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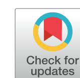
The insecticide permethrin induces transgenerational behavioral changes linked to transcriptomic and epigenetic alterations in zebrafish (*Danio rerio*)



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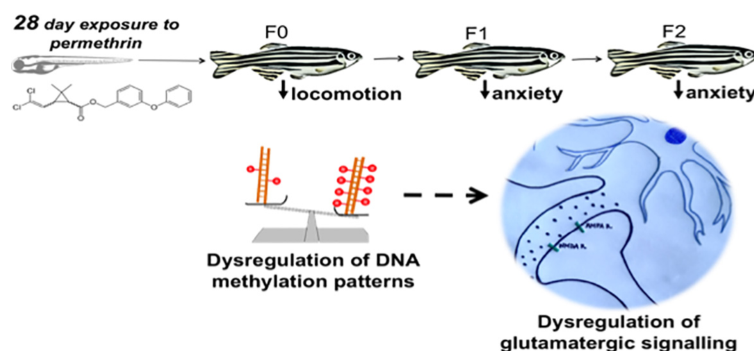
HIGHLIGHTS

- Permethrin impairs behavior of adult zebrafish (F0) following early life exposure.
- F1 and F2 male offspring show a decrease in anxiety-like behavior.
- Transcriptome data show transgenerational glutamatergic signaling (GS) disruption.
- DNA methylation analyses also indicate persistent dysregulation of GS.
- Epigenetic dysregulation of GS may cause transgenerational behavioral alterations.

GRAPHICAL ABSTRACT



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ABSTRACT

The pyrethroid insecticide permethrin is widely used for agricultural and domestic purposes. Previous data indicated that it acts as a developmental neurotoxicant and can induce transgenerational effects in non-target organisms. However, associated underlying mechanisms remain unclear. The aim of this study was to investigate permethrin-related transgenerational effects in the zebrafish model, and to identify possible molecular mechanisms underlying inheritance. Zebrafish (F0) were exposed to permethrin during early-life (2 h post-fertilization up to 28 days). The F1 and F2 offspring generations were obtained by pairing exposed F0 males and females, and were bred unexposed. Locomotor and anxiety behavior were investigated, together with transcriptomic and epigenomic (DNA methylation) changes in brains. Permethrin exposed F0 fish were hypoactive at adulthood, while males from the F1 and F2 generations showed a specific decrease in anxiety-like behavior. In F0, transcriptomic data showed enrichment in pathways related to glutamatergic synapse activity, which may partly underlie the behavioral effects. In F1 and F2 males, dysregulation of similar pathways was observed, including a subset of differentially methylated regions that were inherited from the F0 to the F2 generation and indicated stable dysregulation of glutamatergic signaling. Altogether, the present results provide novel evidence on the transgenerational neurotoxic effects of permethrin, as

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well as mechanistic insight: a transient exposure induces persistent transcriptional and DNA methylation changes that may translate into transgenerational alteration of glutamatergic signaling and, thus, into behavioral alterations.

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1. Introduction

Permethrin is one of the most common domestic and agricultural pyrethroid insecticides worldwide (DeMars et al., 2021; Banks et al., 2014). It is considered a safer alternative compared to many other pesticides since permethrin does not accumulate in the environment and is quickly metabolized and excreted by organisms (Holmstead et al., 1978; Imgrund, 2003; Zhu et al., 2020). Nonetheless, a recent study showed that photodegradation products of permethrin can potentially be more harmful to aquatic organisms than the parent compound (Zhu et al., 2020). Due to increasing usage, pyrethroids are detected in human fluids in the low μg/L range (Barr et al., 2010; Saillenfait et al., 2015), and in aquatic compartments in the ng/L to high μg/L range (Hladik and Kuivila, 2009; Shahsavari et al., 2012; Budd et al., 2020). Exposure to permethrin can elicit a variety of adverse effects in various species, including neurotoxic effects and behavioral deficits (Carloni et al., 2012; DeMicco et al., 2010; Fedeli et al., 2017; Nunes et al., 2019; Saito et al., 2019; Yang et al., 2014; Furlong et al., 2017; Shelton et al., 2014), as well as oxidative stress (Carloni et al., 2012; Dhivya Vadhana et al., 2013; Nunes et al., 2019). Permethrin has also been shown to act as an endocrine disrupter in fish (Tu et al., 2016; Zhang et al., 2017).

In a previous study, we showed that early-life exposure of zebrafish (F0) to permethrin induces transgenerational disruption of larvae behavior in the F1 and F2 generations (Blanc et al., 2020). Permethrin is not known to be mutagenic (Pluijmen et al., 1984; Pednekar et al., 1987), thus, transgenerational effects are more likely to be a consequence of chemically-induced, inherited changes in epigenetic patterns (Nilsson et al., 2019). Epigenetic mechanisms are mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence, and which can further lead to alterations of cell function and impair the physiology of an organism (Holliday, 2014). During fetal development and early life, exposure to epigenetic modifiers is of concern due to high epigenetic plasticity that can affect normal and pathological development later on (Baccarelli and Bollati, 2009; Guerrero-Preston et al., 2010; Stel and Legler, 2015; Vaiserman, 2014). Additionally, cancer, neurological disorders, and several other conditions have been associated with changes in the epigenetic landscape and were linked to chemical exposure (Grandjean, 2013; Prusinski et al., 2016; Zoghbi and Beaudet, 2016). Some of these epigenetic changes may be inherited, and thereby induce adversities in the unexposed offspring, a phenomenon known as transgenerational epigenetic inheritance (Liberman et al., 2019; Skinner et al., 2010; Skinner, 2008). For now, most evidence is based on persistent DNA methylation changes in germ cells of exposed individuals, that can escape the epigenetic reprogramming events occurring at fertilization and sex differentiation. They are further transferred to somatic and germ cells of the developing offspring, and remain «imprinted» in the epigenome over generations (Liberman et al., 2019; Skinner, 2011). However, there are studies showing that other epigenetic marks, such as histone post-translational modifications and non-coding RNA regulation, can also be inherited and likely work in concert to support inheritance phenomena (Blake and Watson, 2016).

A recent work found significant associations between pyrethroid exposure and DNA methylation changes in human blood (Furlong et al., 2020). In addition, previous studies have reported that permethrin affects expression of DNA methyltransferases, the enzymes in charge of DNA methylation, in the striatum of exposed rats (Fedeli et al., 2017) and in zebrafish embryos, along with other epigenetic factors (Blanc et al., 2019). Furthermore, global DNA methylation changes have been observed in the F1 offspring upon permethrin exposure (Bordoni et al., 2015). Finally, specific changes in DNA methylation patterns in sperm were linked to reproductive abnormalities in the F3 offspring of rats exposed to a mixture of permethrin and the insect repellent N, N-diethyl-meta-toluamide (DEET) (Manikkam et al., 2012a).

