyroMark PCR Master Mix, 1  $\mu$ L MgCl<sub>2</sub>, 2.5  $\mu$ L 10x CoralLoad Concentrate, 2  $\mu$ L forward and reverse primer mix (5  $\mu$ M). We followed manufacturer's protocol for PCR conditions. Forward and reverse primers for PCR and sequencing primer for pyrosequencing were designed with software PyroMark Assay Design SW 2.0 on selected DNA sequences. Firstly, we highlighted CpG islands across each sequence including promotor region, exons and introns using MethPrimer 2.0. Then, we used Match<sup>TM</sup> to highlight smaller sequences containing binding sites of transcription factors (TF) whose functions are relevant to our study; central nervous system and sensory organs development, neuronal plasticity, oxidative and cytotoxic stress resistance. We selected sequences of approximately 150 bp, containing CpGs islands and TF binding sites in different regions including promoters, exons and introns (see Table A.2). Pyrosequencing was performed on the Pyromark Q24 instrument using PyroMark Q24 Advanced Reagents (Qiagen) according to the manufacturer's protocol, with a 15  $\mu$ L input of PCR products. Pyrograms were manually interpreted and evaluated using the PyroMark Q24 software.

## 2.9. *Data statistical analysis*

Statistical analyses were performed with the Prism<sup>TM</sup> 8.0.2 (GraphPad software, Inc.). The normal distribution of standard length and molecular variables was analyzed with a Shapiro-Wilk normality test, and the homogeneity of variances was tested with a Barlett's test. If the normality and homogeneity were proved, we realized an ordinary one-way ANOVA followed by a Tukey's multiple comparisons test. Otherwise, if normality and/or homogeneity couldn't be proved, a nonparametric Kruskal-Wallis test was performed, followed by a Dunn's multiple comparison test. For all statistical analysis, p value was fixed to 0.05.

Regarding behavioral analysis, these measurements cannot be considered as independent because two measurements were collected per fish. As we have repeated measures, linear mixed models (LMMs) were used on behavioral variables using “lmer” function from “lme4” R package in R Studio (version 1.4.1103). MeHg condition and rivulus age were entered as fixed effects and individuals as random effects.

### 3. Results

#### 3.1. Methylmercury and inorganic mercury dosimetry

Measured concentrations of MeHg in newly prepared working solutions of 90 and 135  $\mu\text{g/L}$  (nominal concentrations) were  $75.17 \pm 2.17$  and  $120.24 \pm 6.18$   $\mu\text{g/L}$  respectively (mean  $\pm$  SD) (Table 2). We also observed in both MeHg exposure conditions low concentrations of iHg. We were under the LOD of GC/ICP-MS in the control group. In whole larvae exposed during 7 days to 0, 90 or 135  $\mu\text{g/L}$  MeHg, we measured  $<\text{LOD}$ ,  $29.16 \pm 0.86$  and  $47.62 \pm 0.95$   $\mu\text{g/g dw}$  respectively (mean  $\pm$  SD). We observed high concentrations of iHg. By comparing measured MeHg concentrations in fresh working solutions and in whole larvae at the end of the exposure, we obtained MeHg bioconcentration factors of 388 and 396 in 90  $\mu\text{g/L}$  and 135  $\mu\text{g/L}$  MeHg conditions, respectively.

**Table 2: Methylmercury (MeHg) and inorganic mercury (iHg) concentrations in fresh solutions used for exposures and in whole larvae at the end of the 7-days MeHg exposure. Limits of detection (LOD) of GC/ICP-MS are 0.005  $\mu\text{g}$  for MeHg and 0.013  $\mu\text{g}$  for iHg. Mean  $\pm$  SD.**

<b>Fresh working solutions</b>		
<i>Nominal MeHg concentration (<math>\mu\text{g/L}</math>)</i>	<i>Measured MeHg concentration (<math>\mu\text{g/L}</math>)</i>	<i>Measured iHg concentration (<math>\mu\text{g/L}</math>)</i>
0	$< \text{LOD}$	$< \text{LOD}$
90	$75.17 \pm 2.17$	$3.93 \pm 0.41$
135	$120.24 \pm 6.18$	$6.04 \pm 1.86$
<b>Whole larvae (7-dph)</b>		
<i>Nominal MeHg concentration (<math>\mu\text{g/L}</math>)</i>	<i>Measured MeHg concentration (<math>\mu\text{g/g dw}</math>)</i>	<i>Measured iHg concentration (<math>\mu\text{g/g dw}</math>)</i>
0	$< \text{LOD}$	$< \text{LOD}$
90	$29.16 \pm 0.86$	$38.55 \pm 0.84$

### 3.2. Standard length

Rivulus standard length were measured at the beginning of the experiment (1 dph rivulus), at the end of MeHg exposure (7 dph) and after the recovery period (90 dph). We didn't show any significant standard length differences between fish at the beginning of the experiment, and also no significant immediate or delayed effects of MeHg exposure on standard length (Table 3).

**Table 3 : Data monitoring of fish standard length (mm) at different ages. Dph = days post hatching. No significant differences were observed in 1-dph rivulus ( $N_{\text{control}} = 52$ ;  $N_{90} = 42$ ;  $N_{135} = 43$ ), 7-dph rivulus ( $N_{\text{control}} = 24$ ;  $N_{90} = 14$ ;  $N_{135} = 14$ ) or 90-dph rivulus ( $N_{\text{control}} = 26$ ;  $N_{90} = 16$ ;  $N_{135} = 13$ ). Mean ± SD.**

Age (dph)	Control	90 µg/L	135 µg/L
1	4.79 ± 0.26	4.65 ± 0.27	4.61 ± 0.24
7	5.30 ± 0.27	5.17 ± 0.21	5.15 ± 0.27
90	22.2 ± 1.12	21.5 ± 1.62	21 ± 1.87

### 3.3. Locomotor activity assessment

Linear mixed models (LMMs) showed a significant interaction between MeHg condition and rivulus age ( $p < 0.01$ ). All MeHg-exposed larvae exhibited lower locomotor activity relative to control, with 250 ± 99.8 cm travelled by control rivulus, 159 ± 94.8 cm travelled by rivulus exposed to 90 µg/L MeHg and 82.6 ± 65.9 cm travelled by rivulus exposed to 135 µg/L MeHg (mean ± SD). We observed significant dose-dependent effect of MeHg, as activity was reduced by a factor of 1.6 and 3.0 in fish exposed to 90 and 135 µg/L MeHg, respectively, in comparison to controls larvae. On the opposite, no significant effect of MeHg was observed in 90 dph adults that showed activity reduction as larvae, with 141 ± 111 cm, 157 ± 106 cm and 174 ± 138 cm travelled by rivulus exposed to 0, 90 or 135 µg/L MeHg, respectively (Figure 2).

