An environmentally relevant mixture of polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs) disrupts mitochondrial function, lipid metabolism and neurotransmission in the brain of exposed zebrafish and their unexposed F2 offspring

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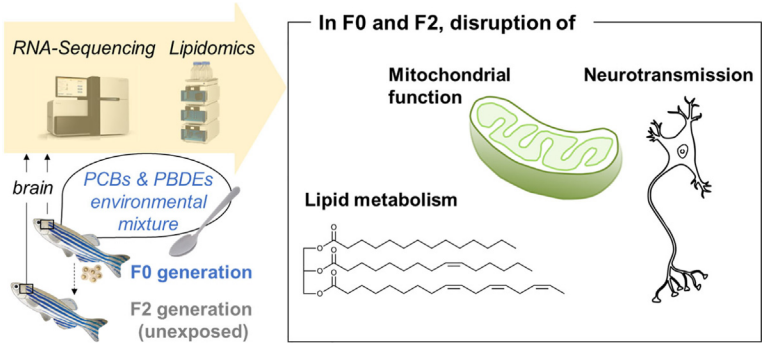
HIGHLIGHTS

- A mixture of PCBs and PBDEs regulated energetic pathways in zebrafish male brain.
- Transcriptomic and lipidomic changes supported the observed phenotypes.
- These were partly inherited in the unexposed F2 generation.
- Dysregulation of epigenetic mechanisms may be implicated in effect inheritance.

GRAPHICAL ABSTRACT



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ABSTRACT

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are persistent organic pollutants still present in aquatic environments despite their total or partial ban. Previously, we observed that an environmentally realistic mixture of these compounds affects energy balance, growth, and reproduction in exposed zebrafish (F0), and behavior in their unexposed offspring (F1-F4). In the present work, we performed lipidomic and transcriptomic analyses on brains of zebrafish (F0-F2) from exposed and control lineages to identify molecular changes that could explain the observed phenotypes. The use of both technologies highlighted that F0 zebrafish displayed impaired mitochondrial function and lipid metabolism regulation (depletion in triacylglycerols and phospholipids) which can explain disruption of energy homeostasis. A subset of the regulated biological pathways related to energetic metabolism and neurotransmission were inherited in F2. In addition, there were increasing effects on epigenetic pathways from the F0 to the F2 generation. Altogether, we show that the effects of an environmental exposure to PCBs and PBDEs on energetic metabolism as well as neurotransmission extend over 2 generations of zebrafish, possibly due to transgenerational epigenetic inheritance.

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1. Introduction

Numerous investigations have shown that persistent organic pollutants (POPs) (bio-)accumulate in ecosystems, which is associated with health risks for humans and wildlife (Bonefeld-Jorgensen et al., 2014; Holma-Suutari et al., 2016; Berghuis et al., 2015). In this study, we focused on the toxicity of two families of industrial POPs: polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). PCBs were used for various applications such as transformers, capacitors, vectors for pesticides (Voogt and Brinkman, 1989) and PBDEs were used as flame retardants in a variety of industrial as well as consumer products (de Wit, 2002). Both groups of compounds were massively commercialized as technical mixtures. Due to their toxicity, regulation was progressively adopted to remove PCBs from the market and to limit PBDE production around the globe (Kemmlein et al., 2009). However, (eco-)toxicological issues related to PCBs and PBDEs remain because of their environmental persistence. Nowadays, they are still detected in humans and in the environment (Holma-Suutari et al., 2016; Suarez-Lopez et al., 2019). Especially, they often end up in the aquatic compartment where aquatic species are most at risk (Häder et al., 2020).

There is extensive literature on the toxicological effects of individual and/or high level PCB and PBDE exposure, especially on the nervous system and hormonal signaling (Hany et al., 1999; Kodavanti, 2005; Lyche et al., 2010; Yu et al., 2010; Di Paolo et al., 2015). Examples of common associated physiological effects are neurobehavioral defects, reduced fertility and dysregulation of lipid metabolism (Berghuis et al., 2015; Suarez-Lopez et al., 2019; Kodavanti, 2005; Wen et al., 2019; Ferrante et al., 2014; Madureira et al., 1993; Daouk et al., 2011; de Cock and van de Bor, 2014; Pean et al., 2013). Lipids are fundamental to fish, and organisms in general, as they act as structural, signaling as well as energy-storing molecules. As such, they are related to a variety of essential biological functions such as growth and reproduction (Tocher, 2003; Ho et al., 2004), and they are major constituents of the brain. Under normal conditions, the brain receives free fatty acids from systemic circulation and further turns them into structural and signaling lipids to ensure proper neurotransmission (Bruce et al., 2017; Bazan, 2005). In addition, a small part is uptaken by the mitochondria for energy *via* fatty acid β-oxidation (Soengas and Aldegunde, 2002). Therefore, interfering with lipid metabolism may have detrimental effects on brain function (Bruce et al., 2017; Bazan, 2005).