Zebrafish is a prominent model organism for ecotoxicological and biomedical studies and is used for various routine testing for the risk assessment of chemicals and complex environmental samples (Scholz et al., 2008). Zebrafish have genetic similarities to mammals and epigenetic pathways are also mostly conserved with some mechanistic differences such as the lack of parental imprinting (Kamstra et al., 2015). Besides, zebrafish are used for several established behavioral tests to assess different endpoints. For example, the novel tank allows to evaluate the activity and exploratory behavior of the fish, which may reflect alterations in anxiety (Egan et al., 2009; Sackerman et al., 2010). Due to their short life cycle, zebrafish is a model for transgenerational inheritance research (Bugel et al., 2014; Kamstra et al., 2017), and previous data showed that transgenerational effects observed in the zebrafish were similar to that observed in mammals (Baker et al., 2014a).

So far, no link between behavior, genetic, and potential epigenetic changes has been investigated following permethrin exposure. There is also no information available on whether these changes may persist in offspring generations and lead to transgenerational effects. In this study, we combined investigations at three different levels of biological organization in F0, F1 and F2 generations of zebrafish. To this end, we investigated in the same fish (1) the presence of a transgenerational behavioral phenotype in adults, (2) whole-brain transcriptomic changes and (3) differentially methylated regions (DMRs) that may be implicated in the heritable phenotype.

2. Material and methods

2.1. Fish exposure and sampling

This work has received approval for research ethics from the Swedish Board of Agriculture, Jönköping, Sweden (#5.2.18-861/15), and a certificate of approval is available upon request. The exposure of the F0 generation and breeding F0, F1, and F2 fish were performed as described previously (Blanc et al., 2020) and additional information is available in supplementary data (Text S1). Briefly, 2-hour-post fertilization (hpf) AB zebrafish embryos (ZFIN ID: ZDB-GENO-960809-7) were transferred to exposure solutions containing 1 μg/L (permethrin low; PL), 10 μg/L (permethrin high; PH) permethrin (Sigma-Aldrich, PESTANAL analytical standard, purity >90% *cis* + *trans* isomers), or dimethylsulfoxide 0.01% (DMSO, Sigma-Aldrich) as solvent control (SC), and kept exposed in a flow-through system until 28 days post-fertilization. 1 μg/L is in the range of the highest permethrin concentrations found in surface water as it tends to bind to sediments where concentrations up to 20 μg/kg can be found (NORMAN EMPODAT Database, 2020). We have further demonstrated that a 28 day exposure to both 1 and 10 μg/L did not induce any observable defect on growth or survival (Blanc et al., 2020). Chemical analysis was performed on water samples at day 0, 4, 14, 21 and 28. The results, presented in (Blanc et al., 2020), excluded cross-contamination of the solvent controls and confirmed exposure, although measured concentrations were approx. 10 times lower than the nominal ones (Blanc et al., 2020).

At 28 days post-fertilization, juveniles were transferred into clean water and bred according to standard protocols (Westerfield, 2007). The F1 and F2 generations were produced by mating exposed F0 males and females and were maintained under standard (unexposed) conditions. When they reached 4 months of age, fish were euthanized in saturated ethyl 4-aminobenzoate solution (500 mg/L, Sigma-Aldrich) and individual brains ($n = 4$ per treatment and per sex) were dissected and snap-frozen in liquid nitrogen, then kept at −80 °C until processing.

2.2. Novel tank diving test

For F0 to F2 generations, locomotor and exploratory behavior in response to a novel environment was investigated in 8–14 fish per sex and condition. Fish were tested at the age of 9 months pf (mpf; F0), 7 mpf (F1) and 8 mpf (F2). The novel tank diving test is commonly used to identify any impact on activity and anxiety level of fish via analysis of travelled distance and vertical positioning (Levin et al., 2007). Fish were transferred to the recording room the day before testing. They were kept in individual 3 L tanks containing system water, in visual contact with each other. Testing was performed between 1 and 5 p.m. Each individual was transferred to a 1.5 L trapezoid tank and vertical positioning was immediately recorded (Basler® acA1300 camera) for 4 min. The test duration was reduced to take into account the absence of prior incubation and the quicker relief of anxiety compared to what was initially described (Levin et al., 2007). Water was exchanged between each run to avoid inter-individual interferences. All behavioral data were automatically analyzed using Ethovision XT 13.0 (Noldus). The tank was divided into 3 virtual zones: top, middle and bottom (Sackerman et al., 2010). A list of measured exploratory variables is presented in Table S1. Statistical analyses were performed using Principal Component Analysis (PCA) (package factoextra, R v3.6.1) on sex-specific datasets (each including 10 metrics, 3 generations, 3 treatments) as preliminary analyses revealed that the sex was a significantly contributing factor (Text S2). One-way Analysis of Variance followed by Bonferroni's Multiple Comparison test was applied on major principal components (PC) as well as on a selection of individual variables related to exploratory behavior (time spent in top third zone; distance travelled in top third zone; average entry duration) and defensive innate behavior (freezing as the frequency of events at a speed <1% of the running average; angular velocity as a mean to represent erratic movements via sharp changes in direction). Data were checked for the presence of significant outliers (Grubbs' test, $p \leq 0.05$). A p-value (p) < 0.05 was considered as statistically significant.

2.3. RNA-sequencing and qPCR validation

Total DNA and RNA from four brains per treatment (PH or SC), generation, and sex were consecutively extracted for Reduced Representative Bisulfite Sequencing (RRBS) and RNA-Sequencing (RNA-Seq), respectively, using the Triprep extraction kit (Macherey-Nagel) according to the manufacturer's instructions. Quality and quantity were spectrophotometrically evaluated using a Biodrop µLITE (BioDrop, UK). One µg total RNA per sample was sent to BGI Europe for RNA sequencing. Library preparation and sequencing were handled by BGI Europe on BGISeq-500 as paired-end 150 bp reads.

We performed technical validation of the RNA-Seq results using qPCR. Within each generation, a set of genes with different biological functions was selected. Both strongly (absolute fold-change ≥2) and moderately regulated (absolute fold-change <2) genes in RNA-Seq analyses were included. Comparisons were performed within generation/sex specific sub-datasets (see Table S3 for full results). Primers (Eurofins Genomics, Germany) were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table S2). cDNA was synthetized from 400 to 500 ng of RNA template and each qPCR reaction included 2× SYBR® FAST MasterMix (KAPA Biosystems), 200 nM of each primer, 2 µL of 1/10 diluted cDNA completed to 12 µL with MilliQ water. Potential gDNA contamination was controlled with purified RNA (no reverse transcriptase control) and each reaction was run in technical duplicates. Results were normalized to the expression of *rpl13a* and *b2m* as they showed the highest stability among 4 investigated genes (i.e. *eef1a1*, *b2m*, *actb1* and *rpl13a*) (data not shown). Fold-changes were calculated using the Pfaffl method (Pfaffl, 2001). The results from the validation are available in Table S3 and revealed an overall correlation of 86% between fold-changes from both techniques (Pearson correlation).