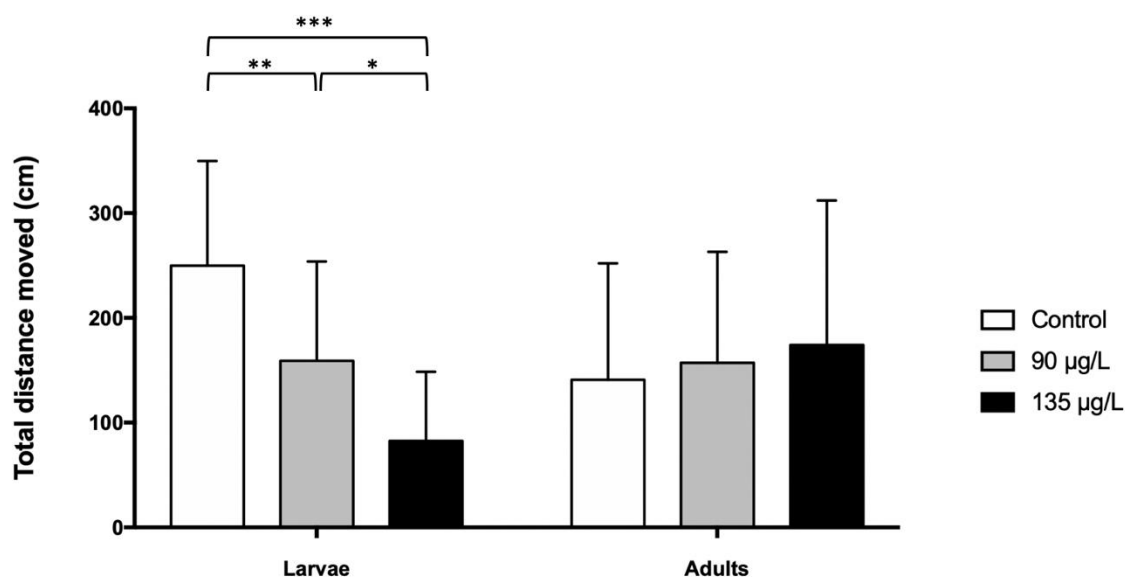


Figure 2 : Total distance moved during 10 minutes of activity assessment test in 7-dph larvae and 90-dph adults exposed from 0 to 7 dph to different concentrations of methylmercury (MeHg). Linear-mixed models shows a significant interaction between MeHg conditions and rivulus age, with a dose-dependent immediate effects of MeHg in larvae ( $N_{\text{control}} = 52$ ;  $N_{90} = 31$ ;  $N_{135} = 31$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ) and no significant delayed effects in adult rivulus ( $N_{\text{control}} = 26$ ;  $N_{90} = 16$ ;  $N_{135} = 14$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Bar charts representing mean  $\pm$  SD.

### 3.4. Thigmotaxis assessment

To evaluate thigmotaxis, we analyzed total time spent in arena central zone. All MeHg-exposed larvae spent significantly more time in the center of the well in comparison to the control, with  $62.2 \pm 49.7$  s,  $107 \pm 102$  s and  $131 \pm 100$  s spent in the center of the arena by control, 90 µg/L exposed and 135 µg/L exposed rivulus, respectively (mean  $\pm$  SD). MeHg-exposed larvae exhibit a lower thigmotaxis behavior than control larvae, corresponding to a less anxious state. On the opposite, no significant effects of MeHg were observed in 90 dph adults, as control, 90 µg/L and 135 µg/L exposed rivulus spent  $40.7 \pm 58.4$  s,  $37.6 \pm 61.2$  s and  $40.9 \pm 65$  s in the center of the arena (mean  $\pm$  SD) (Figure 3).

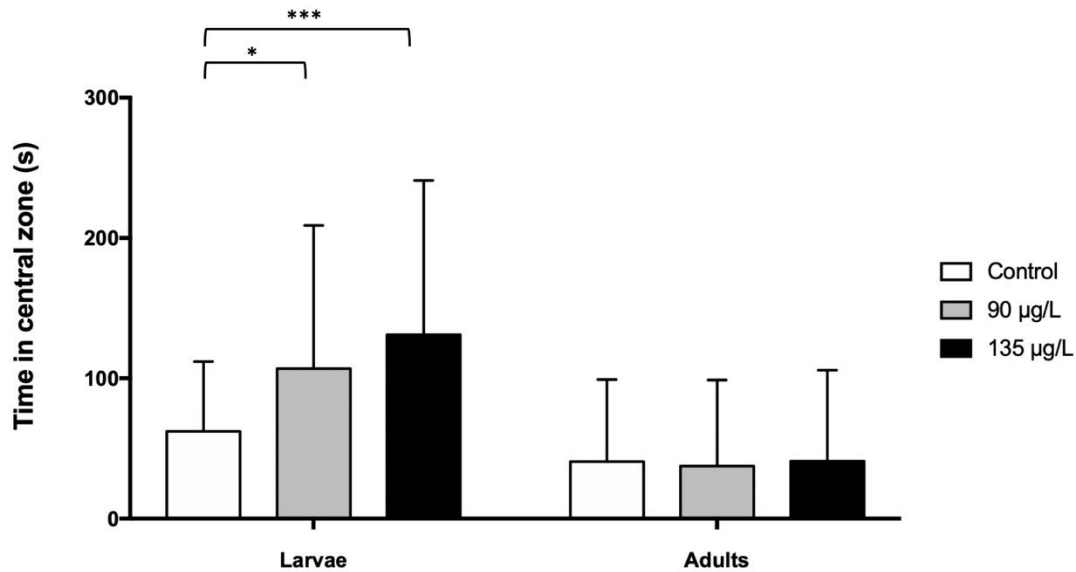
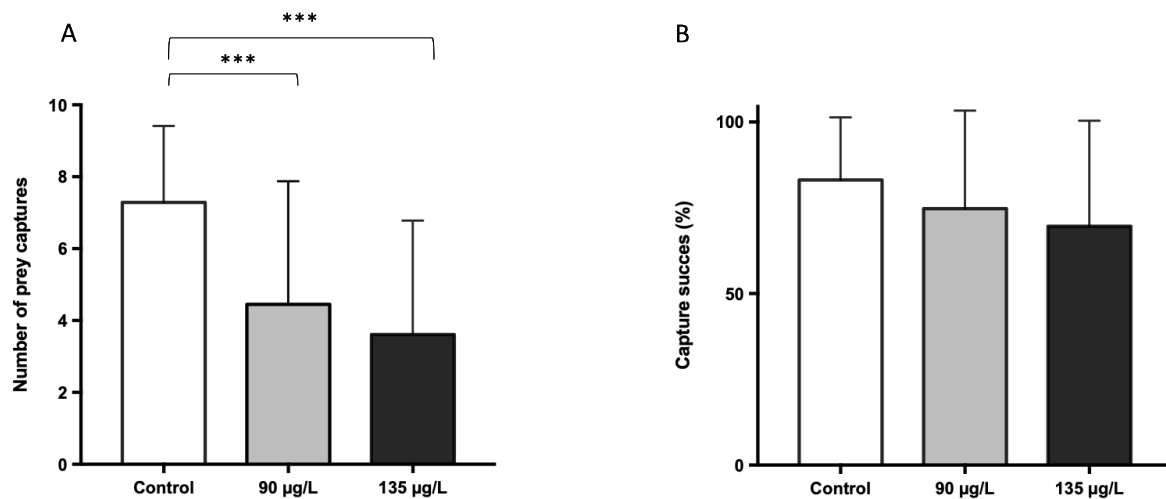


Figure 3: Time spent in the center of the arena during 10 minutes of thigmotaxis assessment test in 7-dph larvae and 90-dph adults exposed from 0 to 7 dph to different concentrations of methylmercury (MeHg). Results show a significant immediate effect of MeHg in larvae ( $N_{\text{control}} = 52$ ;  $N_{90} = 31$ ;  $N_{135} = 31$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ) and no significant delayed effects in adult rivulus ( $N_{\text{control}} = 26$ ;  $N_{90} = 16$ ;  $N_{135} = 14$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). Bar charts representing mean  $\pm$  SD.

