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Impacts of TCDD and MeHg on DNA methylation in zebrafish (*Danio rerio*) across two generations

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Abstract

This study aimed to investigate whether dioxin (TCDD) and methylmercury (MeHg) pose a threat to offspring of fish exposed to elevated concentrations of these chemicals via epigenetic-based mechanisms. Adult female zebrafish were fed diets added either 20 µg/kg 2,3,7,8 TCDD or 10 mg/kg MeHg for 47 days, or 10 mg/kg 5-aza-2′-deoxycytidine (5-AZA), a hypomethylating agent, for 32 days, and bred with unexposed males in clean water to produce F1 and F2 offspring. Global DNA methylation, promoter CpG island methylation and target gene transcription in liver of adult females and in 3 days post fertilization (dpf) F1 and F2 embryos were determined with HPLC, a novel CpG island tiling array containing 54,933 different probes and RT-qPCR, respectively. The results showed that chemical treatment had no significant effect on global DNA methylation levels in F1 (MeHg and TCDD) and F2 (MeHg) embryos and only a limited number of genes were identified with altered methylation levels at their promoter regions. CYP1A1 transcription, an established marker of TCDD exposure, was elevated 27-fold in F1 embryos compared to the controls, matching the high levels of CYP1A1 expression observed in F0 TCDD-treated females. This suggests that maternal transfer of TCDD is a significant route of exposure for the F1 offspring. In conclusion, the selected doses of TCDD and MeHg, two chemicals often found in high concentrations in fish, appear to have only modest effects on DNA methylation in F1 (MeHg and TCDD) and F2 (MeHg) embryos of treated F0 females.

Keywords: Fish; Contaminants, Epigenetics,

1. Introduction

Dioxins and methylmercury (MeHg) are contaminants often found in elevated levels in fish. Dioxins are highly toxic substances mainly released into the environment by industrial activities. Dioxins are fat-soluble, and concentrate in fatty tissues of fish. Fatty fish such as salmon can therefore contain relatively high concentrations of dioxins (Hites et al., 2004). TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) is a persistent environmental contaminant that acts as a reproductive toxicant and endocrine disruptor in nearly all vertebrates (Tillitt et al., 2008). TCDD toxicity in fish is mediated by the aryl hydrocarbon receptor (AHR) pathway. Mercury (Hg) may have natural or anthropogenic origin. In order for Hg to bioaccumulate in fish, it must first be transformed into a bioavailable form of organic mercury, i.e. MeHg. In fish, Hg is therefore predominantly present as MeHg, which often accounts for more than 80% of total Hg (Grieb et al., 1990; Bloom et al., 1992; Kidd and Batchelar, 2011). Hg resides in the entire body – including muscle, liver and kidney (Kidd and Batchelar, 2012). In mammals, MeHg primarily acts as a neurotoxin, while in fish the chemical may also disturb reproductive hormones and act as an endocrine disruptor, mediating development of smaller gonads, delaying spawning and affecting fecundity (Klaper et al., 2006; 2008; Liu et al., 2013). Several studies have also shown that MeHg induces oxidative stress and apoptosis in fish (Gonzalez et al., 2005; Klaper et al., 2008; Cambier et al., 2010; Richter et al., 2011; Olsvik et al., 2011).

Juvenile and larval life stages of fish are highly susceptible to the toxic effects of TCDD (Peterson et al., 1993). While diet is the major source of exposure to TCDD (Nichols et al., 1998) and MeHg (Phillips and Buhler, 1978; Hall et al., 1997) in adult fish, maternal transfer has been shown to be a significant route of exposure to these substances for larval and juvenile fish (Russell et al., 1999; Heiden et al., 2005; Latif et al., 2001; Alvarez et al., 2006). According to Russell et al., (1999), the concentrations of organochlorine chemicals in eggs of oviparous organisms range between 25.1% and 56% of the maternal tissue concentration. Significant amounts of TCDD transferred to eggs can disrupt critical developmental events and can cause cardiovascular dysfunction, edema, hemorrhages, craniofacial malformations, growth arrest, and mortality (Peterson et al., 1993; Cook et al., 2003; Carney et al., 2004; Hill et al., 2005; Heiden et al., 2005; 2008; Tillitt et al., 2008). Less information is available on the effects of MeHg on eggs and larvae of fish species. However, as MeHg is a known endocrine disruptor and a potent neurotoxin, it has the potential to cause variety of abnormalities in the offspring. In eggs of adult

fathead minnows (*Pimephales promelas*) exposed to 8.5 mg/kg of MeHg, the concentrations of MeHg were 35% of the concentrations detected in adults (Hammerschmidt and Sandheinrich, 2005). This suggests that embryos are potentially exposed to about the same effective internal concentration as the maternal organisms from which the eggs originated.

Environmental chemicals can affect the expression of specific genes and pathways not only in the exposed individual but also in the subsequent generations through epigenetically mediated modulation (Skinner et al., 2010; Vandegehuchte and Janssen, 2011). The current study was designed to investigate if dioxin (TCDD) and MeHg can induce DNA methylation abnormalities in the directly exposed F0 female fish and F1 embryo and whether the induced DNA methylation changes can be transmitted to the F2 embryo. To investigate this a single-dose experiment was conducted in which adult female (F0) zebrafish (*Danio rerio*) were exposed to 20 μg/kg TCDD and 10 mg/kg MeHg for 47 days, or 10 to mg/kg 5-aza-2′-deoxycytidine (5-AZA), a hypomethylating agent, for 32 days. The treated F0 female fish were bred with non-exposed male fish to generate the F1 and F2 generations. As the treated females (F0) were transferred to clean water for breading, any transfer of epigenetic abnormalities to the F2 generations must be through the germline. The exposure concentrations were selected based on previous data (TCDD: Heiden et al., 2005, MeHg: Ellingsen, unpublished data, 5-AZA: Aniagu et al., 2008). To evaluate possible effects in the developing fish, three days post fertilization (dpf) embryos were selected for analysis of the F1 and F2 generations. Earlier studies in zebrafish have shown that DNA methylation reprogramming events associated with blastula stage have already been completed by this time and the overall percentage of DNA methylation is similar to adult zebrafish (Fang et al., 2013). Global DNA methylation was assessed with HPLC, genome-wide promoter DNA methylation with CpG island (CGI) tiling array, and transcription of selected genes with RT-qPCR. To our knowledge this is the first attempt to determine whether TCDD and MeHg, two environmental contaminants that accumulate in seafood, can exert effects via DNA methylation across generations in fish.

2. Materials and methods

2.1 Experimental feeds

Four experimental diets were produced by adding solutions of methylmercury (MeHg) solubilized in water (MeHg diet), 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) solubilized in DMSO (TCDD diet), 5-aza-2′-deoxycytidine (5-AZA) solubilized in DMSO (5-AZA diet) or DMSO alone (Control diet) to a commercial zebrafish feed (Aqua Schwarz, Göttingen, Germany). DMSO was added in equal amounts to all four diets, including the

MeHg-containing feed. The diets were produced in batches of 10 g to ensure homogeneous concentrations of MeHg, 2,3,7,8-TCDD, 5-AZA and DMSO. For all the diets three ml solution were added 1 ml at a time to 10 g diet while stirring carefully between the additions to avoid the formation of lumps. The diets were dried at room temperature in a fume hood until a constant weight and stored at -20°C in the dark. Mercury was added as methylmercury-cysteine (MeHg-cys), which was made by mixing (1:1 (v:v)) solutions of methylmercury(II) chloride (Alfa Aesar, Karlsruhe, Germany) and cysteine (L-cysteine; Sigma-Aldrich, Seelze, Germany), to the diet at nominal concentrations of 10 mg Hg/kg. 2,3,7,8-TCDD, analytical standard, was purchased from Sigma-Aldrich (48599 Supelco) as 10 µg dissolved in toulene. Toluene was evaporated after which the 2,3,7,8-TCDD solubilized in DMSO was mixed into the feed at a nominal concentration of 20 µg/kg. 5-AZA was purchased from Sigma-Aldrich (A3656 Sigma) and added at a nominal concentration of 10 mg/kg to the diet. Batches of feed were sampled for later total Hg determination and TCDD determination.