2.4. Reduced Representative Bisulfite Sequencing (RRBS)

RRBS experiments were performed at the Environmental Epigenetics facility of the IHPE research unit, to identify differentially methylated cytosines and differentially methylated regions (DMRs) on DNA (Meissner et al., 2005). 100 ng of purified DNA was digested with *MspI* and libraries were prepared for each sample using the Diagenode RRBS kit Cat No C02030033 (Diagenode, Belgium), according to the manufacturer's protocol. DNA-free water served as negative control. The 48 samples were blindly divided into eight pools to create the libraries as described in the Diagenode protocol. qPCR was used to determine the optimal number of amplification cycles. Relative fluorescence was plotted versus cycle number and the cycle number that corresponds to roughly one-third of the maximum fluorescent intensity was used for PCR amplification (19 cycles for all pools of libraries). Five µl of the PCR products were used to check quality and quantity of libraries by electrophoresis on a 1.2% agarose gel and Midori green direct staining. Purification was done on an IP-Star system (Diagenode) with Ampure XP beads (Beckman Coulter, USA). Libraries were resuspended in 20 µL of elution buffer, and size distribution and concentration were profiled with an Agilent Bioanalyzer High Sensitivity DNA Assay. Libraries were sequenced on two NextSeq550 High Output flow cells (Illumina, USA) as paired-end or single-end 150 bp reads. Randomization was applied to dispatch samples from different generations/sex/treatments on each flow cell.

2.5. Bioinformatic analyses

2.5.1. RNA-sequencing data analysis

Quality of the fastq files was checked using the FastQC software (v0.11.8) (Andrews, 2010) and reads were mapped to the last version of the zebrafish genome (Ensembl GRCz11, annotation GRCz11.97) with StAR (v2.7.0) (Dobin et al., 2013). QC statistics are available in Excel Table S1. Read count was performed with HTSeq (v0.11.2) (Anders et al., 2015) and differential expression analysis with DESeq2 (v1.24.0) (Love et al., 2014). A gene showing an adjusted p-value for multiple comparisons (padj) ≤ 0.05 (Benjamini-Hochberg correction) was considered as a significantly differentially expressed gene (DEG). Gene Set Enrichment Analysis (GSEA v4.0.3) was used for functional enrichment against Gene Ontology (GO, 2019-07-31) and REACTOME (2019-10-02) databases (Reimand et al., 2019; Subramanian et al., 2005). The ranking metric used in GSEA analyses was the Walt statistic given by DESeq2 (Esteve-Codina, 2018). At first, a gene set showing a padj≤0.05 and abs (normalized enrichment score) (NES) > 1.5 was considered as significantly enriched. The statistical threshold was further relaxed to a padj≤0.25 to include borderline yet biologically relevant findings to the analysis, as advised by GSEA documentation.

2.5.2. RRBS data analysis

All analyses were done on the Galaxy instance of the UMR 5244 - “Interactions Hôtes-Pathogènes-Environnements” laboratory http://bioinfo.univ-perp.fr (Afgan et al., 2018). Quality of the sequencing data was evaluated using FastQC (v0.71), and one replicate from F0 males was discarded due to poor sequencing quality. Adaptors were

automatically trimmed using TrimGalore! (v0.4.3.1) (Krueger, 2012) and reads were aligned with Bismark (v0.22.1) (Krueger and Andrews, 2011) to the GRCz11 version of the zebrafish genome, with the adjusted parameter "L,0,-0.6" for optimal alignment (Kamstra et al., 2017). Methylation calling was performed with Bismark extractor. QC statistics are available in the Excel Table S8. Data were normalized to median read count and cytosine positions showing a coverage below 5 and above the 99.9 percentile were discarded. At this stage, we observed a batch effect between the two flow cells that were used in the experiment (Fig. S1A). The built-in methylKit batch correction (R package v1.12) (Akalin et al., 2012) is based on a principal component association of available metadata. This type of batch correction has limited application, particularly with high dimensional datasets, as it focuses on the removal of a whole principal component that may contain more than just variation associated to batch. For this reason, the authors provided ways of extracting and reimporting batch-corrected values into their framework. We tested the built-in version and found that overall batch specific differences were still observed (Fig. S1B). Currently, no fully appropriate batch correction methodology has been published for methylation data. For this reason, we extracted the percentage of methylation for each identified position and used an Empirical Bayes Framework previously developed to correct for batch effects in continuous datasets, ComBat (Johnson et al., 2006). We provided the methodology with the batch information as well as the model matrix relating to sex, treatment, and generation of the fish to remove as much technical information as possible while preserving biological information (Fig. S1C). The resulting matrix was rescaled to 0–100 to be integrated back into a methylKit object. To test whether the correction removed any biological information, we compared the results from a within batch differentially methylated position analysis. This showed that 100% of the regions identified after correction were also identified in the dataset before correction and that there was a > 97% correlation between differential methylation values from both datasets (Fig. S2), showing that the batch correction was successful and did not create false-positive DMRs. However, the normalization step reduced the number of significant DMRs by a factor of approximately 5, potentially leading to underestimation of the amount of methylation changes. The output was used in methylkit to perform differential analyses on artificially segmented 300 bp tiles (Kamstra et al., 2017). DMRs were defined as 300 bp tiles including at least 4 cytosines showing a 5× coverage. An initial cut-off of 10% differential methylation and padj≤0.05 was used for statistical significance, which was further relaxed to a padj≤0.25 as for RNA-Seq data in order to discuss biologically meaningful findings. Significant DMRs were visualized in the Integrative Genome Viewer (IGV v. 2.8.0) (Robinson et al., 2011) to compare methylation levels of specific cytosine positions between the different generations.

DMRs were associated to the closest transcriptional start site using the genomation package with default parameters (v. 1.18.0) (Akalin et al., 2015). Functional enrichment was performed using gprofiler2 (v.0.1.9) (Reimand et al., 2018) against the Gene Ontology and Reactome databases. A custom background containing associated genes from the full list of 300 bp tiles was used. A gene set was considered significantly enriched at a padj≤0.05.

Both RNA-Seq and RRBS data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE154020 (*reviewer access token czwvcykovjajxkj*) and GSE154206 (*reviewer access token sxuhiycgbryxhkv*), respectively.