Previous own results showed that energetic metabolism, growth and reproduction were disrupted in zebrafish after a long-term feeding experiment with an environmentally-relevant mixture of PCBs and PBDEs (MIX) (Horri et al., 2018; Horri, 2018). In addition, we observed that this MIX caused neurobehavioral defects in offspring of zebrafish over 4 generations (Alfonso et al., 2019). Especially, adults F2 displayed increased anxiety, which was not observed in the F0 generation and suggests multi- or transgenerationally-acquired traits (Skinner, 2008; Head, 2019). A few other studies reported multigenerational (F0, F1) effects of PCBs and PBDEs (Chen et al., 2012; He et al., 2011; Yu et al., 2011). However, to our knowledge, none investigated further generations and environmentally relevant concentration scenarios. The aim of the present study was to provide information about underlying mechanisms for the observed physiological effects. As we observed transgenerational neurobehavioral changes, an important objective was to assess whether there were inherited patterns from the F0 to the F2 generation at the molecular level with focus on the brain.

2. Material and methods

2.1. Fish husbandry

The study was conducted using wild type strain TU zebrafish (ZFIN ID: ZDB-GENO-990623-3) under the Approval of the Animal Care Committee of Poitou-Charentes #84 COMETHEA (France), project authorization number CE2012-23, and followed the recommendation of the Directive 2010/63/EU.

Fish were kept at 27 ± 1 °C under a 14 h/10 h light/dark cycle. Physico-chemical water parameters were within recommended ranges over the course of the experiment (Lawrence, 2007). To produce the F0, F1 and F2 generations, eggs were obtained by random pairwise mating using adults of approx. 180 days *post*-fertilization (dpf) of the appropriate experimental condition (*i.e.* Control or MIX, both parents of the same condition) (Fig. S1). Pairs were transferred to spawning boxes containing clean and fresh water, which strongly limited the possibility of F0 egg exposure to relevant concentrations of dissolved MIX. At least 5 replicate aquaria of 50 individuals were reared per condition and generation; each replicate issued from a mixture of 6 different spawns as described previously (Horri et al., 2018).

2.2. Fish exposure and sampling

The preparation of the spiked diet (MIX) and validation of exposure concentration were described in (Horri et al., 2018) (Table S1). The MIX contains 22 PCBs and 7 PBDEs and was prepared with dilution of a MIX stock solution in isooctane solvent for incorporation (Daouk et al., 2011). Control diet consisted of isooctane incorporation only. In the MIX diet, the total concentrations, *i.e.* summed across 22 PCB and 7 PBDE congeners, were 1932.3 ± 90.4 ng/g (mean ± SE) ww for ∑PCBs and 479.8 ± 50.8 ng/g ww for ∑PBDEs (Table S1). In the Control diet, the total concentrations were 245 and 522 times lower, *i.e.* 7.9 ± 3.5 ng/g ww for ∑PCBs and 0.92 ± 0.36 ng/g ww for ∑PBDEs, showing no cross contamination between the two conditions (Horri et al., 2018) (Table S1). Fish from the F0 generation were chronically fed from five dpf until the end of the experiment (135 dpf, time of sampling) with the MIX or Control diet. The F1 and F2 generations were given commercial plain food. In all cases (contaminated or plain), diets were adapted to fish size and consisted in the sequential use of 100, 200 and 300 μm SDS (Special Diet Service; Dietex international, France) followed by Inicio+ 500 μm (Biomar, France) at 70 dpf and onwards. When they reached 135 dpf, fish were euthanized in ethyl 4-aminobenzoate (final concentration 500 mg/l, Sigma-Aldrich), measured and weighted. Sampling was performed between 10 and 12 a.m. after an overnight fasting for all conditions and generations to avoid confounding factors due to physiological status or circadian differences. Brains were dissected and snap-frozen in liquid nitrogen, and then kept at −80 °C until processing. The sampling procedure and time were kept consistent for all generations (Fig. S1).

2.3. Lipidomics

Brains from four males and four females of each condition and per generation (F0 and F2) were prepared for lipidomics. Brains were weighted and lysed in 350 μl of RP1 buffer provided by the Triprep extraction kit (Macherey-Nagel, France). Then, 45 μl aliquots were taken out for total lipid extraction. The all procedure was conducted on ice plates. Each sample was added 60 μl of internal standard (ISTD, 5 ppm PE(17:0/17:0), SM(d18:1/17:0), Cer(d18:1/17:0), PC(17:0/17:0), LPC (17:0), PC(16:0/d31/18:1), TG(17:0/17:0/17:0)), 90 μl of methanol (Fisher Scientific, Sweden), 300 μl of methyl tert-butyl ether (Fisher Scientific) and vortexed. They were incubated on a shaking plate for 1 h, then, 240 μl of MilliQ water (Fisher Scientific) were added and samples were further incubated for 10 min. After a 10 min centrifugation (9000*g*), approx. 250 μl of the upper organic phase were transferred into a LC vial with a 300 μl insert (Waters, USA) and stored at −80 °C until processing. The samples were analyzed using an ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry method (UHPLC-Q-TOF-MS), which has been presented in detail previously (O'Gorman et al., 2017). Additional information is available in Section S2.

2.4. RNA-Sequencing and qPCR validation

Total RNAs from twelve F0 male brains (6 MIX, 6 Control), four F2 male brains (2 Control, 2 MIX –due to limited material available) and eight F2 female brains (4 Control, 4 MIX) were extracted using the RNeasy Plus Universal Mini Kit (Qiagen, France) following manufacturer procedure. Quality and quantity were evaluated by electrophoretic migration and spectrometry. 1 μg total RNA per sample was sequenced by the platform MGX. Library was prepared using a TrueSeq adapter kit. Validation of the library was performed on Fragment Analyzer (Standard Sensitivity NGS kit) and using qPCR (ROCHE Light Cycler 480, France). Total RNAs were sequenced on Illumina HiSeq 2500 generating 50-nucleotide single-end reads. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through the GEO Series accession number GSE146175 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146175).

