Delayed effects of permethrin exposure on personality traits and (epi)genetic associated mechanisms in the mangrove rivulus, *Kryptolebias marmoratus*

MASTER'S THESIS

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August 2023

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Acknowledgments

First of all, I would like to express my gratitude to my Promotor, Frederic Silvestre, for guiding me all through my Master's Thesis, and being so involved and available in order to seeing me, as well as his other Master's students, progressing and succeeding.

I am also thankful to my supervisor, Anthony Mathiron, for his advice and above all, his patience. It also includes Pauline Witsel and Jérôme Lambert, who helped me improving my technical skills on the lab and, in an enthusiastic and encouraging atmosphere. I feel grateful to have received their precious help for my qPCR and Pyrosequencing analysis.

Thanks also to the rest of the LEAP members for the friendly and professional atmosphere I was working with.

I am sending a huge thanks to the other co-students for their support and the pleasant happy hours and lunchtimes we spent together.

Thanks to my friends Charlotte Wouters and Mathilde Hames, with whom we passed through this "University" step of life together, and without whom all of this won't have been possible. Thanks to a series of other good friends for their emotional support.

Above all, I would like to thank sincerely my mother, my aunts, and my brothers, for their encouragement and their trust in me and my capacities.

Abstract

While early life stages (ELS) constitute a crucial period for behavioural individualities' establishment and maintenance, they are also recognized to be sensitive to neurotoxicants such as permethrin (PM). PM neurotoxicity is particularly harmful to fish species and is sometimes manifested as neurobehavioural changes. Yet, the underlying epigenetic contribution to PM persistent effects on behaviour and personality's ontogeny on fishes, remain enigmatic.

The main scope of this review is to investigate PM-related behavioural delayed effects on the self-fertilizing *Kryptolebias marmoratus*, and to identify possible epigenetic related molecular processes. To achieve this goal, we subjected mangrove killifish larvae to a one-week exposure period (from 0 to 7 days post-hatching) under three distinct conditions: absence of PM (0µg/L), low concentration (5 µg/L), and high concentration (200 µg/L). We first explored the impact of exposure on phenotypic attributes encompassing behaviour through the implementation of a shelter test. Subsequently, we evaluated the repercussions on a molecular scale, specifically in terms of relative gene expression and the methylation status of select genes, mainly behaviour related.

After a larval exposure to a high PM concentration, adults' mangrove rivulus exposed to the high PM concentration (200 µg/L), significantly exhibited repeatable behaviours. No other consistent phenotypical differences, either behaviour or life history-trait related endpoints, have been detected. Furthermore, no effect on DNA methylation or relative expression of genes, either related to neurological functions (Nipbl, Mecp2) or involved in DNA methylation (Dnmt3a), has been highlighted. However, a slight tendency of growth compensation might exist following a one-week-200 µg/L permethrin exposure, leading to recovery from the observed decreases in growth, length, and weight in 7-day-old post-hatching larvae. The non-persistent larval neurobehavioural alterations, tend also to demonstrate recovery mechanisms that could be based on neurogenesis.

Overall, this study suggests that PM might trigger the emergence of behavioural individualities (repeatability) on adults *Kryptolebias marmoratus*. Moreover, it shows the high resilience capacity of this neotropical fish model, both at the neurobehavioural and morphological levels, following exposure to a neurotoxicant.

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Foreword

The ensuing composition is structured as a scientific article, encompassing distinct sections including an introduction, materials and methods, results, discussion, and conclusion. Supplementary to the article, a comprehensive array of supplementary data (numbered from S1 to S15) is presented in the appendices. The initial section of these appendices serves to enhance comprehension of the experimental design, particularly pertaining to molecular investigations (from the Supplementary data S1 to S8). Furthermore, supplementary details on the conducted statistical analyses, are also provided (Supplementary data S9 to S10). Additional graphical representations are also included in the appendices (Supplementary data S11), as well as detailed results from the pyrosequencing assay (Supplementary data S12). Conclusively, the subsequent segment of the appendices mainly contains a theoretical summary of the pyrethroid pesticides (Supplementary data S13), followed by a specific section about permethrin (Supplementary data S14). Lastly, fish model specie's ecology, physiology and particularities are regrouped in the last supplementary data (S15).

1. Introduction

Behavioural ecotoxicology is a recent and promising discipline for investigating animal health and the status of environment (Cabrera *et al.,* 2021; Hellou 2011). It has resulted into the behaviours' description of numerous types of organisms including mammals, insects, and fishes (Cabrera *et al.,* 2021). To this day, the definition of behaviour has been much debated within the scientific community and there is still no consensus. It has been proposed that behaviour "is the internally coordinated responses (actions or inactions) of whole living organisms (individuals or groups) to internal ad/or external stimuli, excluding responses more easily understood as developmental changes" (Levitis *et al.*, 2009). In contrast, personality is defined as a consistent individual behaviour over time and across contexts (Cabrera *et al.,* 2021). It is thus referring to a variation of behavioural types (BTs) between conspecifics and includes all the stages of life (Edenbrow & Croft, 2012). Therefore, the ontogeny of personality remains generally poorly understood. Although it seems that genetic and environmental components participate to the establishment and maintenance of personality, it is thus not clear to what extent their contribution (Trillmich *et al.,* 2020). On one hand, BTs are documented to be genetic-based and/or partly hereditary (Li *et al.,* 2020; Mazué *et al.,* 2015). On another hand, environment, especially during the early life stages (ELS), has been reported to be another determinant factor for shaping personality. As proposed by Carion and colleagues, BTs establishment might be profoundly influenced by the chemical environmental (i.e., environmental stressor) (Carion *et al.,* 2020). The exact period in which the personality develops is not either known, but it seems that these ELS are a crucial time for the emergence of individual behavioural variation. In behavioural ecology, this latter has been described thanks to five main axes[1], one of which is called the boldness-shyness continuum, estimating the tendency of individuals to take risks (Mazué *et al.,* 2015). This latter hence mirrors an individual's ability to deal with its environment, making the boldness-shyness traits ecologically relevant. Since it helps determining organism's reaction to the presence of a chemical stress[2] (i.e., presence of a pesticide in the natural habitat), studying more closely these personality traits are of great help in the field of ecotoxicology (Charles *et al.,* 2022; Li *et al.,* 2020).

[1] Being shyness–boldness, exploration–avoidance, activity, aggressiveness, and sociability (Mazué *et al.,* 2015)

[2] Stress can take a variety of forms (chemical, social, etc.) and involves the hypothalamic-pituitary-adrenal (HPA) axis. An intense or prolonged stress can have deleterious effects on an organism's homeostasis (Sánchez-Lafuente *et al.,* 2022).

Since the brain is a central organ that is associated with personality traits, it is even more helpful in the context of assigning behavioural toxicology of neurotoxic compounds (Chudler, 2022).

Among these neurotoxic xenobiotics is permethrin (PM); a pesticide massively used to control pest species in a wide range of fields (i.e., agricultural, industrial, and domestic domains). PM has been related to high neurotoxicity towards wild fish communities (Moniruzzaman *et al.,* 2020; Zhu *et al.,* 2020). Being highly lipophilic and difficult to metabolize by fish, PM tend to bioretain, especially in fatty organs such as the brain, where its mode of action is mainly applied (Zhu *et al.,* 2020).

This leads to deleterious neurobehavioral effects in various fish species, such as depressive-like behaviour (Blanc *et al.,* 2021) and negative thigmotaxis[3](Zhu *et al.,* 2020). Since the brain is particularly vulnerable during the developmental stages of an organism, neurobehavioural side effects due to PM exposure might be dramatic and disrupt neurodevelopment (Carion *et al.,* 2020; Mathiron *et al.*,2023). However, adverse effects, especially delayed ones, induced by an early PM exposure are insufficiently documented on fish species (Blanc *et al.,* 2021). Therefore, it is crucial to understand the mechanisms underlying early PM exposure toxicity for performing a proper risk assessment and ensure a sustainable development of agrochemicals (Voisin *et al.,* 2021; Zhu *et al.,* 2020).

To date, behavioural changes due to an early PM exposure have been suggested to be supported by epigenetic mechanisms (Lindeman *et al.,* 2021; Verhulst *et al.,* 2016; Voisin *et al.,* 2021). Holliday defines epigenetic mechanisms as "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, 2006). An increasing appreciation for a specific epigenetic modification, being DNA methylation, is observed in behavioural ecotoxicology. This DNA modification consists of adding a methyl group on cytosines, mediated by DNA methyltransferases (i.e., Dnmt3) and demethylating enzymes. During development, a series of methylation/demethylation events takes place, illustrating the dynamic character of DNA methylation during prenatal life (Lindeman *et al.,* 2021). Cytosines methylation is associated to an onset of developmental processes including cellular differentiation (Dorts *et al.,* 2016).

[3] Thigmotaxis is a behavioural endpoint typically used as a proxy for estimating fish 'anxiety. It can thus be measured in a shelter test by calculating the proportion of time spent to "'*wall hugging*" (Fontana & Parker, 2022).

The pivotal role of DNA methylation during embryonic stages, in addition to methylome's plasticity, makes this type of epigenetic mark potentially sensitive to xenobiotics at that critical period (Lindeman *et al.*, 2021; Voisin *et al.*, 2021). Moreover, epigenetics might be linked to individual behavioural changes, but information is lacking (Edenbrow & Croft, 2011; Edenbrow & Croft, 2012; Verhulst *et al.*, 2016). Since the brain is reported to be highly methylated and contains many epigenetic actors (i.e., Mecp2), this structure may be particularly vulnerable to PM (Sánchez-Lafuente *et al.*, 2022; Voisin *et al.*, 2021). A growing body of evidence converges towards the hypothesis that PM is indeed able to provoke methylation changes in this structure, being potentially persistent during the life of an organism (Blanc *et al.*, 2021; Voisin *et al.*, 2021). Consequently, it could lead to delayed and/or long-lasting phenotypical effects including behavioural impairments. The association of methylation patterns modifications with neurobehavioural changes (i.e., depressive-like behaviour) has already been point up on adults *Danio rerio* exposed to a 3-week-larval PM exposure (Blanc *et al.*, 2021).

It is necessary to clarify that the interaction between the above mentioned genetic and epigenetic processes is postulated to be much complex than that (Voisin *et al.*, 2021).

Currently, delayed effects on boldness-shyness continuum, genome and methylome, are however poorly documented on aquatic organisms (Sánchez-Lafuente *et al.*, 2022; Verhulst *et al.*, 2016). Since biological levels are closely interconnected, it is essential to identify the toxicity of PM at both molecular and phenotypical scales. On one hand, studying molecular toxicity of a compound is beneficial for understanding processes that may be responsible for the emergence of phenotypical impairments (Erofeeva, 2022).

On another hand, the study of behavioural response is ecologically relevant when investigating the neurotoxicity of a compound, since it reflects the interaction between a living being and its environment. Moreover, it mirrors the integration of external stimuli by the central nervous system (Carion *et al.*, 2020; Hellou, 2011). Behavioural modifications could subsequently impact survival, access to resources, and/or social interactions, that can potentially affect an organism's fitness (Carion *et al.*, 2020; Holtmann *et al.*, 2016). Individual' fitness is a key parameter in ecotoxicology since it determines organism's fate in its natural environment and may change population endpoints such as population density (Erofeeva, 2022). To adequately evaluate the fitness of fishes, other biological endpoints such as growth can be monitored. A diminution of growth and ultimately length, might have negative repercussions on fish' reproductive success and survival as well (Carion *et al.*, 2020; Mathiron *et al.*, 2023).

In addition, in view of the correlation between life history traits and behaviour (i.e., boldness and growth), it is consistent to quantify growth for preventing the presence of confounding factors (i.e., PM exposure and growth) in the current study (Edenbrow & Croft, 2011; Mathiron *et al.*, 2023). Lastly, fecundity related parameter (i.e., probability of laying eggs), is also a useful tool for estimating the fitness of an individual because this decrease of fecundity could compromise an organism's reproductive fitness (Gresham, 2020).

In ecotoxicology, isolating the effect of the environment on the epigenome requires minimizing genetic variability between individuals (Chapelle, 2023). In that perspective, a unique and promising model specie, *Kryptolebias marmoratus*, is of great interest. Also named mangrove killifish, this is one of the two only known clonal vertebrates which naturally generate offsprings with very low interindividual genetic variability. Wild populations are thus mainly composed of hermaphroditic individuals, naturally capable of internal self-fertilization. Recurrent self-fertilization events from the same individual can lead to the formation of an isogenic population. Composed of homozygous individuals, the use of isogenic populations in ecotoxicological studies helps reducing the confounding factor of genetic variability, guaranteeing a certain degree of experimental standardization (Choi *et al.,* 2020; Tatarenkov *et al.,* 2010; Voisin *et al.,* 2021). Scientists 'fascination towards the mangrove killifish is also explained by the outstanding phenotypic plasticity exhibited by a population made of genetically identical individuals (clonemates) (Choi *et al.,* 2020). It has then ensured the specie 'survival over generations despite its genome homogenization. Investigating the phenotypic and (epi)genetic reactions to an environmental stressor, during ELS, could then provide insights about how they counteract genetic homogenization under selection pressure (Bierbach *et al.,* 2017; Choi *et al.,* 2020; Voisin *et al.,* 2021). Moreover, the broad tolerance to environmental conditions, including pollutants 'concentration, also makes this inshore-dwelling organism incredibly interesting to use in this experiment (Choi *et al.,* 2020; Edenbrow & Croft., 2011; Puthumana *et al.,* 2017).

The current study aims mainly to determine (a) how the chemical environmental (i.e., PM concentration) during development may influence the expression and methylation level of a series of genes, mainly implied in neurodevelopment or behaviour, in the brain later at the adulthood, as well as on behavioural individualities and their repeatability[4] (b) to what extent

[4] Repeatability reflects the part of behavioural variation that is. A repeatable behaviour varies significantly between individuals but tend to stay identical over time (Bell *et al.,* 2009).

genetic and epigenetic contribution to personality traits' variation, as well as to environmentally induced life-history traits alterations.

In that perspective, PM delayed effects were investigated on boldness-shyness continuum in adults after an early exposure to PM (0 µg/L, 5 µg/L or 200 µg/L, respectively named "C" group, "L" group and "H" group) during 7 days at juvenile stage. The delayed effects on several life-history traits (mortality-survival, growth, size, weight, and fecundity) were also explored as well as potential (epi)genetic associated mechanisms. The relative genetic expression and methylation level of several genes of interests have been thus investigated via qPCR and pyrosequencing assays. These genes were implied in methylation modification (i.e., Dnmt-3a and Dnmt-3ab), growth regulation (i.e., Igf2-b) and the etiology of behaviour (i.e., DRD4) (DeCourten *et al.*, 2020; Lindeman *et al.*, 2021; Munafò *et al.*, 2008; Verhulst *et al.*, 2016) (See Materials and Methods for additional information on the genes and their full names). Since antioxidant mechanisms are crucial for counteracting pyrethroids 'toxicity, a gene related to redox homeostasis, GSS, was also included in the list of genes 'candidates (Choi *et al.*, 2020). Oxidative imbalance as well as a reduction of growth due to PM has already been documented (Mathiron *et al.*, submitted article). Genes implied in neurobehavioural impairments when dysregulated (Igf2-b, MAOA, Mecp2, Tollip), were also quantified (Connolly et Zhou, 2019; Li *et al.*, 2020; Moniruzzaman *et al.*, 2020; Sélénou *et al.*, 2022). Lastly, a gene related to genome stability (Nipbl) was also included. Nipbl methylation seems to be a plausible target of another EDC's mode of action (EE2), which makes this gene consistent to study (Gao *et al.*, 2019; Voisin *et al.*, 2020).

2. Material and methods

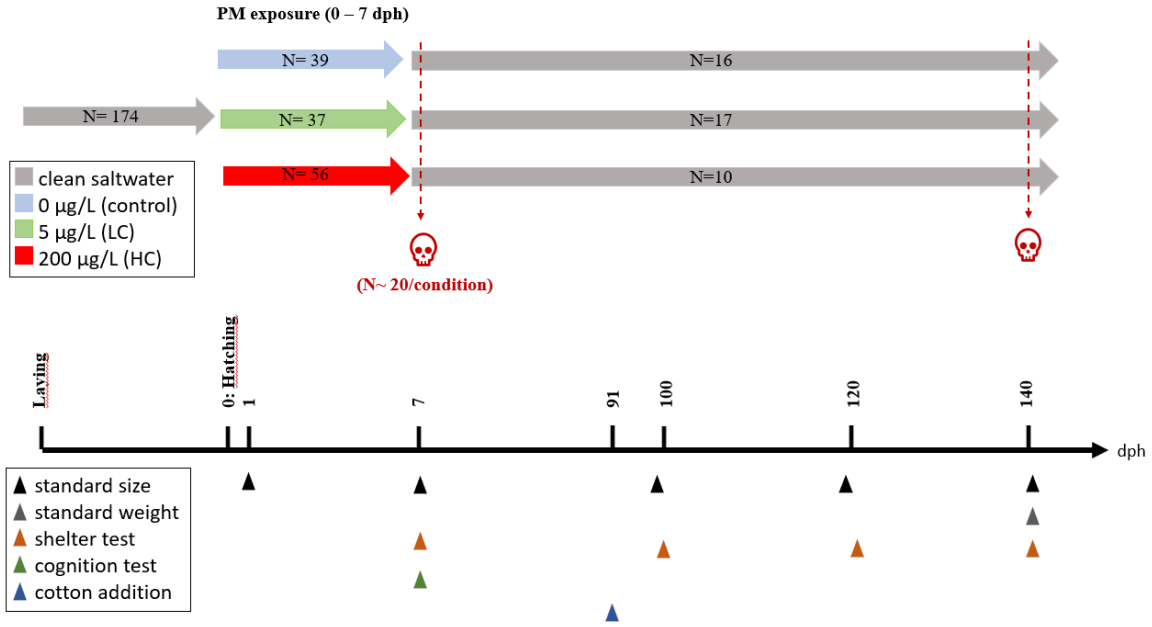

Figure 1: General experimental design of the study that was achieved first by Isabelle Chevalme until J80[5]. Then, I carried out the rest of the experiment (euthanasia and molecular analysis included). Eggs were collected from hermaphrodite *Kryptolebias marmoratus* belonging to the EPP lineage and placed in regularly renewed saltwater. As soon as they hatched (0 dph), individuals were exposed until 7 dph, to one of the three following PM treatments (0 µg/L (control 'C'), 5 µg/L (low concentration 'L') or 200 µg/L (high concentration 'H'). Standard length and weight were monitored at various time points (at 7 and 140 dph for body weight, and at 1, 7, 99, 119 and 140 dph for body size) for studying the immediate and delayed effects of PM exposure on those phenotypic traits. Parameters related to fecundity were monitored since 91 dph (addition of cotton), when the subjects are sexually mature. The fish were checked two times a week for calculating the number of eggs laid and for tracking mortality and survival. To test immediate and/or delayed effects of PM exposure on cognition and personality traits, mobility and thigmotaxis, cognition test was performed at 7dph, and shelter tests were conducted at 7, 100, 120 and 140dph. Molecular analysis was carried out on 7 dph-old juveniles (~ 20/condition) and 140 dph-old adults.