3. Results

3.1. Behavioral effects

Behavioral analyses were performed in permethrin and control fish from all generations using the novel tank diving test. PCA analysis yielded one major component (PC1) representing most of the explanatory variables (i.e., global locomotor activity) and explaining 46% and 53% of the total variability in behavior in males and females, respectively (Table S4). Positive scores were assigned to high exploratory and locomotor activity. In F0 males exposed to 10 μg/L (PH), we observed a significant reduction in PC1, i.e. a lower locomotor activity compared to control fish ($p = 0.024$, Fig. 1A). This was not seen in F0 males from the lower exposure dose (PL). Although not significant, F0 females showed the same tendency ($p = 0.070$, Fig. S3). No effect on global locomotor activity was observed in the F1 and F2 generations (Fig. 1A, S3).

In males, but not in females, PC2 specifically described anxiety-like behavior (distance travelled, and time spent in top third area) and explained 19% of the total variability in behavior (Table S4). Positive PC2 scores were assigned to reduced anxiety. Significant reduction in anxiety-like behavior was shown in PH F2 males ($p = 0.030$) and the same tendency was observed in PH F1 males ($p = 0.094$; Fig. 1B). PH F1 males specifically displayed a significant increase in the total distance travelled in Top area ($p = 0.035$; Fig. 2A) and an increase in the time spent in this zone (p = 0.070; Fig. 2B). Same trends were observed for PL F1 males ($p = 0.089$ for distance in Top and $p = 0.082$ for time in Top). PH F2 males showed a significant increase in the time spent in Top area ($p = 0.048$) but no change in the distance travelled in this zone compared to controls ($p = 0.293$; Fig. 2B). No effect was observed

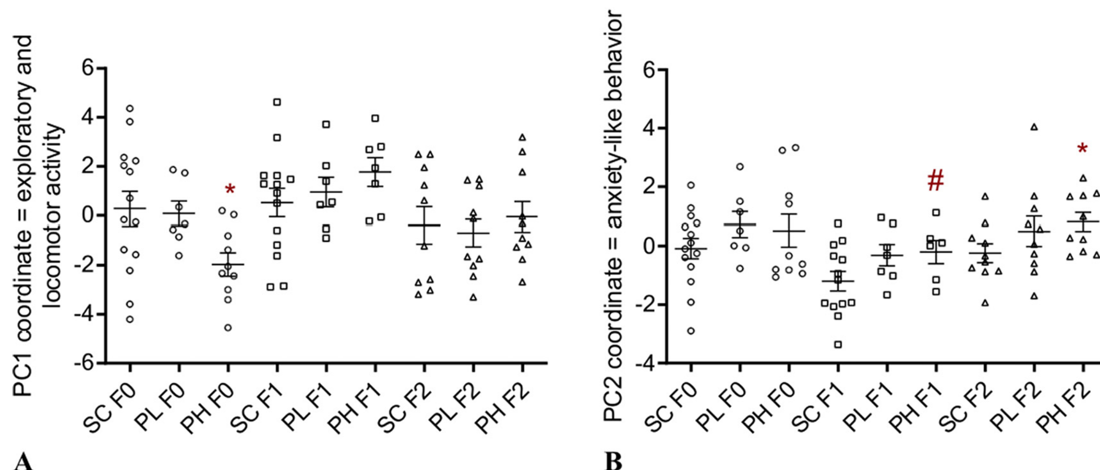

Fig. 1. PC1 (A) and PC2 (B) individual loadings after novel tank diving test in F0, F1, and F2 generations of adult male fish. #: p < 0.1; *: p < 0.05. PL: 1 μg/L Permethrin; PH: 10 μg/L Permethrin; SC: Solvent Control (DMSO 0.01%). Numeric values are available in Table S8.

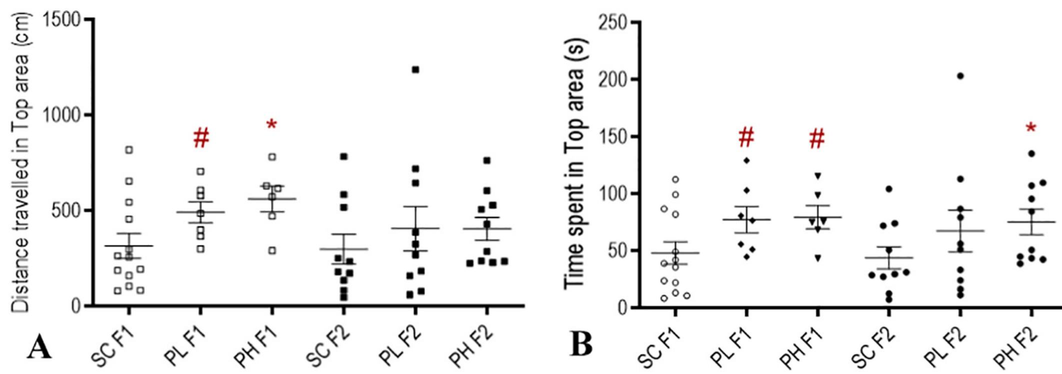

Fig. 2. Univariate analysis related to anxiety-like behavior in males of the F1 and F2 generations. A: Distance travelled in top area; B: Time spent in Top area. #: $p < 0.1$; *: $p < 0.05$. PL: 1 μg/L Permethrin; PH: 10 μg/L Permethrin; SC: Solvent Control (DMSO 0.01%). Numeric values are available in Table S8.

in PL F2 males. Besides, the data collected did not indicate any difference in average entry duration (Fig. S4), nor in freezing episodes and erratic movement frequency as markers for defensive innate behavior measurements (Fig. S5-S6). Overall, exploratory behavior endpoints suggest that both F1 and F2 PH males were less anxious than their SC congeners. No effect on behavior was observed in F1 and F2 females (Fig. S4-S8).

3.2. Transcriptional changes in zebrafish brain

RNA-Seq analyses were performed in zebrafish whole brains from each generation (F0, F1, F2) to investigate transcriptomic changes that may be associated with the behavioral defects. The results showed an increasing number of significant differentially expressed genes (DEGs) and enriched gene sets in permethrin-exposed fish compared to controls from the F0 to F2 generation (Fig. 3). Overall, there was little overlap in significant DEGs or pathways between generations (Fig. 3B-C). In the F0 generation, a limited number of genes, 22 in males and 27 in females, were significantly dysregulated (Fig. 3). Nonetheless, GSEA analysis revealed 95 and 69 gene sets significantly enriched in male and female, respectively (Fig. 3); out of this, 19 were common to both sexes and related to transcription/translation processes, eye development, and glutamatergic synaptic activity (unblocking of NMDA receptors, glutamate binding and activation) (Fig. S9). Further, in zebrafish F0 males specifically, transcriptional upregulation of NMDA receptors was accompanied by upregulation of genes linked to AMPA glutamate receptor activity (Table 1).