Biological validation of RNA-Seq results was performed using quantitative Polymerase Chain Reaction (qPCR) on individuals that were produced independently from the ones used for RNA-Seq analyses (Section S3).

2.5. Statistics and bioinformatics

Lipidomic analysis: Concentrations in lipids were normalized to brain weight and data were log-transformed and mean-centered before statistical analysis. Data visualization was performed using Metaboanalyst® 4.0 (Chong et al., 2019). Lipids were also gathered into 14 biological groups as follow: all phospholipids (PL), triacylglycerols (TG), diacylglycerols (DG), monoacylglycerols (MG), sphingomyelines (SM), phosphatidic acids (PA), lysophosphatidylethanolamines (lysoPE), lysophosphatidylcholines (lysoPC), phosphatidylethanolamines (PE), phosphatidylcholines (PC), phosphatidylserines (PS), phosphatidylinositols (PI), cholesteryl esters (CE) and ceramides (Cer) (Table S2). Statistical analysis was performed in Metaboanalyst 4.0 using *t*-tests to identify differences between MIX and Control fish of the respective sex and generation. An adjusted p-value for multiple comparisons (padj) < 0.05 was considered as a statistically significant difference and a p-value < 0.05 was used to show trends. The results were displayed in a heatmap generated using displayr (www.displayr.com).

RNA-Sequencing analysis: Quality of the fastq files was checked using the FastQC software (v0.11.8) (Andrews, 2010). TrueSeq adapters were removed using Cutadapt (v2.1) (Martin, 2011). Clean reads were aligned to the last version of the zebrafish genome (Ensembl GRCz11, annotation GRCz11.97) with STAR (v2.7.0) (Dobin et al., 2013). The number of reads mapping to each gene were counted with HTSeq (v0.11.2) (Anders et al., 2015). Differential expression analysis between MIX and Controls of the respective sex and generation was performed using R (v3.6.1) and RStudio (v1.2.1335), package DESeq2 (v1.24.0) (Love et al., 2014). Genes showing a padj < 0.05 were considered as significantly differentially expressed genes. Identification of enriched biological pathways was performed against Gene Ontology (GO) and Reactome databases. Overall, the method followed the guidelines presented in Reimand et al. (2019). Gene Set Enrichment Analysis (GSEA, v4.0.3) was used against GO terms related to biological processes, molecular and cellular functions, and Reactome pathways, retrieved from g:profiler server (release 2019-07-31 and 2019-10-02 respectively). The ranking metric used in GSEA analyses was the Walt statistic given by DESeq2 (Esteve-Codina, 2018). Only gene sets containing 15–200 genes were selected to limit the results to the most informative pathways. Then, GO genes sets displaying a padj < 0.05 were used as an input in REVIGO (Supek et al., 2011) in order to remove redundant information due to the hierarchical organization of GO terms. The final list of enriched terms (including both up- and downregulated gene sets) was restricted to a similarity coefficient > 0.5.

3. Results

3.1. MIX affects the lipid content in the brain in a sex-specific fashion

In male F0 MIX, we observed a slight but significant decrease in PC (average fold-change 0.90) and SM (0.80), and a sharp reduction in TG (0.53) (Fig. 1). We further observed general trends to a lower content in PA (0.80) and DG (0.82) (p-value < 0.05, Fig. 1). No significant effect was observed in F2 males but a tendency for accumulation of TG (1.57) and Cer (1.23); and for depletion in MG (0.64) and PS (0.87) (p-value < 0.05, Fig. 1). There was no significant effect on lipid profiles in females whatever the generation (Fig. S3).

3.2. Mitochondria and neurotransmission related pathways are regulated in F0 and F2 male fish brain after exposure to the MIX

Since lipid metabolism was only significantly affected in male fish, transcriptomic analyses were performed in males. Differential expression analysis revealed strong regulation triggered by the MIX in F0 brains. In total, 1696 genes were significantly upregulated and 1588 were significantly downregulated (Fig. 2A). Rather limited changes were observed in F2 MIX male brains with a total of 41 significantly upregulated genes and 57 significantly downregulated genes, including 12 genes already regulated in F0 MIX brains (5 commonly downregulated, 1 commonly upregulated and 6 with opposite regulation) (Table S5).