[5] It includes eggs collection and development, PM exposure, fish' breeding, standard length, and weight measurements (at 1 dph and/or 7 dph), mortality and survival monitoring, as well as shelter and cognition test at 7 dph.

2.1. Ethic statement

The project (20 365 KE) has been approved by the Belgian Ethics Committee in animal experimentation. The agreement number of the laboratory for fish experiments is LA1900048. All *Kryptolebias marmoratus* were reared in the respect of the ethic legislation.

2.2. Fish' collection and breeding

All fish were obtained from hermaphrodites of the EPP lineage that was initially collected by Valentine Chapelle and Frédéric Silvestre in 2019 at Emerson Point Preserve in the Florida Keys (N27°53'29.80'', W82°62'55.01''). The sampled organisms were then transported to Frederic Silvestre's laboratory in the Biology Department of University of Namur and acclimated. They were raised individually in 500 mL capacity plastic container filled with 200 mL of 12 ± 1 parts per thousand (ppt) reconstituted saltwater (Instant Ocean™ sea salt) and housed in climate room at 26 ± 1 °C and 12:12 light: dark. Individuals were fed everyday *ad libitum* with living *Artemia salina.*

2.3. Eggs collection and development

Eggs were collected from hermaphrodite individuals (N=57) in February 2022. They were individually maintained in cell culture (Cellstar®), 24-well plates, filled with 2mL saltwater (12 ± 1 ppt) and maintained at 27 ± 1 °C, 12:12 light: dark until hatching (i.e., 15-30 days post-fertilization). To provide stable conditions, water was renewed every 3-4 days.

Thanks to a technique developed by Ivan Blanco (PhD at UNamur), it was possible to induce synchronized eggs' hatching. To do so, the microplates containing eggs were incubated in Eiplock bags already containing an Anaerocult™ P sachet (purchased from Merck, Inc.). This sachet is a microbiological reagent that chemically bind to oxygen and creates an anaerobic space that have been shown to favor hatching in *K. marmoratus* (Wells *et al.*, 2015).

2.4. PM exposure

In order to investigate the delayed effects of a PM early exposure on personality traits and associated molecular mechanisms in *K.marmoratus*, newly hatched individuals were placed in 12-well plates (4mL) and exposed during 7 days to one of the following treatments: control (0µg/L of PM; N=39), low concentration (5µg/L of PM; N=37) and high concentration (200 µg/L of PM; N=56).

While the low concentration tends to reflect environmental detected concentrations in mangrove killifish's habitat, especially in the Florida Keys (State of California, USA) (Nillos *et al.*, 2010; Tang *et al.*, 2018), the high concentration of PM has been chosen to increase visible effects of PM exposure. Previous experiment has shown that exposure to 200µg/L of PM did not affect survival of mangrove rivulus from the DC4 lineage (UNamur, unpublished data). The PM exposure duration has been fixed at one week as it was estimated to be sufficient for inducing (epi)genetic or phenotypic effects. Since PM is a persistent molecule in the environment, such an exposure time also permits to highlight the relevant compound 'toxicity towards the model specie (US EPA, 2009).

PM stock solutions were prepared in 99% Dimethyl Sulfoxide (DMSO) to increase water solubility of lipophilic neurotoxic. PM working solutions were prepared with Instant Ocean™ sea salt (12 ± 1ppt). Whatever the treatment, the final DMSO's concentration was 0.1%.
Water was fully renewed every day during PM exposure to keep treatment conditions as constant as possible. Real PM concentrations of working solutions (nominal 200 µg/L and 5µg/L) were measured *a posteriori* following a High Performance Liquid Chromatograph (HPLC) method (additional information in Supplementary data S1). Mean ± SE measured concentrations in PM working solutions for individuals exposed to high concentration were 147 ± 4 µg/L (N=8). However, values from 5µg/L nominal PM working solutions (low concentration treatment) were below the detection limit of the method.

2.5. Delayed effects on boldness-shyness personality traits, activity and thigmotaxis

To explore the delayed effect of early PM exposure on fish personality traits, mobility and thigmotaxis, a "shelter test" system was used. This latter consists in an apparatus composed of an open arena filled with 1.5 L of water (12 ±1 ppt; 27±1°C) and an opaque plastic door that is used for blocking the shelter entrance (see **Figure 2**). Individuals were introduced in an opaque tube (the shelter part) covered with a lid and maintained during 10 minutes of acclimation, video was launched, and the door was removed. Fish could then circulate in the shelter part as well as in the open space (the arena). Three repetitions were done for each individual at adult stage, respectively at 100, 120 and 140 dph. Tests were performed preferably in the morning, between 9 AM and 1 PM. The measured parameters include the total distance moved, the cumulative duration in the arena (intern and extern) and the first latency to the extern and the inter parts of the arena.

The activity (total distance moved corrected by the length measured the day before the test and the total time spent in the arena (relative TDM)), the thigmotaxis (cumulative time spent in the intern part of the arena, divided by the cumulative time spent in the entire arena (ratio I/A)) and boldness/shyness (the cumulative time spent in the shelter and the first latency in the arena) were estimated thanks to the software Noldus Ethovision XT™ 15.

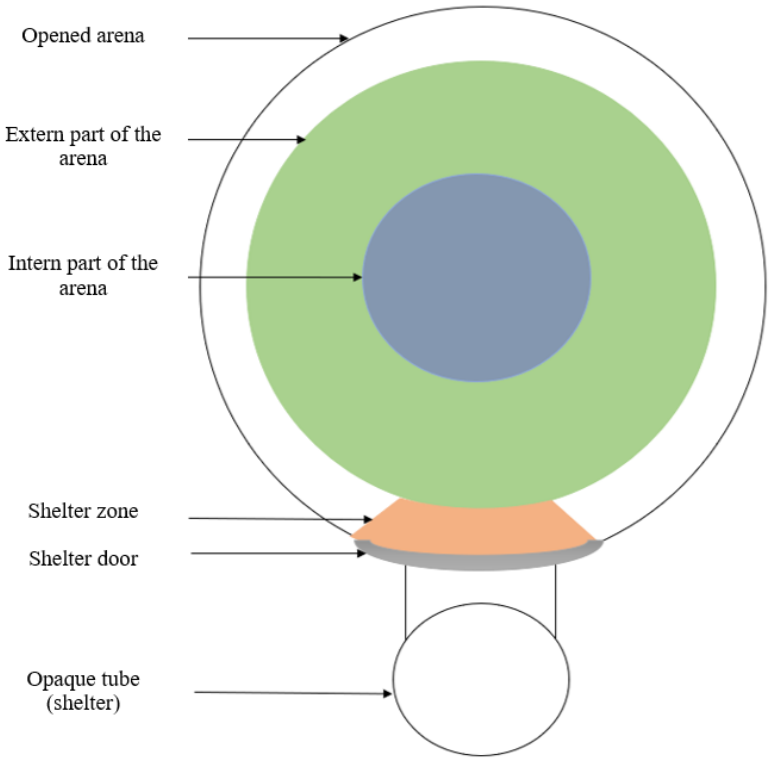

Figure 2: Schematic representation of the different compartments of the shelter test, being associated to a specific colour. This behavioural test mainly helps estimating boldness-shyness continuum of fish. It is divided into two main parts: the arena and the shelter, that are interconnected thanks to a removable plaque.

Delayed effects on life history traits

To investigate potential delayed effect of early PM exposure on life-history traits, *K. marmoratus*' size was first measured (i.e., from snout to tail). The day preceding the first and second repetition of shelter test (i.e., 99 and 119 dph). To do so picture of individual disposed in a petri dish filled with water were taken with a One Plus Nord cell phone. Photos were then processed and analysed with the software ImageJ ®.

The last measure of size occurred at 140 dph after fish euthanasia (see below). The body mass was also calculated at the same moment thanks to a laboratory balance.

Moreover, to determine delayed effects of early PM exposure on reproduction, a piece of cotton was added to each tank at 91 dph to allow fish to lay eggs. Number of eggs laying was quantified twice a week until the end of experiment (140 dph). Lastly, individual mortality was noted during all procedures for investigating survival and mortality among each treatment.

2.6. Molecular analysis (qPCR and pyrosequencing)

For information, detailed preparation of the various mastermix (MM) used either for the qPCR or pyrosequencing assays, are regrouped in Supplementary data S2.

Euthanasia

Fish were sacrificed in the afternoon, after the last shelter test (i.e.,140 dph). An overconcentration (150µg/L) of the anaesthetic, Tricaine methanesulfonate (MS222), was used for euthanasia. MS222 acts on the nervous system by blocking sodium ions channels and leads to nerves influx inhibition and immobility. Opercular movement are stopped causing finally hypoxia and death (Ferreira *et al.*, 2022). The next step may be referred to the previous point, which was size and weight measures. After that, the fish was decapitated using a blade. The animal head was then fixed and observed with a binocular to continue the procedure. Then, skull's opening allowed brain extraction with the help of dissection tools. Brains were directly placed in annotated microtubes and put in contact with liquid nitrogen for a better preservation of the organ. Lastly, the microtubes were conserved at -80°C until molecular analysis was conducted. Material was disinfected between each dissection.

RNA and genomic DNA isolation

The RNA and genomic DNA extraction from brain's samples was ensured by following the instructions of manufacturers in the Quick-DNA/RNA Microprep Plus Kit (Zymo Kit) from Zymo Research®.

Genes of interest

The several genes of interest are:

- Monoamine oxidase A (MAOA)
- Toll interacting protein (Tollip)
- Methyl CpG binding protein 2 (Mecp2)

- Dopamine D4 Receptor (DRD4)
- Nipped-B-like protein (Nipbl)
- DNA Methyltransferases genes (Dnmt-3ab and Dnmt-3a)
- Insulin-like growth factor 2 (Igf2-b)
- Glutathione antioxidant system gene (GSS)

A brief description of their implication in fish's neurodevelopment and/or behaviour is described in the Supplementary data S3.

2.6.1. qPCR

Additional information about DNase and Reverse Transcriptase treatments, are reported in Supplementary data S4.

DNase and Reverse Transcriptase (RT) treatments

After the DNA and RNA extraction, a rigorous DNAse treatment was performed to eliminate any potential DNA contamination in RNA samples. The kit utilized in this step is named "In vitrogen ThermoFisher, DNA-free kit DNase Treatment & Removal" from Zymo Research®. DNA concentration and quality was assessed using 1% agarose gel electrophoresis. After that, a RT treatment was conducted to ensure the first strand cDNA synthesis based on RNA samples (Thermo Scientific RevertAid RT Kit®).

Samples and standards preparation

The final volume in the plate 2 is equal to 20 µL. In order to have a sufficient volume of samples for the next steps, 80 µL of nuclease-free water was put in each well (x5 dilution).

The standards 'preparation, based on a 4x dilution, were respectively diluted: 5x (Standard1), 20x (Standard 2), 80x (Standard 3), 320x (Standard 4) and 1280x (Standard 5).

Efficacity test

Before starting qPCR on genes of interests, two efficacity tests (qPCR) have been set up to select the efficient primers. The samples 'volumes were pipetted from plate 2. The volume of either sample/standard/RT-/NTC was 5µL and the identical volume of the adequate primer was loaded. Five µL of SYBR was added in each well. The forward and reverse primers 'sequences, as well as criteria used for considering a primers 'pair as efficient, are regrouped in the supplementary data S5. Design and Analysis software 2.6.0 from Thermofisher® was used for visualizing and analysing efficacity tests (as well as qPCR) results.

The selected primers are: Nipbl, Mecp2 and Dnmt3aa1. The two selected housekeeping genes are ef1a1 and B-actine.

qPCR on the selected primers

Three 384-well plates were needed for ensuring the qPCR. The samples were diluted x10 in that test due to the lack of volume. The relative gene expression (REG) per condition is represented in the Table 3.

2.6.2. Pyrosequencing

Genes sequences and samples' selection

The candidate genes for pyrosequencing were the ones that gave consistent qPCR results (for gene expression) in the preceding section. Among these, only Mecp2, Nipbl and Dnmt3aa1 were chosen because primers for these genes had been already designed for another study conducted by Valentine Chapelle and were functional (Chapelle, 2023). Primers' sequences are detailed in the Supplementary data S6. In brief, after targeting the position of the promoter region and introns within each gene thanks to NCBI, the software Methprimer 2.2. ® helped for predicting CpG islands on the genes of interests and so selecting interesting sequences for pyrosequencing. The primers' design was possible thanks to the software PyroMark Assay Design of Quiagen®.

Since three strips of eight wells each can be placed in the Pyromark Q24 Advanced (one strip / gene), eight samples were selected (2 samples for "C" and 3 for "L" and "H"). For this purpose, the concentration and quality of RNA were investigated using the Thermoscientific® Nanodrop 2000c Spectrophotometer. Among the remaining samples, shelter test results were analysed for identifying two individuals who tend to behave differently than the average (i.e., one subject who tend to be bolder and another one shyer than the others). For the "H" treatment, the third selected candidate was characterized by boldness/timidity variables that were closed to mean values. This selection allows to consider the variability present in fish within each treatment.

Bisulfite treatment

Bisulfite treatment was conducted as per the manufacturer's protocol from Zymo Research ® (EZ DNA Methylation ™kit – Streamlined bisulfite conversion of DNA) (see Supplementary Data S7).

This step was realized on the target 5'-Cytosine- phosphate -Guanine-3' dinucleotides (CpG sites), and consists of converting non-methylated cytosine into uracil, thanks to sodium bisulfite modification. It consequently generates single strand DNA whose cytosines are all methylated (Poulin *et al.,* 2018).

PCR

A PCR was performed according to the protocol PyroMark ® PCR Kit (from Quiagen®) on the desulphonated, and bisulfite-recovered samples resulted from the previous step. A 2% agarose gel electrophoresis was conducted for confirming the correct amplification of the target sequences thanks to forward and reverse primers.

Pyrosequencing

A pre-run assay has been completed on the PyroMark Q24 Advanced® software to obtain the quantity of PyroMark Advanced CpG reagents and nucleotides necessary for a future step.

PCR chain was filled with mastermix (MM3) and placed on an agitating plate for 10 minutes. Biotinylated PCR products were then loaded in the PCR chain before being vortexed for another 10 minutes to avoid streptavidin's precipitation.

Following these steps, 6 cartridge's compartments were filled with a volume of one of the Q24 PyroMark Advanced CpG reagents (E-mix and S-mix), or nucleotides (dnTP) previously calculated by PyroMark software Q24 Advanced®. Once the cartridge was placed in the PyroMark Q24 Advanced instrument, the PCR plate and the sequencing primers, previously diluted with Annealing Buffer (final concentration: 0.375 µM), were placed in the PyroMark Q24 Workstation. Ethanol (70%), denaturing solution (NaOH), Washing Buffer and MilliQ water were also deposited in this latter. The PCR product was then extracted and fixed on the Vacuum Tool without the Streptavidin Sepharose beads. After that, the aspiration tool was sequentially deposited in the different solutions during a determined time. The volumes and roles of each solution present in the PyroMark Q24 Workstation, as well as specific duration time in each compartment are specified in the Supplementary data S8. The PCR product was finally put in contact with the sequencing corresponding primer and the resulted solution was placed in the Pyromark Q24 Advanced.

Statistical analysis

Behavioural and molecular results were analysed on software R (version 4.2.2).

Data's normality was estimated with shapiro test and transformed if necessary to achieve normal distribution, using the package "bestNormalize". Linear Mixed Models (LMMs) were performed on behavioural variables with "lme4" package. Model selection method, as well as the selected models are described in Supplementary Materials S9). Generalized Linear Models (GLMs) were computed on variables with a random factor (fish) and binomial distribution, being death variable and the probability of laying. Lastly, survival analysis and curves were performed respectively via the "survdiff" and "survfit" functions (packages "survminer" and "survival").