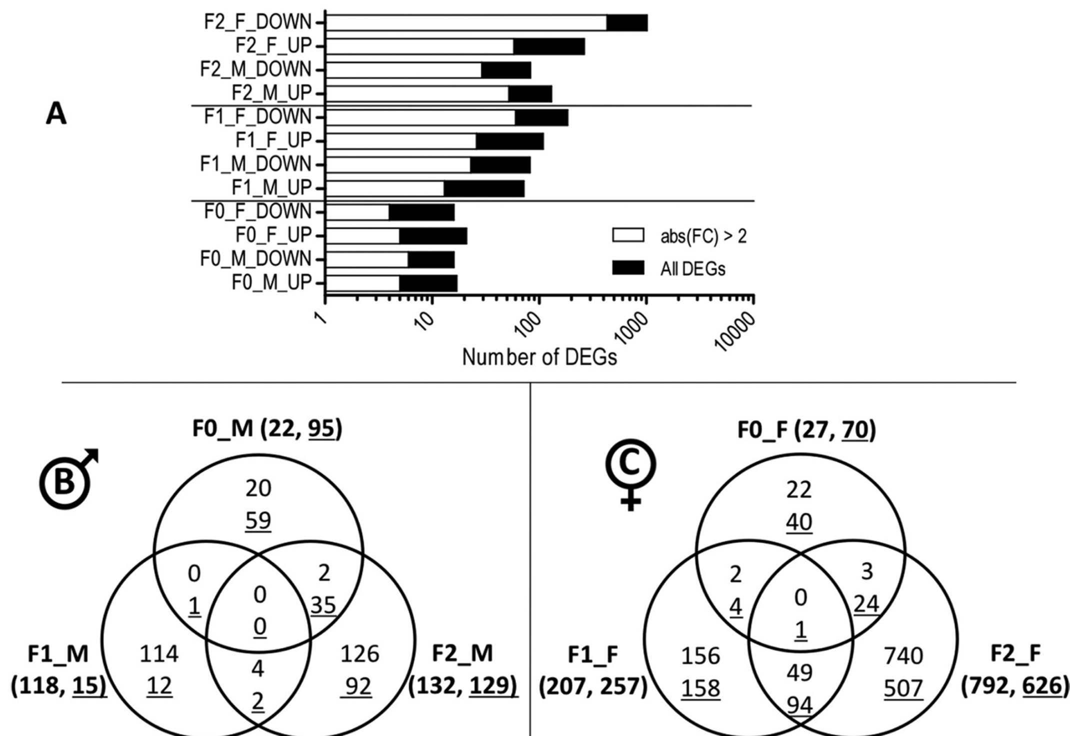

Fig. 3. A. Distribution of differentially expressed genes (DEGs) between upregulation (UP) and downregulation (DOWN) in F0 males (F0_M), F0 females (F0_F), F1 males (F1_M), F1 females (F1_F), F2 males (F2_M) and F2 females (F2_F) following F0 early exposure to permethrin 10 μg/L (n = 4). The graphic displays the total number of DEGs as well as the proportion of transcripts showing an absolute fold change above 2. B. Venn diagram showing the number of differentially expressed genes and overlapping between the 3 generations of males (padj≤0.05). Underlined numbers show significantly enriched gene sets according to Gene Set Enrichment Analysis (GSEA) results (padj≤0.05). C. Venn diagram showing the number of differentially expressed genes and overlapping between the 3 generations of females (padj≤0.05). Underlined numbers show significantly enriched gene sets according to Gene Set Enrichment Analysis (GSEA) results (padj≤0.05).

Table 1
Transcriptional enrichment in glutamate signaling related gene sets in brains from all generations of males ($n = 4$). This table shows the Normalized Enrichment Score (NES) and associated adjusted p-value (padj) as given by the Gene Set Enrichment Analysis (GSEA) output. Headings refer to F0 males (F0_M), F1 males (F1_M) and F2 males (F2_M). Complete results from differential gene expression analyses and GSEA are available in the Excel Tables S2–S7 and S15–S17.

Gene set	NES (padj) F0_M	NES (padj) F1_M	NES (padj) F2_M
Unblocking of NMDA receptors, glutamate binding and activation	2.18 (0.001)		
NMDA selective glutamate receptor complex	1.89 (0.044)		
Activation of NMDA receptors and postsynaptic events	1.98 (0.018)		
AMPA glutamate receptor activity	1.92 (0.032)	1.76 (0.19)	−1.70 (0.22)
NMDA glutamate receptor activity	1.81 (0.087)		
Trafficking of GluR2-containing AMPA receptors		1.79 (0.17)	−1.72 (0.21)
Glutamate binding, activation of AMPA receptors and synaptic plasticity		1.77 (0.19)	−1.72 (0.21)
Synaptic transmission, glutamatergic			−1.72 (0.2)
Trafficking of AMPA receptors		1.73 (0.21)	−1.71 (0.22)
Regulation of synaptic transmission, glutamatergic			−1.86 (0.13)
Activation of AMPA receptors	1.80 (0.095)		

In the F1 and F2 generations, we observed larger transcriptomic changes in females compared to males, even though the measured behavioral changes were only observed in the latter. At first, no significant effect on glutamatergic neural circuits was observed in the F1 or F2 generation (padj≤0.05). However, when relaxing the statistical cut-off to an acceptable threshold for GSEA studies ($p \leq 0.05$, padj≤0.25) (Subramanian et al., 2005), we observed transcriptomic enrichment in gene sets related to glutamatergic receptor activity in all generations of males, but not in females (Table 1). The persistence of this effect was specific to glutamatergic signaling as other enriched gene sets were different between generations (Excel Tables S15–S17). The direction of regulation was however opposite between F0/F1 (up) and F2 (down) (Table 1).

Of note, we also reported significant transcriptomic enrichment in epigenetic mechanisms: histone acetylation (F0_M, F2_M, F1_F, F2_F) and histone methylation/demethylation (F0_F, F1_F) pathways (Fig. S10).

3.3. Epigenetic alterations in zebrafish brain

As a possible mechanism for inheritance of behavioral defects, we investigated DNA methylation changes induced by permethrin exposure, and their stability over generations in zebrafish brain using RRBS. We observed a generation-specific clustering (F0 vs F1–F2) of the methylation profiles that was independent of sex and treatment (Fig. S1C). This may be explained by the higher genetic and epigenetic variability among the F0 fish group compared to F1–F2 groups due to the breeding set-up, since we included a higher number of contributing spawns in the F0 generation compared to F1 and F2 (18 vs 7) (Blanc et al., 2020). This, however, did not prevent from assessing intra-generation and inter-condition changes in DNA methylation and further compare DMRs between generations.