### 3.5. Foraging efficiency

The foraging efficiency test on 10 *Artemia salina* nauplii per 7-dph larvae showed a significant decrease in the number of prey captures in both MeHg exposure groups, as control larvae caught  $7.29 \pm 2.13$  preys, 90 µg/L exposed larvae caught  $4.45 \pm 3.42$  preys and 135 µg/L exposed larvae caught  $3.61 \pm 3.17$  preys in 10 minutes (mean  $\pm$  SD,  $p < 0.001$ ) (**Erreur ! Source du renvoi introuvable.A**). MeHg-exposed larvae also tried less to capture preys in comparison with control, with  $9.08 \pm 3.09$ ,  $5.65 \pm 3.57$  and  $4.84 \pm 3.56$  prey capture tried by control, 90 µg/L and 135 µg/L larvae, respectively (mean  $\pm$  SD,  $p < 0.001$ ). As exposed larvae caught less preys and also tried less to capture preys in comparison with control, percentage of prey capture success (prey capture/capture attempt) shows no significant differences between condition, with  $83.1 \pm 18.2$  %,  $74.8 \pm 28.6$  % and  $69.6 \pm 30.8$  % of capture success for control, 90 µg/L and 135 µg/L larvae, respectively (mean  $\pm$  SD) (**Erreur ! Source du renvoi introuvable.B**).



**Figure 4: Immediate effects of MeHg exposure on foraging efficiency on 10 *Artemia salina* nauplii per 7-dph larvae. (A) Number of prey capture trials and (B) the prey capture success of rivulus larvae. A Kruskal-Wallis test showed a significant decrease in the number of capture trials for both groups exposed to MeHg but not significant effect of MeHg on prey capture success ( $N_{\text{Control}} = 52$ ;  $N_{90} = 31$ ;  $N_{135} = 31$ ;  $P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Bar charts representing mean  $\pm$  SD.**

### 3.6. Gene expression

Relative expression analyses in whole larvae revealed a reduction in expression of DNMT3a, NipBL, MeCP2 and MAOA in 90 and 135 µg/L MeHg-exposed groups compared to controls (Figure 5A). We also highlighted a significant increased expression of GSS in larvae exposed to 90 and 135 µg/L MeHg, with a relative expression in control, 90 µg/L MeHg and 135 µg/L MeHg groups of  $0.97 \pm 0.32$ ,  $2.51 \pm 1.36$  and  $2.57 \pm 0.77$  respectively (mean  $\pm$  SD) (Figure 5B). Finally, we did not show any significant effect of MeHg exposure on relative expression of Tollip. Relative expression analyses in brain of 90-dph adults exposed to MeHg from 0 to 7 dph showed no significant delayed effects of MeHg on expression of all genes of interest (Figure 6).



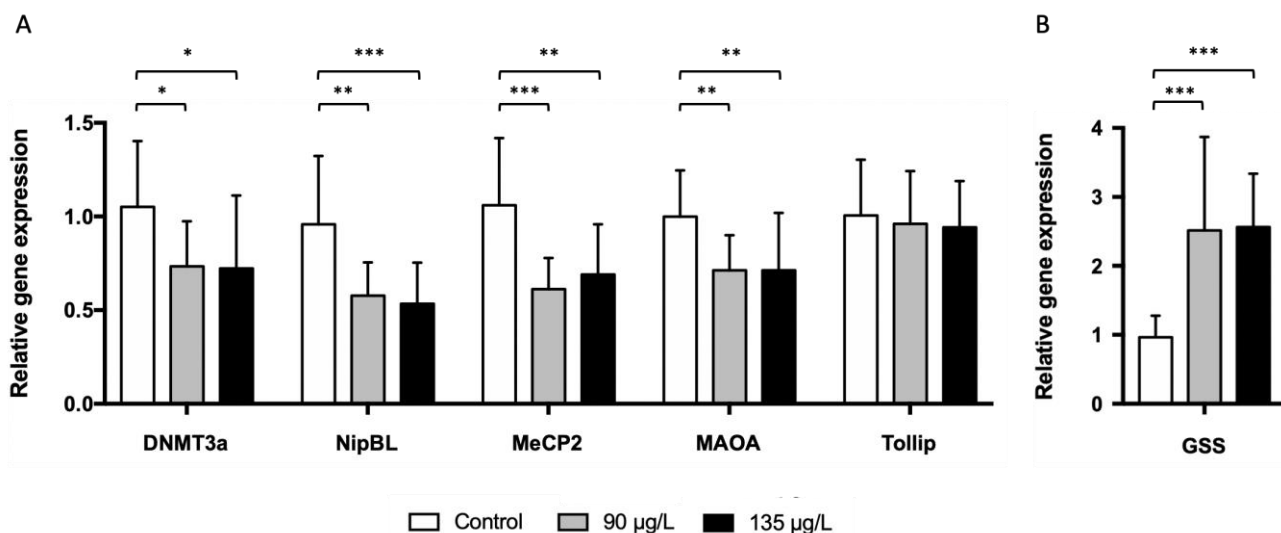


Figure 5: Relative gene expression (RGE) in whole body of 7-dph larvae rivulus in response to MeHg exposure from 0 to 7 dph. Results are presented as mean  $\pm$  SEM. Housekeeping genes used are Ubiquitin-conjugating enzyme E2 A (UBE2A) and selenoprotein 15 (Sep15). (A) MeHg exposure creates a decreased expression of DNA methyltransferase 3a (DNMT3a), Nipped-B-like protein (NipBL), methyl CpG binding protein 2 (MeCP2), monoamine oxidase A (MAOA), and no significant effects on Toll interacting protein (Tollip). (B) MeHg exposure creates an increased expression of Glutathione synthetase (GSS) ( $N_{\text{Control}} = 22$ ;  $N_{90} = 11$ ;  $N_{135} = 11$ ;  $P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Bar charts representing mean  $\pm$  SD.