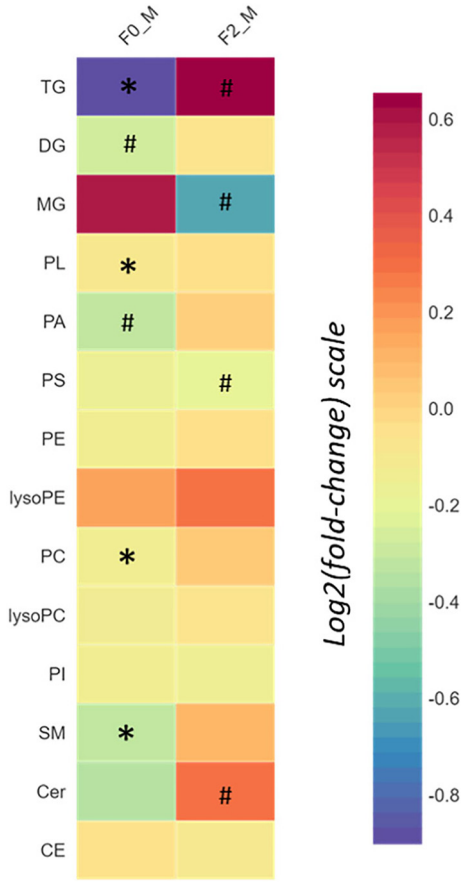
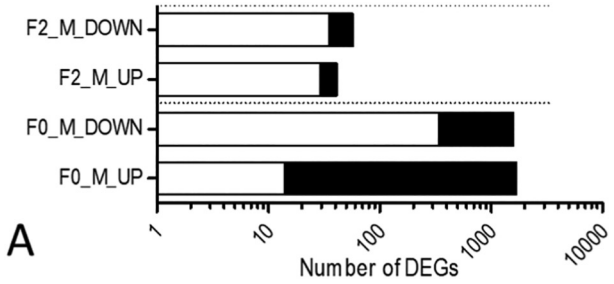


Fig. 1. Heatmap displaying the changes in lipid profiles in brain of fish exposed to MIX. Results are shown as log2(fold-change) (MIX/Control) for lipid classes (rows). Rows indicate lipid classes: triacylglycerols (TG), diacylglycerols (DG), monoacylglycerols (MG), all phospholipids (PL), phosphatidic acids (PA), phosphatidylserine (PS), phosphatidylethanolamines (PE), lysophosphatidylethanolamines (lysoPE), phosphatidylcholines (PC), lysophosphatidylcholines (lysoPC), phosphatidylinositols (PI), sphingomyelines (SM), Ceramides (Cer), and cholesteryl esters (CE). Columns indicate F0 (F0_M) and F2 males (F2_M). Symbols indicate statistical significance with *: padj < 0.05 and #: p-value < 0.05 ($n = 4$).



B

	GO_MF	GO_CC	GO_BP	REACTOME
F0_M	30	18	39	132
F2_M	20	14	40	33

Fig. 2. A. Distribution of differentially expressed genes (DEGs) between upregulation (UP) and downregulation (DOWN) in F0 males (F0_M) and F2 males (F2_M) (MIX/Control). The graphic displays the total number of DEGs as well as the proportion of transcripts showing an absolute fold change > 2 (abs(FC) > 2). B. Distribution of significantly enriched GO (Gene Ontology) terms in the different datasets, between molecular function (MF), cellular component (CC) and biological process (BP), and Reactome gene sets.

Analysis of GO-Reactome enrichment results highlighted 219 and 107 significantly enriched terms in F0 and F2 MIX male brains, respectively (Fig. 2B). In the F0 generation, the most upregulated gene sets were related to translation and mitochondrial respiratory chain (Fig. 3A). Terms related to additional energetic pathways were also significantly enriched such as gluconeogenesis and interleukin-1 signaling (Table S6). In contrast, terms related to cell signaling and more specifically synaptic transmission (GABAergic) were downregulated (Fig. 3B). In F2 males, we observed opposite patterns, with the upregulation of terms related to cell signaling and the synapse (dopaminergic, glutamatergic); and the downregulation of translational processes (Fig. 3A, B). A list of all significantly regulated gene sets that relate to neurotransmission is available in Table S7.

A list of 30 gene sets was regulated in both F0 and F2 MIX males including 17 for RNA regulation and translation, 6 for mitochondrial integrity and function, 4 for proteasomal activity and 1 for synaptic structure (Table S8). However, almost all were downregulated in F2 while they were upregulated in F0 (Fig. 4). Supplementary analysis of the transcriptome of F2 MIX females confirmed that a significant amount of the changes appeared “inherited” from MIX fish of the F0 generation to the offspring (Table S9).

Persistent alteration of epigenetic mechanisms may be involved in the inheritance of the pathways that were simultaneously affected in F0 and F2 generations. We reported that expression of epigenetic factors was significantly regulated by the MIX in both generations. Three REACTOME gene sets were significantly enriched in F0 MIX, with 14 epigenetic factors among the DEG list (Fig. 5). In F2 MIX males, and despite that no epigenetic factor-encoding gene was part of the DEG list, GSEA analysis revealed that 4 gene sets related to epigenetic mechanisms were significantly enriched (Fig. 5).

Biological validation of the results using qPCR showed an overall fit of 54% between fold-changes from RNA-Seq and qPCR (Pearson correlation). Especially, there was a 76% correlation in the F0 generation and 52% in the F2 generation (Table S4). Overall, genes that were disagreeing between qPCR and RNA-Seq analyses are part of translation process or pathways that are less relevant to the major findings of this study. Genes that were directly involved in lipid metabolism and mitochondrial function (*acsl4a*, *ifi45*), behavior and neurotransmission (*fosaa*, *scn1lab*), and epigenetics (*dnmt3ba*, *kdm6bb*) were in agreement between both methods.