A one-way analysis of variance ("aov" function) was achieved on growth and relative genes expression data. For information, relative genes expression results were obtained using Pfaffl method with one reference gene (beta-actine). In case significative results were obtained with a type 2 analysis of variance, a post hoc test was realized (Tukey test for LMMs and Dunn test GLMs with "emmeans" or "dunn.test" packages). Potential multicolinearity within the model was investigated based on the Variance Inflation Factor (function "vif()"). Models were then validated by verifying residuals 'distribution (and homoscedasticity, independence of residuals versus individual explanatory variables for LMMs). Concerning repeatability, rpt() function (from "rptR" package) and general ICC scores help determining the presence of personality among each treatment. Parametric bootstrapping (n =1000 bootstraps) has been computed to estimate R parameter with an alpha-error probability of 0.05 (Carion *et al.,* 2020; Edenbrow & Croft, 2012). This parameter helps determining if a group would stand out and contain fishes that are described by a non-negligible intraindividual variability. Principal components analysis (PCA) on behavioural results was realized to detect the presence or absence of loading between the four response variables previously described (function "dudi.pca" from "ade4" and "factoextra" packages). A second PCA was performed on molecular data (relative genetic expression) to describe a potential tendency of the studied genes to be up or down regulated. Correlations between response variables was assessed via a correlation matrix thanks to "Hmisc", "corrplot", and "PerformanceAnalytics" packages (see script in supplementary data S10).

3. Results

3.1. Life history traits

Mortality

A significant effect of PM exposure on *K.marmoratus* was found on mortality (ANOVA type II, GLM with binomial distribution: Chisq= 12.546, df=2, p < 0.001; **Figure 3**[6]). There is a significant difference of mortality in the "H" treatment compared to the "C" and "L" treatments (Dunn's post hoc test: p-value < 0.001). While no case of mortality is observed in the control and low dose treatments, only 60% of individuals from the 'H" group survived.

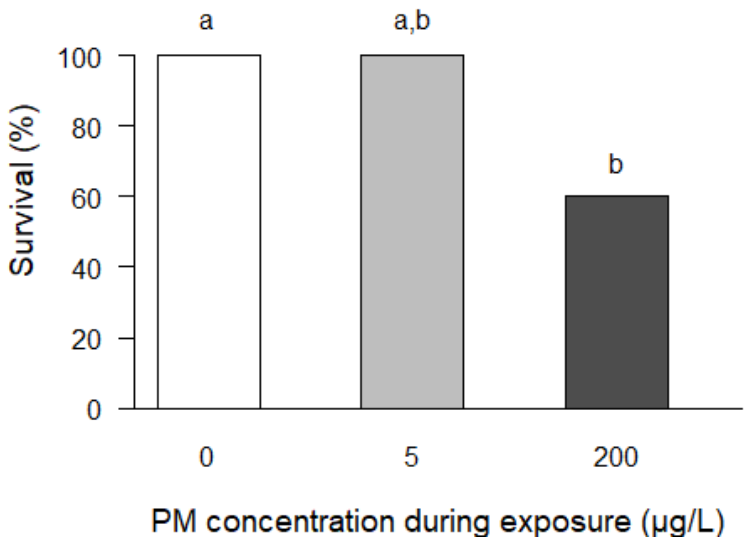

Figure 3: Delayed effects of early exposure of PM on percentage of adults' survival of *K.marmoratus* (N=16, 14 and 10 for 0, 5 and 200 µg/L, respectively) after the treatment period (0-7 dph). PM concentration during exposure (0, 5, 200) does respectively refers to the following treatments: "C", "L" and "H". Different letters indicate significant effect between treatments.

Survival

There were significant effects of PM on the survival curves among treatments (survdiff test; Chisq= 8, df=2, P < 0.05; **Figure 4**). On average, individuals exposed to the "H" treatment survive less time than the other treatments. Among the "L" and the "C" treatments, individuals survived until euthanasia.

[6] Given the 0% of mortality in two treatments, Figure 3 represents the survival percentage instead of the mortality percentage.

The survival curves for the “L” and “C” treatments are constant (survival probability = 1). The high concentration’s survival curve decreases from 91 dph until reaching a survival probability value of 0.6 (120dph).

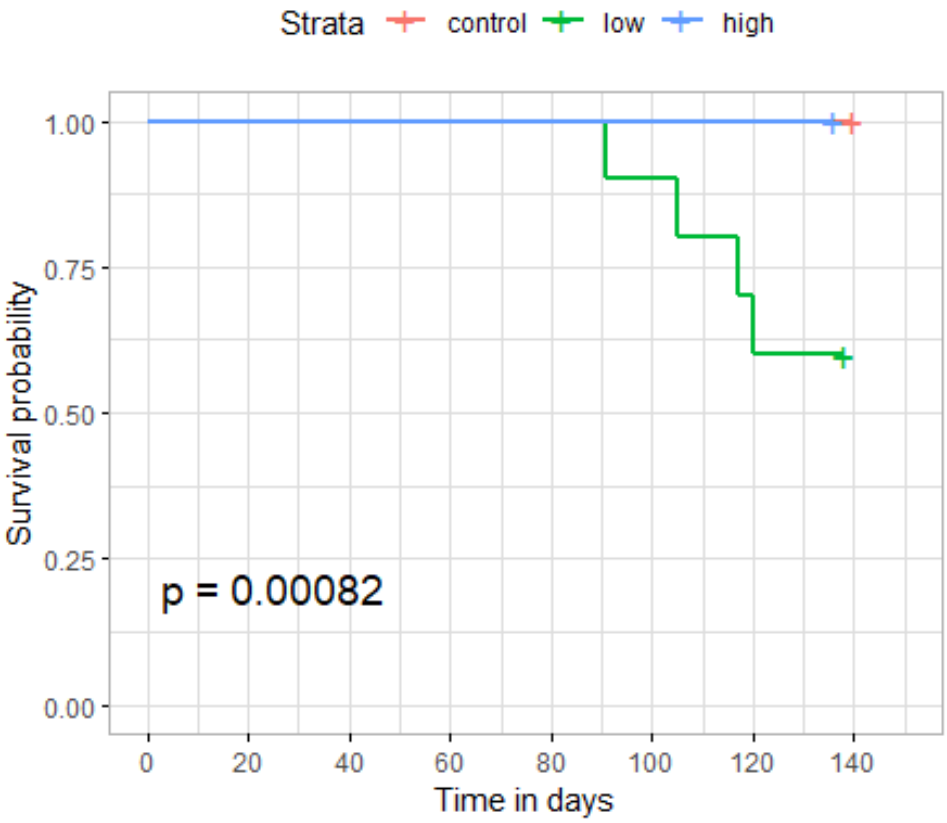

***Figure 4*:** Delayed effects of early PM exposure to three PM on *K.marmoratus*’ survival curves of individuals per treatment between 90 and 140 dph (N=16, 14 and 10 for 0, 5 and 200 µg/L, respectively). The P-value is indicated on the graph and is equal to 0.00082.

Growth

PM exposure had no notable effects on growth between 7 to 140 dph (ANOVA type II, gaussian distribution: F= 2.462, df=2, p >0.1; **Figure 5**). Mean growth is quite similar among groups.

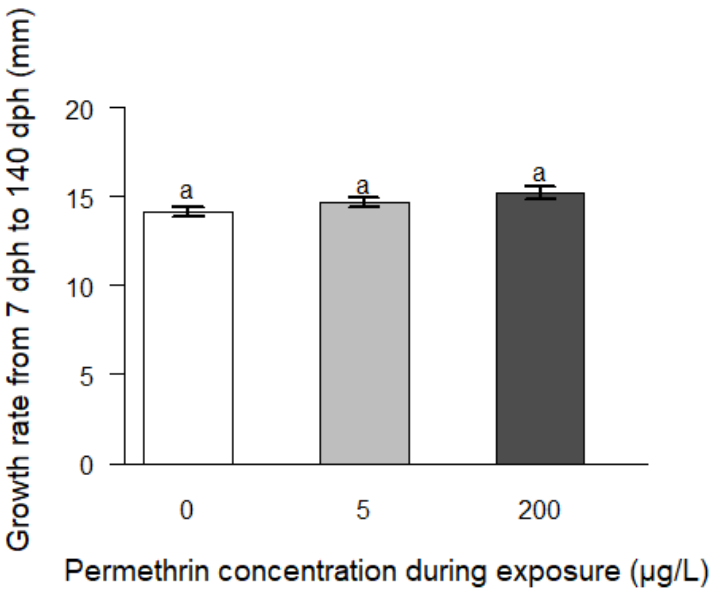

***Figure 5*:** Delayed effects of early PM exposure (N=16, 14 and 6 for 0, 5 and 200 µg/L, respectively) on adults *K.marmoratus'* mean (± SE) growth. Growth is calculated by subtracting the standard length at 140 dph, from the standard length at 7dph.

Standard length and weight

There is no significative effect of the treatment on either the standard length (F= 3.214, df=2, p >0.05) or weight (F= 3.096, df=2, P> 0.05) (ANOVA type II, gaussian distribution, **Figure 6**).

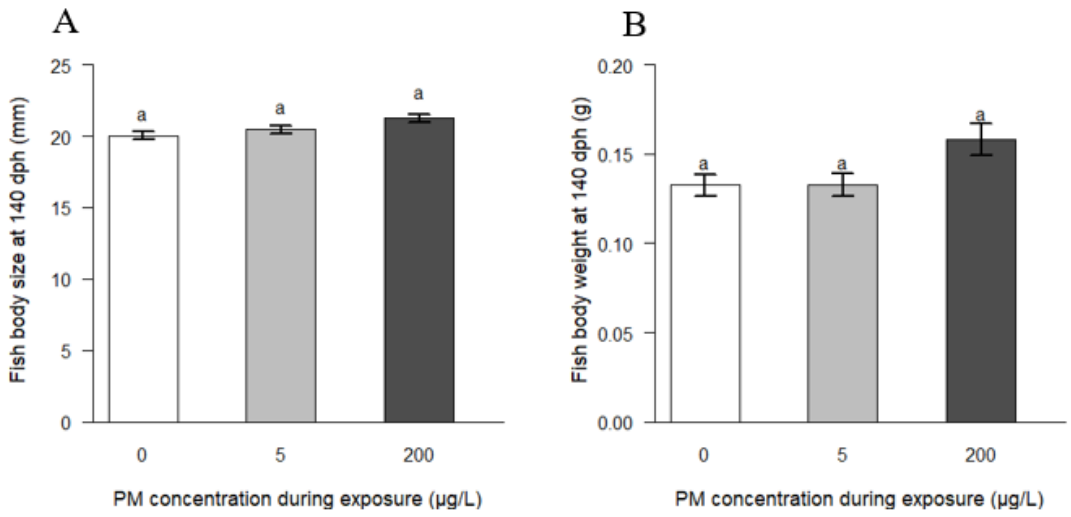

***Figure 6*:** Delayed effects of early PM exposure (N=16, 14 and 6 for 0, 5 and 200 µg/L, respectively) on adults *K.marmoratus'* mean (± SE) on A) mean (± SE) standard size B) mean (± SE) standard weight at 140 dph. Different letter indicates significant differences.

Similarly, growth, standard length and weight do not exhibit significative differences between the three groups.

Fecundity

The probability of laying eggs is not significantly affected by PM exposure (ANOVA type II, GLM with binomial distribution: Chisq= 4.895, df=2, p = 0.09; **Figure 7**). Despite the absence of statistical significative differences, fishes that were not treated with PM ("C"), laid on average half as many eggs as those exposed to 5 µg/L ("L"), and four times less than those exposed to 200 µg/L ("H").

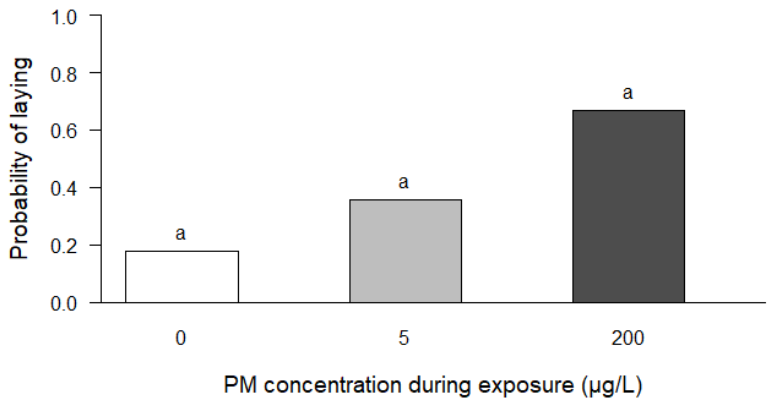

Figure 7: Delayed effects of PM exposure on the probability of laying of *K.marmoratus* (N= 17, 14 and 6 respectively in 0, 5 and 200 µg/L groups). PM concentration during exposure (0, 5, 200) does respectively refers to the following treatments: control, low concentration, and high concentration.

Among the fishes that laid eggs, three are part of the control treatment, five of the "L" group and four fishes are part of the "H" group. Consequently, the restricted number of individuals that laid egg(s) (N= 12) all treatments combined, limited further analysis to descriptive statistics.

Total eggs production

From 91 to 140 dph, fishes that were not exposed to PM laid on average less eggs (mean ± SE: 6.33 ± 3.93) than fishes exposed to a PM treatment. The total mean eggs production is higher for the "L" fishes (10 ± 3.92) compared to the "H" (7 ± 1.78).

Age at the first laying

On average, age at the first laying is earlier for individuals from the "C" treatment (132.7±1.86) than the two other treatments. The age of the first egg laying is rather similar for "L" (135.6±2.48) and "H" (136.0±0.71), being approximatively 3 days later than the "C" group.

3.2. Behavioural variables

Cumulative time spent in the shelter

The time spent in the shelter did not vary significantly between treatments (LMMs with gaussian distribution). By contrast, there was a significant effect of repetition (Chisq=7.966, df=2, $P < 0.05$; **Figure 8**) on the cumulative time in the shelter.

The Tukey test demonstrates a significative difference of the cumulative time spent in the shelter between repetitions one and two.

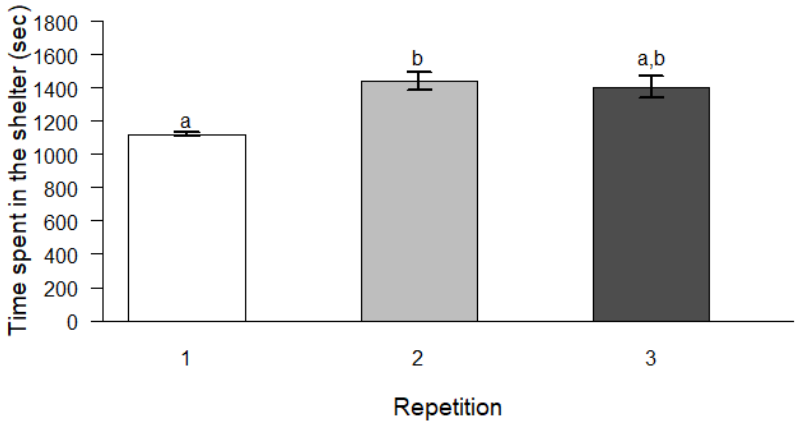

Figure 8: Impact of the repetition on the mean (± SE) cumulative time spent in the shelter by *K.marmoratus* (N=35 for each repetition). Different letters indicate significant effect between repetitions.

First latency in the arena

The first latency in the arena is not significantly impacted by PM exposure (LMMs with gaussian distribution). As observed for the cumulative time spent in the shelter, the repetition impacts significantly the first latency in the arena (Chisq= 7.650, df=2, $P < 0.05$; **Figure 9**). A significative difference between the first and third repetitions has been detected (Tukey post hoc test; F=68, df =2, $P < 0.05$). Fishes tend to take longer to exit the shelter on the third shelter test than on the first one.

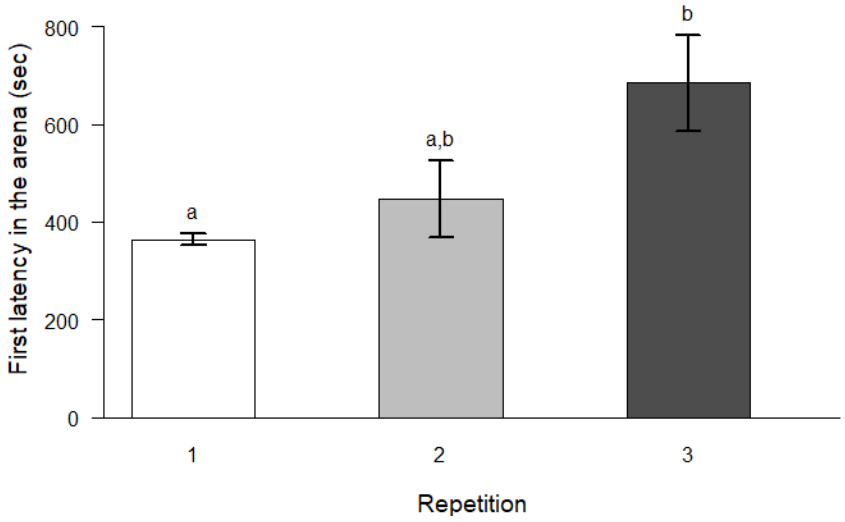

***Figure 9*:** Impact of the repetition on the first latency in the arena of *K.marmoratus* (N=35 for each repetition). Different letters indicate significant effect between repetitions. Bar charts representing mean ± SEM.

The ratio I/A

The ratio I/A is defined as the time spent in the intern part of the arena divided by the time spent in the whole arena.