2.2. Exposure experiments and sampling

The exposure experiments were initiated September 9, 2011, in which 360 adult TAB strain females were randomly distributed into 24 5-liter tanks, with 15 individuals in each tank. The F0 adult females were exposed to the experimental diets for 47 days until sampling (MeHg, TCDD and Control diets) with six tanks per diet (n=6). The F0 females exposed to 5-AZA and intended for chemical, gene expression and DNA methylation analyses were sacrificed for welfare reasons after 32 days of exposure, because some of the fish from this exposure group showed skin blood lesions, most likely as a result of the treatment. The remaining 5-AZA exposed females, intended for reproduction, were fed the un-contaminated control diet for 15 days until breeding. No fish with blood lesions were used for any of the analyses or for later breeding. The zebrafish were fed 1.5% of their body weight daily by hand three times a day. The light:dark photoperiod was 14h:10h. The feeding trials were done at the National Institute of Nutrition and Seafood Research (NIFES), Bergen, Norway, approved by the Norwegian Animal Research Authority and performed according to national and international ethical standards. The fish were held in Multi-Rack System for zebrafish (Aquatic Habitats Inc., Apopka, FL, USA), kept constant at 28°C in standard conditions as described elsewhere (Westerfield, 2000). After the exposure period, 5 female fish were crossed to unexposed adult male fish in water containing no test chemicals, and eggs were pooled to raise progeny of the F1 generation. The F1 fish were fed a non-enriched commercial zebrafish diet (Aqua Schwarz, Göttingen, Germany) before 5 adult F1 female fish were crossed to unexposed adult male fish, and eggs were pooled to raise the F2 generation. Similarly, the F2 fish were fed a non-enriched

commercial zebrafish diet (Aqua Schwarz, Göttingen, Germany) using the same setup as for the F0/F1 experiment. Additionally embryos (pools of ten, 3 dpf) and whole adult fish were collected from the F1 and F2 generations. The liver and embryo samples were collected for global DNA methylation and RT-qPCR analyses, and whole fish for total Hg and TCDD determination. An overview of the experimental setup is shown in Figure 1. The trials were conducted in accordance with the Norwegian Animal Welfare Act no. 73 of 20 December 1974 and the Regulation on Animal Experimentation of 15 January 1996.

2.3. Total Hg determination

Total content of Hg in whole zebrafish and in the feeds was analyzed by using a Milestone® DMA-80 (Shelton, CT, USA), which combines the techniques of thermal decomposition, catalytic conversion, amalgamation, and atomic absorption spectrophotometry. Briefly, homogenized and lyophilized whole-body samples or un-treated feeds were dried and thermally decomposed. Hg was then reduced to its elemental state and trapped with gold-amalgamation. When the amalgamator is heated, all of the trapped Hg is released to the atomic absorption spectrophotometer. The absorbance measured at 253.7 nm is proportional to Hg content in the sample. This method has been validated for solid and liquid tissue matrices in US EPA Method 7473 (U.S. EPA 2007). All samples were analyzed as duplicates. The minimum instrument detection limit (LOD) for the method is 0.02 ng, while the limit of quantification (LOQ) is 0.08 ng. LOD was calculated as the standard deviation of 30 blind samples, while the LOQ was calculated as 10 times the standard deviation. For samples within the linear area (1.5 ng - 1000 ng), the recovery is between 80 - 120%.

2.4. TCDD determination by HRGC/HRMS

The analytical method used for dioxin determination is accredited in accordance with ISO 17025 by the Norwegian accreditation authorities and described in detail by Berntssen et al., (2010). Briefly, feed and whole fish test samples were freeze-dried and well homogenized prior to analyses. The test samples were extracted, cleaned up and concentrated, and analyzed according to US EPA Method 1613 (US EPA 1994.). Sample material (scaled to give approx. 25 pg sum toxic equivalence (TEQ), was pressure solvent (hexane) extracted with a layer of acidic silica gel (Merck) on a Dionex accelerated solvent extractor (ASE 300™/350 Dionex, USA, at 100°C and 1500 psi). Surrogate 13C labeled internal standards were added prior to extraction (EDF8999, EC-4937, Cambridge Isotope Laboratories, Andover, MA, USA). The extract was purified and separated in a Power-Prep System™ (Fluid Management System, Waltham, MA, USA) using a sequence of columns (multilayered silica, basic alumina and

carbon columns), and eluted with different solvents. After elution, the samples were concentrated by pressurized evaporation (Turbovap II™ Zymark, USA). Prior to analysis, a mixture of 13C labeled performance standards (EDF 5999, EC-4979, Cambridge Isotope Laboratories, Andover, MA, USA) was added and then the extract was purified by acid treatment. Analysis of TCDD was performed by high-resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS) using a DFS-MS, HRGC-HRMS (Thermo Fisher Scientific, Bremen, Germany), equipped with an RTX-5SILMS fused silica capillary column (Restek, Bellefonte, USA). LOD for TCDD was automatically determined by the software and was estimated to equal three times the background noise in the individual samples, while LOQ was determined as three times the LOD.

2.5. DNA and RNA extraction

Liver tissue from adult zebrafish and three-day-old embryo were thoroughly homogenized before nucleotide extraction using ceramic beads (AH Diagnostics, Oslo, Norway) and the Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA and DNA were extracted from the same sample according to the Trizol protocol (Invitrogen, Life Technologies, Carlsbad, USA). Adult liver RNA was also isolated by using the BioRobot EZ1 workstation following the provided protocol from Qiagen (Qiagen Oslo, Norway).

2.6. Global DNA methylation determination by HPLC

Global methylation of enzymatically hydrolyzed DNA was determined using a modified HPLC method based of Ramsahoye (2002) as previously described by Skjaerven et al. (2014). The nucleotide standards were combined in a standard mix based on the composition of the nucleotides in the DNA sample. Adenine (dAMP; 2'-deoxyadenosine 5'-monophosphate, Sigma D6375), guanine (dGMP; 2'-deoxyguanosine 5'-monophosphate, Sigma D9500), cytosine (dCMP; 2'-deoxycytidine 5'-monophospate, Sigma D7750), and uracil (U; 2,4-dihydroxypyrimidine, Sigma U0750) were obtained from Sigma-Aldrich. Methylated cytosine (5mdCMP; 5-methyl deoxycytidine 5'-monophosphate, disodium salt, and thymine (dTMP; 2'-deoxythymidine 5'-monophosphate, were obtained from Reliable Biopharmaceutical Corporation, St. Louis, MO, USA. The theoretical concentration of each nucleotide was checked spectrophotometrically prior to combining the standards into one mixed solution. Uracil was included in the standard mix as a reference for RNA, to ensure no RNA in the DNA samples.

The HPLC system consisted of a quaternary pump (Dionex Ultimate 3000, with a degasser), a UV-detector (Thermo Separation Products TSP, UV 1000), a diode array detector (Thermo

Separation Products TSP, UV 6000 LP) and A/D converter (PE Nelson 900 Interface, Perkin Elmer). The auto-sampler (Thermo Separation Products TSP, AS 3000) injected 50 µL of each sample into the column (ACE 5 C18-AR 250x4.6 mm, advanced chromatography technologies, Aberdeen, Scotland). The column was cooled to 6°C by a column oven (Dionex TCC-3000SD). The mobile phase (20 mM orthophosphoacid, pH 2.5) was filtered and degassed prior to use, and run through the HPLC system for approximately 16 h at 0.2 mL/min prior to each sample series to stabilize the system. During analysis the flow rate of the mobile phase was set to 1 mL/min. After each sample series the HPLC system was washed with 5% acetonitrile in water for at least two hours. Chromeleon software program (Thermo Scientific, CA, USA) was used for data processing.

