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ABSTRACT

Transgenerational effects induced by environmental stressors are a threat to ecosystems and human health. However, there is still limited observation and understanding of the potential of chemicals to influence life outcomes over several generations. In the present study, we investigated the effects of two environmental contaminants, coumarin 47 and permethrin, on exposed zebrafish (F0) and their progeny (F1–F3). Coumarin 47 is commonly found in personal care products and dyes, whereas permethrin is used as a domestic and agricultural pyrethroid insecticide/insect repellent. Zebrafish (F0) were exposed during early development until 28 days post-fertilization and their progeny (F1–F3) were bred unexposed. On one hand, the effects induced by coumarin 47 suggest no multigenerational toxicity. On the other hand, we found that behavior of zebrafish larvae was significantly affected by exposure to permethrin in F1 to F3 generations with some differences depending on the concentration. This suggests persistent alteration of the neural or neuromuscular function. In addition, lipidomic analyses showed that permethrin treatment was partially correlated with lysophosphatidylcholine levels in zebrafish, an important lipid for neurodevelopment. Overall, these results stress out one of the most widely used pyrethroids can trigger long-term, multi- and possibly transgenerational changes in the nervous system of zebrafish. These neurobehavioral changes echo the effects observed under direct exposure to high concentrations of permethrin and therefore call for more research on mechanisms underlying effect inheritance.

1. Introduction

In the last decades, many studies reported that early-life exposure to low concentrations of chemicals affects the physiology of the organism later in life. The concept of the Developmental Origin of Health and Disease (DOHaD) hypothesizes that during fetal and childhood development the environment may influence the risk for many chronic diseases with late-onset including neurodevelopmental defects (Sturza et al., 2016; Tran and Miyake, 2017). It was also shown that these adverse effects could persist in subsequent generations without the need for additional exposure (Anway et al., 2006; Knecht et al., 2017; Vera-Chang et al., 2018). These findings raise great concern about health-associated risks caused by chemical exposure that are not taken into consideration by current legislations. Besides, studies about transgenerational effects of environmental chemicals are still limited. Nonetheless, there is evidence for the involvement of epigenetic dysregulation as an underlying mechanism (Tran and Miyake, 2017). Especially, exposure to epigenetic modifiers during fetal development and early life is a concern due to high cellular plasticity at these stages that can affect normal and pathological development later in life (Stel and Legler, 2015; Vaiserman, 2014).

In a previous study, we found that two chemicals, coumarin 47 and

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permethrin, induce strong changes in the expression of epigenetic factors during embryonic development of zebrafish (Blanc et al., 2019). Coumarin 47 (7-diethylamino-4-methylcoumarin) is used in personal care products and paper industry as a whitening agent. Not much attention has been paid to this chemical as an environmental contaminant so far. Nonetheless, coumarin 47 was recently identified as a highly potent anti-androgenic compound at an environmental concentration of 13.7 μg/L in the Holtemme River, Germany (Muschket et al., 2018). Besides, relatively high concentrations (250–2000 μg/L) induce a decrease in survival and affect heart rate as well as activate the oxidative stress pathway in zebrafish embryos (Jung et al., 2012).

Permethrin is a synthetic pyrethroid insect repellent and insecticide used for a variety of applications: agriculture, clothing, human and veterinary applications. Biomonitoring of the main pyrethroid metabolite, 3-phenoxybenzoic acid, in human urine has confirmed widespread and repetitive exposure in the general population (Saillenfait et al., 2015). In the environment, typical reported concentrations are in the low ng/L range with the most contaminated areas showing values from 20 to 150 ng/L in urban and agricultural runoffs as well as in storm waters (Hladik and Kuivila, 2009; Weston and Lydy, 2010, 2012). However, permethrin tends to degrade quickly in water and to bind to sediments, therefore environmental concentrations may be underestimated (Hladik and Kuivila, 2009; Kuivila et al., 2012; Weston et al., 2005). Numerous negative health effects were associated with permethrin exposure. As a primary mode of action, permethrin is neurotoxic to insects, fish, and honeybees *via* disruption of voltage-gated sodium channels at high concentrations (Silver et al., 2014). In addition, several studies reported the impact of prenatal and early-life exposure to pyrethroids on neurodevelopment and cognitive abilities later in life, possible through dopamine metabolism (Carloni et al., 2012; Furlong et al., 2017; Saito et al., 2019; Shelton et al., 2014). There is also evidence that lipid metabolism is a key-pathway in permethrin toxicity, promoting accumulation of triglycerides in hepatic cells and the development of insulin resistance-associated pathologies (Kim et al., 2014; Liang et al., 2020; Yang et al., 2019). Permethrin associated with the insect repellent N,N-diethyl-meta-toluamide (DEET) negatively affects the reproductive capacity of F2–F3 generations of rats, giving unique evidence on the implication of permethrin in inducing transgenerational effects in organisms (Manikkam et al., 2012).