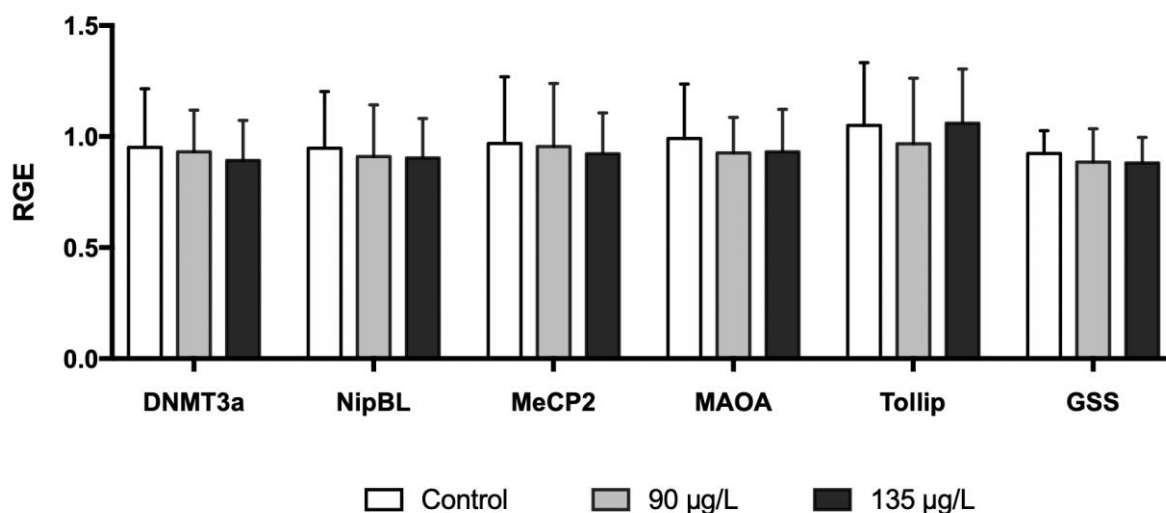


Figure 6: Relative gene expression (RGE) in brain of 90-dph rivulus in response to MeHg exposure from 0 to 7 dph. Results are presented as mean  $\pm$  SEM. Housekeeping genes used are Ubiquitin-conjugating enzyme E2 A (UBE2A) and selenoprotein 15 (Sep15). No significant delayed effects were observed in adults rivulus on expression of DNA methyltransferase 3a (DNMT3a), Nipped-B-like protein (NipBL), methyl CpG binding protein 2 (MeCP2), monoamine oxidase A (MAOA), Toll interacting protein (Tollip) and Glutathione synthetase (GSS) ( $N_{\text{Control}} = 24$ ;  $N_{90} = 13$ ;  $N_{135} = 13$ ;  $P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Bar charts representing mean  $\pm$  SD.

### 3.7. Gene-specific DNA methylation

Analysis of gene-specific methylation in whole larvae showed no significant effects of MeHg on methylation of targeted CpGs in all genes of interest (Figure 7). We can observe a global high level of methylation of DNMT3a and GSS targeted CpGs, as larvae showed an average of  $71.08 \pm 2.58$  % of methylation at targeted CpGs in DNMT3a, and  $83.82 \pm 1.79$  of methylation at targeted CpGs in GSS. In the opposite, we can observe a global low level of methylation of MeCP2 and MAOA targeted CpGs, as larvae showed an average of  $2.21 \pm 0.82$  % of methylation at targeted CpGs in MeCP2, and  $3.60 \pm 0.36$  of methylation at targeted CpGs in MAOA. Finally, NIPBL targeted CpGs showed intermediate levels of methylation, with  $23.65 \pm 2.25$  % of methylation (mean  $\pm$  SD). The same analysis was performed on 90 dph adult brains to search for possible delayed effects of MeHg on targeted CpGs methylation level. We showed no significant effects of MeHg on targeted CpGs methylation in any genes of interest (Figure 8). As for larvae, we can observe a global high level of methylation of DNMT3a and GSS, a global low level of methylation of MeCP2 and MAOA, and an intermediate level of methylation of NIPBL, with very similar values.

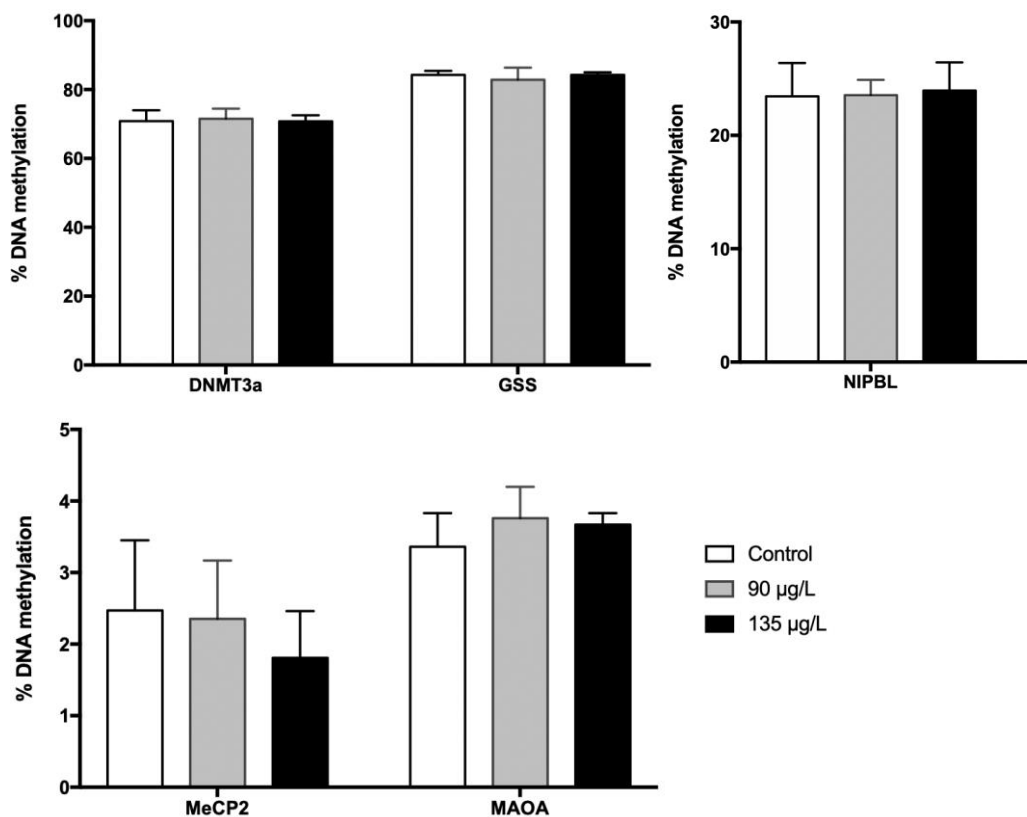


Figure 7: Mean percentage of methylation at targeted CpGs in DNMT3a, GSS, NIPBL, MECP2 and MAOA in whole body of 7-dph larvae rivulus exposed to MeHg from 0 to 7 dph ( $N_{\text{Control}} = 3$ ;  $N_{90} = 3$ ;  $N_{135} = 3$ ;  $P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Bar charts representing mean  $\pm$  SD.