2.7 DNA immunoprecipitation

DNA samples were dissolved in TE buffer (1 µg in 50 µL) and fragmented with *Mse* I digestion (New England BioLabs, Ipswich, MA, USA) at 37 °C for 3 h and inactivated at 65 °C for 20 min to generate 200-1000 base pair products. Lengths of the generated fragments were checked on a 1% agarose gel. The final concentration of the digested DNA was adjusted to 0.1 µg/µL. Methylated fragments of DNA were separated from the un-methylated fragments using the MagMeDIP kit (Diagenode, Seraing, Belgium) according to the manufacturer's protocol. The optimum starting amount of DNA for each MeDIP reaction is 1 µg. As the recommended starting amount of DNA prior to MeDIP for methylation based microarray experiments is 5 µg, whole-genome DNA amplification of the MeDIP products was conducted to generate the sufficient starting material for DNA methylation microarrays. Immunoprecipitated DNA fragments were amplified using the GenomePlex Complete Whole Genome Amplification (WGA2) Kit following the protocol from Sigma (Sigma-Aldrich, Seelze, Germany).

2.8 CpG island tiling microarray

Zebrafish gene promoter regions, arbitrarily defined here as the regions between -1500 and +1000 bases of the putative transcriptional start sites (TSS), were downloaded from Ensembl (Ensembl genes 67; *Danio rerio* genome assembly Zv9). CpG islands were identified using cpgplot via Jemboss (EMBOSS 6.3.1) using default criteria (window 100; min length 200; o/e 0.6; min %GC 50). Under these criteria, 9468 promoter CpG islands were identified in 7170 zebrafish genes. Identified CpG island sequences were input to the Genomic Tiling application of Earray (Agilent) and 54933 60-mer probes were designed for 6813 CpG islands, an average of 8 per CpG island (CGI). Zebrafish and control probes were printed on G3 microarray slides in 8x60k format (Agilent) and named 8x60k Promoter CGI array for zebrafish genome release

Zv9, design ID 041519. The array design has been uploaded to ArrayExpress with accession number A-MEXP-1813.

Zebrafish DNA samples were labeled using a SureTag DNA labeling kit (Agilent) according the manufacturer's protocol. All immunoprecipitated samples were labeled with Cy5, an untreated genomic DNA sample was labeled with Cy3, 200 ng DNA was used in each case. Two-color hybridizations were carried out, each of one Cy5-labeled MeDIP sample versus one Cy3-labeled genomic DNA sample, using the Agilent Oligo aCGH/Chip on chip hybridization kit according to the manufacturer's protocol, similar to methods employed previously (Mirbahai et al., 2011). Slides were scanned and data captured using a Surescan High Resolution DNA microarray scanner with FE software (Agilent). Sample processing was carried out at The University of Birmingham Functional Genomics Facility. Data were obtained for 56 samples in 11 sample groups (F0 Ctrl n=6, 5-AZA n=4, MeHg n=6, TCDD n=3; F1 Ctrl n=5, 5-AZA n=5, MeHg n=5, TCDD n=5; F2 Ctrl n=6, 5-AZA n=6, MeHg n=5). Data were lowess normalized and analyzed within Genespring (GX 7.3; Agilent) and MeV (Saeed et al., 2006). Gene annotation analyses were performed using the 'Batch Query' facility of the Comparative Toxicogenomics Database (CTD) at http://ctdbase.org/tools/batchQuery.go, employing a multiple testing correction (Davis et al., 2009).

2.9 Quantitative real-time RT-qPCR

PCR primer sequences used for quantification of the transcriptional levels of the selected genes in zebrafish are shown in Supplementary file 1, Table 1. In total 16 target genes were quantified with RT-qPCR. BLASTX or BLASTN was used to determine PCR assay specificity. The reaction specificity of each assay was checked by examining the melting curves generated with a dissociation protocol from 65 to 97 °C. A two-step real-time RT-PCR protocol was used to quantify the transcriptional levels of the selected genes as previously described by Olsvik et al. (2013). Target gene mean normalized expression (MNE) was calculated using a *geNorm* normalization factor based upon the reference genes eukaryotic translation elongation factor 1 alpha 1 (*eef1a1),* ubiquitin A-52 residue ribosomal protein fusion product 1 *(uba52)* and tubulin, alpha 1c *(tuba1c)* (Vandesompele et al., 2002). For F0 liver samples, the *eef1a1* and *uba52* combination was used, while for F1 and F2 embryo samples a combination of three reference genes was used to calculate MNE. The geNorm stability index (M-value) was M<0.1 for the F0 reference genes, M<0.48 for the F1 reference genes and M<0.19 for the F2 reference genes.

2.10 Statistics

Significant differences among treatments were assessed with 1-way analysis of variance (ANOVA). Post hoc testing of significant differences was assessed by using the Tukey's multiple comparisons test. In case of significantly different standard deviations, as determined by the Bartlett's test, the data was log transformed before ANOVA analysis. Outliers were removed according to Rout test (Q=1.0%). The GraphPad Prism 6.0c software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses of the transcriptional data. Correlation analysis was performed using the program Statistica 8.0; 2008 (Statsoft Inc., Tulsa, OK, USA). A significance level of $P<0.05$ was used for all tests.

3. Results

3.1 Exposure experiment

Of the 15 fish in each tank, six were used for DNA and RNA extraction and four for chemical determination, of which two were used for total Hg quantification and two for TCDD quantification. The remaining fish from each tank were used for reproduction. The total numbers of fish lost (i.e. sacrificed because of skin blood lesions (5-AZA group), found dead in the tanks or escaped) prior to planned sampling were 3 from the control group, 23 from the 5-AZA group, 14 from the MeHg group and 10 from the TCDD group. All F1 generation larvae from F0 fish fed TCDD died 1-2 weeks post hatching, even though visual inspection suggested no embryonic or larval malformation before 5 dpf. No further efforts were made in this initial screening experiment to phenotypically characterize the offspring. Because of this no F2 generation were obtained from this treatment group.

Fish exposed to the 5-AZA diet for 32 days weighed significantly less (Figure 2A, one-way ANOVA, $P<0.001$) compared to the fish fed the control diet. These fish were sampled after 32 days of exposure versus 47 days of exposure for the other groups, possible explaining the observed difference. No significant weight differences were observed between the control fish and fish exposed to MeHg or TCDD. The fish exposed to 5-AZA were significantly longer than fish from the other treatment groups (Figure 2B, $P<0.001$), while there were no significant differences in length between the control fish and fish exposed to MeHg or TCDD. The condition factor (CF) of fish exposed to 5-AZA and TCDD was significantly lower than the control fish and fish exposed to MeHg (Figure 2C, $P<0.001$).

3.2 Feed and carcass concentrations of Hg and TCDD

Total Hg concentrations in the feeds and in whole adult F0 female zebrafish are shown in Figure 3A. One feed sample per experimental diet was analyzed, while pools of two fish from each fish

tank were used to determine the total concentration of Hg in F0 fish (n ranging from 4 to 6 per treatment depending on the number of available fish). The concentration of total Hg in the experimental MeHg feed was 10.98 mg/kg wet weight (ww) or about 110 fold higher than in the other three feeds (0.10±0.01 mg/kg ww, n=3). In whole zebrafish the concentration difference of total Hg between the MeHg group and the other three groups were about 44 fold, with a mean Hg concentration in whole zebrafish of 3.1±0.3 mg/kg ww (n=6). The feed to fish bioaccumulation ratio for MeHg was 28%.

Figure 3B shows the concentrations of 2,3,7,8 TCDD in the experimental feeds and in whole adult F0 female zebrafish. The concentration of TCDD in the experimental feed given to the dioxin fed fish was 19.18 µg/kg ww or about 30,500 fold higher than in the other three feeds (0.63±0.18 ng/kg, n=3). In whole zebrafish the concentration difference of TCDD between the dioxin group and the other three groups were about 1770 fold, with a mean TCDD concentration in whole zebrafish of 11.54±0.11 µg/kg ww (n=6) and a feed to fish bioaccumulation ratio of 60%. The concentrations of 5-AZA in feeds and whole fish were not measured.