4. Discussion

In this study, we described the effects of chronic exposure to an environmentally relevant mixture of PCBs and PBDEs on brain lipid profile and transcriptome of exposed F0 zebrafish and their unexposed offspring. Previous results indicated physiological effects of the MIX diet on energetic metabolism, with F0 fish displaying reallocation of energy to promote somatic maintenance to the expense of reproduction (Horri et al., 2018; Horri, 2018). However, underlying mechanisms were unknown. The present results show mobilization of lipid storage (TG) in male brain and hyper-activation of several pathways related to energy, suggesting a need for higher energy production compared to control fish. Lipidomic analyses revealed that direct exposure to the MIX induced several significant changes in the brain lipid content and males appeared more strongly affected than females. Interestingly, endocrine disruptors, such as PCBs, are known to sex-differentially interfere with lipid metabolism (Lyssimachou et al., 2015; Li et al., 2019). These differences may be exacerbated in males due to differences in the endocrine system between sexes (Li et al., 2019; Levin et al., 2010; Suzuki et al., 2006). Another explanation may be the differences in bioenergetics or feeding behavior inherent to males and females (Collison et al., 2012; Nielsen et al., 2019; Rennie et al., 2008) that were, however, not reflected in sex-specific contaminant body burden (Horri et al., 2018). Metabolism of TGs in the brain relies both on exchanges with systemic circulation as well as endogenous lipolysis. It acts as a sensor to adjust systemic energy homeostasis (Bruce et al., 2017). Thus, the present results suggest an overall reduction in lipid storage in the F0 generation. At the transcriptome level, we observed upregulation of a large number of pathways related to mitochondrial respiration, as well as energy-related routes such as gluconeogenesis and interleukine-1 signaling, all of which associated with TG mobilization (Holly et al., 2006; Scherer et al., 2016; Matsuki et al., 2003; Chen et al., 2017; Espinosa Ruiz et al., 2019; Leijs et al., 2017). This is in agreement with another study, which highlighted that PCB exposure increased the mobilization of TGs as a consequence of higher energetic demands (Madureira et al., 1993). Thus, one hypothesis is that the MIX increases the energy cost for basic maintenance and growth, which will further lead to a higher energetic allocation toward this compartment (Horri, 2018). This may explain the observed slower growth rate in MIX fish (Horri et al., 2018).

The initial motivation for investigations on the brain was to draw links between molecular changes and persistent behavioral effects (Alfonso et al., 2019). These chemicals can be transported directly to the brain (Mitchell et al., 2012) and acute exposure induces neurotoxicity *via* disruption of calcium homeostasis and induction of oxidative stress (Westerink, 2014). In the present study, we also observed effects of the MIX on voltage-gated calcium channels and oxidative stress, and both lipid and transcriptome changes further suggested an impact of the MIX on neuron integrity and function *via* additional mechanisms. Phosphatidylcholine content is associated with proper cognitive function (Troen et al., 2008; Chung et al., 1995; Sabogal-Guáqueta et al., 2018). Sphingomyelins are key lipids for electrical insulation of axons as well as for axon extension and neuronal survival (Avila et al., 2007; Olsen and Færgeman, 2017; Svennerholm et al., 1994). In addition, the results indicated a slight decrease in PA content, known to regulate neurite and dendrite outgrowth *via* the modulation of the activity of Rho GTPase and serine/threonine kinase enzymes that are crucial to actin cytoskeleton reorganization (reviewed in Ammar et al., 2014). Regulation of sphingolipid and PA metabolism were further supported by pathway enrichment resulting from transcriptomic data. However, significant enrichment in phosphatidylinositol signaling pathways was not associated with any significant change in PI content. Despite these molecular