We could identify several DMRs within each generation, including significant overlaps between generations (Fig. 4). In the F0 generation, 110 and 121 DMRs (out of 17,456 identified regions) were identified in males and females, respectively, out of which 28 were common to both sexes (Table S5).

When looking at DMR inheritance, a total of 13 and 8 DMRs were overlapping between all generations in males and females, respectively (Fig. 4, Table S6). In males, these overlapping DMRs were consistently either hyper- or hypomethylated across generations with only one exception, whereas results in females suggest more complex interactions with half of the DMRs being hypomethylated in one generation and hypermethylated in the next (Fig. 5).

Visualization of all inherited regions using the Integrative Genome Viewer confirmed that the most robust DMR in both sexes was on chr24 and associated to *vim*, encoding for a structural protein expressed in all body cell types, including in brain glial cells (Schnitzer et al., 1981; Cerdà et al., 1998) (Fig. S11). This region showed inheritance of epigenetic alteration of eight cytosine positions from F0 to F2 generations. Within the list of genes associated with inherited DMRs in males only, from F0 to F2 generations, *fmr1* (chr14, translational regulator within the nervous system), and *pnocb* (chr20, regulation of nociception via opioid-receptor binding activity) appeared as interesting candidates as they may be relevant to behavioral alterations (Demin et al., 2018; Ng et al., 2013) (Fig. S12-S13). Whereas *fmr1* showed inheritance of DNA hypermethylation at the exact same cytosine positions, effects on *pnocb*-associated DMR were involving different cytosines and directions of regulation across generations (Fig. S13).

We further performed pathway analysis using the list of genes associated to the DMRs (Fig. S14). In F0 males, the gene set “AMPA glutamate receptor activity” was significantly enriched, as observed in RNA-Seq data (Table S7). When slightly relaxing the statistical cut-off ($p \leq 0.05$, padj≤0.25), the results indicated that DNA methylation changes were linked to additional pathways related to glutamatergic signaling in F0 and F2 males, but this could not be identified in F1 (Table S7).

4. Discussion

Developmental exposure to pyrethroids such as permethrin has been shown to induce neurobehavioral deficits in various species (Nunes et al., 2019; Saito et al., 2019; Furlong et al., 2017; Shelton et al., 2014). However, if and how these effects are inherited across generations is unknown. In this study, we show that early-life exposure of zebrafish to permethrin induces transgenerational behavioral alterations as well as persistent epigenetic and transcriptional modifications that are linked to glutamatergic signaling disruption.

Permethrin exposure induced a late onset reduction in overall swimming activity, findings that are in line with a recent study performed in zebrafish (Nunes et al., 2020). The results further showed that males were more sensitive, as previously observed in mammals (Imanishi et al., 2013; Saito et al., 2019).

Acute exposure to permethrin induces overactivation of voltage-gated sodium channels in the nervous system, which in turn leads to constant depolarization of nerve cells, increase in the excitatory postsynaptic potential, tremors, and further paralysis and death (Vijverberg and van den Bercken, 1990). Sodium channels of invertebrates and fish are particularly sensitive to acute pyrethroid exposure compared to mammals (Bradbury and Coats, 1989), which makes pyrethroid use a specific threat to aquatic communities. However, mechanisms underlying delayed neurotoxicity after low-dose exposure to permethrin are not well known and were poorly investigated in fish. In mammals, possible molecular changes were linked to neurodegeneration and synaptic morphology (Carloni et al., 2012; Carloni et al., 2013). In addition, early-life exposure of rodents to permethrin was shown to impair glutamatergic signaling in vitro and in vivo (Carloni et al., 2012; Shafer et al., 2008), and another study linked overactivation

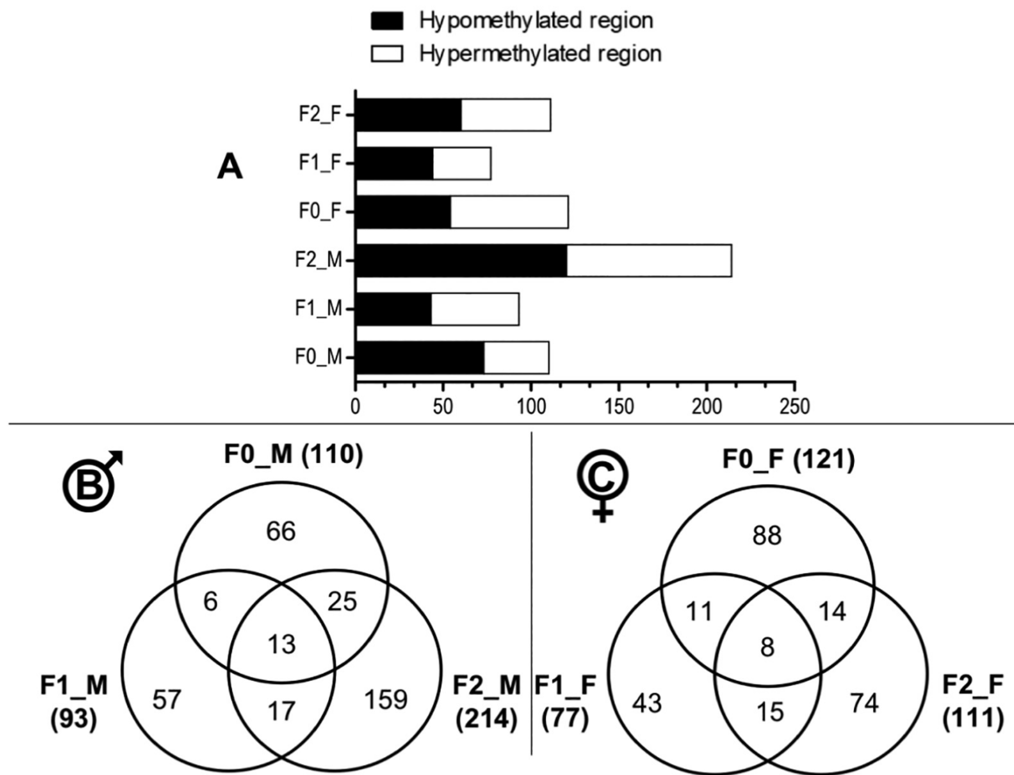

Fig. 4. Changes in DNA methylation in F0, F1 and F2 generations of zebrafish following F0 early-life exposure to permethrin 10 μg/L (n = 4; except for treated F0 males where $n = 3$). A. Distribution of the Differentially Methylated Regions (DMRs) between hypo- and hypermethylation within each dataset (padj≤0.05): F0 males (F0_M), F0 females (F0_F), F1 males (F1_M), F1 females (F1_F), F2 males (F2_M) and F2 females (F2_F). B. Venn diagram showing the number of DMRs and overlaps between the 3 generations in males (padj≤0.05). C. Venn diagram showing the number of DMRs and overlaps between the 3 generations in females (padj≤0.05).

of glutamatergic NMDA receptors to a depressive-like behavior (i.e. reduced mobility) in rats (Cattani et al., 2017). Altogether, this indicates that upregulation of genes related to glutamatergic synaptic activity may support the behavioral effects observed in the F0 generation.