3.3 Global DNA methylation

Figure 4 shows the global DNA methylation level in liver of adult female fish (F0), and in 3 days old F1 and F2 embryos. Compared to the control, none of the treatments mediated a significant effect on the global DNA methylation levels in the F0 fish (Figure 4A), although there was a trend toward lower levels in the 5-AZA group compared to the controls (uncorrected Fisher's LSD, P=0.0528). The global DNA methylation level in liver of fish exposed to 5-AZA was significantly lower than in fish exposed to MeHg and TCDD (uncorrected Fisher's LSD, P=0.0035 for both). No significant effects on global DNA methylation were observed between the treatments groups for the F1 (Figure 4B) or F2 (Figure 4C) embryo groups.

3.4 Microarray results

MeDIP microarray data were analyzed both by individual probe and by CGI, using the mean value for all probes designed to bind each CGI. One-way ANOVA with a multiple testing correction (Benjamini and Hochberg, 1995) for a false-discovery rate (FDR) of <0.05 identified no probes revealing significant differential methylation between control and treated groups within each generation. Similarly, ANOVA at FDR<0.05 failed to identify any CGIs significantly differentially methylated between control and treated groups within each generation and principal component analyses (PCA) did not separate treated from control samples. In contrast, extensive DNA methylation differences were detected between F0 liver tissue and F1 and F2 embryo tissues. 21,826 probes, or 3,517 CGIs, were significantly

differentially methylated (FDR<0.05) between liver and embryo (Supplementary File 1; Table 2). The non-parametric rank products test (Breitling et al., 2004) did identify a number of probes (Supplementary File 1; Table 3) that significantly differed between test and control fish of the same generation. The majority of these (110 probes) were hypomethylated in F0 5-AZA exposed fish in comparison with F0 controls (Table 1). Five genes were chosen for RT-qPCR analysis, ((fumarylacetoacetate hydrolase domain containing 1 *(fahd1)* - hypermethylated F1 5-AZA; AP2 associated kinase 1 *(aak1)* - hypomethylated F0 TCDD; sterol regulatory element binding transcription factor 2 (*srebf2)* - hypomethylated F0 5AZA; potassium inwardly-rectifying channel, subfamily J, member 2 (*kcnj2)* - hypomethylated F0 and F1 MeHg; and B-cell CLL/lymphoma 2 *(bcl2)* – unchanged)). The rank products test was also applied to the mean values for each CGI and identified 148 CGIs that were differentially methylated in comparison with the relevant controls (Table 2, Supplementary File 1; Table 4). These differences in CGI methylation were modest in extent, with few exceeding a 1.5-fold change. As with the analysis by individual probes, *kcnj2* was the only protein-coding gene whose promoter was significantly differentially methylated in two generations following the same treatment (MeHg F0 and F1). Annotation analyses (Supplementary File 1; Table 5) highlighted functions associated with the genes shown in Table 4. 5-AZA differentially methylated genes involved in morphogenesis in F0, and hypomethylated G-protein coupled receptor pathways in F1. MeHg affected genes of the neurological system and gap junctions in F0, G-protein coupled receptor pathways and hypotension in F1. TCDD treatment resulted in hypermethylation of genes responsive to glucose in F0. There were no significant associations with annotation for F2 fish.

3.4 RT-qPCR results

Six individual samples from each exposure group were used for the RT-qPCR analyses, except for the F1 embryo group from progeny exposed to TCDD which had n=5. As explained above, since no F1 larvae from fish exposed to TCDD survived, no RT-qPCR data from the F2 group were available. Figures are not provided for genes whose expression did not change. Based on the microarray results, five genes with altered promoter DNA methylation of detoxification interest were selected for transcriptional analysis (Figure 5). Of these, significant differences between the treatment groups were only observed for *fahd1* in F0 females (Figure 5D) and for *aak1* in F1 embryo (Figure 5B). *fahd1* was significantly higher expressed in liver of F0 females exposed to 5-AZA (one-way ANOVA, P<0.01) compared to the controls, whereas *aak1* was significantly higher expressed in F1 embryo exposed to 5-AZA (P<0.05) compared to the controls. Figure 5 also shows the transcriptional levels of three genes selected as markers for

TCDD exposure. Cytochrome P450, family 1 genes (*cyp1a1*) and (*cyp1b1*) both showed a distinct response to exposure to TCDD. *cyp1a1* was 24-fold higher expressed in liver of adult zebrafish exposed to TCDD compared to the control fish (Figure 5G, one-way ANOVA, P<0.0001), while *cyp1b1* was 7.6-fold higher expressed in the TCDD group (Figure 5J, P<0.0001). For *cyp1a1*, the difference was even greater in the F1 embryo, where this transcript was 27-fold higher expressed in offspring of TCDD exposed female fish (Figure 5K, P<0.0001). *cyp1b1* was 2.3-fold higher expressed in F1 embryo (Figure 5L, P<0.001). Also the aryl hydrocarbon receptor 1A gene (*ahr1a*) was significantly higher expressed in liver of adult females compared to the controls and fish from the 5-AZA and MeHg treatment groups (Figure 5M, P<0.0001). Contrary to the *cyp1a1*, *cyp1b1* and *ahr1a* results, TCDD exposure down-regulated the transcription of vitellogenin 1A (*vtg1a*) (Figure 6J, P<0.0001), whereas MeHg exposure appeared to have induced its expression in liver of adult female zebrafish (P<0.05). The expression levels of the *vtg1a* gene were too low in 3 dpf zebrafish embryo to be measured with the currently used PCR assay. Figure 6 further shows that selenoprotein P, plasma, 1 (*sepp1a*) was significantly higher expressed in liver of female zebrafish exposed to TCDD (Figure 6A, P<0.001), but did not respond to MeHg exposure. Both heme oxygenase 1 (*hmox1*) (Figure 6D, P<0.05) and tumor protein p53 **(***p53*) (Figure 6G, P<0.0001) were significantly higher expressed in liver of adult female zebrafish exposed to the hypomethylating agent 5-AZA, but none of these three transcripts were differentially expressed in F1 or F2 embryo. No significant effects of any of the treatments were observed on the transcriptional levels of three DNA (cytosine-5-)-methyltransferase genes (*dnmt1*, *dnmt3a* and *dnmt3b*) in liver of exposed adult female zebrafish or in F1 or F2 offspring (data not shown).

Tank-based correlation between accumulated concentrations of 2,3,7,8 TCDD and Hg in whole-fish and the 16 quantified target transcripts in liver of adult F0 females was performed in search of additional effects of the three chemical exposures (Supplementary file 1; Table 6). Chemical measurements and gene transcription analysis were conducted on different fish obtained from the same exposure units. Spearman's rank-order correlation analysis showed a strong correlation between whole-fish concentrations of 2,3,7,8 TCDD and liver transcription of *cyp1a1* (R=0.71), *cyp1b1* (R=0.71) and *ahr1a* (R=0.71), all well-established markers of dioxin exposure, and several other transcripts (i.e. *fahd1*, *sepp1a* and *vtg1a*). Accumulated concentrations of Hg in whole fish correlated positively with transcription of *vtg1a* (R=0.52) and negatively with transcription of *hmox1* (R=-0.54) in liver tissue.