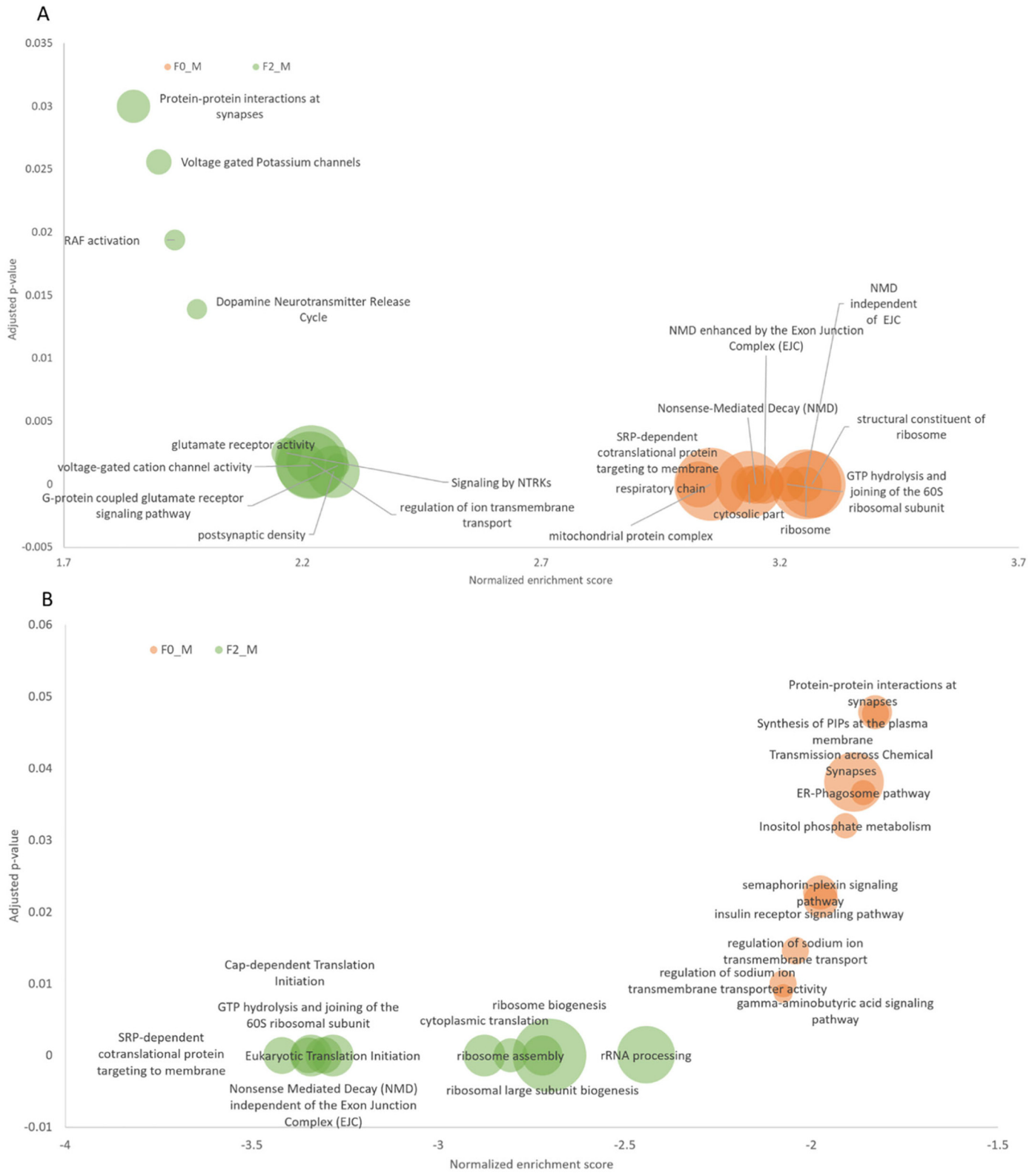


Fig. 3. Visualization of the 5 most significantly enriched GO Biological Processes and the 5 most significantly enriched REACTOME gene sets according to GSEA and REVIGO results. A. Upregulated sets in brains of MIX male F0 (F0_M) and F2 (F2_M) B. Downregulated sets in brains of MIX male F0 and F2 The size of the bubbles is related to the number of genes included in the gene set.

alterations, no disrupted behavioral phenotype was observed in F0 MIX fish (Alfonso et al., 2019). In addition to the results presented by Alfonso et al. (2019), we observed no impact of the MIX on daily activity patterns and personality traits (unpublished data). It may be that the molecular changes described above affect cognitive endpoints such as memory and learning, as previously observed with PCBs (Schantz et al., 2001; Pessah et al., 2019) but which were not investigated in the present study. This hypothesis is supported by previous research

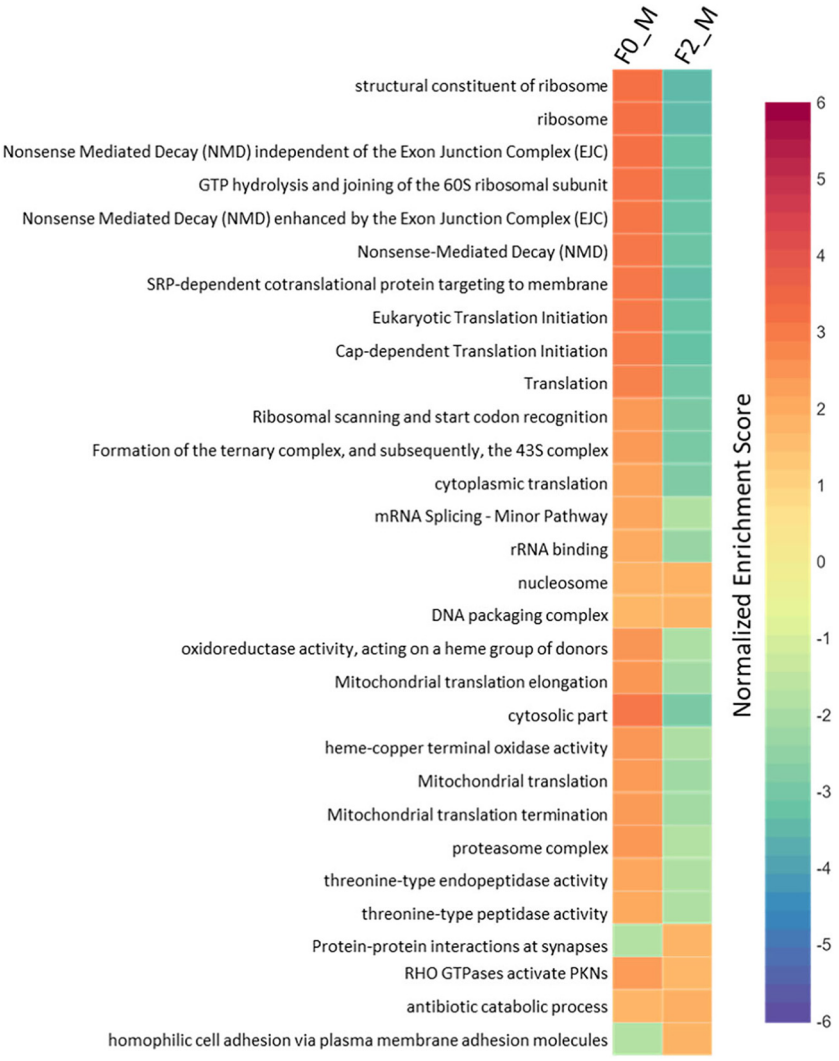


Fig. 4. Heatmap displaying normalized enrichment score (MIX/Control) of GO and REACTOME gene sets (rows) that were significantly enriched in both F0 and F2 generations of males (columns).

showing association between cognitive defects, energetic metabolism and mitochondrial function (Wong and Giulivi, 2016; Ferrer, 2009). Finally, one can also hypothesize that molecular changes anticipate behavioral defects, which would be observed in older stages as the brain gets more sensitive to injury with age.