Further, we showed for the first time that the indirectly exposed F1 and the unexposed F2 generations of male offspring were also affected, as they displayed a reduction in their anxiety-like behavior. While the data did not indicate a change in defensive innate behavior endpoints, other parameters that relate to anxiety, such as time spent and/or

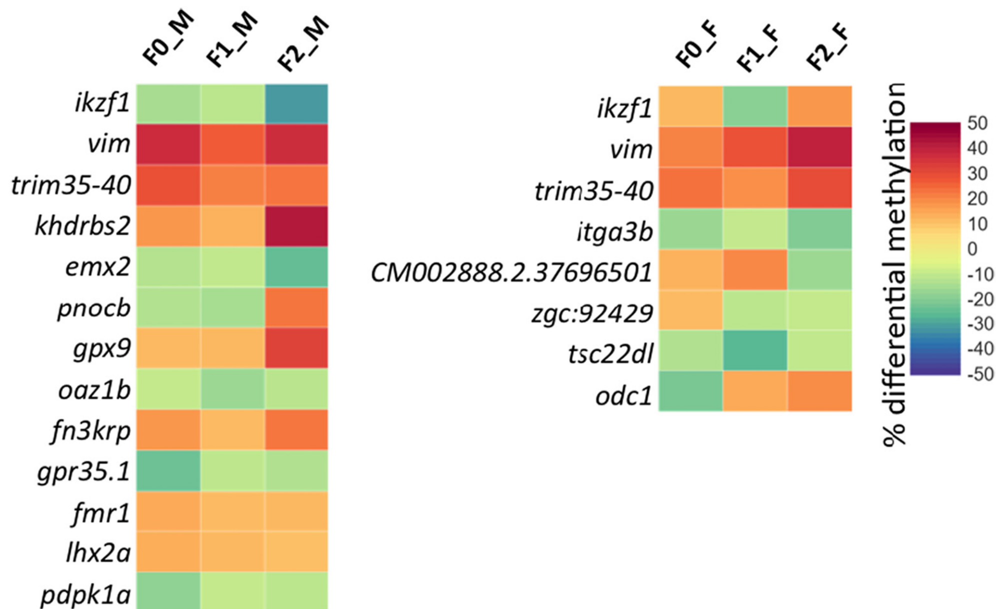

Fig. 5. Heatmap showing inherited differentially methylated regions (padj≤0.05) in F0, F1, and F2 generations of males (left) or females (right) (n = 4; except for treated F0 males where n = 3). Row headings refer to genes associated to DMRs. If none, it refers to the DMR as chromosome name.starting position. Column headings refer to F0 males (F0_M), F0 females (F0_F), F1 males (F1_M), F1 females (F1_F), F2 males (F2_M) and F2 females (F2_F). Numeric values are available in Table S6 and Excel Tables S9–S14.

distance swam in the Top area of the tank, were altered (Cachat et al., 2010). This effect was significant in the highest tested concentration, whereas there was only a trend suggesting that similar alterations may happen in the low concentration. This result supports the presence of a transgenerational phenotype of developmental origin caused by F0 exposure to permethrin that confirms previous data from zebrafish larvae (Blanc et al., 2020). Sex differences in transgenerational effects were observed following other exposure scenario and may suggest a different capacity for adaptation and recovery (Baker et al., 2014b; Crews et al., 2007; Vera-Chang et al., 2018). In this regard, gene expression data showed that in F1 and in F2, the number of DEGs was higher in females compared to males, which may highlight sex-specific adaptive or compensatory mechanisms at play.

These results further support the notion that direct effects of developmental exposure (i.e. effects in F0) can be partly dissimilar to the ones resulting from germ cell exposure (F1) or transgenerational inheritance (F2) as described in previous studies (Alfonso et al., 2019; Bell and Hellman, 2019; Schmitt et al., 2020; Blanc et al., 2021). In this study, F0 males were hypoactive, while more specific effects on anxiety relief were identified in F1 and F2 males. At the molecular level, gene expression and epigenetic changes were however similar between all three generations, and no specific similarity relevant to behavior could be identified between both F1 and F2 only. Thus, we hypothesize that behavioral alterations were partly inherited from F0 to F1 and F2, and that the hypoactive phenotype in F0 prevented from measuring more subtle behavioral changes, such as alterations in the anxiety level of F0 males. This is further strengthened by the identification of additional molecular effects specific to the F0 generation that could explain the aggravated phenotype.

Pathway analysis in F1 and F2 generations strengthened the hypothesis that behavioral alterations may be linked to glutamatergic synapse activity, as the related transcriptional changes were also specific to males. However, more investigation into the impact of the transcriptional regulation of this pathway is needed, in particular as glutamate-related pathways were upregulated in F1 but downregulated in F2, while observing similar behavioral phenotypes. We can only speculate on how these opposite patterns could lead to similar phenotypes. It is possible that despite opposite transcriptional changes, similar alterations in these pathways at the level of protein expression or function are induced in both generations. Furthermore, F1 and F2 may show similar transcriptional changes in specific brain areas, which cannot be addressed in this study as the analyses were performed on whole brains, and thus represent overall changes. For these reasons, further investigations focusing on protein expression and functionality as well on specific brain regions may clarify the effects of permethrin on the glutamatergic system of zebrafish.