4. Discussion

Global DNA methylation levels in F1 and F2 embryos of MeHg exposed F0 female adults were not significantly affected compared to controls, although a number of gene promoters were identified as differentially methylated after MeHg exposure of adult F0 liver and in their F1 embryo offspring. We have previously exposed adult zebrafish to 10 mg/kg MeHg for 10 weeks without detecting any lethality or other overt signs of toxicity (Ellingsen, unpublished data). For MeHg, a carcass concentration of 3.1±0.3 mg/kg w/w is environmentally relevant, and corresponds to the upper limit of what can be expected to be found in tissues of wild-caught fish (Olsvik et al., 2013). In this experiment we did not determine the concentration of the contaminants in the embryo, however after exposing female zebrafish to 10 mg/kg MeHg for six weeks, concentrations of 0.3 mg/kg Hg have typically been measured in three dpf old embryo (Ellingsen, unpublished data). Since most of the Hg (80-100%) in muscle tissue of contaminated fish normally is present as MeHg (Kidd and Batchelar, 2011), the total Hg measurements of whole zebrafish carcasses will, to a large degree, reflect the MeHg concentrations. In liver, the MeHg to total Hg ratio is normally lower in wild-caught fish (Batchelar et al., 2013; Olsvik et al., 2013). At an environmentally realistic body concentration for fish, the present study therefore indicates that MeHg has no effects on DNA methylation in F2 generation embryo offspring of adult female zebrafish exposed to the contaminant. This finding may in part be due to the selected exposure concentration and limitations of the experimental design (breeding of exposed F0 female zebrafish with non-exposed F0 male zebrafish). Recent studies also suggest that paternally inherited DNA methylation contribute to embryonic methylation (Jiang et al., 2013; Potok et al., 2013).

Unfortunately, no F2 offspring from the TCDD-exposed females were available for DNA methylation evaluation in this study. The TCDD exposure concentration selected for this experiment (20 µg/kg), although relatively high, was based on previous fish studies indicating sub-lethal effects on adult fish. For example, after chronic dietary exposure of zebrafish resulting in the whole-body TCDD accumulation of 1.1–36 µg/kg fish, Heiden et al. (2005) found no signs of overt toxic response. Follow-up experiments need to determine the maximum TCDD exposure concentration that can be given to adult F0 females and allowing offspring to survive to adulthood. As for 5-AZA, female F0 fish exposed to TCDD had significant lower condition factor compared to the control fish, suggesting a phenotypic response. The transcriptional data for phase I detoxification enzymes presented here clearly suggest that TCDD is maternally transferred to F1 embryo, suggesting a significant exposure to F1 embryo. In line with findings from earlier fish studies (Nichols et al., 1998; Tietge et al., 1998; Jones et al., 2001; Halden et al., 2011), maternal transfer from the ovary to the eggs constitutes a major source of TCDD exposure in fish embryo. Halden et al. (2011) found highly correlated

concentrations of TCDD in zebrafish ovarian tissues and eggs with carcass tissues (including skeletal muscle), with egg concentrations reaching 6% of carcass TCDD concentrations. Thus, a female carcass TCDD concentration of about 12 μg/kg ww TEQ as reported here is high enough to affect the survival of F1 generation larvae. Even within the F1 generation embryo, originating from oocytes that may have been exposed to TCDD of the F0 females, TCDD exposure induced no significant effect on global DNA methylation and very few CpG-island DNA methylation changes. As a positive control based on earlier studies (Lantry et al., 1998; Martin et al., 1999; Aniagu et al., 2008), feed spiked with 10 mg/kg 5-AZA was expected to decrease genome-wide DNA methylation. However, no significant effects were observed on global DNA methylation levels of the F1 and F2 embryos compared to the control fish. We were thus not able to reproduce the significantly lowered hepatic global methylation levels previously observed in the liver of female three-spine stickleback (*Gasterosteus aculeatus*) exposed to 5 mg/kg 5-AZA (Aniagu et al., 2008). However, 5-AZA exposure did induce the highest number of gene specific hypomethylated and limited hypermethylated regions in the F0 females. For example, promoter regions for extracellular leucine-rich repeat and fibronectin type III domain containing 1 (*elfn1*) and protease, serine, 23 (*prss23*) genes were hypomethylated while collagen, type II, alpha 1 (*col2a1*), adrenoceptor alpha 2A (*adra2a*) and dipeptidyl-peptidase 10 (*dpp10*) genes were hypermethylated in 5-AZA exposed adult females (F0) while frizzled family receptor 9 (*fzd9b*), carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8 (*chst8*) and netrin G1A (*ntng1a*) were hypomethylated in F1 embryos (Table 2). These results are consistent with the findings of a study by Tryndyak et al. (2011) where changes in DNA methylation of these genes were also detected in the liver of mouse (*Mus musculus*) on a methyl-deficient diet. Interestingly, female fish exposed to 5-AZA were significantly longer than fish from the other three treatment groups, resulting in a significantly reduced condition factor. Whether or not this was a direct result of 5-AZA exposure is unknown. Some of the F0 female fish exposed to 10 mg/kg 5-AZA for 32 days displayed skin blood lesions, and none of these individuals were included for weight and length calculation or any other analyses.

Promoter CpG-island DNA methylation profiling by microarray was effective at discovering differences between zebrafish liver tissue and embryos (Supplementary File 1; Table 2). This data may provide a useful resource to facilitate discovery of tissue-specific and developmental stage-specific epigenetic changes. In contrast DNA methylation changes in response to chemical exposure were modest in extent. However, analysis of promoter methylation by CGI rather than by individual probes did identify a number of genes with differentially methylated CGIs (Table 2). Gene promoter region methylation is often considered to be inhibitory of transcription, but this is only one of the complex series of events that can affect transcription (Gronbaek et al.,

2007). The genes chosen for RT-qPCR did not show an inverse relationship between promoter methylation and transcription, indeed, the biological significance of small alterations in promoter methylation is as yet uncertain. To identify changes as transgenerational, they would have to clearly persist to the F2 generation in fish. In this experiment no detected methylation change fitted this criterion (Supplementary File 1; Table 3). However, a number of different methylation changes were detected in the F0 and F1 fish. These provide an indication of the role of CGI methylation changes in response to chemical exposure.

While promoter methylation change does not necessarily affect transcription in all genes, it is interesting to note that several of the genes for which differential promoter methylation was detected in response to MeHg or TCDD treatment in this study (Table 2) have previously been found to respond to these compounds transcriptionally. MeHg hypomethylated the pyrimidinergic receptor P2Y, G-protein coupled, 6 (*p2ry6*) and CDC42 effector protein (Rho GTPase binding) 4 (*cdc42ep4*) promoters in F1 zebrafish and induced transcription of orthologous genes in other vertebrates (Toyama et al., 2011; Robinson et al., 2011), while somatostatin (*sst*), hypermethylated in F1, was transcriptionally repressed by copper, another heavy metal (Song et al., 2009). For TCDD, lecithin retinol acyltransferase (*lrata*) and THUMP domain containing 1 (*thumpd1*) promoters were hypomethylated in F0 and cadherin-related family member 1 (*cdhr1*) in F1 and these genes can be transcriptionally activated by TCDD (Hogberg et al., 2003; Thornley et al., 2011; Fracciolla et al., 2011). Conversely TCDD hypermethylated promoters of tight junction protein 1 (*tjp1*), keratin 5 (*krt5*), SPRY domain containing 4 (*spryd4*), *adra2a* and *col2a1* in F0 fish, and TCDD repressed transcription of these genes in other systems (Thornley et al., 2011; Heiden et al., 2007; Boutros et al., 2011; Rowlands et al., 2011; Dong et al., 2012). The functional significance of the modest promoter methylation changes detected is difficult to state with certainty but annotation enrichment analyses (Supplementary File 1; Table 5) provide indications of the biological systems that could potentially be affected. For example MeHg exposure affected methylation of genes associated with cardiovascular and neurological system effects, both of which are known consequences of exposure to mercuric compounds (Virtanen et al., 2007).