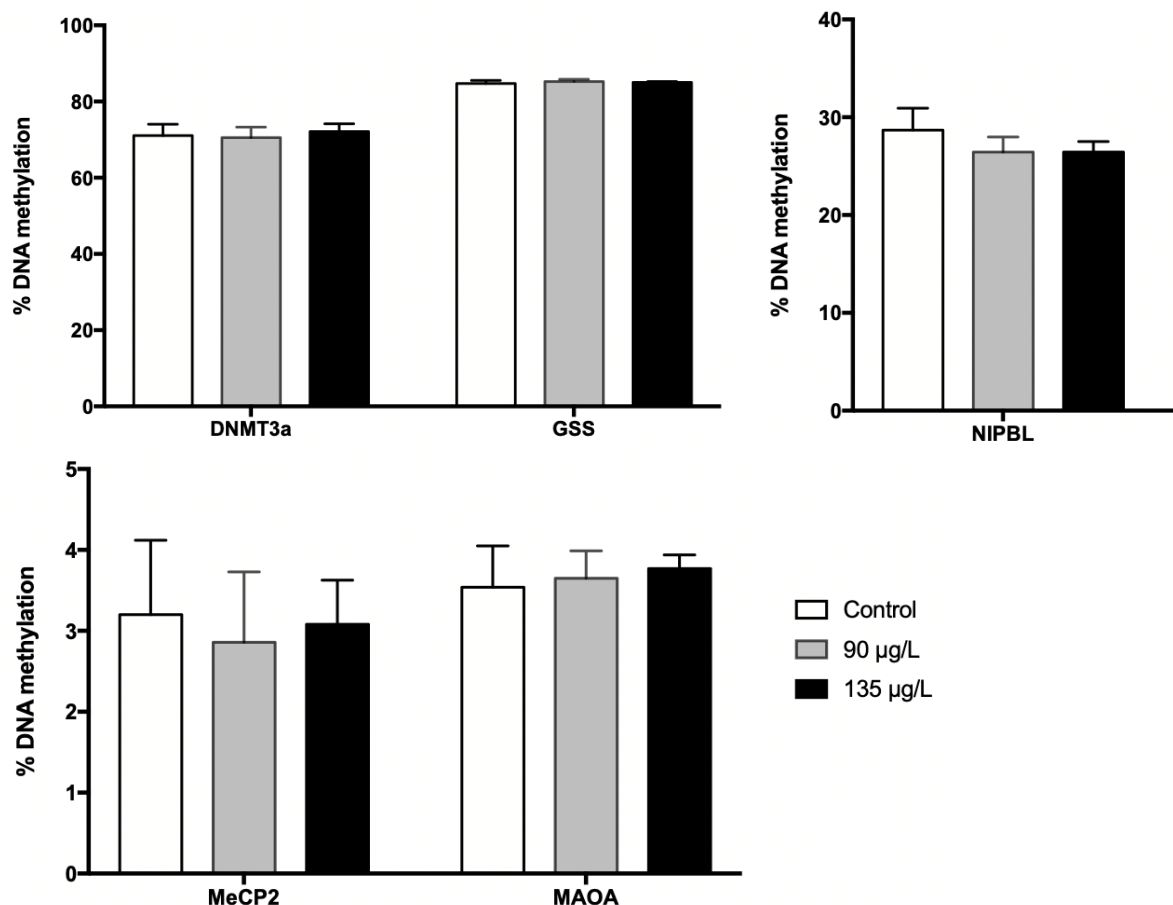


Figure 8: Mean percentage of methylation at targeted CpGs in DNMT3a, GSS, NIPBL, MECP2 and MAOA in brains of 90 dph rivulus exposed to MeHg from 0 to 7 dph ( $N_{\text{Control}} = 3$ ;  $N_{90} = 3$ ;  $N_{135} = 3$ ;  $P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Bar charts representing mean  $\pm$  SD.

## 4. Discussion

### 4.1. Mangrove ecosystems and bioconcentration

Mangrove rivulus is a relevant model species to assess MeHg exposure consequences as mangroves are considered as MeHg hotspots due to their large amount of organic matter and their physico-chemical characteristics, so as other wetland ecosystems (Lei et al., 2019). MeHg was found in water, sediments, and resident species of South Florida mangroves, as described in the introduction section. Our dosimetry results showed bioconcentration factors (BCF) of 388 and 396 in 90 µg/L and 135 µg/L MeHg conditions, respectively. Comparison of BCF with other studies is delicate as it is linked to bioavailability, which is influenced by different environmental factors such as dissolved organic matter, salinity, dissolved oxygen concentration and pH (Berzas Nevado et al., 2003; Kidd and Batchelar, 2011). BCF varies also according to

organisms that allow the entry of MeHg into the aquatic food web (Wu et al., 2019). Mangrove rivulus are predators, with various terrestrial and aquatic invertebrates forming the bulk of the diet including gastropods, various crustaceans, dipterans, formicids, and juvenile fishes, sometimes from its own species (Taylor, 2012). In mangroves biota, the reported THg levels were 100 ng/g dw in snails and crabs (Cheng and Yap, 2015), 50 to 280 ng/g dw in polychaetes (Alam et al., 2010), 100 to 940 ng/g dw in bivalves (Saha et al., 2006) and 22 to 617 ng/g dw in fish from the Sundarbans mangrove systems in Bangladesh (Borrell et al., 2016). Into the wild, mangrove rivulus is thus not an entry of MeHg into the aquatic food web, but is rather at a higher trophic level where bioaccumulation occurs. It is worth noting that MeHg concentration in whole 7-dph larvae bodies exposed from 0 to 7 dph is the results of detoxification processes, as we showed high iHg concentration. Demethylation and detoxification processes are discussed further.

#### 4.2. *Immediate effects of MeHg on rivulus behaviors, physiology and ecology*

We showed that MeHg exposures significantly decreased rivulus larvae locomotor activity in a dose dependent manner. This hypoactivity is consistent with studies carried out on other fish species such as zebrafish *Danio rerio* (Zhu et al., 2020), yellow perch *Perca flavescens* (Mora-Zamorano et al., 2017) and grass carp *Ctenopharyngodon idella* (Baldissera et al., 2020) exposed to MeHg. In contrast to our results, MeHg can also create hyperactivity as observed in 6 dpf zebrafish exposed to 10 and 30 nM at  $\leq 2$  hpf for 24 hours (Mora-Zamorano et al., 2016). These differences in locomotor activity outcomes between but also within species may be attributable to MeHg concentrations, way of exposure (waterborne, diet, etc.), exposure duration and stages as some developmental stages are more critical than others.

Locomotor activity can influence foraging, dispersal and antipredator behaviors, which have important consequences on individual's fitness as it influences reproductive success, food access, survival and social interactions (Réale et al., 2007). In our study, we highlighted a significant decrease in the number of prey capture, but no significant effect on capture success (prey capture/capture attempt) in rivulus larvae. It means that MeHg-exposed larvae tried less to capture preys, but when they try, they succeed

as well as the control ones. These results interpretation is complex as feeding behavior depends on different factors such as visual acuity, learning capacities, locomotor activity and growth. In our case, MeHg seemed to primarily reduce prey capture by larval activity alteration, which has a direct influence on prey encounter rates. As it has been reported that MeHg can create visual deficit (Carvan et al., 2017; Liu et al., 2016) and learning impairments (Xu et al., 2016), these causes of decreased foraging efficiency are not ruled out and should be deeper investigated.