Besides behavior, neurological circuits are in control of reproduction *via* the hypothalamo-pituitary-gonadal (HPG) axis, and reproductive success was reduced in MIX fish (Horri et al., 2018; Horri, 2018). Interestingly, inhibition of GABAergic signaling, as observed in F0 fish, leads to a decrease in activation of the HPG axis (Song et al., 2017). In addition, F0 MIX fish showed a decrease in circulating sex hormones (17β-estradiol and 11-ketotestosterone), as reported previously with semicarbazide which alters the GABAergic system (Horri, 2018; Yu et al., 2017). Therefore, this could represent a mechanism for the observed decrease in spawning success following MIX exposure. However, this would require further investigation since the whole brain was used in the present study, which does not allow direct conclusion on effects on hypothalamic neurons.

In a previous article, we reported no effect on reproduction or growth in the F2 generation; however, MIX fish were more anxious compared to controls (Alfonso et al., 2019). Therefore, it was not surprising to observe different molecular changes in both generations. The low number of F2 males due to technical issues limited comparisons between F0 and F2 generations because of restricted statistical power. However, glutamatergic signaling and dopamine release were strongly stimulated in the F2 generation, which was not observed in the F0 generation and could explain the behavioral phenotype (Alfonso et al., 2019). In addition, transcriptomic analyses were performed in F2 females, which displayed similar behavioral alterations than males, and they confirmed the significant stimulation of glutamatergic signaling in this generation. Both glutamate and dopamine were shown to control anxiety *via* the corticotropin-releasing hormone receptor (CRHR1) (Refojo et al., 2011). It was also shown that the balance between glutamate and GABA in the brain is critical to the development of anxiety disorders (Wierońska et al., 2011), overall stressing the role of glutamate signaling in the observed neurobehavioral effects.

While the effects observed in F0 relate to direct exposure, the effects in the F2 generation reflect an indirect effect on the nervous system conveyed by epigenetic mechanisms and/or disruption in germ cells in F1 embryo resulting from maternal transfer of MIX to the eggs. Multi- and transgenerational effects are of high concern for long-term health of human and wildlife, and have been associated with epigenetic changes such as DNA methylation and histone modifications (Constantinof et al., 2016; Skinner et al., 2010; Xavier et al., 2019; Youngson and Whitelaw, 2008). Environmental chemicals can interact with epigenetic mechanisms and, thus, induce transgenerational effects (Baccarelli and Bollati, 2009; Aluru, 2017; Jacobs et al., 2017). These