There is increasing evidence that endocrine-disrupting compounds, such as MeHg and TCDD, can modify the epigenome (Anway et al., 2005; Dolinoy et al., 2007; Skinner et al., 2010; Bernal and Jirtle, 2010). Ceccatelli et al. (2013) found that MeHg exposure induced long-lasting DNA hypermethylation in the nervous system of offspring of female mice exposed to MeHg. Manikkam et al. (2012) studied the transgenerational effects of pesticides (permethrin and insect repellant DEET), plasticisers (bisphenol A and phthalates), and hydrocarbon (jet fuel and JP8) mixtures, and TCDD, on F1, F2 and F3 offspring of gestating female rats (*Rattus norvegicus*).

These studies showed evidence of transgenerational epigenetic effects of TCDD in F3 generation sperm, with 50 differentially methylated regions, indicating that dioxins may affect offspring through epigenetic mechanisms. The authors suggested, however, that classic endocrine disruptor actions are likely not involved in the F2 and F3 generation, but only in the F1 generation (Manikkam et al., 2012). To which degrees the epigenome of F2 offspring in fish are affected by direct exposure is uncertain, as it is difficult to clearly demonstrate germ cell-dependent epigenetic mechanisms mediating intergenerational effects (Patti, 2013). In mammals, exposure of gestating viviparous females provides direct exposure of the F0 generation female, the F1 generation embryo, and the germ line that will generate the F2 generation (Skinner, 2008). Endocrine-disrupting exposure of the germ line inside the mammalian F1 embryo may therefore directly affect the F2 generation. In non-eutherian fish, however, it can be argued that only the F0 and the gamete/oocyte of the F1 generation are directly exposed. Mirbahai and Chipman (2013) suggested that, after elimination of the potential exposure directly to the eggs following spawning, it would be possible to categorize any epigenetically inherited phenotype in the F2 generation as a true transgenerational epigenetic effect. Future chemical assessment with fish should therefore focus on responses in F2 and preferably also F3 embryo and adults.

Surprisingly, no significant effects of MeHg exposure were found on oxidative stress (glutathione peroxidase 1 (*gpx1*), *hmox1*) and apoptosis (*bcl2*, *p53*) markers in liver of F0 female fish. The only transcript that was significantly higher expressed in liver of F0 female fish exposed to MeHg compared to the controls was *vtg1a*. Contrary to this finding, vitellogenin gene expression has been shown to significantly decrease in liver of female fathead minnows following dietary MeHg exposure (Klaper et al., 2006). Since vitellogenin is an essential yolk protein, these changes likely have downstream effects on egg quality. As a potent aryl hydrocarbon receptor (AHR) agonist, the relatively strong induction of *cyp1a1* in liver of F0 female zebrafish exposed to TCDD was expected. The equally strong induction of *cyp1a1*, as well as a significantly higher level of *cyp1b1* compared to the controls, clearly suggests a substantial TCDD exposure in F1 embryo. In F0 females, 5-AZA exposure significantly induced the transcription of *hmox1*, *fahd1*, and *p53*, while *aak1* was significantly induced in F1 embryo. For the adult females, increased transcription of *hmox1*, which is regulated by the transcription factor NRF2 (nuclear factor erythoid-derived 2 related factor 2), suggests 5-AZA exposure induced an oxidative stress response in liver of F0 females. Increased transcription of *fahd1* in adult females suggest 5-AZA effects on acylpyruvate hydrolase activity, while effects on *p53* transcription might suggest several types of cellular stress, including apoptosis. Increased transcription of *aak1* in the F1 embryos indicate altered protein kinase activity. None of the 15

transcripts evaluated with RT-qPCR showed any differential expression in F2 embryo, including the three DNA methyltransferase transcripts encoding enzymes important for the cellular methylation status, further supporting the general lack of response in F1 and F2 embryos to these contaminants with the selected experimental design.

In conclusion, in this work we show that dioxin (TCDD) and MeHg, often present at relatively high concentrations in fish, have modest effects on DNA methylation in adult zebrafish, and in F1 (MeHg and TCDD) and F2 (MeHg) offspring at the concentrations tested.

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Figure legends

Figure 1. Overview of the experimental design, time of sampling and age (days post-fertilization, dpf) of the zebrafish in the exposure trial.

Figure 2. A) Weight (W), B) Length (L) and C) Condition factor (formula CF = W*100/L*L*L) of F0 exposed female fish. Number of fish in each group: Control n=89, 5; AZA n=49; MeHg n=76, TCDD n=80. Mean±SEM. Means with different letters are significantly different (1-way ANOVA with Tukey's posthoc test, $P<0.05$). Shared letters indicate no significant difference.

Figure 3. Concentrations of A) total Hg and B) 2,3,7,8 TCDD in experimental fish feeds (n=1) and in whole F0 female zebrafish. For total Hg in F0 female zebrafish, Control n=4; 5-AZA n=5, MeHg n=6, TCDD n=5. For TCDD in F0 female zebrafish, Control n=6, 5-AZA n=3, MeHg n=6, TCDD n=6. Mean±SEM. Significance levels between the whole fish samples are given (comparison to the controls). **** $P<0.0001$.

Figure 4. Global DNA methylation in A) F0 adult female liver, B) 3 days old F1 embryo or C) 3 days old F2 embryo. Mean±SEM. Means with different letters are significantly different (1-way ANOVA with Tukey's posthoc test, $P<0.05$). Shared letters indicate no significant difference.

Figure 5. Mean normalized expression (MNE) of potential marker genes of 5-AZA and MeHg exposure selected from the microarray results in liver of F0 female zebrafish and in 3 dpf F1 and F2 zebrafish embryo. A) F0 *aak1*, B) F1 *aak1*, C) F2 *aak1*, D) F0 *fahd1*, E) F1 *fahd1*, F) F2

fahd1, G) F0 *cyp1a1*, H) F1 *cyp1a1*, I) F2 *cyp1a1*, J) F0 *cyp1b1*, K) F1 *cyp1b1*, L) F2 *cyp1b1*, M) F0 *ahr1a*, N) F1 *ahr1a*, and O) F2 *ahr1a*. Analyzed with 1-way ANOVA with Tukey's posthoc test. * P<0.05, ***P<0.001, ***P<0.001. n=6 for all groups except for the F1 embryo TCDD group where n=5. Mean±SEM.

Figure 6. Mean normalized expression (MNE) of potential phase I detoxification and endocrine disruption marker genes of MeHg and TCDD exposure in liver of F0 female zebrafish and in 3 dpf F1 and F2 zebrafish embryo. A) F0 *sepp1a*, B) F1 *sepp1a*, C) F2 *sepp1a*, D) F0 *hmox1*, E) F1 *hmox1*, F) F2 *hmox1*, G) F0 *p53*, H) F1 *p53*, and I) F2 *p53* and J) F0 *vtg1a*. Analyzed with 1-way ANOVA with Tukey's posthoc test. * P<0.05, ***P<0.001, ***P<0.001. n=6 for all groups except for the F1 embryo TCDD group where n=5. Mean±SEM.

Supplementary file (a single excel file with six worksheets)

Table 1. PCR primers, accession numbers, amplicon sizes and PCR efficiencies.

Table 2. Probes significantly changing between zebrafish (*Danio rerio*) livers and embryo.

Table 3. Probes indicating differential DNA methylation following chemical treatments.

Table 4. CpG Islands showing differential DNA methylation following chemical treatments.

Table 5. Annotation associated with genes whose CpG Islands showed differential DNA methylation following chemical treatments.

Table 6. Spearman's rank-order correlation matrix.

Table 1. Number of probes differing from controls.

Generation	Group	Hypomethylated	Hypermethylated
F0	5-Aza	110	0
F0	MeHg	36	0
F0	TCDD	1	0
F1	5-Aza	32	7
F1	MeHg	2	5
F1	TCDD	0	0
F2	5-Aza	0	1
F2	MeHg	0	34

Number of individual probes significantly differing (Rank Products test $q<0.05$) from controls of the same generation.

Table 2. Genes with differentially methylated CpG Islands.