Zebrafish represents an established model for vertebrates in medical and toxicological research (Langheinrich, 2003; Mudbhary and Sadler, 2011). Its short life cycle and large progeny facilitates the application of multi- and transgenerational studies. Finally, since embryonic development is external, it is possible to investigate transgenerational effects from the F2 generation in most exposure scenarios, instead of going to the F3 as in mammals (Baker et al., 2014; Head, 2019). In the present study, we hypothesized that early-life exposure to permethrin or coumarin 47 would lead to delayed toxic effects in adult F0 zebrafish. We further tested whether these effects were inherited in their descendants over 3 generations by looking at growth, reproduction, behavior, and alteration of lipid metabolism.

2. Material and methods

2.1. Fish maintenance

The laboratory animal facilities at MTM (#5.2.18.-12707/17) hold permission to use (5.12.18-12628-17) and breed (5.2.18-12630-17) laboratory animals; these permits are affiliated with the Swedish Board of Agriculture, Jönköping, Sweden. Moreover, the facilities hold ethical permission (5.2.18–861/15) affiliated with the same board in Linköping, Sweden. Adult zebrafish (AB strain; ZFIN ID: ZDB-GENO-960809-7) were purchased from Karolinska Institute (Stockholm, Sweden) and kept on a 14 h:10 h light:dark cycle. Maintenance of adult zebrafish and egg production were according to standard protocols (Westerfield, 2007). Fish were kept in a recirculating system where conductivity (350 ± 40 μS), pH (7.2 ± 0.2), and temperature (26 ± 1 °C) were regulated by a ProfiLux 3.1 controller (GHL Advanced Technology, Germany). Carbon hardness, nitrates, nitrites (EasyTest stripes, JBL, Germany), and ammonia (Tetra, Germany) contents were monitored once a week. Fish were fed twice a day with flakes (TetraRubin, Tetra) and freshly hatched artemia *nauplii* (Ocean Nutrition, Canada) in the morning and afternoon, respectively. The amount of food given to each tank was controlled: fish were fed *ad libitum* and no difference in feeding behavior was observed between tanks and conditions.

2.2. Fish exposure and breeding

Permethrin ($C_{21}H_{20}Cl_2O_3$, 52,645-53-1, Pestanal analytical standard), Coumarin 47 ($C_{14}H_{17}NO_2$, 91-44-1, >99%) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich, Germany. Commercial powders were dissolved in 100 % DMSO to prepare stock solutions at 5 g/L (coumarin 47) and 1 g/L (permethrin) that were stored in amber glass vials (VWR, USA) at −20 °C throughout the study. Preliminary experiments were performed to identify concentrations suitable for long-term exposures using the fish embryo toxicity test (FET) according to the OECD Technical Guideline no. 236 (OECD, 2013). We also included the larval photomotor response (LPMR) and daily visual observation of swimming activity as additional endpoints (section S1, Figure S1 and S2). Subsequently, batches of approx. 200 fertilized eggs resulting from mixing 18 independent spawns were exposed to permethrin at 10 μg/L (PH), 1 μg/L (PL), to coumarin 47 at 10 μg/L (CH), 1 μg/L (CL) or DMSO 0.01% (SC) as the carrier solvent. All solutions were diluted in ISO water, prepared according to ISO 7346–2 (1996) (294 mg/L $CaCl_2 \cdot 2\ H_2O$, 123.3 mg/L $MgSO_4 \cdot 7\ H_2O$, 63 mg/L $NaHCO_3$, 5.5 mg/L KCl). Exposure started at 2 h post fertilization and until 4 days post-fertilization (dpf), embryos were exposed in 3 L tanks filled with 500 ml exposure solution with 50% daily renewal. From 5 dpf onwards, larvae were transferred into pre-exposed 40 L breeding tanks. Exposure was semi-static, with a daily exchange of 25% of the total volume until 28 dpf. Selected concentrations had no impact on fish survival throughout the study. Then, fish were kept one week in the exposure system with progressive exchange to regular water ("depuration" period). According to Schimmel et al. (1983) and the European chemical agency, the selected compounds are unlikely to bioaccumulate in organisms. From day 35 onwards, seventy-five fish per treatment were kept in regular conditions in a 40 L tank as described in sub-section 2.1, with additional activated carbon treatment before water recirculation. The F1 and F2 generations were obtained by pair-cross spawning (7 contributing pairs per treatment) using 4-month-old F0 and F1 fish, respectively. They were bred under regular conditions, i.e. they were not exposed to the chemicals. Fertile adults (120 dpf for F0 and F2, 105 dpf for F1) were euthanized in saturated ethyl 4-aminobenzoate solution (500 mg/L, Sigma-Aldrich) and subsequently weighed, measured, and dissected. Ten fish per treatment, generation and sex were used for analysis of body length and weight, except for F2 PL, which included 4 fish.