Interestingly, MeHg reduced locomotor activity and prey capture in rivulus larvae, but has no significant immediate or delayed effects on growth. These results could be explained by energy allocation modification. MeHg induces detoxification responses that are energetically costly to exposed organisms (Handy et al., 1999). This energetic alteration could modify the energy allocation to other physiological processes, creating impairment of growth, reproduction, development and locomotor activity. Studies have associated locomotor alteration of zebrafish larvae (Andrade et al., 2016) and rainbow trout *Oncorhynchus mykiss* (Handy et al., 1999) with the energy needed to detoxification of carbendazim and cooper, respectively. Our results indicate that this possible energy allocation trade-off between MeHg detoxification processes and locomotor activity occurs without concomitant reductions in growth. As detailed in Handy et al. (1999), where they obtained similar results, locomotor activity of exposed fish could be reduced as a metabolic 'sparing effect' to maintain growth, activity being the major component of energy expenditure. This observed stable growth across MeHg conditions and time points is consistent with other ecotoxicological studies on mangrove rivulus where exposure to 20 µg/L and 15 mg/L of β-N-Methylamino-L-alanine (BMAA) from 0 to 7 dph created no immediate effects on growth at 7 dph (Carion et al., 2018) and no delayed effects on growth at 50 and 170 dph (Carion et al., 2020). In contrast, mangrove rivulus exposed to 4 and 120 ng/L of 17-α-ethinylestradiol (EE2) from 0 to 28 dph were significantly smaller in standard length than the control group at 28 dph, but underwent compensatory growth and reached a similar size as controls at 90 dph (Voisin et al., 2016). These results provide insight into the diversity of energy allocation by mangrove rivulus under chemical stresses.

In addition to locomotor activity and prey capture reduction, MeHg exposure also creates thigmotaxis reduction in exposed larvae, as they spent significantly more time

in the center of the arena. Thigmotaxis (or “wall hugging”) is a validated index of anxiety that has been shown in several fish species (Dadda et al., 2010; Schnörr et al., 2012; Sharma et al., 2009) but has not yet been characterized in mangrove rivulus. Here we provide evidence that larval rivulus as young as 7 dph show thigmotaxic behavior as control larvae strongly avoid the center of the arena by spending most of their time close to the walls during the locomotor activity assessment ( $89.64 \pm 8.29$  %). These results are consistent with what was found in zebrafish larvae as they spent  $79.10 \pm 2.36$ % of the thigmotaxis test in the outer zone (Schnörr et al., 2012). These findings suggest that thigmotaxis develops early in rivulus life and appears to be already expressed in an adult-like manner in larvae as adults rivulus from our control group spent  $93.22 \pm 9.73$  % of their time close to the walls during the locomotor activity assessment. We characterized one innate behavior of mangrove rivulus, that have a natural tendency to remain at the edge of its environment for shelter seeking, foraging and predator avoidance (Taylor, 2012). In our experiment, MeHg exposure decreased this anxiety-like behavior in rivulus larvae, altering the normal preference of the periphery in favor of the central area. This change induced by MeHg exposure may be a threat to the survival of these larvae in terms of predator avoidance and prey capture. Compelled at reduction of locomotor activity and prey capture, MeHg can significantly affect rivulus survival and fitness.

#### 4.3. *Mechanisms underlying immediate phenotypic effects of MeHg*

Besides MeHg effects on rivulus behaviors and growth, we investigated possible underlying mechanisms by studying MeHg effects on expression of six genes and on their methylation level at targeted CpGs. Our hypothesis is that MeHg could modify these epigenetic marks, creating environmentally-induced epimutations, which could explain gene expression modification induced by MeHg exposure.

In our study, we showed that MeHg exposure significantly decreased DNMT3a, NIPBL, MAOA and MECP2 expression, and increased GSS expression in larvae. However, there is no significant effect of MeHg on DNA methylation of targeted CpGs located in those genes. On the one hand, DNA methylation changes could occur at other locations in the genome, which is supported by the observed downregulation of DNMT3a expression in MeHg-exposed larvae, the enzyme responsible for *de novo*

DNA methylation. One interesting perspective would be to use a Reduced Representation Bisulfite Sequencing (RRBS) or Whole Genome Bisulfite Sequencing (WGBS) for genome-wide analysis of DNA methylation modifications induced by MeHg exposure. On the other hand, changes in gene expression could be due to other epigenetic mechanisms altered by MeHg such as post-translational modification of histones, supported by the downregulation of NIPBL expression in MeHg-exposed larvae. NIPBL initiates deacetylation of lysine 9 of histone 3 (H3K9) and recruits histone deacetylases. This process of histone deacetylation allows histones to wrap the DNA more tightly and creates a compaction of nucleosome structure, preventing transcription (Gao et al., 2019). As MeHg exposure decreased NIPBL expression in rivulus larvae, it could facilitate the dissociation of DNA and relaxation of nucleosome structure, allowing various transcription factors to bind to DNA and activate gene transcription. The observed DNMT3a and NIPBL downregulations are in line with previous studies where MeHg exposure decreased histones H3 and H4 acetylation, decreased global DNA methylation and DNMTs activity (reviewed in Culbreth and Aschner, 2019). The functional consequences of these epigenetic modifications are not entirely understood. However, these examples show that epigenetic modifications are an attractive mechanistic target and illustrate the potential of MeHg to impact the epigenome.

As mentioned above, we highlighted an increased relative expression of Glutathione synthase (GSS) in MeHg-exposed larvae. MeHg is known to interact with either thiols or selenols groups including glutathione (GSH; a major thiol antioxidant). This complex formation, GS-HgCH<sub>3</sub>, is a common mechanism for MeHg metabolism and clearance from the body as it can be transported out of the cell and excreted in the bile (Dutczak and Ballatori, 1994). Increased MeHg exposure competes with redox buffering for available GSH, resulting in oxidative stress (Farina and Aschner, 2019). Moreover, clearance of MeHg is delayed due to this competition for GSH, extending organism exposure duration. Pro-oxidative properties of MeHg could explain the observed behavioral impairments, as recent evidence suggests the involvement of brain oxidative damage in mercury-mediated motor dysfunction (Santana et al., 2019; Teixeira et al., 2019). It is worth noting that, according to the redox/methylation trade-off hypothesis, oxidative stress would result in global hypomethylation (Carvan, 2020;

Farina and Aschner, 2019), as S-adenosylmethionine (methyl donor used by DNMTs to methylate DNA) and GSH share a common synthesis pathway from homocysteine.

MeHg exposure also modified expression of genes involved in neuronal functions and behaviors. MeHg-exposed larvae showed significant decrease of monoamine oxidase A (MAOA) relative expression. In fish, monoamine oxidase regulates levels of monoamine neurotransmitters such as dopamine, serotonin, and noradrenaline in the nervous tissue. These neurotransmitters are involved in neurofunctional behaviors such as fight-or-flight response, reward-motivated behaviors, attention, emotion, cognition and motor activity (Beyrouy et al., 2006; Newman et al., 2005). Enzymatic degradation catalyzed by MAO is thus required for proper neuronal development and behaviors (Beyrouy et al., 2006). It has been proposed that MeHg disrupts these pathways by reducing brain MAO activity, leading to an increased concentration of these neurotransmitters and ultimately creates neurotoxicity in various brain regions (Bridges et al., 2017; Sallinen et al., 2009). Reduction in MAO activity due to MeHg exposure has been associated with hyperactivity in fathead minnow *Pimephales promelas* (Bridges et al., 2017), but also with hypoactivity in Atlantic salmon *Salmo salar* (Berntssen et al., 2003). This implies that reduction in MAO activity could be a widespread mechanism of MeHg neurotoxicity, and that the behavioral consequences vary across studies. A study of loss of function in MAO supports the hypoactivity hypothesis, as *mao*<sup>-/-</sup> zebrafish showed decreased locomotor activity, but also abnormal serotonergic and dopaminergic systems (Baronio et al., 2022). An interesting perspective would be to evaluate dopamine, serotonin and noradrenaline concentrations in brain of MeHg-exposed rivulus to determine if this exposure would cause their accumulation in rivulus brain, which could be associated with the observed hypoactivity and reduced foraging efficiency.