There are no notable effects of PM exposure on the ratio I/A (LMMs with gaussian distribution; Chisq= 5.257, df = 2, P = 0.07: **Figure 10**). On the other hand, repetition does significantly impact the ratio I/A (Chisq= 35.528, df = 2, P < 0.001). The first repetition is described by a ratio I/A (mean ± SE: 0.21±0.003) significantly different to the second repetition (mean ± SE: 0.14± 0.023; Dunn's post hoc comparison test: P< 0.001) and third repetition (mean ± SE: 0.11 ± 0.012; Dunn's post hoc comparison test: P < 0.001).

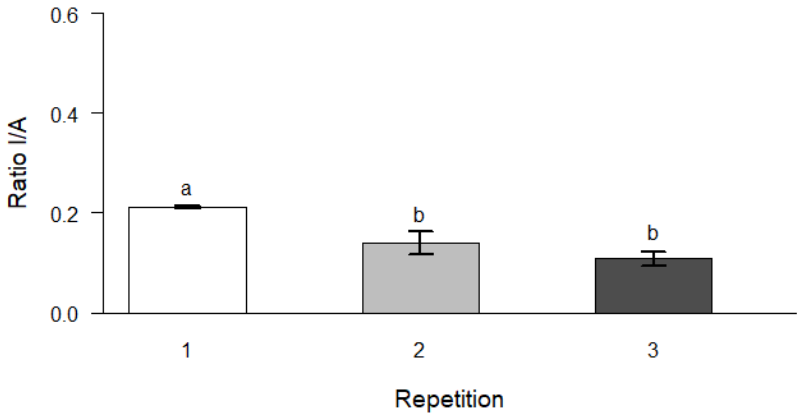

***Figure 10*:** Impact of the repetition on the ratio I/A (N=31 for each repetition). Different letters indicate significant effect between repetitions. Bar charts representing mean ± SEM.

Relative TDM

The total distance moved has been divided by the length of the individual, and by the cumulative time in the arena. This variable whose unit is [sec^{-1}], will be named by the abbreviation "relative TDM". This latter does not display significative differences among treatments (see **Table 1**). It is not significantly impacted by other variables either (size or repetition) (N=31).

***Table 1*:** Relative TDM (mean ± SE) expressed in seconds for the three groups.

relative TDM in "C" group (mean ± SE)	relative TDM in "L" group (mean ± SE)	relative TDM in "H" group (mean ± SE)
1.22± 0.07	1.94 ± 0.8	0.74 ± 0.08

Repeatability

The general intraclass coefficient correlation (ICC) represents the ratio between variability among groups and the total variability (that includes variability between individuals). The ICC values are all under 0.3, meaning it is unlikely that there is a personality within the individuals (all groups combined).

The conditional adjusted repeatability (R) for fish has been performed on the shelter tests dataset (see **Table 2**). Individuals were then aged between 100 (first repetition) and 140 dph. R

parameter has been separately calculated for each treatment, on the four variables (i.e., cumulative time in the shelter, first latency in the arena, ratio I/A and relative TDM).

The cumulative time spent in the shelter, the ratio I/A and the relative TDM show consistent individual differences within the "L" group, whereas the first latency to the arena was significantly repeatable for "H" group. The significative p-value tends to indicate that the locomotion (relative TDM), as well as the thigmotaxis, are repeatable in adults rivulus coming from the "L" group.

In other words, "L" subjects are described by a low within-individual variability compared to inter-individual variability. Regarding the shyness-boldness continuum, this latter remains consistent within exposed-individuals ("H" for the first latency to the arena, and "L" for the cumulative time spent in the shelter).

Table 2: ICC, conditional repeatability, and p-value of each group for the four behavioural variables[7] studied during the shelter tests.

Variable	ICC	R for fish	P-value
Time spent in the shelter	0.28	"C": 0.018 "L": 0.658 "H": 0.182	"C": 0.451 "L": 9.63e-06 "H": 0.229
First latency in the arena	0.28	"C": 0.262 "L": 0.183 "H": 0.445	"C": 0.0473 "L": 0.128 "H": 0.0357
Ratio I/A	0.27	"C":0.168 "L": 0.551 "H": 0.08	"C": 0.139 "L": 0.00044 "H": 0.368
Relative TDM	0.23	"C": 0.225 "L": 0.44 "H": 0	"C": 0.0749 "L": 0.00343 "H": 1

[7] The detailed models of these four behavioural variables are regrouped in data supplementary S9.

Principal component analysis (PCA)

The percentage of behavioural variation explained by dimensions one and two is equal to 73.7% (**Figure 11**). The four variables contribute sufficiently to the plan. Although some individuals particularly contribute to the analysis (i.e., individuals 16, 83, 93), no group tends to contribute more to the variability of data distribution than another (**Figure 11A**). Ratio I/A, CT shelter and FL arena tend to explain the same data dispersion (dim1). Potential correlations might then exist within these variables (see correlations matrix in Supplementary data S11). PCA results demonstrates a negative loading between FL arena and TDM.

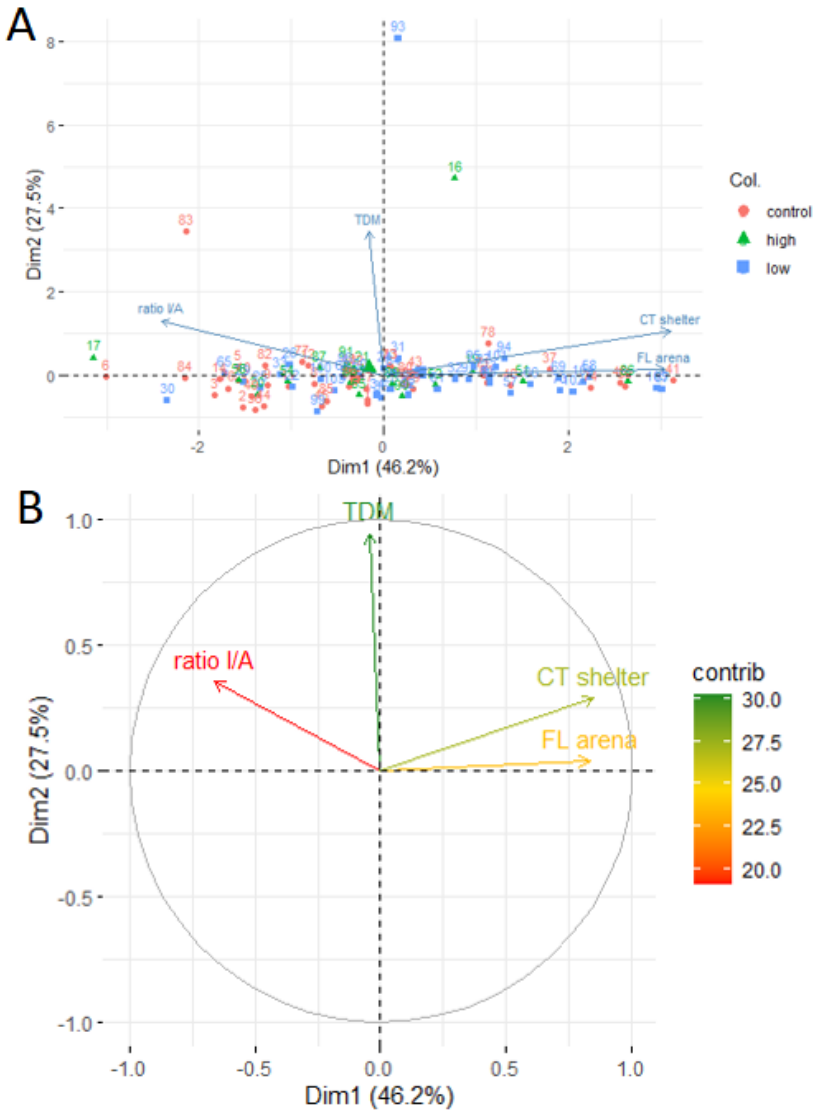

Figure 11: Principal component analysis (A:biplot of individuals and explanatory variables; B: correlation circle on the four explanatory variables) on behavioural data collected during the three repetitions of shelter test on *K.marmoratus* (N=35 for each repetition). CT shelter" and "FL arena" respectively refer to the cumulative time spent in the shelter, and the first latency in the arena. A: The form and colour of the labels show which treatment the individual refers to. The labels 'size is proportional to the contribution of the individual in the analysis. B: A gradient of colouring is applied according to the contribution value. Vectors 'lengthreflects the quality of variables 'representation within the plan. X and y axis correspond to dimensions 1 and 2 used for defining the plan. Vectors pointing to orthogonal directions reflect a weak, or an absence of correlation between variables. Variables are positively, or negatively loaded, if their vectors are respectively separated by an angle of 0°, or 180°.

3.3.Relative genes expression

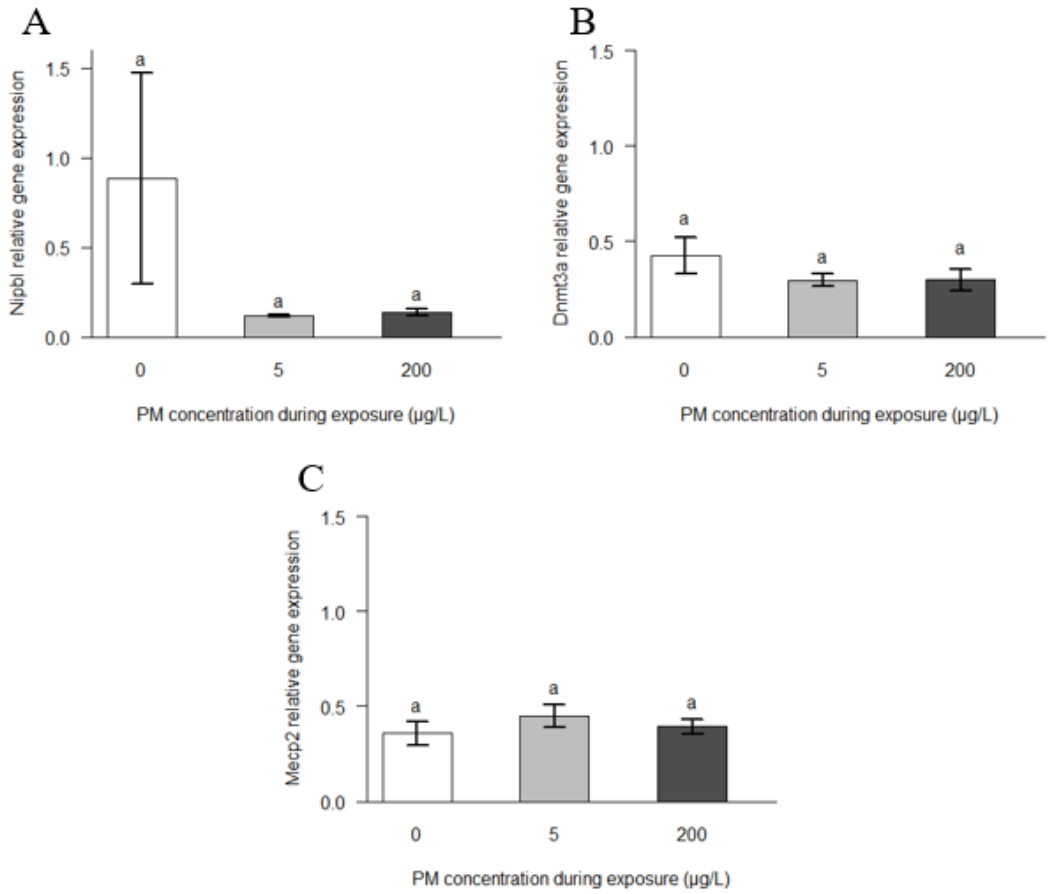

Figure 12: Delayed effects of PM exposure on the relative gene expression (RGE) (mean ± SE) of: A) Nipbl, B) Dnmt3a, C) Mecp2 in the brain of *K.marmoratus* at 140 dph. Barcharts represent mean ± SEM. PM concentration during exposure (0, 5, 200) does respectively refers to the following treatments: “C” (N=13), "L” (N=12) and “H” (N=7). Different letters indicate significant effect between treatments.

There is no significant effect due to PM exposure on any of the genes’ relative expression (ANOVA type II, df=2, overall P >0.5 for the three genes; **Figure 12**). Despite this, Nipbl shows a strong REG decrease between the non-exposed (~0.9) and exposed individuals (~0.1).

Table 3: RGE (%) of three genes (Nipbl, Dnmt3-a and Mecp2) expressed on mangrove rivulus'brain at 140 dph for the 3 different treatments ("C", "L" and "H").

Gene	RGE in"C" group (mean ± SE)	RGE in"L" group (mean ± SE)	RGE in"H" group (mean ± SE)
Nipbl	0.88 ± 0.59	0.12 ± 0.07	0.14 ± 0.02
Dnmt3-a	0.42 ± 0.09	0.3 ± 0.03	0.3 ± 0.06
Mecp2	0.36 ± 0.06	0.45 ± 0.06	0.39 ± 0.04

Principal component analysis (PCA)

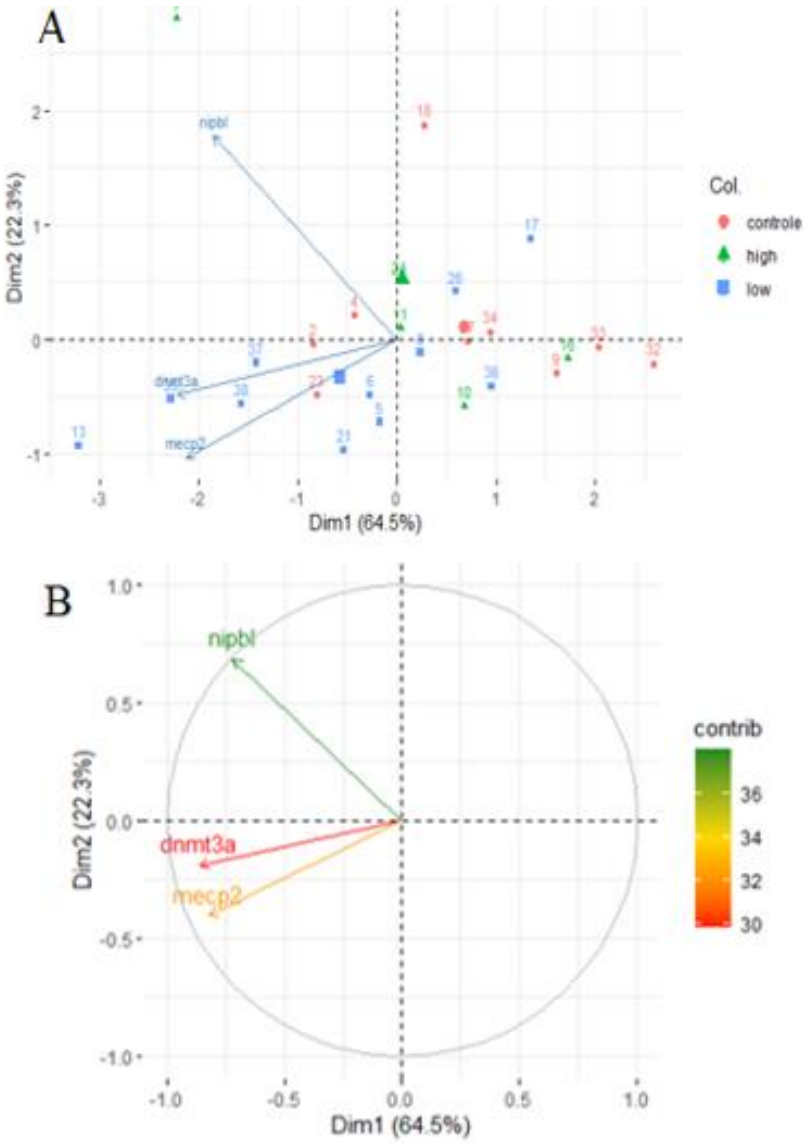

***Figure 13*:** Principal component analysis (A:biplot of individuals and explanatory variables; B: correlation circle on the four explanatory variables) on genetic data obtained from the qPCR analysis on *K.marmoratus*'brain at 140dph (N=25 for each repetition). A: The form and colour of the labels show which treatment the individual refers to. The labels'size is proportional to the contribution of the individual in the analysis. B: A gradient of colouring is applied according to the contribution value. Vectors'length reflects the quality of variables'representation within the plan. X and y axis correspond to dimensions 1 and 2 used for defining the plan. Vectors pointing to orthogonal directions reflect a weak, or an absence of correlation between variables. Variables are positively, or negatively loaded, if their vectors are respectively separated by an angle of 0°, or 180°.

The percentage of variability explained by axis one and two is equal to 86.9% (**Figure 13**). Any group tend to contribute more to the data distribution than another one (**Figure 13A**). The set of genes do contribute significantly to the ACP. While Dnmt3a and Mecp2 looks positively correlated, the absence of loading between Nipbl and Mecp2 is observed (see correlation matrix in Supplementary data S11).

3.4. Methylation level of CpG sites in Dnmt3a, Mecp2 and Nipbl

Results are not shown for Dnmt3a because the Pyrosequencing assay has failed and is explained by important differences between the designed theorical sequence, and the real one. Due to sequencing errors, data is available only for the first four CpG of Mecp2 and the first eleven CpG of Nipbl (see Supplementary data S12 for methylation level of each CpG sites for both Mecp2 and Nipbl).

The results from pyrosequencing assays are limited, for the reasons previously mentioned, which justifies that only descriptive statistics have been run.

Table 4: Mean methylation level (%) of Mecp2 and Nipbl genes on adults aged of 140dph to 3 conditions (control, 5µg/L, and 200µg/L of PM).