Generation	Group	Genes with hypomethylated CGIs	Genes with hypermethylated CGIs
F0	5-AZA	*pax9, elfn1, c2cd4c, sptbn4, mab21l2, gpr25, ghitm, prss23, cldn20, herc1, irs4*, 1 uncharacterized, 4 microRNAs, 2 rRNAs, 5 ncRNAs	*p2y4l, col2a1, adra2a, prdm1b, c1orf216, card14, ppp3ccb, slc5a3, cdk4, bmp7a, dpp10, gas2, klhl29, dlgap4*, 3 uncharacterized, 2 snRNAs, 16 ncRNAs,
F0	MeHg	*znf710, lpar6l, mab21l2, zbtb39,* ***kcnj2****, scrt1a, plkhm3, dlgap1b, rbms3, prss23, gpr25, cx52.7, tmem299b, fbxw7*, cldn20, sptbn4, kctd4, rho, elfn1, socs9, cx34.4, herc1, zbtb46, foxe1, c2cd4c, pou6f2*, celsr2, taar20p*, dnah9*, 9 uncharacterized, 2 microRNAs, 2 ncRNAs	none significant
F0	TCDD	*lrata, thumpd1*	*tjp1, usp5, krt5, spryd4, adra2a, col2a1*, 2 uncharacterized, 2 ncRNA
F1	5-AZA	*nmur1, cxcr1, chst8, triobp, ntng1a, mgat1, hrh1, tmem169, htr1bd, c16h1orf172, pacs1, fzd9b, lphn3, purb, foxe1,* ***kcnj2****, ighv1-3, klhl23*, 5 uncharacterized, 1 ncRNA	*taar20p*, rbms3, sart3*, 2 uncharacterized, 1 snoRNA
F1	MeHg	*npy8br, fut9,* ***kcnj2****, pacs1, b3galt6, lim2.5, p2ry6, chst8, cdc42ep4, hrh1, htr1bd, nmur1, fam43b, b3galt1, triobp, tmem169, c16h1orf172*, 5 uncharacterized, 1 rRNA, 1 ncRNA	*taar20p*, adra2a, sst1.1*, 1 uncharacterized, 1 rRNA
F1	TCDD	*cdhr1*, 1 uncharacterized	*taar20p**
F2	5-AZA	1 uncharacterized	*adra2a*
F2	MeHg	none significant	1 rRNA

Genes with differentially methylated CG islands (Rank Products test q<0.05) in comparison with controls of the same generation. Asterisk denotes potential ambiguity; *kcnj2* is highlighted in bold text. Further details are shown in Supplementary Table 4. Uncharacterized denotes uncharacterized zebrafish protein coding genes, rRNA ribosomal RNA, snRNA small nucleolar RNA, ncRNA non-coding RNAs including pseudogenes, antisense and long intergenic RNAs.