We also showed that there is significant decrease of Methyl-CpG-binding protein 2 (MeCP2) relative expression in MeHg-exposed rivulus larvae. MeCP2 is a transcription factor that can act as gene expression repressor or activator (Guy et al., 2011). Once bound, MeCP2 modulates gene expression by recruiting protein complexes involved in histone modification and chromatin remodeling (Skene et al., 2010). MeCP2 is found in high concentrations in neurons and is associated with maturation of the central nervous system and in forming synaptic contacts (Guy et al., 2011). Consequently,



MeCP2 dysfunction impacts brain anatomy and neuronal structure and function, and can create several neurodevelopmental diseases associated with autism spectrum disorders (ASD), including Rett Syndrome (RTT). Patients with RTT suffer from pronounced alterations of their motor systems, learning disabilities and social behavioral deficits (Sansom et al., 2008; Weaving, 2005). This can be associated with autistic-like features, as dysfunction of the reward system and avoidance of tactile stimulation (Belmonte et al., 2004). MeCP2 knockout zebrafish had decreased locomotor activity and velocity, and has an impact on thigmotaxis as mutants tends to stay more in the arena center in comparison to wild type (Pietri et al., 2013). In our experiment, MeHg-exposed larvae showed decreased MeCP2 expression, thigmotaxis and locomotor activity, which is consistent with RTT phenotypes created in MeCP2 knockout zebrafish. [Al-Mazroua et al. \(2022\)](#) evaluated MeHg effects on autism-like behaviors in BTBR autistic mouse model. MeHg administration aggravated existing behavioral abnormalities in BTBR mice with increased stereotypic, repetitive, and impaired social behaviors. Interestingly, studies on MeCP2 knockout mice highlighted other dysfunctions, as defects in the different aminergic systems including the dopaminergic system (Panayotis et al., 2011). This is in line with the hypothesis that one of the MeHg neurotoxicity mechanisms involve aminergic system, by disturbing expression of genes regulating these pathways including MAOA and MeCP2. Dysfunction of the aminergic system could create the observed behaviors alterations including autistic features (reduction of interest in environment exploration, avoidance of tactile stimulation from the wall, etc.).

#### *4.4. Delayed effects and detoxification of MeHg*

Immediate effects of MeHg on locomotor activity, prey capture, thigmotaxis and gene expression in 7 dph larvae are no longer observed later in life, after 83 days of recovery. Several studies evaluated reversibility of MeHg effects after a detoxification period in fish. MeHg exposure during fish embryogenesis can create delayed impairments in the feeding behavior, swimming activity, learning abilities, and a delayed mortality syndrome (Samson, 2001; Xiaojuan Xu, 2012), but can also create transitory impairments at similar concentrations (Weis and Weis, 1995). Post-hatching but still early developmental exposure can also have delayed consequences on

swimming activity and related dopaminergic neurons functionality in zebrafish (Huang et al., 2016). These studies highlight the complexity of MeHg effects that may be reversible/transient in some cases, and irreversible in others. The delay between MeHg exposure and behavioral testing on 90 dph adults rivulus might have allowed time for protective and repair mechanisms to operate despite a relatively high exposure concentration. MeHg mechanisms of neurotoxicity could be countered later in life as the mercury is detoxified.

Detoxification of MeHg includes demethylation that takes place at different organs including gut and liver (Wang et al., 2017). In our study, detoxification by demethylation process is supported by our dosimetry results, as 43 % and 54 % of THg were in the form of MeHg in 90 µg/L and 135 µg/L MeHg exposed larvae, respectively. These results are in line with the study of (Gonzalez et al., 2005) where MeHg represented 66% of THg in the liver of zebrafish, and decreased to 36% after 63 days of MeHg exposure. Once exposure ends, fish could enter in depuration stage and efficiently eliminate accumulated iHg. Wang et al. (2017) showed that during depuration of 30 days in the marine fish *Acanthopagrus schlegeli*, whole-body concentrations of iHg also decreased significantly to the same level to the beginning of their experiment. Our study showed that there could be a considerable amount of iHg generated from demethylation and accumulated by rivulus larvae during the exposure. During the recovery period, rivulus possibly eliminate accumulated iHg, which could progressively diminish MeHg toxic effects until significant effects observed in 7 dph rivulus disappear.

#### 4.5. *Mangrove rivulus as ecotoxicological model species*

Mangrove rivulus is the only known self-fertilizing hermaphroditic vertebrates (Costa et al., 2010). Consistent self-fertilization is an extreme form of inbreeding and it consequently produces isogenic lineages. By naturally minimizing genetic noise in scientific studies, researchers can investigate how the environment influences the phenotype (Kelley et al., 2016). Moreover, this mating system provides an ideal model for the identification of true cause-effect relationships between the environment, the epigenome and the phenotype, including the role of epigenetic mechanisms in toxicity and ecotoxicity. A growing body of work on mangrove rivulus supports its value as a

new model species for studying the potential lasting effects of environmental stressors through epigenetic modifications. Voisin et al. showed that early life exposure of rivulus to an endocrine disrupting compound, 17- $\alpha$ -ethinylestradiol, can induce delayed effects on the adult phenotype, proteome and epigenome (Voisin et al., 2021, 2016). Carion et al. highlighted immediate effects of the neurotoxin  $\beta$ -N-Methylamino-L-alanine (BMAA) on rivulus larvae behaviors, and delayed effects of BMAA on expression of genes involved in glutamate turnover, intracellular dopamine levels and astrocyte protective mechanisms (Carion et al., 2020, 2018). These studies join ours in showing that the mangrove rivulus seems more resistant to pollutants, at least in terms of its behavioral phenotype and mortality compared to other fish species. In comparison, a delayed mortality syndrome was observed in zebrafish larvae after 72h of exposure to 15  $\mu$ g/l MeHg (Samson, 2001) while no mortality was observed in our experiment. Mangrove rivulus may have more effective protective mechanisms that could come from its high plasticity, an essential prerequisite for adaptation to mangroves environmental conditions. At first sight, high phenotypic plasticity would be paradoxical considering rivulus reproductive system. Consistent selfing is an intense form of inbreeding and produces isogenic lineages with high homozygosity level, which reduces fitness by increasing the risk that deleterious and recessive alleles are expressed and exposed to selection or by reducing any benefits due to heterozygote advantage (Davenport, 1908). This phenomenon is known as inbreeding depression, and is often associated with an increased vulnerability to environmental stress (Fox and Reed, 2011). Such inbreeding-by-environment interaction might imply that inbred organisms have a lower capacity for adjusting their phenotype to environmental variation, corresponding to a reduced phenotypic plasticity (Reed et al., 2012). Several empirical studies showed alterations of developmental plasticity (Auld and Relyea, 2010) and phenotypic plasticity of morphological or life history traits (Schiegg et al., 2002; Swillen et al., 2015) due to inbreeding. This interplay between inbreeding depression, phenotypic plasticity and environmental variation should be further investigated to understand how declining natural populations respond to environmental stress. This paradox of robustness and inbreeding makes the rivulus an interesting ecotoxicological model to consider, as it can highlights protective mechanisms and phenotypic plasticity that are highly solicited, while showing MeHg phenotypic effects encountered in other species but with a low genetic variability among individuals.