2.3. Chemical analysis

Water samples were taken at day 0 before exposure of eggs, and at day 4, 14, 21, and 28, before medium exchange (one replicate for permethrin, duplicates for coumarin 47). Permethrin analysis was performed by Eurofins Food and Feed Testing Sweden AB (Lidköping, Sweden). Briefly, water samples were concentrated through C2/ENV + SPE columns and permethrin was eluted in methanol/ethyl acetate before analysis using Gas Chromatography-Mass Spectrometry (limit of quantification: 0.03 μg/L; standardized Eurofins method). Coumarin 47 was analyzed by liquid chromatography-high resolution mass spectrometry using a Thermo Ultimate 3000 LC system coupled to an ion trap-Orbitrap instrument (Thermo LTQ Orbitrap XL) with electrospray ionization (ESI). Additional information is provided in section S2.

2.4. Fertility assessment

In the evening before spawning, groups of 1 female:2 males were transferred to 3 L spawning tanks with insert (Tecniplast®) and left overnight. The day after (approx. 9 a.m.), spawns from treated and control groups were collected and fish were transferred back to the maintenance system. No significant effect on spawning success, fertilization rate, and 24 h embryo survival was observed (data not shown); therefore, fertility was further evaluated as the average number of eggs laid per female only. For each generation, fertility was assessed for approx. 10 weeks between 3 and 5.5 month of age, 4–7 replicates/treatment/week. Compared to F1 and F2 generations, the analysis of F0 fertility included much less early spawns, which lead to a higher baseline count of eggs laid per female. In total, an average number of 44 ± 18 spawns per treatment and generation were included in the analysis.

2.5. Behavioral analyses

Behavior of larvae was investigated in F0, F1, F2, and F3 generations using the larvae photomotor response test (adapted from Schnörr et al. (2012)). Eggs were collected within 2 h. Fifty fertilized zebrafish embryos were transferred to glass beakers filled up with 50 ml of reconstituted ISO water, or in the case of the F0 generation, to 50 ml of exposure solution, which 50% were daily replaced to ensure stable exposure. At 4 dpf, they were gently transferred to 96-well plates with the same number of control and treatment individuals on each plate. At 5 dpf, locomotor activity in response to light change was recorded using DanioVision® (Noldus, The Netherlands). The behavior of larvae was camera-recorded (Basler®) for 15 min, comprising of 5 min of light (LON 1), 5 min of darkness (LOFF) and 5 min of light (LON 2) after 5 min acclimation in full light. Data were analyzed as total distance travelled during each 5 min period (cm). An average number of 7 ± 2 plates per treatment and generation, representing biological replicates, were included in the analysis.

2.6. Tissue-specific lipidomic measurements

Brains, livers, and testes were lysed in 350 µL of RP1 buffer (Triprep extraction kit, Macherey-Nagel), ovaries in 700 µL. For each sample, 45 µL aliquots of the lysate were used for total lipid extraction. Briefly, 60 µL of internal standard (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)) were added to the aliquots for quantification, followed by 90 µL of methanol (Fisher Scientific) and 300 µL of methyl tert-butyl ether (Fisher Scientific). Samples were incubated for 1 h, then 240 µl of MilliQ water (Fisher Scientific) were added. After 10 min incubation and 10 min centrifugation (9000 × g), approx. 250 µL of the upper organic phase was transferred into an LC vial with insert (Waters). All samples were stored at −80 °C until processing on an ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry instrument (UHPLC-Q-TOF-MS) (section S3). Lipid concentrations were normalized to weight for brains and gonads, and protein content for livers. Between 4 and 6 biological replicates per treatment, generation and sex were analyzed. Concentrations from individual lipids were summed up to evaluate enrichment in the following 14 functional lipid groups: all phospholipids (PL), triacylglycerols (TG, further divided into low saturation (TG4, 0–4 double-bounds), intermediate saturation (TG5, 5–6 double-bounds) and high saturation (TG7, ≥7 double-bounds)), lysophosphatidylcholines (lysoPC), sphingomyelins (SM), phosphatidic acids (PA), phosphatidylethanolamines (ALLPE), phosphatidylcholines (ALLPC), cholesteryl esters (CE), ceramides (Cer), phosphatidylinositols (PI) and phosphatidylserines (PS).