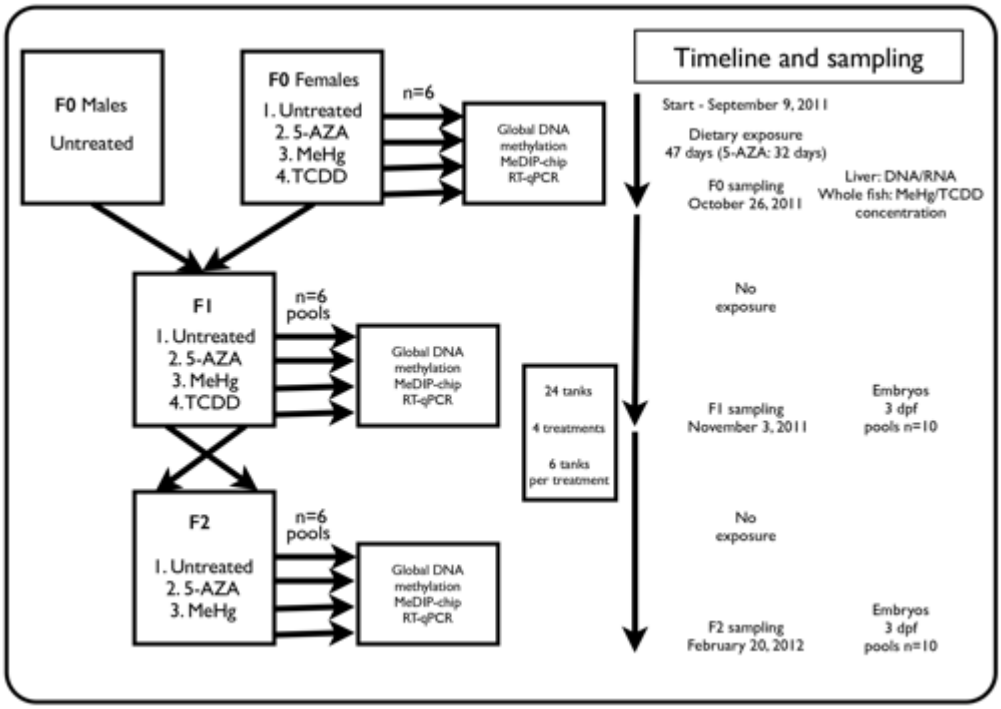

Figure 1

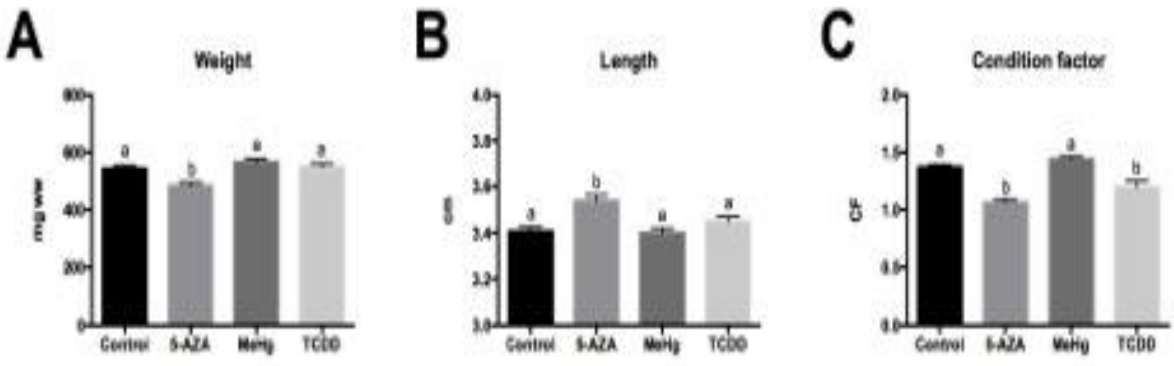

Figure 2

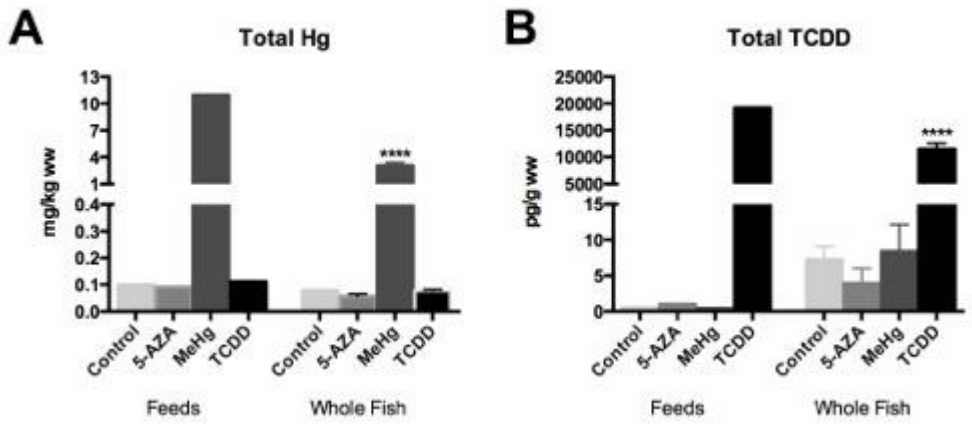

Figure 3

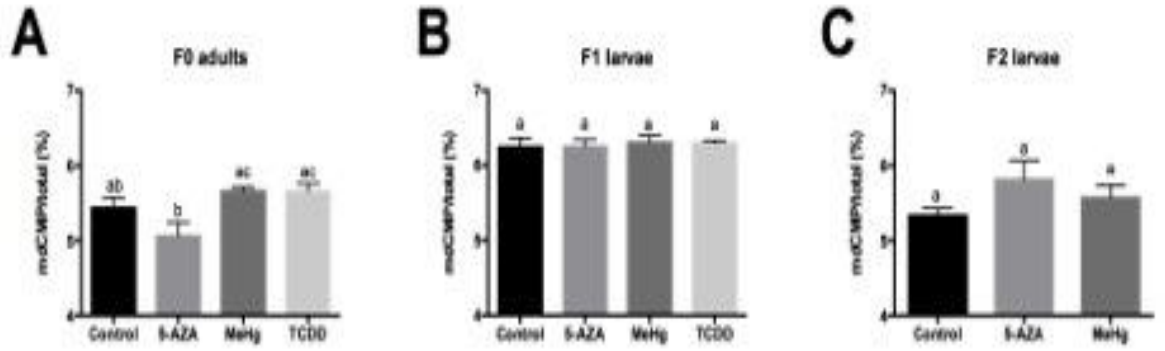

Figure 4

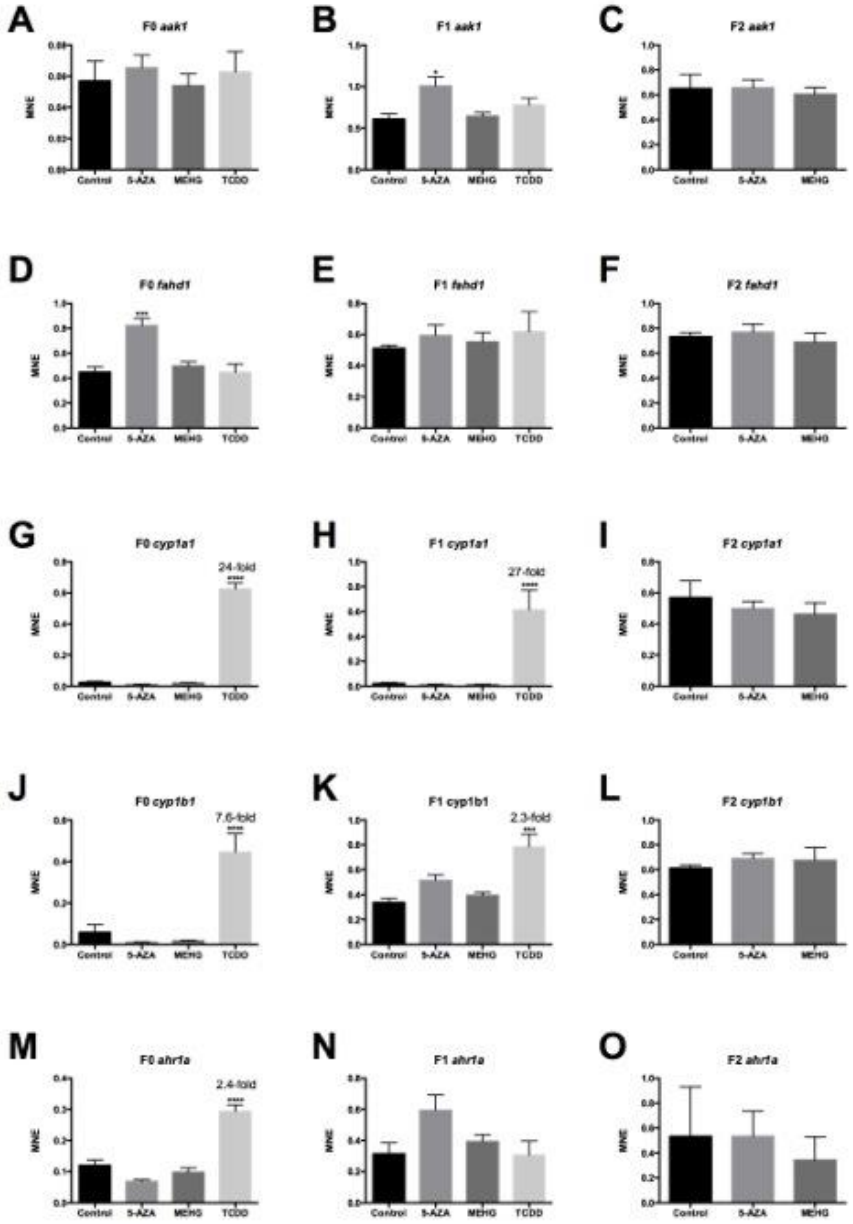

Figure 5

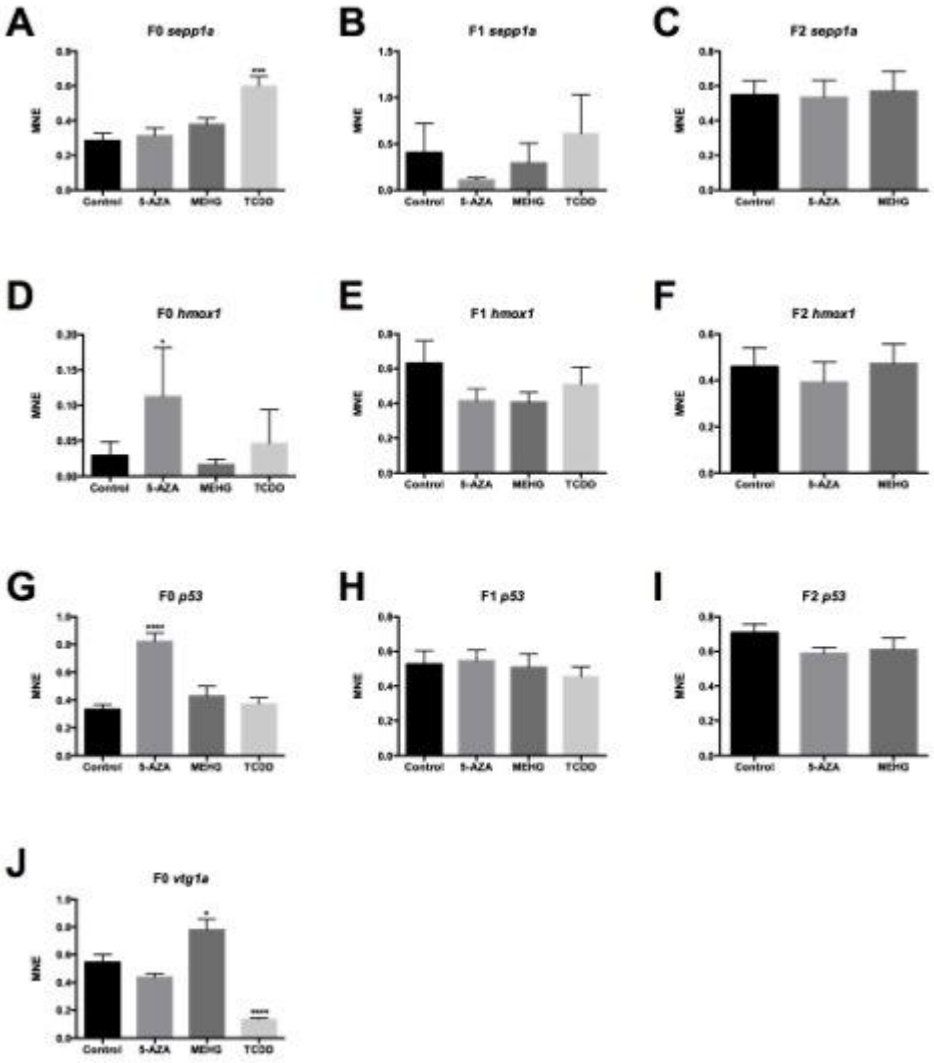

Figure 6