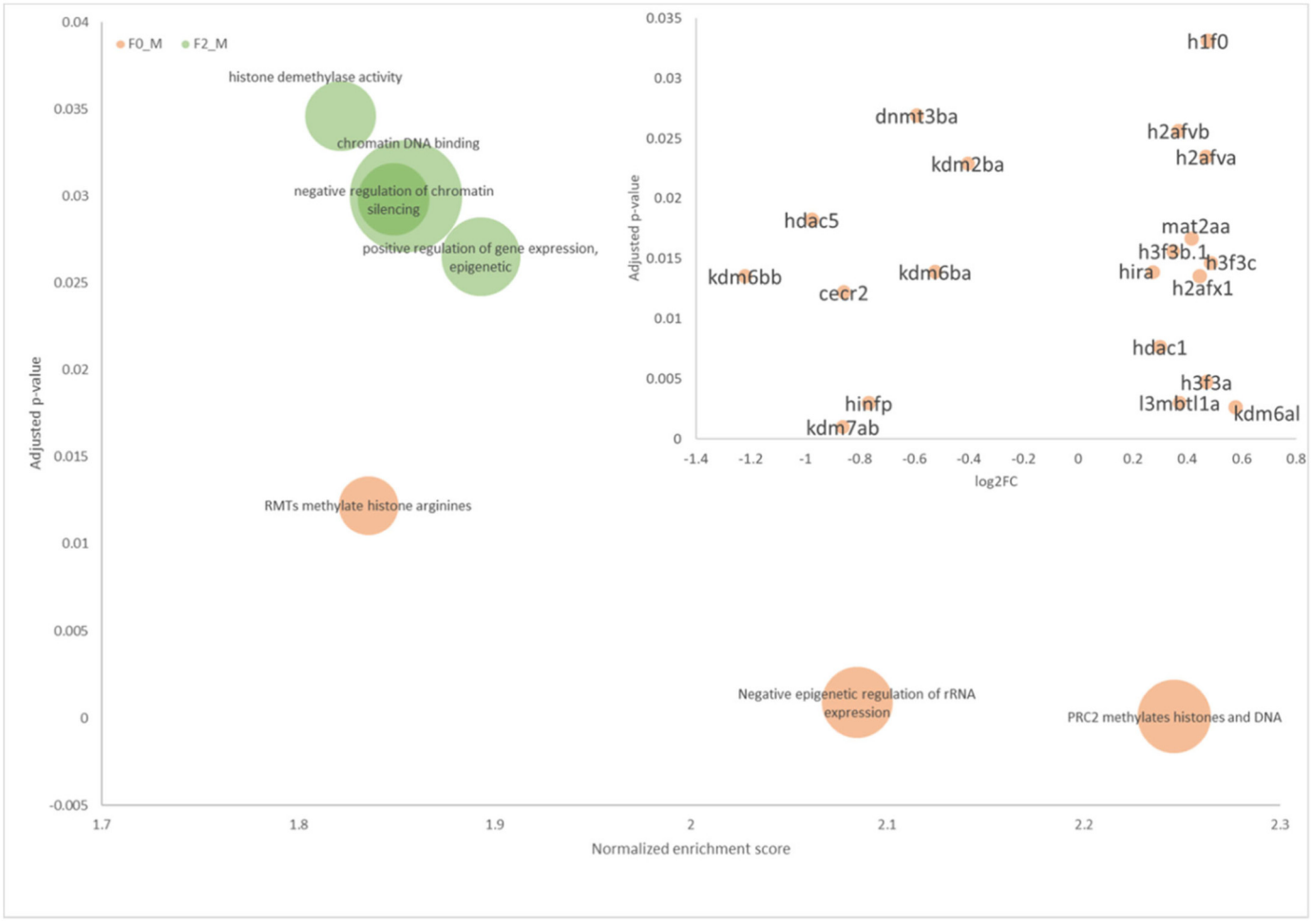


Fig. 5. Visualization of significantly enriched gene sets related to epigenetic mechanisms in MIX male F0 (F0_M) and F2 (F2_M), according to GSEA and REVIGO results. The size of the bubbles is based on the number of genes included in the gene set. Top right: significantly enriched genes part of the epigenetic machinery in male F0 exposed to MIX.

changes will directly impact gene expression in offspring generations and may further have physiological adverse consequences. Modes of action of neurotoxic chemicals encompass a wide range of molecular mechanisms (reviewed in Legradi et al., 2018). In addition, there is some evidence that epigenetic disruption is linked to neurodegeneration or neurobehavioral changes (Legradi et al., 2018). Here, we reported that behavior of MIX larvae and expression of the DNA methyltransferase *dnmt3ba* were affected until the F4 generation, showing the potential of the MIX to induce transgenerational effects (Alfonso et al., 2019). In the present study, epigenetic mechanisms were significantly regulated in F0 and F2 adult brains, although no direct investigation on epigenetic marks was done. Especially, *dnmt3ba* expression was significantly downregulated in F0 adult brains, as observed in F1 larvae (Alfonso et al., 2019). It is noteworthy that pathways which expression was modified in both F0 and F2 generations displayed opposite regulations, and we observed the same pattern for larvae behavior and *dnmt3ba* expression (Alfonso et al., 2019). This suggests the implication of more complex effects than direct epigenetic inheritance, which may be explained by the exposure scenario. In fact, we cannot exclude that the observed effects aren't completely due to stable epigenetic changes as germ cells giving rise to the F2 generation may have been shortly exposed to the MIX. However, besides inducing "adverse epigenetic changes", environmental cues may indirectly trigger epigenetic modifications in the F0 germ line as an adaptive mechanism for their offspring to better cope with the continuous presence of the stressor. Therefore, the inherited changes would lead to opposite effects in the offspring upon stressor removal -whether these effects persist in further generations remains to be known (Radford et al., 2014; Sales et al., 2017). Especially, additional research is needed on whether epigenetic marks are affected in F0, transferred to F1 and F2 and whether they can functionally relate to transcriptomic changes.

In conclusion, multi- and transgenerational effects of environmentally-relevant chemical mixtures such as the MIX on neurotransmission and mitochondria related pathways are important to highlight because they may trigger ecological consequences for natural fish population and ecosystems (Legradi et al., 2018; Mills and Chichester, 2005; Forbes and Depledge, 1992). However, based on the results, functional experiments are needed to relate specific changes in gene expression to the observed outcomes, and would help confirming some of the results of this study that suffered from technical limitations. Particularly, limited agreement between RNA-Seq and qPCR results in the F2 generation may be a direct consequence of a lower number of replicates used for RNA-Sequencing. However, it could also be due to heightened biological variability in response to multi and transgenerational effects compared to direct exposures. Besides, this study would benefit from analysis of transcriptomic changes in F0 females; and investigations in other organs than the brain, *e.g.* the liver, may also provide with further insight especially on energetic metabolism. In addition, further research is warranted on the role of the epigenetic component in POP-induced transgenerational effects, in which context adversity *vs* adaptability appears as an exciting research perspective.

CRediT authorship contribution statement

MLB and XC designed the project. MLB, XC, SK and MB planned the experiments. SA, CB, XC and MB performed the experiments. TH and MB analyzed the data. MB wrote the manuscript with input and comments from SA, MLB, TH, SK and XC.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supporting Information on chemical analysis, lipidomic methods, RNASeq QC statistics, qPCR validation, differential gene expression analysis and pathway enrichment (word file).

Lists of individual lipids concentrations, differentially expressed genes, and significantly enriched gene sets (Excel file) Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.142097.

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