2.7. Statistical analyses

Statistical analyses of data from each generation independently were performed using analysis of variance (ANOVA) in R (v3.6.1). Residuals from models derived from all datasets were normally distributed, only slight deviation from normality was tolerated due to the large sample size. Multiple ANOVA (MANOVA) with 3 factors (generation, treatment and sex) was applied on morphometric variables (body length, weight). ANOVA 2 factors (treatment, generation) was performed on reproduction data. Due to independency between LPMR experiments, ANOVA 3 factors (generation, control/treated, and light status) was applied separately on each of the 4 treatments. For all, results showing a p-value ≤ 0.05 between treated and control samples in *post-hoc* analysis (Tukey's method with p-value adjustment for multiple comparisons) were reported as significant. Lipidomic data were analyzed using basic partial correlation analysis on the whole dataset following normalization (mean-centering and log-transformation). Then, ANOVA 3 factors (treatment, generation, sex) was applied on organ-specific data. P-values ≤ 0.05 (Tukey's adjusted) were considered as significant correlations/differences. Figures were generated using either Graphpad Prism 5, DisplayR, or Metscape (v3.1.3) and Cytoscape (v3.7.2) (Karnovsky et al., 2012; Shannon et al., 2003).

3. Results

3.1. Quantification of chemicals in exposure solutions

Measured values for permethrin in water were on average 10 times lower than the nominal concentrations and declining over time (Figure S3A). Measured concentrations of coumarin 47 were in the range of nominal values, with accumulation during the first four days of exposure in plastic tanks, and progressive decrease over time in glass tanks until 28 days (Figure S3B-C). Biotransformation products of coumarin 47 showed an increase over time, especially Desethyl-coumarin 47 (Figure S3B-C). Average measured concentrations in water were of 0.70 µg/L (PH), 0.15 µg/L (PL), 10.5 µg/L (CH) and 1.4 µg/L (CL).

3.2. Growth

MANOVA revealed significant differences in body length and weight according to sex, generation, and treatment, however, *post-hoc* analyses did not allow clear conclusions. In fact, only CL treatment had a significant effect on fish growth in the F2 generation (Section S4, Figure S4 to S6).

3.3. Reproductive capacity

Results from ANOVA revealed that the generation had a main effect on the number of eggs laid per female, which was expected due to the differences in age at the time of spawn collection between F0 and F1/F2 generations. Besides, there was a significant effect of the treatment dependent on the generation (Section S5). *Post-hoc* analysis showed that exposure to PH, CH, and CL had no effect in any of the generations examined (Figure S7). In the F0 generation, PL-exposed females spawned on average significantly fewer eggs when compared to the control (Fig. 1). No significant effect on spawn size was observed in the F1 and F2 generations.

3.4. Larvae neurobehavioral disruption

ANOVA revealed that effects of each treatment were depending on the generation and on the light period (Section S6). In the F0 generation, none of the treatment induced a significant change in larval locomotor activity (Figure S8). In contrast, PH and PL treatments induced significant changes in F1 individuals. Exposure to PH significantly decreased

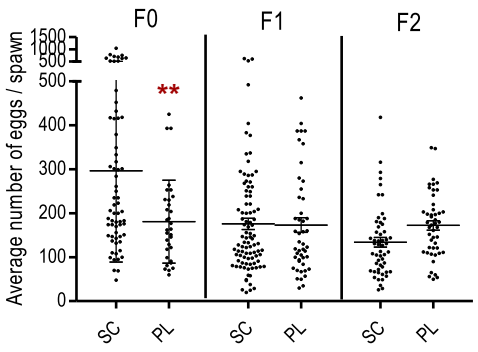

Fig. 1. Number of eggs spawned per female per spawn in the PL treatment across generations (mean ± SD). Individual dots represent the different spawning event replicates. **: p<0.01. PL: Permethrin 1 µg/L; SC: Solvent Control (DMSO 0.01%).

larvae activity only during light periods, i.e. LON 1 and LON 2, and PL significantly decreased their activity over all periods (Fig. 2). In the F2 generation, the activity was reduced over all periods in the PH treatment, and during LOFF in the PL treatment (Fig. 3). Exposure to CH and CL did not induce any significant effect on behavior in F1 or F2 (Figs. 2–3).