Gene	Methylation in "C" group (%mean ± SE)	Methylation in "L" group (%mean ± SE)	Methylation in "H"group (%mean ± SE)
Mecp2	6.5±0.75	6.83±0.67	6.88±0.62
Nipbl	32.77±2.68	26.27±0.46	27.06±2.08

Mecp2

On average, the methylation level, all CpG sites combined (the four presented), is lower for “C” individuals (mean ± SE: 6.5±0.75) than for exposed fishes (“L”: 6.83±0.67; “H”: 6.88±0.62).

Nipbl

The opposite tendency is observed for Nipbl. The studied CpG sites tend to be more methylated on fishes from "C" treatment (32.77±2.68), being around 5-6 % higher compared to individuals exposed to a PM treatment. The level of methylation of these CpG sites are quite similar between the "L" (26.27±0.46) and "H" (27.06±2.08) treatments.

4. **Discussion**

Fishes' early life stages are known to be extremely sensible to pyrethroids' exposure, whereas it constitutes a crucial period for brain development. Consequently, developmental toxicity of pyrethroids might be reflected in neurobehavioural disruptions (Carion *et al.,* 2020; Verhulst *et al.,* 2016). To date, significant modifications of behavioural patterns have been reported on various fish species after a larvae PM exposure (e.g., depressive-like behaviour in adults' *Danio rerio* after 10µg/L PM between 0-28 dph) (Blanc *et al.,* 2021).

In this experiment, no behavioural disturbances have been highlighted in the adult killifish rivulus. Despite this observation, adult individuals from the low dose group tend to express repeatable behaviours, except for the first latency in the arena. However, repeatable first latency for high dose treatment is debatable since Harris and colleagues wrote that "a randomly moving individual within the shelter could well be more repeatable in terms of exit latency than in terms of time in the hesitancy zone simply because of spatial effects" (Harris *et al.,* 2010). Excluding this variable, the results tend to indicate the potential emergence of an individuality in fish exposed to one-week developmental exposure to 5 µg/L of PM. As proposed in the literature, the interaction between the environment and factors that are intrinsic to the individual (*i.e*., genome an epigenome) could be drivers of personality (Martin & Réale, 2008; Mazué *et al.,* 2015; Verhulst *et al.,* 2016). Since behavioural consistency is only detected in exposed individuals, the present results might reinforce this hypothesis. The chemical environment, especially during ELS, might potentially shape repeatability among these EPP killifish rivulus through genetic and epigenetic phenomenon (Trillmich *& al.,* 2020). Being known for triggering methylation changes in the brain, PM early-life exposure could consequently impact the expression of genes associated to behavioural individualities (Blanc *et al.,* 2021; Voisin *et al.,* 2021). Considering that this epigenetic mechanism mediates chromatin state and hence accessibility to CpG sites, differences of methylation level may lead to changes in genetic expression (Voisin *et al.,* 2021; Sánchez-Lafuente *et al.,* 2022). Depending on the position of the differentially methylated region, it might cause gene silencing or activation. For example, DNA methylation within a regulatory sequence lead in general into gene transcriptional silencing, that could be potentially behaviour-related (Chapelle *et al.,* 2023) (**Figure 14**). Environmentally-induced epimutations[8] (PM exposure) could have conceivably persisted until

[8] Heritable change in gene activity that is not associated with a DNA mutation, but rather with the gain or loss of DNA methylation or other heritable modifications of chromatin (Chapelle *et al.,* 2023).

K.marmoratus reached adulthood, contributing to variations in repeatability among the PM treatments (Blanc *et al.*, 2021; Chapelle *et al.*, 2023; Voisin *et al.*, 2021)

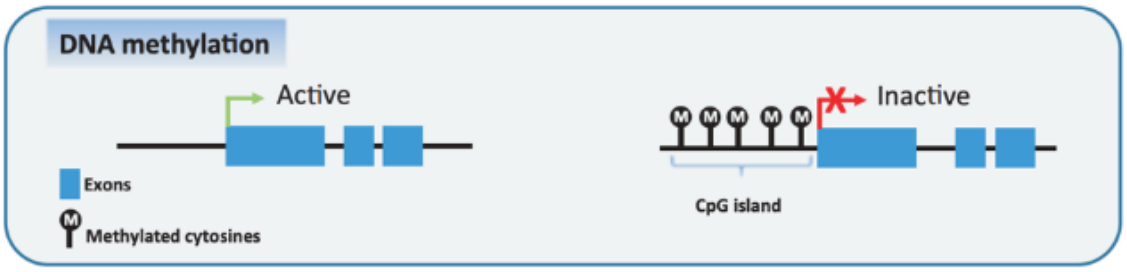

Figure 14: Representation of DNA methylation on a CpG island situated upstream a gene sequence. The addition of methyl groups on CpG-rich region (dark part) mostly led to a repressive effect on the gene regulation (Chapelle *et al.,* 2023).

Moreover, since PM is considered as a neurotoxicant and an EDC, the nervous system might trigger neuroendocrine-behaviour-related processes by changing hormonal production in response to PM larval exposure (Trillmich *et al.,* 2020). The magnitude of the implication of neuroendocrine, genetic and epigenetic mechanisms, and their interaction with the environment during the ontogeny, is still unclear (**Figure 15**).

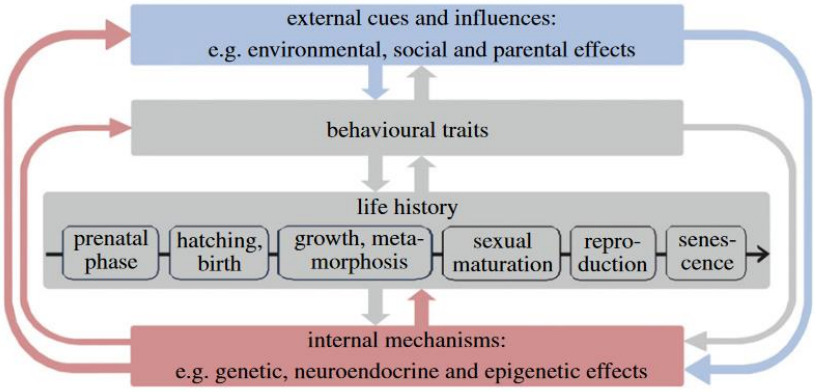

Figure 15: Representation of both internal (red), and external (blue) mechanisms underlying personality development during their lifetime (Trillmich *et al.*, 2020). Life history traits are also represented, and personality refers to the behavioural traits box. Arrows are indicating interactions between the different actors playing a role in behavioural individualities establishment.

PM could then potentially be a driver of behavioural consistency establishment, depending on several factors such as the timing and length of exposure.

Further investigations must be conducted to provide insights about the potential link between a developmental exposure to a neurotoxicant such as PM, and the emergence of individualities. Also, if behaviours tend to be similar over time, but different between individuals in the low dose group, this could hence lead to differences in fish fitness (Bell *et al.,* 2009; Holtmann et al., 2016). In the natural environment, such observations could have evolutionary consequences through natural selection. However, overall, it should be recalled that there seems to be no personality within this subpopulation of rivulus (ICC < 0.3), and that no significant effect of PM was detected on the 4 parameters monitored during the shelter tests (see **Figures 8 to 10** in Section 3.2). Therefore, we cannot claim that PM shapes personality in this subpopulation of this model specie, but is a potent trigger for repeatable behaviours in K.marmoratus. We acknowledge the potential limitation of our sample size and highly recommend conducting a similar experiment on a larger sample size for guaranteeing a sufficient statistical power.

On the other hand, neurobehavioural impairments were potentially found on larvae rivulus exposed to the high concentration of PM (part of the experiment conducted by Isabelle Chevalme). A light and dark test was performed on rivulus aged of 7 dph. H-exposed individuals do not seem to detect the light change. While the C and L subjects tend to express a higher mean mobility (%) and relative total distance moved in the presence of light, high dose group do not react to the light stimulus. Thus, light signal 'reception might be disrupted from a certain concentration of PM (in this case, 200 µg/L). It is also conceivable that there is no photoreception issue of the light stimuli, and the fish simply does not respond to the stimulus. In a future study, specific experimental protocols could be established to investigate and validate these hypotheses. On one hand, further genetic expression analysis should be conducted on mangrove rivulus larvae, on genes related to light stimuli reception. Even though its visual system has not been fully detailed yet, opsins genes[9] might be attractive candidates for molecular investigations on larvae from the different PM treatments, directly following a light stimulus. Quantifying the genetic expression level of a series of photoreception-related genes in this neotropical fish 'retina, such as opsin genes, could help determining if the exposed larvae sense light (Carleton *et al.,* 2020). Conversely, the mechanisms by which PM induces the lack of significant larvae's response to the light stimuli (during the light and dark test performed by Isabelle Chevalme), might be linked to neurobehavioural impairements, as initially proposed. The optic tectum (OT) is a brain structure involved in light signal analysis

[9] Opsins genes encode light-sensitive proteins (opsins) that are present in both cons and rods photoreceptors and are absorbing a specific wavelength (Carleton *et al.,* 2020).

and behavioural light signal responses (Ewert, 1974). Visual acuity and performances have been associated with OT neurogenesis, as reviewed by Rossi and colleagues (Rossi *et al.,* 2022). In the literature, neurogenesis does refer to "a complex and multistep process by which functional neurons are generated from resident neural stem/progenitor cells. In fact, neurogenesis encompasses the birth, the maturation and the migration of new neurons that integrate into existing neuronal networks" (Pelligrini *et al.,* 2016). The existence of a phenotypic plasticity of *K.marmoratus*' visual system has been pointed out, notably in presence of environmental cues such as an air or water acclimation (Rossi *et al.,* 2022). During early life stages, this plasticity might be neurogenesis-based and notably translated into cellular proliferation within OT structure. Since the visual system is a highly energy-intensive process, even in the basal state, visual system plasticity might be an adaptative tool that help adjusting individual fitness (Rossi *et al.,* 2022). Therefore, it is fair to wonder whether larval exposure to 200 µg/L PM concentration, might have considerably limited the neurogenesis process within OT structure to counterbalance energy loss due to this environmental stressor. To verify this theory, it might be legitimate to monitor neurogenesis process, by quantifying cellular proliferation, within OT, during and at the end of the different PM conditions (0, 5µg/L and 200 µg/L).

A series of other phenotypic effects are not sustained at the adult stage. No significative differences were observed for either thigmotaxis or relative total distance moved in adults. The growth (7-140 dph), the standard size and weight (at 140 dph), were also not significantly impacted by PM treatment. Despite the absence of consistent delayed effects of the PM treatment on these patterns, immediate impacts of PM on individuals (7dph) were detected. First, a consistent decreasing mean of the percentage of time spent in the central part of the arena, among high dose exposed subjects, compared to non-exposed ones, is depicting a relatively higher anxiety for the high dose group compared to the control group. The same tendency is observed in larvae for depressive-related patterns. The maximum velocity and acceleration, as well as the mobility, is significantly lower for the high dose group. Lastly, a significant decrease of growth (0-7 dph), standard length (7dph) and standard weight (7dph) on high dose larvae compared to control larvae is observed. The loss of significant effects for depressive and anxiety-like behaviours, and for these life-history traits, in young mature adults rivulus, suggests the existence of recovery mechanisms. The lack of detection of neurobehavioural deficits in adults, both at the genetic (i.e., Mecp2) and phenotypic levels (the ratio I/A and the relative total distance moved), may further support this hypothesis of repair phenomena. The period after the larval exposure during which the fishes where not exposed to

any pollutant, could have been a key moment for recovering from behavioural damages observed at 7dph by Isabelle Chevalme. Neurogenesis has been documented to be an important regenerative mechanism present in teleost fishes, during both the early stages of life and the adulthood (Pellegrini *et al.,* 2016). Thereby, neurogenesis helps them adapting themselves to environmental changes and recovering from neurological damages all through life (Pellegrini *et al.,* 2016; Zupanc, 2009). This phenomenon could explain the absence of neurobehavioural effects in the adults *K.marmoratus*. In teleost, neurogenesis has been pointed up to be a steroid-based process, in which Cytochrome P450 aromatase (aromatase) is a key player. Highly expressed in the brain, the enzyme aromatase is producing oestrogens (e.g., oestradiol) that modulate neurogenerative processes. Aromatase is encoded by highly conserved genes among vertebrates, being *cyp19a1* genes (Pellegrini *et al.,* 2016). To confirm this hypothesis, molecular investigations (RGE of *cyp19a1 genes*) should be conducted with a similar experimental protocol, on individuals at different time points following PM exposure, until the adult stage, to understand if the notable larval neurobehavioural deficits caused by PM, have gradually disappeared thanks to neurogenerative mechanisms. While the absence of neurobehavioural effects on adults might be based on neurogenesis, the absence of morphological effects (i.e., growth (7-140 dph), standard length and standard weight), on adults might be based on a growth compensation phenomenon. This latter is described as an accelerated growth that occurs once favourable environment returns after a period of growth depression due to stressing conditions, allowing individuals to catch up in size with non-stress conspecifics (Ali *et al.,* 2003). The growth, standard length, and standard weight of the 7-day-old larvae exposed to the highest concentration of PM showed a decline during (growth) or at the end of the exposure period (standard length and weight). However, this decrease in growth was not observed in the 140-day-old adult rivulus. Despite the absence of delayed PM exposure effects on the adult fish, the group exposed to the high dose exhibited a trend towards higher growth rates and slightly greater length and weight. These observations tend to indicate the occurrence of a compensatory growth phenomenon following the neurotoxicant (PM) exposure. These results also provide insight on the presence of a growth resilience within this vertebrate, long after the environmental stressor (in this case, a one-week 200 µg/L PM exposure) (Mathiron *et al.,* 2023). Moreover, the findings carried out by Mathiron and colleagues (Mathiron *et al.,* 2023) tend to demonstrate that growth resilience could be lineage-specific within this teleost specie. Their experimental design shares similarities with this current study (i.e., same PM concentration and duration of PM exposure) but *K.marmoratus* individual were issued from a different lineage: DC4. The immediate effects of PM on both lineages (DC4 and

EPP) tend to follow the same trends. PM early exposure does significantly reduce mobility, activity (relative total distance moved), and thigmotaxis on larvae subjects. In addition, the growth, the standard length, and the standard weight are also negatively impacted in exposed individuals. In contrast, the delayed effects on life-history traits, observed in both lineages, are different. It appeared that PM early exposure does exclusively impact growth, standard size, and weight in adults 'individuals from DC4 population. The absence of significant delayed effects of PM exposure on morphology-related traits on EPP lineage is similarly observed in this study. It is plausible to hypothesize that growth compensation is regulated by epigenetic mechanisms. As a reminder, *Kryptolebias marmoratus*' lineages are isogenic, meaning that there is a low genetic variability among individuals within the same lineage (Tatarenkov *et al.,* 2010). Additionally, this species is known for its high phenotypic plasticity and resilience to environmental stress (Choi *et al.,* 2020; Puthumana *et al.,* 2017). Given the significant genetic uniformity within each lineage, it has been suggested that these evolutionary advantages may be attributed to epigenetic modifications. In the literature, the methylome has already been reported to be environment sensitive. In view of the changing environmental habitat conditions and the isogeneity of K.marmoratus, researchers have suggested that DNA methylation serves as a fundamental support for the adaptive capabilities of this self-fertilizing species (Chapelle, 2023; Verhulst *et al.,* 2016). Epimutation does refer to the variation of DNA methylation of an organism (especially in developmental stages) that can be provoked by environmental cues (Angers *et al.,* 2010; Chapelle *et al.,* 2023). Environmentally induced epimutations might then have been triggered on each of the individual at the origin of the DC4 and EPP laboratory lineages, throughout their lifespan preceding their catching. Before being collected on the field, those two individuals were living under specific environmental conditions present either at Dove Creek (for DC4 lineage), or at Emerson Point Preserve (for EPP lineage), in Florida (Chapelle *et al.,* 2023). These wild changing environments might have provoked the emergence of a methylation profile that provide them adaptative benefits to survive in their living habitat. Since epimutations appearing on germ cells have been reported to be stable and transgenerational (Blanc *et al.,* 2021; Chapelle *et al.,* 2023), the reared EPP and DC4 lineages produced by self-fertilization, could be both characterized by a unique set of identical epimutations. Consequently, lineage-dependent methylation marks are probably existing. Thus, they could justify the potential presence of growth compensation mechanisms within the EPP individuals.

While most of the significant effects of PM during the larval stages seem to be attenuated at the adult stage, it must be mentioned that PM significantly affects the mortality and the survival of individuals, in both larvae and adult individuals. Mortality cases, at both stages, are mainly (adult stage), if not totally (larval stage) present in the H-exposed group. The results reflect a lethal concentration of PM (i.e., 200 µg/L). Since a huge selection pression has been applied on the H-exposed subjects, only resistant individuals survive. It could probably prevent a significant immediate or delayed effect of the H-treatment on phenotypic patterns (i.e., boldness-shyness). This could then also justify that adult killifish rivulus coming from the low dose group tend to express repeatable behaviours, except for the first latency in the arena. In that perspective, considering a lower PM concentration as the high (sublethal) concentration treatment would prevent the loss of individuals and ultimately increase the statistical power.