DNA methylation analyses also pointed to effects related to glutamatergic signaling, which persisted across all three generations. Changes in DNA methylation have been previously linked to late onset and transgenerational effects and have a functional role in disease etiology, development, and behavior in various species (Liberman et al., 2019; McGhee and Bell, 2014; Tran and Miyake, 2017). They could therefore be the mechanism underlying inheritance of the observed behavioral defects in male fish (Andersen et al., 2013; Bordoni et al., 2015; Manikkam et al., 2012b). Observing sex-specific inheritance of effects in the domesticated zebrafish AB strain, which does not possess known genetic determination of sex, is somewhat unexpected. However, Vera-Chang et al. (2018) observed such effect in response to fluoxetine exposure, another compound affecting anxiety levels in fish (Vera-Chang et al., 2018). Fluoxetine exposure led to distinct behavioral changes in zebrafish males compared to females across generations, which could partly be attributed to sex-specific levels of sex steroids. Interestingly, permethrin has also been shown to interfere with the male hormone system in rodents (Kim et al., 2005), and with the endocrine system of zebrafish embryos (Zhang et al., 2017). In our study, no monitoring of steroids was performed, but transcriptomic and epigenetic alterations pointed to sex-specific changes in glutamate signaling. Interestingly, it was shown before that the methylation status of glutamate receptors was only involved in regulating the depression-like behavior of male rats, but not females, supporting that mechanisms are sex-specific (Lin et al., 2018). Thus, it is possible that similar epigenetic alterations induce sex-specific behavioral alterations in organisms, although mechanisms require further clarification. In the present case, however, most of the methylation changes were also sex-specific. Several DMRs, inherited from the F0 to the F2 generation of males, were associated to genes implicated in the control of behavior and linked to glutamatergic signaling: *vim*, *pnocb*, and *fmr1*. Vimentin (*vim*) is an intermediate filament protein expressed in glial cells such as astrocytes (Cerdà et al., 1998), which is important for synapse reorganization and neuron regeneration after brain injury (Krohn et al., 1995; Zupanc and Clint, 2003). In addition, vimentin misexpression in rat prefrontal cortex was linked to locomotor and anxiety-like behavior changes following adolescent social isolation (Sun et al., 2017), and interactions between glutamatergic signaling and vimentin expression were reported in other studies (Kawakami, 2000; McNearney et al., 2009). Further, astrocyte dysregulation was incriminated in developmental neurotoxic effects of permethrin in rat (Saito et al., 2019), overall suggesting that transgenerational epigenetic dysregulation of vimentin may have profound effects on astrocyte function within the tripartite glutamatergic synapse. Prepronociceptin b (*pnocb*) is responsible for modulating nociception and locomotor behavior (Demin et al., 2018). Nociceptin signaling can disturb the GABA/glutamatergic balance and favor the development of Parkinson's disease, for which permethrin is under suspicion as a causal agent (Mercatelli et al., 2019). Finally, glutamatergic signals regulate the trafficking of the FMR-protein encoded by *fmr1* at dendrites and synapses in vitro (Antar et al., 2004), and knock-out of *fmr1* produces an anxiolytic-like phenotype and impairment of avoidance learning in zebrafish (Ng et al., 2013). Methylation changes associated to the described genes and pathways implicate epigenetic regulation of neuronal development and glutamatergic synapse activity in the observed transgenerational and sex-specific behavioral effects. However, we could not well correlate DEGs and predicted cis-regulated genes from DMRs, which limits our conclusions. The lack of correlation between methylation and transcription changes is acknowledged within the field (Kamstra et al., 2018; Falisse et al., 2018) and both technical and biological factors may be at cause. As observed in other studies (Falisse et al., 2018; Kamstra et al., 2017), most of the identified DMRs were located at intergenic regions (≈70% in average, data not shown). Thus, the restrictive association of DMRs to potential cis-regulated genes, and the limited scope of RRBS analyses (screen of 3–5% of the methylome) likely underestimated functional associations. Besides, the relationship between genomic structure and gene activity is not fully understood yet and surely involves different levels of epigenetic remodeling which were not all measured in the present study (Bemer, 2017).

Nonetheless, this study identified several candidate DMRs that were inherited over three generations of zebrafish and that may have implications in the behavioral effects. However, it cannot be excluded that the observed transgenerational effects are not subsequent to DNA methylation changes. Other epigenetic mechanisms could be involved, such as micro-RNAs or histone modifications (Blake and Watson, 2016). Histone modifications are important epigenetic marks for proper nervous system development and are the target of neurotoxic compounds (Lilja et al., 2013; Song et al., 2010). To which extent these modifications can contribute to epigenetic inheritance in vertebrates is a very active domain of research (Rose and Klose, 2014; Lismer et al., 2020). We reported previously that the histone demethylase Kdm5ba was dysregulated in zebrafish embryos exposed to permethrin (Blanc et al., 2019) and we also observed enrichment in histone modification pathways in the present work. Another study suggested the involvement of histone modifications on H3 and H4 rather than DNA methylation changes to support permethrin epigenetic effects (Fedeli et al.,

2017). Besides, Valles et al. (2020) recently showed sex-specific inheritance of histone modifications in zebrafish following ancestral exposure to arsenic; thus, suggesting that chemical exposure can induce sex-specific epigenetic alterations, which may turn into sex-specific behavioral changes. Therefore, identifying DNA regions enriched or depleted in specific histone marks may provide further insight into the epigenetic component of the observed alterations.

In conclusion, early-life exposure to permethrin induced late-onset behavioral defects in F0 adult zebrafish and a transgenerational decrease in anxiety-like behavior in males. Transcriptional and epigenetic data indicated inherited molecular changes linked to dysregulation of glutamatergic signaling as a potential mechanism underlying the delayed and transgenerational neurobehavioral alterations in males. Functional analyses are now warranted to confirm the role of the identified pathways in the behavioral alterations, especially since some of the effects in our study were not strongly significant, possibly due to the limited number of replicates per condition. It would also be of interest to extend the phenotypical characterization to cognitive defects – learning and memory, as they could help to clarify the role of some molecular changes observed in both males and females, or the ones that are generation-specific (Feng et al., 2010; Saito et al., 2019; Shelton et al., 2014). In addition, future research may also focus on the identification of critical windows of exposure within the first 28 days, and on distinguishing between paternal and maternal contributions to germline mediated transgenerational epigenetic inheritance. In fact, the study of epigenetic changes in parental gametes would help characterizing the role of epigenetics in mediating the observed transgenerational effects, by understanding 1) how they could carry on information to the next generation, and 2) how these could translate into persistent sex-specific brain alterations. Nevertheless, the present results show that neurobehavioral effects of pyrethroid exposure previously identified in other organisms may not be restricted to exposed generations and may therefore have profound long-term consequences on environmental health.

CRediT authorship contribution statement

Mélanie Blanc: Conceptualization, Methodology, Investigation, Formal analysis, Funding acquisition, Writing – original draft. **Philipp Antczak:** Methodology, Formal analysis, Writing – review & editing. **Xavier Cousin:** Conceptualization, Methodology, Writing – review & editing. **Christoph Grunau:** Formal analysis, Writing – review & editing. **Nikolai Scherbak:** Conceptualization, Methodology, Writing – review & editing. **Joëlle Rüegg:** Conceptualization, Methodology, Writing – review & editing. **Steffen H. Keiter:** Conceptualization, Methodology, Resources, Funding acquisition, Supervision, Project administration, Validation, Writing – review & editing.

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

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