The behavior of larvae from the F3 generation was also investigated to see whether the effects observed in F2 would be further transferred. There was no longer a significant effect in the PH treatment, however, the PL treatment still induced behavioral changes in the F3 generation. Besides, larvae from CH and CL groups displayed significant changes in their behavior, which could not be explained (Figure S9).

3.5. Lipidomic analyses

We analyzed lipid profiles in brains, livers, and gonads of PL and PH adults. Partial correlation analyses revealed that permethrin exposure was overall correlated with a reduction in lysoPC content (Fig. 4A, corr. coeff ρ=−0.126). However, additional statistical analysis did not allow drawing clear conclusions on the effect of the treatment on lysoPC concentrations (Fig. 4B, Section S7). It is also of note that permethrin exposure was significantly correlated with fish weight and size (Fig. 4A). Details on data are provided in Appendix B. Other lipid classes did not show any significant change upon exposure.

4. Discussion

In this study, we investigated the multi- and transgenerational effects of coumarin 47 and permethrin in zebrafish with a focus on growth, reproduction, behavior, and lipid metabolism. The selection of these two compounds was based on their ability to induce changes in expression of epigenetic factors in zebrafish embryos that may further give rise to epigenetically-inherited effects in the progeny (Blanc et al., 2019). Exposure to coumarin 47 induced limited effects in exposed fish and their offspring. It was previously described that coumarin 47 acts as an anti-androgenic chemical in spiggin-gfp transgenic medaka larvae at concentrations as low as 10 µg/L (Muschket et al., 2018). In the present study, no substantial impact on reproductive capacity, sex ratio, growth, or larval behavior was observed in zebrafish at a similar concentration suggesting no consequence of transient early-life endocrine disruption at the physiological level. Nonetheless, the growth of CL F2 fish was significantly increased; however, we faced unexpected lethality over breeding in this treatment, which likely promoted growth *via* fish density decrease.