Besides, the assumption that the strong selection pressure applied by the high PM concentration would hide some significative effects, might be also applied to molecular results. RGE of Nipbl, Mecp2 and DNTM3a have not been significantly modified by the PM treatment, even after the high PM concentration exposure. Even though only descriptive statistics were performed on the pyrosequencing results, it must be reminded that no clear tendency seem to appear between PM groups for Mecp2 (less than 0.4% of differences between exposed and control adult fish). The mean methylation percentage of CpG sites for Nipbl for control individuals is slightly higher (5-6%) compared to the other conditions. Since qPCR and pyrosequencing assays were performed on the whole brain tissue, it may also be possible that consistent changes appear in specific brain structures and were not detected (Poulin *et al.,* 2018). The absence or slight differences of CpG sites' methylation percentages between treatments, on both specific sequences within Mecp2 and Nipbl genes, might also be due to methodological aspects. First, the minor dissimilarities of single nucleotide polymorphisms (SNPs) between the three groups could also be explained by the strand-specificity of this DNA sequencing method (Poulin *et al.,* 2018). Thymine nucleotides are complementary to both methylated and non-methylated cytosines, meaning that thymine present on the amplified strand (matrix strand) could hide SNP(s) that are not detected by pyrosequencing. The bisulfite treatment applied on the DNA sequences of interest also constitute another methodological limitation. As previously described, the bisulfite treatment applied on the matrix strand ensures the transformation of non-methylated cytosines into uraciles, which leads to the formation of three-base-DNA sequences. Recovery and redundance phenomenon of DNA regions might then exist within each gene, that leads to a decrease of specificity between the primers and the bisulfite-treated-

matrix strand (Poulin *et al.*, 2018). Furthermore, since pyrosequencing technology targets short sequences of nucleotides (~ 150-250 nucleotides), PM might potentially act on cytosines methylation within other region(s) in the gene(s) of interest (Poulin *et al.,* 2018). Assessing first a global analysis of CpG sites in Dnmt3a, Nipbl and Mecp2 for the three groups, thanks to a whole-genome technology, could counteract this limitation and might point up potential CpG methylation biomarkers related to PM exposure within each gene. For example, reduced Representation Bisulfite Sequencing (RRBS) could highlight sequences that are differentially methylated among the three treatments. As cited in Yuan and colleagues' publication, "RRBS is a cost-effective channel for studying genome-wide patterns of DNA methylation" (Yuan *et al.*, 2016). Using RRBS at the scale of each gene of interest could provide essential insights that might be useful for targeted sequencing.

Concerning RGE, it might be then relevant to maintain Dnmt3a, Mecp2 and Nipbl in the genes' candidates for further molecular analysis on this selfing fish. This idea might be reinforced by the following information. As it is observed in this experiment, Dnmt3a tends also to be downregulated on embryo of another model specie, the zebrafish, after a developmental PM exposure (Blanc *et al.,* 2019). Nipbl does follow the same tendency, even though any significative differences of gene expression were detected between the three groups. To this present, Nipbl has not been pointed up on the mangrove rivulus yet. However, in view of its key role in genome stability, and its sensibility to other endocrine disruptors (i.e, EE2), Nipbl activity should be more investigated to determine if this gene is potentially targeted by PM or not (Voisin *et al,* 2021).

Since there are no significant effects of PM on both biological levels, it is difficult to estimate a potential (epi)genetic contribution to personality traits' variation in this isogenic EPP population. Based on the literature, potential impacts of PM on behavioural individualities, and (epi)genetic-related effects, are still very unclear, especially on the long-term. Behavioural parameters (i.e., first latency in the arena and cumulative time in the shelter), as well as (neurogenesis-based) molecular analysis should be investigated on larval individuals. As previously mentioned, the delayed effects, especially at the phenotypical level, should be performed on a higher sample size, by using a lower "high PM concentration".

It could thus reveal new insights on what extent early PM exposure' effects, and more especially on the link between developmental exposure to PM, DNA methylation and behavioural disruptions.

5. Conclusion

This research provides insights about a potential causal relationship between a permethrin early-life exposure and the delayed emergence of behavioural individualities within *K.marmoratus*' EPP isogenic lineage. The results tend also to confirm the known resilience of the mangrove rivulus that is notably translated into a high phenotypic plasticity. Since mangrove killifish populations are described by a high genetic homogeneity, the evolutionary processes allowing this specie to survive and adapt to its environment, might be epigenetic based. In this study, the absence of consistent delayed phenotypic effects of permethrin exposure on adults, converge towards the hypothesis that the existence of recovery and compensatory mechanisms might be implied in *K.marmoratus*'resilience and associated to DNA methylation. Neurogenesis could be a key process in this outstanding capacity, by limiting the delayed neurobehavioural effects of a developmental neurotoxicant stress. Furthermore, a growth compensation mechanism might also be triggered after an intense environmental stress (one-week 200µg/L permethrin larval exposure) and be linked to epimutations.

Further molecular investigations should be conducted to better understand to what extent the role of DNA methylation in *K.marmoratus*' resilience mechanisms, with a particular interest to growth compensation and neurogenesis. Assessing the effects of permethrin exposure on mangrove rivulus'neurogenesis, could provide new information about the pillars of behavioural individualities establishment, and their interaction with neurotoxic compounds.

6. Supplementary data

6.1. Supplementary data S1: HPLC

HPLC is a quantitative method able to measure the quantity of each isomer of a molecule, being, in that case, cis and trans PM isomers (García *et al.*, 2001).

All solvents were HPLC grade quality, purchased from Merck and the standard of PM (21.8% and 75.5% of cis and trans- isomers, respectively) was purchased from Sigma. A Dionex HPLC system provided with a GP50 gradient pump, an automatic injector (UltiMate 3000 RS) and a Shimadzu SPD-6AV UV-VIS spectrophotometric detector were used. The chromatographic analyses were performed on a 5 µm NUCLEODUR C18 (Macherey-Nagel, Germany) column (125x4.6 mm) kept in a Dionex column oven at 35°C. Mobile phase consisted of a gradient acetonitrile–H3PO4 (0-2 min: 50 / 50%; 2-30min: 100/ 0%, 30-40min: 100/ 0%) at a flow rate of 1 mL/min and an injection volume of 0.2 mL. UV detection was performed at 210 nm and peaks were identified with retention times as compared with standards (16.4 and 17.0 min respectively for trans and cis isomers). The standard curve ranged from 125 to 1000 µg/L. Analysis showed that the mean (± S.E.) concentration of samples from the 200µg/L nominal PM working solutions was 157±22µg/L (N=8), while the mean concentration from 5µg/L nominal PM working solutions were below the detection limit of the method based on visual evaluation. Based on these results, nominal concentration was kept throughout the text.

6.2. Supplementary data S2: Mastermix preparation for molecular analysis

MM1: a mix of DNAse enzyme (2µL/sample) and DNAse I buffer (2.5 µL /sample).

MM2: a mix of 5X Reaction Buffer (4µL/sample), 10 mM dNTP Mix (2µL/sample), RiboLock RNase Inhibitor (1µL/sample) and Revert Aid Rt (1µL/sample).

MM3: a mix of PyroMark Binding Buffer (40µL/sample), Streptavidin Sepharose beads (1µL/sample), High-purity water (29 µL/sample).

6.3. Supplementary data S3: Genes' description

MAOA is a gene coding for the protein monoamine oxydase A, involved in behavioural regulation and electrical activity in the CNS (Moniruzzaman *et al.*, 2020). This latter plays a role in the degradation of essential neurotransmitters, being serotonin, dopamine, and norepinephrine in fish.

It leads to a pigment's production, neuromelanin, which can trigger neurodegenerative processes (Moniruzzaman *et al.,* 2020; Rodrigues *et al.,* 2022; Ziegler *et al.,* 2016). In addition, it appeared that MAOA is related to depressive-like behaviour when this protein is deficient (Jones & Raghanti, 2021). MAOA's methylation level of certain CpG sites might be a risk factor for neurologic disorders (i.e., hypomethylation of particularly three CpG sites in exon1/intron 1 is accompanied with panic disorder (PD)). The known methylation plasticity within MAOA in response to environmental changes could then explain the apparition of neurologic disorders (Ziegler *et al.,* 2016). Its usefulness for estimating neuronal impairments makes thus this gene interesting to investigate in the context of this study (Moniruzzaman *et al.,* 2020).

Mecp2 and Tollip are two other genes involved in neurology, more specifically in neurologic and neurodegenerative disorders (Connolly & Zhou, 2019; Li *et al.,* 2020). Firstly, stress-associated diseases and depression do imply the neurotransmitter methyl CpG binding protein 2. This transcriptional regulator is known for being highly methylated in the brain and regulates a series of genes (Sánchez-Lafuente *et al.,* 2022). Impaired genetic expression (mutations, duplications, etc.), gain-of-function and loss-of function of Mecp2 can lead to neurologic diseases such as Rett syndrome or MECP2 duplication syndrome (MDS) (Connolly & Zhou, 2019). Secondly, Tollip is a ubiquitous protein playing a role in a set of various intracellular mechanisms. This latter promotes autophagy, vacuole transport, and is implied in inflammatory processes. This intracellular mediator molecule is related to pathogenesis when dysregulated (Li *et al.,* 2020). The previous explanations highlight the involvement of these two genes in physiological pathways and/or certain behaviours that are sometimes pathological if the gene is abnormally regulated. In this current study, assessing their level of relative genetic expression and their level of methylation of target CpG might then help explaining a potential personality traits' variation between treatments.

A growing body of evidence tends also to demonstrate the genetic and epigenetic contribution to personality traits of another gene: DRD4. This one is highly polymorphic and has been related to novelty seeking within diverse vertebrates, including birds (Munafò *et al.,* 2008; Verhulst *et al.,* 2016). In addition, an experiment conducted by Verhulst and colleagues on *Parus major,* had led to the hypothesis that DRD4's methylation level does also explain variation in exploratory trait (Verhulst *et al.,* 2016). Even though DRD4's role in other personality traits remains unknown, studying the methylation level of interesting CpG among this gene could provide new insights about the role of this latter.

An essential regulatory factor, Nipbl, oversees genome 3D organization and functionality and so developmental processes and genome stability (Gao *et al.*, 2019). Concerning another target genes of this experiment, DNA methyltransferase genes (i.e., Dnmt-3a and Dnmt-3ab) regulate the methylation level of genomic DNA (DeCourten *et al.*, 2020). More specifically, they permit the apparition of methylation patterns on unmethylated DNA (Morris & Monteggia, 2014). Lastly, studies have already highlighted the impact of PM on these epigenetic regulators on rats, which makes consistent to choose these genes for molecular analysis (Blanc *et al.*, 2019). Regarding Dnmt-3a, its implication in postnatal neurogenesis, more specifically neuronal differentiation and maturation, has also been recorded (Feng *et al.*, 2005).

Igf 2 is a gene responsible to produce the growing factor Igf2 that ensures protein synthesis and subsequently plays a role in development and growth (DeCourten *et al.*, 2020). Following birth, expression level of Igf2 drops in most tissues except the brain. Within this structure, Igf2 is involved in memory and the regulation of the number of neuronal cells. Studies carried out on rodents have point up the importance of genetic expression regulation of Igf2 for neurologic homeostasis and avoiding neurodegenerative diseases'apparition. Misregulation of this gene has been linked with depression, anxiety, and autism within individuals (Sélénou *et al.*, 2022).

Finally, it is recognized that neurologic toxicity is linked to the overload of oxidative stress in fish species. As a reminder, oxidative stress is a condition in which body's cells are damaged by reactive oxygen species (ROS). Permethrin, and the pyrethroids in general, induce brain dysfunctions in fish via the increase of free radicals (Moniruzzaman *et al.*, 2020; Rodrigues *et al.*, 2022). It hence DNA damages and disrupt protein and lipidic structures. Fortunately, glutathione system (GSH) consists of antioxidant proteins that helps maintaining redox equilibrium (Rodrigues *et al.*, 2022). The GSH has been suggested to be adapted biomarkers in brain for monitoring the neurotoxicity of pyrethroids (Moniruzzaman *et al.*, 2020). This latter exists thanks to glutathione synthetase (GSS) protein encodes by GSS gene. This enzyme plays a role in the body's defense against oxidative stress by helping to produce the antioxidant glutathione. It is possible that exposure to PM may lead to changes in the expression or activity of the GSS gene, potentially affecting the body's ability to defend against oxidative stress (Choi *et al.*, 2020; Moniruzzaman *et al.*, 2020).

6.4.Supplementary data S4: additional information about DNAse and RT treatments

DNase Treatment

On a 96-well plate (plate 1), a volume of 4.5 µL of mastermix (MM1) is added, followed by 20.5 µL of RNA sample, for a reaction size of 25 µL. This specific volume of RNA to pipette was chosen to obtain up to 5 µg of total RNA in each sample. For that purpose, the concentration (and quality) of RNA in the samples was previously estimated using the Thermoscientific® Nanodrop 2000c Spectrophotometer.

The plate was then homogenized and incubated in the thermocycler QuantStudio 5 Real-Time PCR instrument from Thermo Fisher scientific to undergo a DNAse program lasting 30 minutes. At the end of the program, 5 µL of DNAse Inactivation Reagent were added in each well.

From this plate, the RT- (negative control) is prepared in a nuclease-free tube by drawning 1 µL of each sample and 200 µL of nuclease-free H2O.

Reverse Transcriptase (RT) treatment

For this purpose, a new 96-well plate (plate 2) is used. On each well, 1 µL of hexamer primer, as well as 11 µL of one of the 39 RNA samples that underwent the DNase treatment (plate 1), were loaded. After a centrifugation and a 5-minute incubation in the Thermocycler (Dry Bath) of the plate 2, 8 µL of mastermix 2 (MM2) is added. Finally, a last centrifugation was done before launching the RT program.

6.5.Supplementary data S5: Primers' sequence and efficacity

The primers pairs (see **Table 6** below) are considered effective when they meet the main following criteria:

- Mean Cycle Threshold (CT) (per replicate): should preferably not exceed 30. The triplicate must follow a similar amplification and the three amplification curves should be equally spaced.
- Coefficient of determination (R^2): equal or greater than 98%.
- Efficacity (%): should be between 90 and 110%.
- Melting curve: only one peak should appear.

Table 5: Set of the studied primers for qPCR analysis. Genes whose primers were estimated to be efficient by the efficacity tests are marked in green. Housekeeping genes are highlighted in bold.

Gene of interest	Primer	Sequence
GSS	F	AGACTTCCTGCAAGAGGCTTTAGC
	R	GTAGTCGGACCGATTGAGACCCA
Mecp2	F	TAAGATGCCCTTTGGCAAGACA
	R	GGACTTGGCAGGTGGAGTAG
Dnmt3-a	F	TACACGAGATCAAAAAGAAGACCAGGA
	R	CATCGCTCCTACGAAGAGAGGAT
Dnmt3-ab	F	TCTTTATGTGGTGTCCTGGTTCAAC
	R	TGTGCATCAAACCTCACTTTGGA
Tollip	F	ACTGTCGGATCAGGCTGGGT
	R	CGTCCATCGAAAACGCTCTCTCA
MAOA	F	ACTGATACTCGAGGGAAAAGATCG
	R	GCTCCATGATATGCGTTTGTGTG
Nipbl	F	CAAACTCTACTGTCCATGAACCCCA
	R	CTGGGCTCCCATGAACTTGATTCT
Igf2-b	F	CGGAGAACAGCAGAATAAAGGTCA
	R	CCACAACGTAGAGCGTGAGA
DRD4	F	TGCCGCTCTTCGTTTACTCC
	R	CAATACGGCGATGAACCTGCT
Ef1-α	F	CAGGAAGGATGGTAGTGCG
	R	GCTTCAGTATGCCCGTTTCC
β-actine	F	CTTGCGGAATCCACGAGACC
	R	CCAGGGCTGTGATCTCCTTCTG

6.6.Supplementary data S6: Primers' sequence for pyrosequencing assays

***Table 7*:** Forward, reverse and synthase primers'sequences used for pyrosequencing experiment. Primers' ID and biotinylation are precised as well.

ID	Primers	Biotinylated	Sequences (5' -> 3')
Dnmt3a_3_CREB	Forward	No	TGATATTTGGAGTGTATATTAGGTAGT
	Reverse	Yes	ACCAAAACTTCAATCTACAACACAT
	Synthase	No	GGTAGTTAGGTATAAAGTTTTTA
Nipbl_1_Oct1	Forward	No	GTTATAGTTATTTTTGAGATGGTAAAGG
	Reverse	Yes	ACAAAATCTATCACTTCCCACAAA
	Synthase	No	TTTTGAGATGGTAAAGGT
MeCP2	Forward	No	AAAAGGTAGTTGGTTTAAGAAGTTTATATA
	Reverse	Yes	AATTTTATATTAAAAAAACTCCAAACATCT
	Synthase	No	GTTGGTTTAAGAAGTTTATATAT

6.7.Supplementary data S7: Bisulfite treatment

Firstly, a volume of DNA sample varying between 3 and 10 µL, was withdrawn and put into strips, to obtain a quantity of 500 ng of DNA. For ensuring DNA denaturation, 5µL of M Dilution Buffer was added to the strips as well as a variable volume of water for attaining a final volume of 50 µL. After 15-minute-incubation at 37°C, a reaction mixture (CT conversion reagents) of 100 µL was added to the strips. As this latter is sensitive to the light, the preparation was incubated in the dark for up to 16 hours.

After that, the samples were exposed to a last incubation at low temperature (0-4°C). In parallel, 400 µL of M-Binding Buffer was loaded in the Zymo-Spin™ IC Column that was then placed into a Collection Tube. When the above incubation is finished, the samples were loaded in the Column. This latter was mixed and centrifuged, and the flow was discarded for purifying DNA. The same step was realized after the addition of M-Wash Buffer to the column. Finally, the Column was placed in a microtube and, following the addition of 10 µL of M-Elution Buffer, a last centrifuge was conducted for the elution of DNA.