Another interesting characteristic of mangrove rivulus is its inter-individual variability in a large range of phenotypic traits. Although rivulus used in this study are from the same isogenic lineage, we still observe high individual differences within each condition. For instance, the total distance moved by rivulus larvae during the activity/thigmotaxis test has a coefficient of variation (CV) of 40 %, 60% and 80% in control, 90 µg/L and 135 µg/L MeHg group respectively, with a significant difference between variances in control group vs 135µg/L group (F test,  $F = 2.29$ ,  $DFn = 51$ ,  $Dfd = 30$ ,  $p < 0.05$ ). In comparison, total distance moved by AB line zebrafish exposed to 0 or 10 µg/L MeHg showed CV of 20% and 17%, respectively (Zhu et al., 2020). Thus, there is greater behavioral variability between rivulus generated by selfing from one unique ancestor than between zebrafish from AB line, which is known to have low level of genetic variability (Coe et al., 2009). Two main observations can be drawn from these rivulus activity data: there is a naturally high inter-individual variation in control group and this variation increases with MeHg concentrations. The increase of variance could be the first step in the adaptation of a population in a changing environment (Orlando and Guillette, 2001). As reviewed in Nikinmaa and Anttila (2019), variability should always be included as an endpoint in data analysis as it would bring new information about the responses of organisms to environmental contamination. Therefore, mangrove rivulus is naturally suited to investigate sources of phenotypic variation that are not genetic, as environmental influences, and environmentally-induced or random epigenetic modifications.

## **5. Conclusion**

We used a new vertebrate model species, the mangrove rivulus fish, to investigate immediate and delayed effects of MeHg at different endpoints (behaviors, gene expression and DNA methylation). We reported for the first time that MeHg exposure during early life stages can significantly impair behaviors and gene expression in rivulus larvae, but we didn't find these effects after a detoxification period of several months. Although neurotoxic effects of MeHg on brain have been extensively studied, their underlying mechanisms are not fully understood. Based on our results, we suggested implications of aminergic system and its neurotransmitters, redox/methylation trade-off and other epigenetic mechanisms. It should be kept in mind that no single process can explain the multitude of effects observed in MeHg-exposed

organisms. The use of natural isogenic lineages of mangrove rivulus in ecotoxicological studies could help overcome this challenge, as it permits explicit examination of environmental and epigenetic effects on the phenotype by reducing the genetic variation in the experiment.

### **CRedit authorship contribution statement**

**Valentine Chapelle:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration.

**Jérôme Lambert:** Methodology, Formal analysis. **Thomas Deom:** Investigation, Formal analysis. **Emmanuel Tessier:** Methodology, Formal analysis, Resources.

**David Amouroux:** Resources. **Frédéric Silvestre:** Supervision, Methodology, Formal analysis, Resources, Writing - review & editing, Funding acquisition.

### **Declaration of Competing Interest**

The authors declare that no competing interests exist.

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# APPENDIX

Table A.1: Newly designed primers for gene expression analysis via RTqPCR.

Gene	Gene ID	Forward primer sequence	Reverse primer sequence	Efficacy (%)
SEP15	108244479	TCAAGGGTCTTCAGATCAAGTACG	TCGGTGTCCACTTGAGGATG	92,46
UBE2a	108231206	ACACCTTTTGAAGATGGAACCTT	GGTCGGACTCCAACGATTCTG	106,33
DNMT3a	108233456	TACACGAGATCAAAAAGAAGACCAGGA	CATCGCTCCTACGAAGAGAGGAT	96,31
NIPBL	108231013	CAAACCTACTGTCCATGAACCCCA	CTGGGCTCCCATGAACTTGATTCT	91,76
TOLLIP	108251745	ACTGTCGGATCAGGCTGGGT	CGTCCATCGAAAACGCTCTCTCA	99,64
GSS	108233054	AGACTTCCTGCAAGAGGCTTTAGC	GTAGTCGGACCGATTGAGACCCA	108,63
MAOA	108241385	ACTGATACTCGAGGGAAAAGATCG	GCTCCATGATATGCGTTTGTGTG	96,36
MECP2	108229207	TAAGATGCCCTTTGGCAAGACA	GGACTTGGCAGGTGGAGTAG	99,40

Table A.2: Newly designed primers for gene-specific methylation analysis via pyrosequencing.

Gene	Gene ID	Forward primer sequence	Reverse primer sequence	Synthesis primer sequence	Target regions
DNMT3a	108233456	TGTGGATTATTTGGTTT ATGAGATAATAG	/5Biosg/ACTACTTCTACA CCAAATTTATACTCATT A	ATTTGGTTTATGAGATAAT AGAT	Promotor
		AGGAAATTTGTTAAGTT TGATTTGTGTG	/5Biosg/AACTAACAACTA CCCTAACACAAT	TTTTATTTTTTTGTTAATA AATAT	Intron 1
		TGATATTTGGAGTGTAT ATTAGGTAGT	/5Biosg/ACCAAACTTCA ATCTACAACACAT	GGTAGTTAGGTATAAAGT TTTTA	Intron 2
		GGTGGAAATTTTGTGTT ATAATGAGA	/5Biosg/TCTCCCAACCA CATCACCCA	ATTTTTAGTTTTGTGTTA ATTAGG	Intron 4 - Exon 5
NIPBL	108231013	/5Biosg/GGGATTAGTTG TAAATTGATGATATAAA	CCCTAAAATTTCTTCTT CTTTCTTTTAAC	TTTATTTACCTACACAAAC TA	Promotor
GSS	108233054	GTTATAGTTATTTTGA GATGGTAAAGG	/5Biosg/ACAAAATCTATC ACTTCCCACAAA	TTTTGAGATGGTAAAGGT	Promotor
MAOA	108241385	GTGTTGTTTTAGTTTTA TAGAGGGTAATA	/5Biosg/AACCTTTTCCCT CCAATATCAATATTTTC A	GGGTTTAAGTTAATTTTTA AT	Exon 1
MECP2	108229207	AAAAGGTAGTTGGTTTA AGAAGTTTATATA	/5Biosg/AATTTTATATTA AAAAAACTCCAAACATC T	GTTGGTTTAAGAAGTTTAT ATAT	Promotor
		GGGGAGGTGTGTAGTA TAGATGTTTG	CAACCAATTTTACTCAA CAACAC	AAATTATTTATTTAAATAA AAATGG	Intron 1
		GGGGAGGTGTGTAGTA TAGATGTTTG	CAACCAATTTTACTCAA CAACAC	GGGAATAAGTTATTAATTT AGGG	Exon 3

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