Exposure of zebrafish to permethrin revealed more pronounced

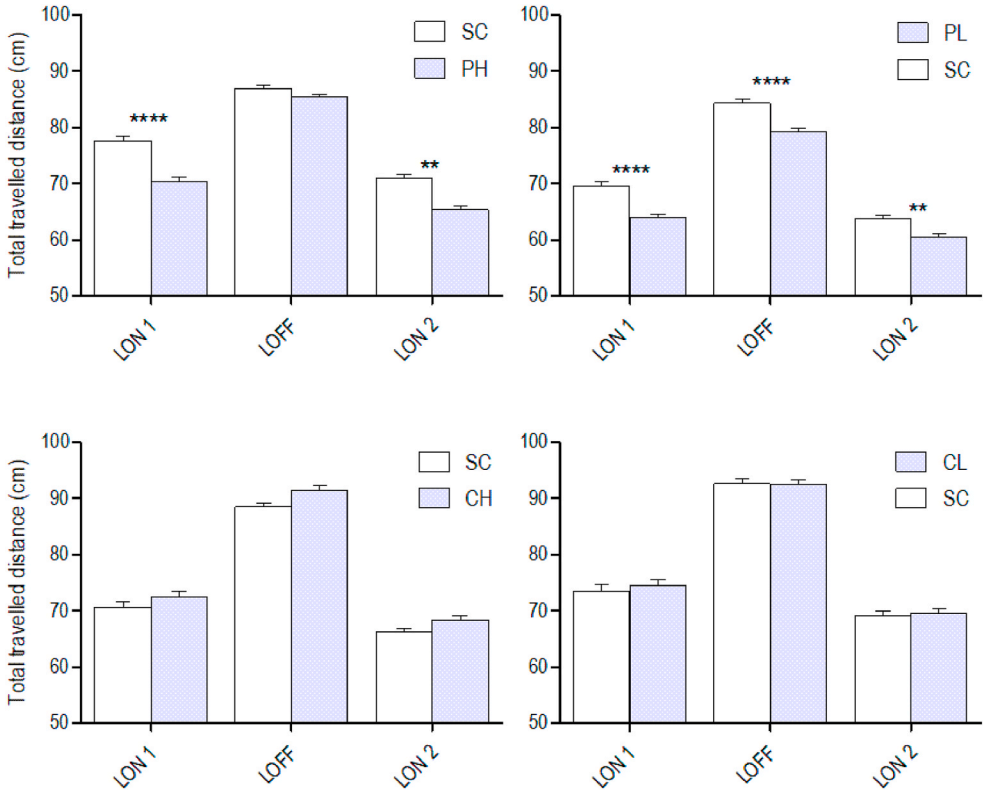

Fig. 2. Total distance travelled per F1 larvae over 3 successive periods of 5-min recording in permethrin and coumarin treatments. Data are shown as mean ± SEM. Stars indicate significant differences between treatments within time periods with **: p<0.01; ****: p<0.0001. PH: 10 µg/L Permethrin; PL: 1 µg/L Permethrin; CH: 10 µg/L Coumarin 47; CL: 1 µg/L Coumarin 47; SC: Solvent Control (DMSO 0.01%).

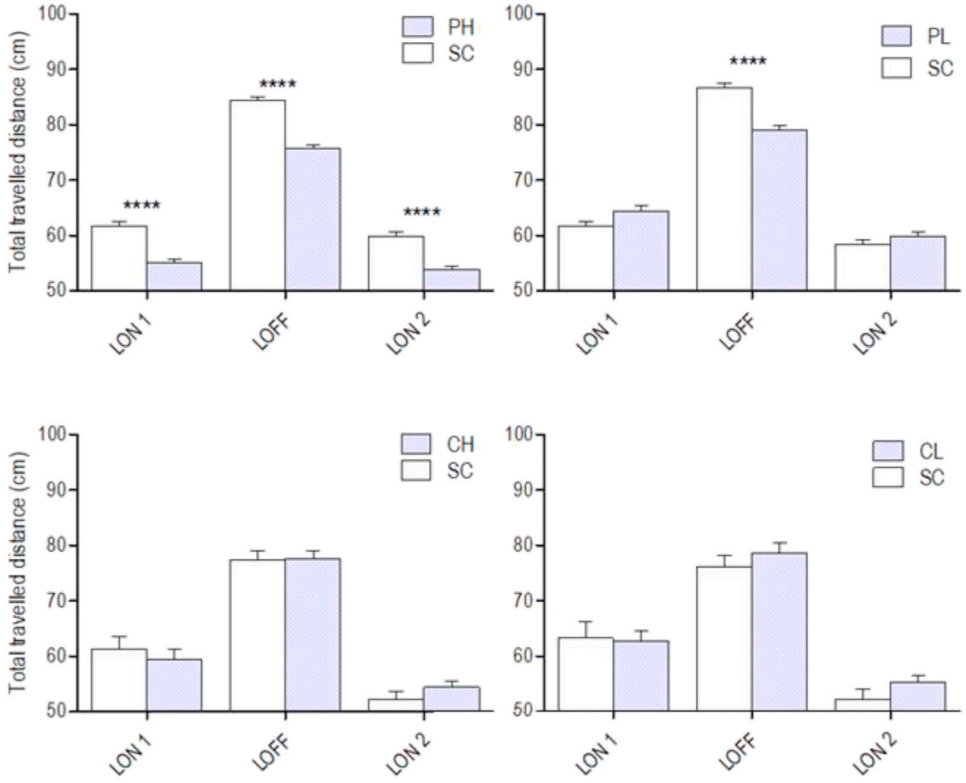

Fig. 3. Average total distance travelled per F2 larvae over 3 successive periods of 5-min recording in permethrin and coumarin treatments. Data are shown as mean ± SEM. Stars indicate significant differences between treatments within time periods with ****: p<0.0001. PH: 10 µg/L Permethrin; PL: 1 µg/L Permethrin; CH: 10 µg/L Coumarin 47; CL: 1 µg/L Coumarin 47; SC: Solvent Control (DMSO 0.01%).