6.8. Supplementary data S8: PyroMark Q24 Workstation complementary information

Table 8: Volumes of the different solutions used in the Pyromark Q24 WorkStation. Ethanol and the denaturing solution were used for removing potential leftover products from the subsequent steps. Moreover, NaOH also plays a role in PH stabilization.

Tray	Solution	Volume (mL)	Time (sec)
1	Ethanol 70%	40	5
2	NaoH 0.2M	40	5
3	10X Wash Buffer	50	10
4	MilliQ water	70	5
5	MilliQ water	70	5

6.9.Supplementary data S9: LMMs selection for behavioural variables

For selecting the more accurate LMMs for the analysis, a first complex model containing all the potential explanatory variables and interactions, was created. An anova was then performed for eliminating the most non-significative factor (firstly within the interactions, and later within the explanatory variables). A non-significative explanatory variable should not be deleted from the model if this latter is part of a significative interaction. By proceeding sequentially that way, the created models were simplified for finally containing only consistent interaction(s) and/or explanatory variable(s). An anova was then computed on the whole models. Finally, the model quality was measured via Akaike information criteria (AIC). The best model is described by the lowest AIC.

Cumulative time in the shelter

lmer(predict_cumul_shelter~ repetition + length + length:repetition +(1|fish), data=m, REML=T)

First latency in the arena:

lmer(predict_first_latency_arena~repetition+ (1|fish), data=m, REML=T)

Ratio I/A

lmer(predict_ratio ~treatment + repetition +(1|fish), data=m, REML=T)

Relative TDM

lmer(predict_rtdm~repetition + (1|fish), data=m, REML=T)

nb: "Predict_" notifies that the response variable has been transformed to meet normality assumptions.

6.10. Supplementary data S10: Script

Full access to R script and datasets used for this study by clicking on this link: https://github.com/clievin/Master-s-Thesis-UNamur-LEAP.git

6.11. Supplementary data S11: Correlation matrix for behavioural variables and for relative genetic expression of Mecp2, Nipbl and Dnmt3a

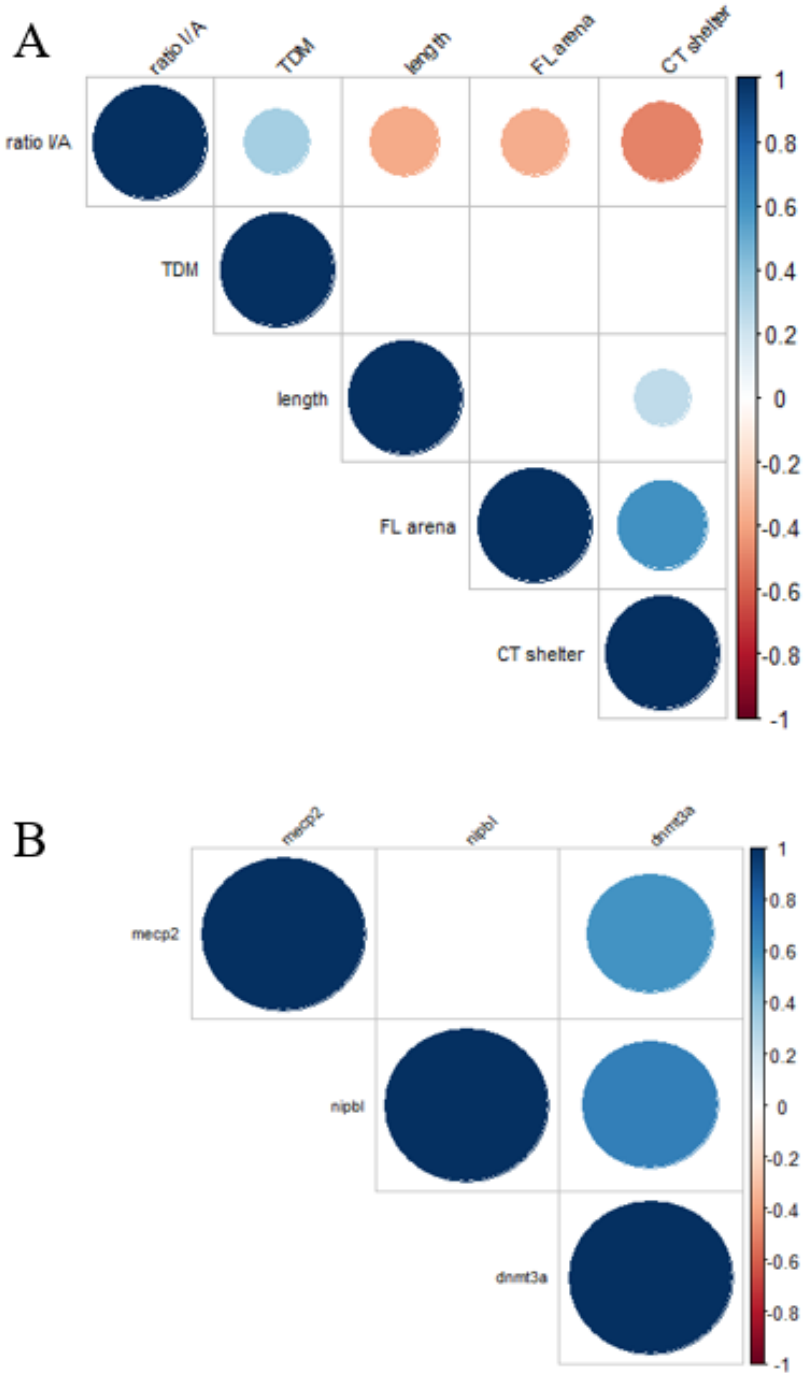

Figure 17: Graphical representation of the correlation coefficients of (A) the four behavioural variables and (B) the relative genetic expression of Mecp2, Nipbl and Dnmt3a genes of *Kryptolebias marmoratus*.

6.12. Supplementary data S12: Methylation level (%) of CpG sites for Mecp2 and Nipbl

For information, samples 3 and 5 do refer to the "C" individuals. While samples 14, 15 and 16 are associated to the "L" condition, samples 9, 12 and 13 are related to the "H" treatment.

Mecp2

Table 9: Methylation level (%) of the first four CpG sites situated on the targeted Mecp2 region, for each mangrove killifish individual (N=2,3, and 2, respectively in 0, 5 and 200 µg/L groups). The sequencing quality follows this ascending order: "Fail" (not shown), "Passed" or "Check". "Meth (%)" describes the percentage of methylation observed on the specified CpG site.

Position	*1*	*2*	*3*	*4*
Sample 3				
Quality	Check	Check	Check	Check
Meth (%)	7	7	8	7
Sample 5				
Quality	Check	Check	Check	Check
Meth (%)	6	5	6	6
Sample 9				
Quality	Passed	Passed	Passed	Passed
Meth (%)	7	5	7	6
Sample 13				
Quality	Check	Check	Check	Check
Meth (%)	8	7	9	6
Sample 14				
Quality	Check	Check	Check	Check
Meth (%)	6	5	6	5
Sample 15				
Quality	Check	Check	Check	Check
Meth (%)	8	8	8	6
Sample 16				
Quality	Check	Check	Check	Check
Meth (%)	8	7	8	7

Nipbl

Table 10: Methylation level (%) of the first eleven CpG sites situated on the Nipbl targeted region, for each mangrove killifish individual (N=3,2, and 3, respectively in 0, 5 and 200 µg/L groups). The sequencing quality follows this ascending order: "Fail" (not shown), "Passed" or "Check". "Meth (%)" describes the percentage of methylation observed on the specified CpG site.

Position	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Sample 3											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Meth (%)	40	38	34	45	33	27	30	18	15	16	35
Sample 5											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Check
Meth (%)	36	44	39	51	45	34	37	27	22	22	33
Sample 9											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Meth (%)	33	41	36	46	34	25	29	15	12	16	33
Sample 12											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Meth (%)	26	26	27	32	30	18	23	19	13	13	25
Sample 13											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Meth (%)	35	38	34	46	35	25	27	15	14	17	35
Sample 14											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Check
Meth (%)	27	33	29	42	28	22	27	20	14	16	35
Sample 15											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed	Passed
Meth (%)	29	35	22	40	28	19	28	20	10	13	35
Sample 16											
Quality	Passed	Passed	Passed	Passed	Passed	Passed	Check	Check	Check	Check	Check
Meth (%)	29	32	27	39	35	24	29	16	14	17	33

6.13. Supplementary data S13: Pyrethroids pesticides overview

Context

In the middle of the 20th century, a new class of insecticides, believed of low toxicity, emerged: pyrethroids pesticides (Abubakar *et al.*, 2020). Other advantages such as their low application rate, high efficiency and biodegradability have encouraged their worldwide use (Park *et al.*, 2021). Thus, they rapidly gained popularity leading to their international commercialization starting in 80s. Considered as an important weapon in the context of global public health, they also helped to limit the spread of various vector-control diseases (*i.e.*, malaria[10]) (Ogunah *et al.*, 2020). Nowadays, pyrethroids pesticides are the most used category of agricultural and domestic pesticides (Furlong *et al.*, 2020). They are still predominant in the public health insecticides market. In 2018, these neurotoxic agents' global market value was estimated to worth $1633.03 million (Tang *et al.*, 2018).

[10] Infectious disease of tropical and subtropical regions caused by a unicellular eukaryote, *Plasmodium sp.*, that might be propagated via female *Anophele sp* (Ogunah *et al.*, 2020).

Even though they save humans 'lives by avoiding or limiting famines and the spread of vectors 'diseases, scientists' community has highlighted an environmental toxicity caused by these compounds (Tang *et al.*, 2018; Zhu et al., 2020*)*. Entering easily entering water media via spray drifts and runoffs, deleterious effects for non-target species, specifically aquatic organisms, have been frequently reported. Classified as endocrine disruptors compounds (EDCs), pyrethroids are known to be responsible for hormonal imbalance and affects reproductive systems of aquatic species (Zhu *et al.*, 2020). Negative impacts on behavioural traits have also been documented about these neuropoisons and might be associated to (epi)genotoxicity. It is assumed that early life stages (ELS) are highly sensitive to pyrethroids 'exposure (Blanc *et al.*, 2021; DeCourten *et al.*, 2020; Furlong *et al.*, 2020).

Despite these elements, they have been only subject to some restrictions and their contribution is even seeing a steady rise since the start of 21st century (**Figure 18**). Residues in the environment are becoming increasingly important over time, particularly in the sediments of aquatic media (Yang *et al.*, 2007). Among this class of insecticides, PM has been one of the most detected worldwide pyrethroids, and that makes it a necessary substance to study. Overall, there are raising concerns about this agrochemical-associated toxicity towards highly exposed aquatic populations, being typically bottom-dwelling and epibenthic species (US EPA, 2009). At present, there is scarce information regarding genetic, epigenetic, and behavioural toxicity towards fishes 'communities (Hellou, 2011).

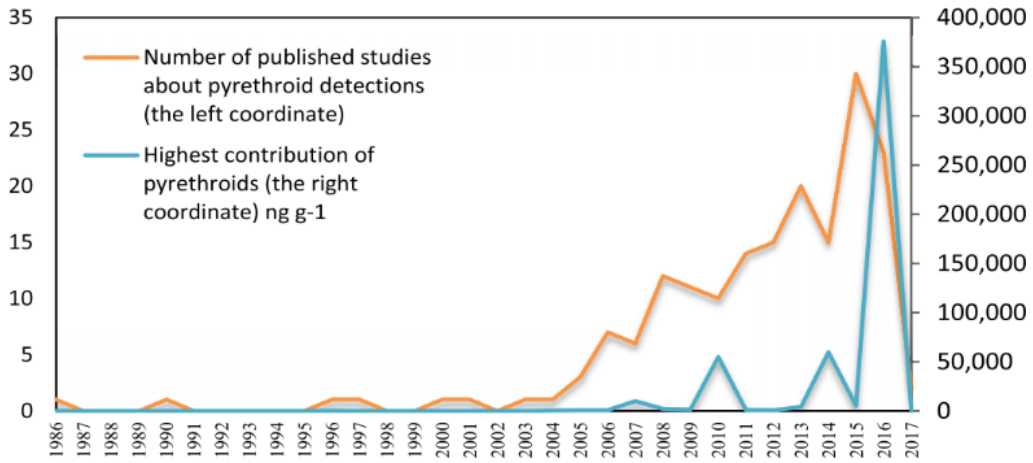

Figure 18: Evolution of pyrethroids contribution (highest found concentration (blue) and detection in scientific literature (orange) over the three last decades. The x axis referred to time scale (years) while y axis indicates pyrethroids concentration (left; $ng.g^{-1}$) and the number of published studies (right; no unit) (Tang *et al.*, 2018).

General description and physico-chemical properties

Pyrethroid pesticides are defined as a class of organochlorine synthetic pesticides. These neurotoxins have insecticidal properties that come from the presence of a modified form of natural molecules, pyrethrins, isolated from *Chrysanthemum cinerariaefolium*'s flowers (Zhu *et al.,* 2020). Pyrethroids pesticides are organic molecules currently used in various domains, ranging from residential to industrial or agricultural purposes (Tang *et al.,* 2018). The main objective is still similar, no matter the application: killing noxious insects' species. Chemically, pyrethroids molecules are esters of some acids (e.g., chrysantemic acid) and do contain a cyclopropane ring. Several asymmetric centres and/or alcohol moiety might be present and be responsible for the existence of various stereoisomers (WHO, 1990). Based on their chemical structure, two subdivided groups of pyrethroid pesticides exist: type I (presence of a a-cyano-group) et and type II (absence of a a-cyano group). Therefore, both types have their own specificities in terms of toxicological symptoms. It is assumed that type I molecules, such as PM, contain different alcohol and acid fractions, and the presence or not of acetylated cyclopropyl. Conversely to first-generation, second-generation of pyrethroid pesticides are photostable but toxicity and mode of action remain identical between both generations (Zhu *et al.,* 2020).

Concerning their physico-chemical properties, their high hydrophobicity is a major characteristic playing a role about their fate within the environment. The high value of octanol-water partition coefficient (Kow), are situated around 4,5 and 7, reflecting their non-polarity (e.g., 6,5 for PM) (Zhu *et al.,* 2020). Their low water solubilities values (0.001-0.01 ppm) and high adsorption constant on organic carbon (Koc) can explain pyrethroid pesticides tendency to be adsorbed to soil or sediments' particles (Zhu *et al.,* 2020). Based on Laskowski's data, molecular mass of pyrethroids is in the order of 380-670 $g.mol^{-1}$ (e.g., 391,3 $g.mol^{-1}$ for PM) (Laskowski, 2002). In addition, they are characterized by a low vapor pressure at 25°C (1.8 x 10-7 to 1.8 X 10^{-11} mm Hg) and low Henry's law constant values, highlighting the low volatility of pyrethroids (Laskowski, 2002).

Presence in the environment

Due to their past and current massive use, typically in countries agriculturally active, (California), severely touched by vector-borne disease (Africa) or described by a high density of population (China), pyrethroids are now ubiquitously present in the environment (Tang *et al.,* 2018; WHO, 1990; Xie *et al.,* 2022; Zhu *et al.,* 2020).

This broad distribution also includes places where those pesticides were not necessarily used (Tang *et al.*, 2018). It might be explained by the slow turnover of these neurotoxins slow compared to the important arrival of pesticides in the environment, enhancing their continuous environmental presence and circulation (Laskowski, 2002). Highest pyrethroids 'concentration ever detected has been measured in Pakistan, in the cultivated area of Okara. Some of them are sometimes less biodegradable than initially believed (US EPA, 2009). In general, the mean half-life values are ranging from to tens to hundreds of days, especially for second-generation pyrethroids such as cypermethrin (US EPA, 2009; Zhu *et al.*, 2020). An extremely rapid degradation is therefore possible for photolabile chemical compound when they are exposed to sunlight radiations (Zhu *et al.*, 2020).

6.14. Supplementary data S14: Permethrin

Main structure and characteristics

Permethrin (PM) is the first synthetic pyrethroid pesticide, that has been developed in 1973 and marketed four years later. In 1990, 600 tons of PM were produced each year, mainly for cattle and crops protection such as cotton plants. This substance was also used as an acaricide in residential applications and as a mean of controlling pests' infestations in the context of paludism (WHO, 1990). PM is currently the second most worldwide detected pesticide, especially in areas subject to intense agricultural activities (*e.g.*, China and the state of California) or to vector-control diseases such as dengue (Tang *et al.*, 2018).

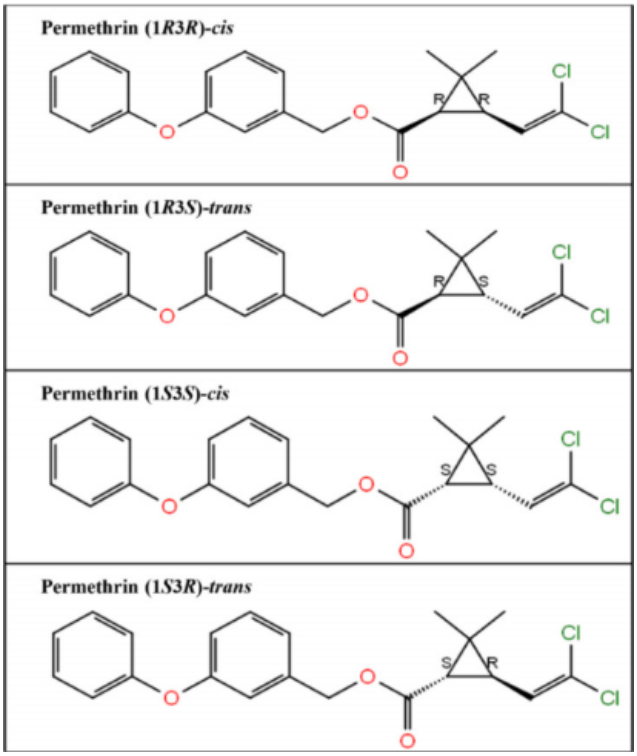

***Figure 19**:* Example of a pyrethroid pesticide structure. Four stereoisomers in two dimensions of PM, which are respectively permethrin (1R3R)-cis, permethrin (1R3S)-trans, permethrin (1S3S)-cis, and permethrin (1S3R)-trans (Sheikh *et al.*, 2021).

Its chemical formula is C21H20Cl2O3 and its complete name is (3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2 dimethylcyclopropane carboxylate. As already mentioned, this is a type I and second-generation pyrethroid. This non-systemic biocide is found on the market as a racemic mixture of the trans and cis isomers (**Figure 19**). This yellowish liquid of low density is usually prepared as a powder or as emulsifiable concentrate (US EPA, 2009; WHO, 1990). Its solubility is equal to 0,2 mg/L at 30°C and its Kow has been measured as 6,5 (WHO, 2020).

In view of its high Koc, low vapor pressure (2.15x10-8 mm Hg), some interpretations can be made about PM's behaviour in the environment.

This latter has been thus considered as a persistent and extremely immobile molecule that does not tend to volatilize from any type of surface (US EPA, 2009).

Regardless of the PH, PM is not very prone to degradation by hydrolysis, as well as photolysis. A study indicates that the half-life of this molecule, in absence of light, at various PH (3-9), reached between 125-175 days depending on O2 saturation percentage.

Moreover, it also indicates that it has a potential for bioconcentration in organisms and partition to soils and sediments' organic particles (US EPA, 2009).

Mode of action

PM is a stomach and contact pesticide and consequently, might enter insects 'organisms by dietary or non-dietary intake. The mode of action of these neurotoxins is based on targeting neuronal cells 'electrophysiology, generating dysfunctions within the nervous system of various noxious insects. In both the central and peripheric nervous system, they are disturbing cellular influx by interfering with voltage-gated sodium channels. This latter highly contributes to the mediation of action potentials, especially by opening when membrane potential is getting positive. PM is characterized by a biphasic mode of action. Voltage-gated sodium channels alteration in the peripheral nervous system is thereby impacting cholinergic signalling within the central nervous system (Blanc *et al.,* 2021; Corbel *et al.,* 2006).

- Peripheric nervous system (PNS)

By affecting the duration of axonal voltage-gated sodium channels 'opening, cellular ionic homeostasis in neuronal cells is lost. In other terms, PM is a voltage-dependant channels 'agonist. It can thus extend the opening 'duration of these transmembrane proteins via conformational changes. A continuous influx of sodium ions is present within the nerve axonal membrane, leading ultimately to a depolarization impossible to counteract. This channels' overactivation causes an overexcitability of the motor or sensory neurons (Blanc *et al.,* 2021; Corbel *et al.,* 2006).

- Central nervous system (CNS)

This neurotoxic effect on voltage-gated sodium channels results into an increased liberation of a neurotransmitter in the SNC, essential for nerve impulses transmission: acetylcholine. Due to a change of presynaptic neuronal activity within the SNC, a huge acetylcholine secretion and accumulation in the synaptic cleft of interneurons are observed. Based on a feedback mechanism, it leads to an activation of presynaptic muscarinic receptor which ultimately inhibits acetylcholine molecules 'liberation from this presynaptic neuron. The absence of acetylcholine totally prevents the well-transmission of signals such as cholinergic transmission (Corbel *et al.,* 2006).

The described mechanisms of PM's action on insects are thereby associated with a series of symptoms of various severity (agitation and loss of the legs, behavioural deterioration, and

paralysis) that generally lead to death. A wide class of insects are sensitive to PM, especially Lepidoptera and developmental stages in general.

Note that chloride, calcium, and potassium channels have also recently been recognized to be also disrupted by pyrethroids (Blanc *et al.*, 2021).

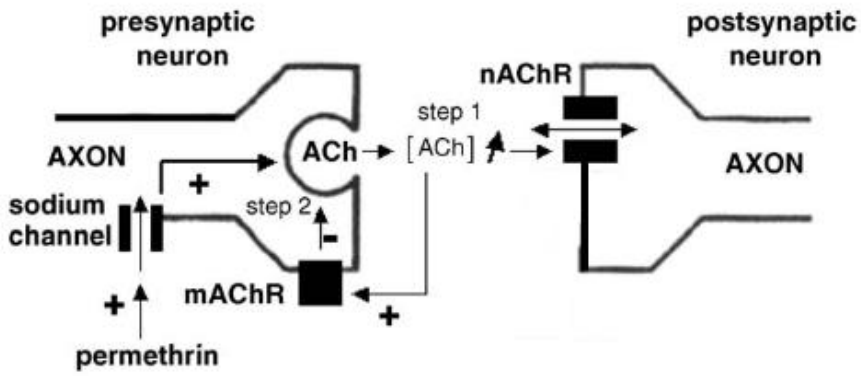

Figure 20: Retroaction mechanism following PM's exposition on the SNC of one of this neuropoison's target, the mosquito Culex quinquefasciatus. An overactivation of sodium channel creates an abundant arrival of acethylcholine in the synaptic clef, released by presynaptic neuron. The muscarinic receptors of this latter (mAChR) are then stimulated in their turn, stopping acetylcholine's liberation from presynaptic nerve cell. In the absence of acetylcholine, post-synaptic cholinergic receptors are not anymore activated, leading to a disruption of cholinergic signals (Corbel et al., 2006) (simplified diagram)

Arrival of permethrin in the aquatic system

There are multiple ways of PM's arrival within the aquatic system. Sprayed on farmlands, the agrochemical stays quite stable on the cultivated vegetation Nevertheless, it does not stop a certain quantity of PM from being taken up by the plant and another amount from becoming lodged in the soil. A cause-effect relationship has been established between the quantity of applied PM on crops, and the residual amount of pesticide found on the whole concerned agricultural field.

Rainfall helps the remaining non-absorbed PM's transfer into the soil's crop and potentially to other nearby water sources. Even though PM is not likely to leach the ground, erosion and leaching can cause the arrival of PM's spray drifts into water bodies (US EPA, 2009; WHO, 1990).

In urban areas, domestic PM's application (*i.e.,* treatment for lice and scabies) is mainly indoors (*i.e.,* healthcare treatment for lice and scabies) and residues join wastewater treatment plants. Most importantly, another indoor residential use of PM is related to humans 'protection against vector-borne diseases (Ogunah *et al.,* 2020). Huge amounts of this product are annually used for epidemiologic control of paludism in subtropical and tropical regions. In 2006, a report from the Environmental Protection Agency (EPA) has assumed that "The majority of PM, approximately 70%, is used in non-agricultural settlings" (US EPA, 2009). For example, used as a mechanical weapon of prevention, mosquito nets are coated with these products. PM is part of the insecticide-treated bed nets (ITNs) approved by the World Health Organization's Pesticide Evaluation Scheme (WHOPES) (Zhu *et al.,* 2020).

The route of residual PM's initially included in some household compounds (anti-lice shampoos, etc.) finally results into their arrival in wastewater treatment plants from which they can be potentially discharged to surrounding water bodies. Furthermore, humans are inevitably exposed to this residential PM-based product. Residual PM's metabolites are found in urines and join effluent. In summary, pesticide's residues can infiltrate into water plants from different sources, such as agricultural run-offs, effluents, and wastewaters (US EPA, 2009).

Preceding its entry in water bodies, PM might be already adsorbed onto particles. Otherwise, its partition rapidly followed the chemical's arrival in the water plant (Xie *et al.,* 2022). its bioavailability within aquatic media is transitional, estimated to have a duration of 48h (Yang *et al.,* 2017). As a highly non-polar substance, PM has a low affinity for water molecules and is more likely to adhere to sediments 'particles (Tang *et al.,* 2018; Zhu *et al.,* 2020). This irreversible partition can lead to PM's accumulation in sediments and might be a threat for the surrounding pyrethroids-sensitive life, typically fishes (Blanc *et al.,* 2021). Both marine and freshwater are concerned by the environmental pollution linked to pyrethroids such as PM. Marine ecosystems are therefore less threatened due to higher water quantities than in lakes or rivers, leading to pollutant 'dilution (Tang *et al.,* 2018). Another possible fate of PM is entering food chain. Being bioretained by organisms through ingestion of contaminated preys or through its fixation on fishes 'gills, are both ways of contamination by this chemical.

6.15. Supplementary data S15: *Kryptolebias marmoratus*

Morphology

Mangrove rivulus is a small killifish measuring only 17mm at the adult stage (3-6 months) (Edenbrow & Croft, 2011). Other morphological features of the neotropical rivulus is its flattened head and upturned mouth. For information, its growth rate has been proved to be temperature dependant and negatively influenced by poor water quality (dissolved oxygen) or surrounding density of other congeners (Taylor, 2012).

Three sexual phenotypes exist for this species: hermaphrodite, cryptic males and traditional males. As presented in **Figure 21**, coloration patterns differ between hermaphrodites, cryptic males, and classic males (Kelley *et al.*, 2016). The three sexual phenotypes differ externally from their body and caudal fin pigmentation, and internally from their reproductive organs. As indicated by a red narrow, hermaphrodites do have a melanic coloration and an ocellus at the base of caudal (**Figure 21**). In contrast, primary males are not characterized by the presence of a dark black spot. However, they do have a homogeneous orange coloration on caudal peduncle. Lastly, secondary males have rather orange spots distributed non-uniformly on the whole body (Marson *et al.*, 2019).

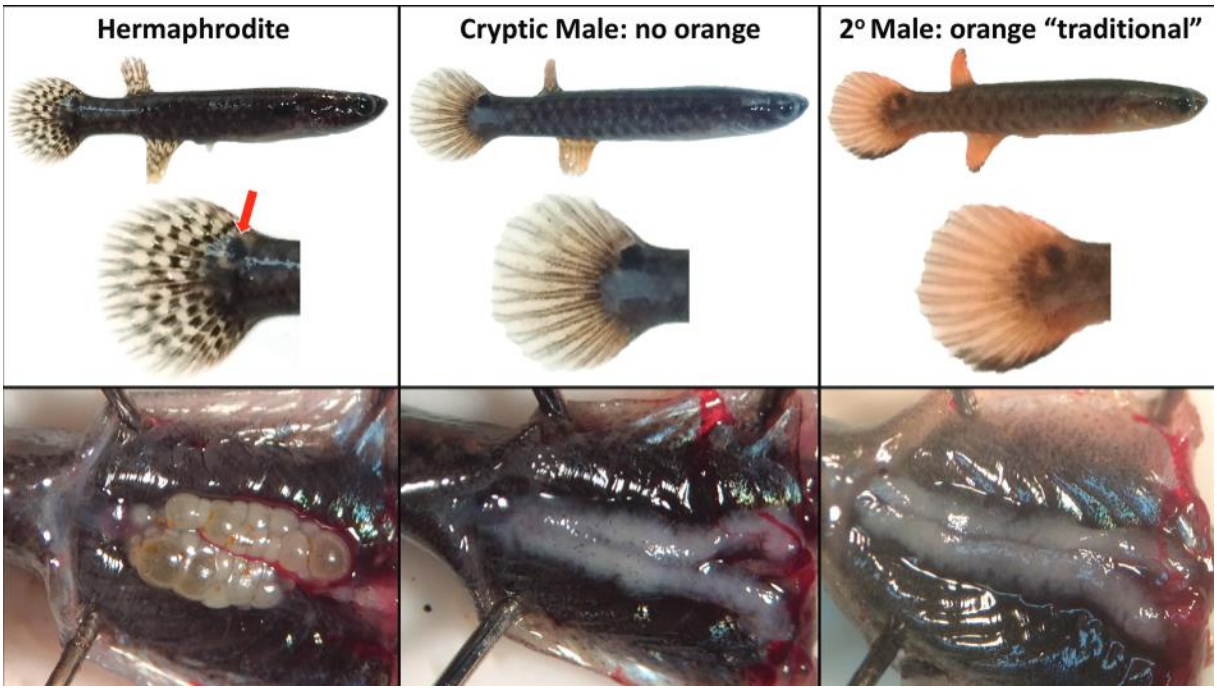

Figure 21*:* Representative of each existing *K. marmoratus* sexual phenotypes (hermaphrodite on the left, cryptic male in the middle and traditional male on the right). While full body is

represented on the top panels, the bottom photographs show the opened internal cavity on the ventral side of each sexual phenotype. Reproductive system is clearly visible (ovotestes and testes for hermaphrodite and only testes for the two other sexes) (Marson *et al.*, 2019).

Ecology

- Geographic distribution, living habitat and dietary

Mangrove killifish is characterized by an important geographic distribution among tropical and subtropical areas. Present in mangroves in South and Central America, as well as in the USA (Florida) and the Caraibes, this vertebrate is mainly occupying shallow and varied salinities areas (Edenbrow & Croft, 2011; Tatarenkov *et al.*, 2010; Taylor, 2012). This dwelling Rivulidae used to live in cryptic habitats in intertidal and estuarine zones. In fact,it hides in areas where interspecific competition is limited. Wet places where vegetal litter or degenerating mangrove trunks are found, are then typical microhabitats (Tatarenkov *et al.*, 2010; Taylor, 2012). Evidence tends to confirm this hypothesis. Even though few species like *Cyprinodon spp.* and *Poecilia spp.*, have been recorded in *K.marmoratus*' ecological niches, the only confirmed sympatric species is *Rivulus tenuis*. Some parts this model species' living habitat helps to limit predation. A strategy for avoiding predators is emersion in drier place when water level is decreasing. By changing its location, it can move away from areas of high predation risk such as the edge of the mangroves. In the same perspective, this Killifish may also temporally escape into crab burrows. For information, the mangrove water snake, *Nerodia asciata compressicauda*, is a common predator (Taylor, 2012).

This organism is particularly tolerant to environmental conditions. Indeed, it can live in a wide range of temperature (7-45°C) and support depleted dissolved oxygen level. Furthermore, this euryhaline specie can physiologically adapt itself to a variety of salinity levels, especially hypersalinity (up to 70ppm) (Rhee *et al.*, 2012). Even though this species has been known to support pure freshwater, recent literature suggest that *K.marmoratus* does not live in water of salinity inferior to 10 ppm (Taylor, 2012; Tatarenkov *et al.*, 2010). Its resistance to high level of toxic compound such as hydrogen sulphides (H2S; 0.4–0.7 ppm) and ammonia (>10 mmol/L) should be mentioned (Taylor, 2012).

Its diet is mainly composed of various types of invertebrates including larvae stage. Adding to mosquitoes' larvae, crustaceans and polychaete are part of their dietary intake. Cannibalism has been reported among this neotropical fish. Their feeding behaviour is characterized by a low rate of feeding.

It might be due to a depleted of food supply within their feeding habitat. As explained above, the abiotic and biotic conditions in their living area tend to be extreme (e.g., repeated periods of drought, anoxia, etc) and might restrict feeding opportunities (Taylor, 2012; Rhee *et al.,* 2012).

- Reproductive system

As already mentioned in the introduction, it is part of the scarce organism able to self-fertilize (Edenbrow & Croft, 2011). In fact, hermaphrodites can both be fertilized by a pure male and self-fertilize. While a major proportion of the populations develops an ovotestis (hermaphrodites), some individuals do not (males) (Edenbrow & Croft, 2011; Tatarenkov *et al.,* 2010). Within ovotestis structure, tissues linked to male and female are entangled Those proportions are not stable between populations. Mangrove rivulus' wild populations are then called androdioecious (Tatarenkov *et al.,* 2010).

Between three and six months after hatching, sexual maturity is reached, and internal fertilization may occur. The moment of the first reproduction and egg laying is quite fluctuant between individuals. Note that there is no oviposition cycle so egg laying can happen anytime during its lifetime. The generation time is quite rapid, ranging from 3 to 5 months (Edenbrow & Croft, 2011; Kelley *et al.,* 2016).

- Particularities

Many arguments can be cited for justifying that mangrove rivulus is a popular experimental fish. Firstly, the easy autofecondation and the high of the organisms enhances its use in laboratory by reducing mortality risks due to potential (and sometimes inevitable) changes in abiotic conditions during the experiment's period (Edenbrow & Croft, 2011; Rhee *et al.,* 2012). Its broad range of salinities tolerance may especially constitute a positive element encouraging its use for studies about hyper salinity adaptation (Taylor, 2012). In some specific works, the absence of oviposition cycle also facilitates investigations that include fecundation parameters. When they become adults, there is no need to wait for a specific period for measuring egg laying rate.

In addition, the geographic widespread brings a representative dimension to, for example, ecotoxicology experiment ((Edenbrow & Croft, 2011; Taylor, 2012; Tatarenkov *et al.,* 2010).

Furthermore, in view of the high exposition of dwelling organisms to PM, the study of an ecotoxicological risk is much more reliable when using a model species like the described *K.marmoratus*. Most importantly, the ability of producing isogenic lineages is an indescribable asset for strongly reducing genetic variations in experiments. Thanks to the presence of genetic uniformity and phenotypic plasticity among the populations, it helps to assess the impacts of a chemical on this organism (Tatarenkov *et al.*, 2010). In fact, the reasons of genetic variations are then linked to environmental conditions (Tatarenkov *et al.*, 2010). In the context of this study, changes in genes expression can then be associated to PM exposure.

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