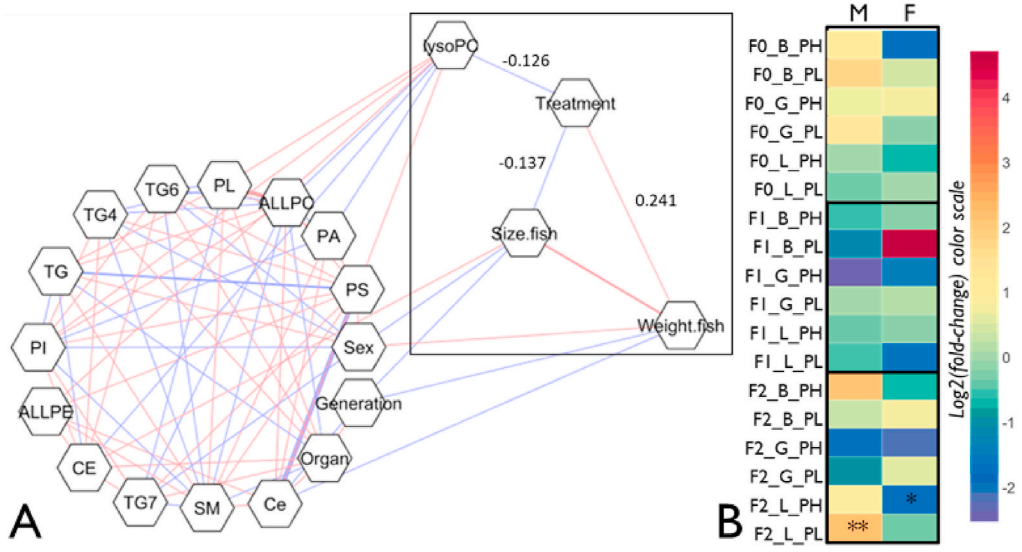

Fig. 4. A. basic partial correlation results based on lipidomic and morphometric data from PH, PL and SC treatments. Squared: zoom on features significantly correlated with treatment concentration. As shown, treatment concentration (PH, PL, SC) is negatively correlated with concentration in lysoPC (ρ=−0.126; p=0.021; padj=0.057), and positively correlated with fish weight (ρ=0.241; p<0.0001; padj<0.0001) and length (ρ=−0.137; p=0.012; padj=0.04). B. heatmap displaying log2(fold-change) in lysoPC concentrations in male (M) and female (F), in the different tissues (brain=B, liver=L, gonads=G), generations (F0, F1, F2) and treatment (PH, PL) relative to the respective solvent controls. *:p≤0.05, **:p≤0.01 (taken from ANOVA).

effects over generations. To begin with, PL –but not PH, influenced the fertility of F0 fish. Despite the absence of bioconcentration data in the present study, previous work showed no significant difference in bio-accumulative potential of permethrin between both concentrations (Tu et al., 2014). This indicates that reproductive toxicity of permethrin is due to non-monotonic action rather than reflecting a higher permethrin uptake in the low exposure scenario. A decrease in egg production and fertility in zebrafish at low compared to higher concentrations was previously reported for ED chemicals (Chen et al., 2017). Permethrin possesses acknowledged ED properties and can impair reproductive health in adult human males (Brander et al., 2016; Saillenfait et al., 2015, 2016). In zebrafish, it can affect endocrine signaling, including sex hormones at similar concentrations as used in the present study (Zhang et al., 2017). These results suggest that early-life development is a critical window for exposure to permethrin and is sufficient to elicit adverse effects later in life, as observed for other pesticides (Sturza et al., 2016; Vaiserman, 2014). Besides, the correlation of permethrin treatment with fish growth over generations may also be a consequence of ED (Wang et al., 2018), however, this result was not confirmed in ANOVA, possibly due to a limited number of replicates and complex factor

interactions.

As the most pronounced effect of permethrin in this study, we reported a transgenerational neurobehavioral phenotype in F1 and F2 zebrafish after early-life exposure of F0 to PH (Norouzitallab et al., 2019; Skinner et al., 2008). F1 and F2 larvae were less active than controls and there were strong similarities across the two tested concentrations. Interestingly, F0 larvae did not display any behavioral alteration, likely due to the short-time exposure window of 4 days. Nonetheless, the nervous system is extremely sensitive to external factors such as chemical exposure during embryonic development, which can influence behavior later on (Saito et al., 2019). Epigenetic changes were also proposed as a mechanism to explain the development of delayed neurodevelopmental disorders (Tran and Miyake, 2017). Therefore, it is possible that permethrin induced epigenetic changes in F0 primordial germ cells that were inherited in F1 and F2 descendants and were responsible for behavioral alterations (Head, 2019). Reduced locomotor activity was observed previously after exposure to a model neurotoxicant, Trimethyltin chloride, which triggers neuronal degeneration (Chen et al., 2011). Effects were stronger in periods of light, as observed in F1 PH larvae, and this may relate to increased alteration of visual circuits or overall heightened predatory avoidance response (Chen et al., 2011). However, it is also possible that the hypoactive behavior of F1 and F2 larvae is an adaptive mechanism inherited from F0 to counteract the effects of permethrin on hyperactivation of voltage-gated sodium channels as observed under acute exposure (Silver et al., 2014). In this regard, the environmental "mismatch hypothesis", described as a conflict between early reprogramming by the chemical and later adult environment, appears as an interesting research perspective in the case of transgenerational exposures (Nederhof and Schmidt, 2012; Schmidt, 2011). Further investigation of adult behavior and molecular changes in the brain will give important clues to identify the neuronal mechanisms involved.

Finally, previous research suggested that lipid metabolism is a key-pathway in permethrin toxicity that could be associated with neurotoxic effects in mammalian models (Liang et al., 2020; Xiao et al., 2015). Compared to previous studies investigating effects of direct exposures (Liang et al., 2020; Xiao et al., 2015), the effect of permethrin on lipid profiles in zebrafish brain were limited. This may be due to recovery following the delay between the end of exposure and the sampling, or higher concentrations may be required to trigger major effects. Nonetheless, we reported a significant correlation of permethrin exposure with lysoPC content. LysoPC are pro-inflammatory molecules. Their dysregulation appears critical in the development of cardiovascular or neurodegenerative diseases (Law et al., 2019; Semba, 2020) and there is evidence that early-life exposure to permethrin can affect both (Carloni et al., 2012; Dhivya Vadhana et al., 2013). Therefore, alterations in lysoPC content may be an important actor that may trigger behavioral defects *via* neurodegeneration, which supports effects observed by Chen et al. (2011). The effects were organ-, generation- and sex-specific. Differences between males and females were observed previously following permethrin exposure in mammals and may be due to fundamental differences in endocrine systems (Bordoni et al., 2015; Imanishi et al., 2013). As lipids were quantified in adult organs and behavior analyzed in larvae, further investigations should include additional measurements at both stages to better evaluate the impact of (sex-specific) lysoPC dysregulation in the observed adverse outcomes. This would help overcoming the limited agreement between correlation and ANOVA analyses reported here.

Chemical analysis revealed lower concentrations of permethrin in water than expected. A few studies reported similar compound loss overtime and attributed it to adsorption to glass or plastic containers (Tu et al., 2014, 2016; Wheelock et al., 2005). In addition, the average half-life time range for degradation of permethrin to non-toxic metabolites in water is between 19 and 27 h (Holmstead et al., 1978; Imgrund, 2003; Toynton et al., 2009). Therefore, the applied semi-static test design may not be sufficient to compensate for compound loss and lead to lower exposure concentrations. However, water samples were taken before daily renewal, thus, chemical analysis results may underestimate permethrin concentration. In addition, biological effects observed in the preliminary study confirmed previous results that were expected in the range of nominal concentrations (DeMicco et al., 2010; Zhang et al., 2017). This result suggests that fish were exposed to expected concentrations and that compound loss may be due to sampling, storage, transport and/or analysis.

Finally, investigations on larval behavior in the F3 generation led to unexpected results for all treatments. Especially, unexpected was that CH and CL F3 larvae displayed sporadic hyperactivity whereas F1 and F2 larvae behavior was similar to that of controls. In fact, according to the study design, we would expect similar changes in F2 and F3 individuals since both generations were completely free from exposure (Head, 2019; Skinner, 2008). The appearance of a treatment-related effect from the F3 generation in zebrafish (or from the F4 in mammals because of *in utero* embryonic development) has never been described before. However, there is, to our knowledge, no study investigating the effects in F3 when they were null in F2. One experimental limitation of this study is the use of one tank per treatment, which may have favored between-tanks sporadic differences. Two SC tanks were bred in both F0 and F1 generation to control for systematic between-tanks variability. No difference in behavior between F1 and F2 larvae from these tanks was observed supporting the treatment specificity of the results in these generations (data not shown); however, causes underlying effects appearing in the F3 generation remain unidentified. These results point out that organisms may be submitted to accumulating molecular changes over generations and/or may be able to trigger adaptive mechanisms to reset their physiological state after a certain number of generations (Beck et al., 2017).

In short, the results described above show that low concentrations of permethrin can elicit transgenerational changes in zebrafish behavior that may relate to neurodegeneration, whereas no such effect was observed for coumarin 47. This emphasizes the long-term consequences that exposure to certain endocrine disrupters can have on health later in life, especially when exposed during the critical - albeit short, window of embryonic development.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111348.

Author contribution

Mélanie Blanc: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. Bettie Cormier: Investigation. Tuulia Hyötyläinen: Formal analysis, Writing - review & editing. Martin Krauss: Investigation, Formal analysis, Writing - review & editing. Nikolai Scherbak: Writing - review & editing. Xavier Cousin: Methodology, Writing - review & editing Steffen Keiter: Conceptualization, Methodology, Funding acquisition, Writing - review & editing.

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