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PROJET DE MÉMOIRE

A Study of early and delayed effects of permethrin, a neurotoxic pesticide, on phenotypic traits of the mangrove killifish *Kryptolebias marmoratus*

Research Unit in Environmental and Evolutionary Biology (URBE)

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Année académique : 2020-2021

Alternative title: Phenotypic variability of the mangrove rivulus, *Kryptolebias marmoratus*, exposed to the neurotoxic compound permethrin

Abstract

The constant interactions with the environment can have major effects on the phenotype of animals and thus their fitness. The early developmental stages are most vulnerable to disturbances of any kind. However, in fish, little research has been done to study the impact of environmental stresses experienced during development on the phenotype of individuals. Here, we studied how early exposure to permethrin, a pyrethroid neurotoxic compound found in numerous insecticides, influences developmental phenotypic plasticity of juvenile mangrove rivulus *Kryptolebias marmoratus* and the delayed effects on them in adulthood. This species is unique among vertebrates due to its ability to self-fertilize, thus producing natural homozygous individuals within isogenic lineages while maintaining a high degree of phenotypic plasticity. By working with minimal genetic variation, this species can provide valuable insights into how the environment including chemical pollutants can impact both morphological and behavioural phenotypes without a change in the genotype.

We exposed *K. marmoratus* larvae to different concentrations of permethrin (PM) (0, 5 and 200 µg/L) to study the effects on life-history traits, behaviour, and hormones levels in 7-day-old subjects. In addition, we studied the possible effects on growth, aggressive behaviour, and hormone levels in 148-day-old *K. marmoratus* adults. Size measurements from four timepoints (Day 1, 7, 70, 149 post-hatch) showed that growth was impacted. In fact, from day 1 to 70 post hatch (dph), individuals exposed to 200µg/L of PM grew significantly less than those of the two other groups (5µg/L and control), however from day 70 to 149, individuals exposed to 200µg/L grew significantly more than those of the control group providing evidence of compensatory growth.

PM exposure during development significantly affected the behaviour of juveniles, hypoactivity and a reduced anxiety-like behaviour for PM exposed individuals compared to control fish was observed. The resulting smaller, slower, and less mobile fish also proved to have less predation success. However, no effect on pre-fight cortisol, pre-fight testosterone, aggressive behaviour or fight outcome was found in adults during “combats” between two adult hermaphrodites. We therefore provided evidence that PM has adverse immediate effects on phenotype and behaviour in *Kryptolebias marmoratus* larvae that seem to recover over time before reaching adulthood. We have also investigated DNA methylation levels and differential expression of certain target genes which will be discussed in a subsequent study.

Key words: phenotypic plasticity – neurotoxicity – permethrin – behaviour – hormones – *Kryptolebias marmoratus*

1. INTRODUCTION:

Animals display a fascinating range of behavioural traits that can vary even within the same species and population (Hau & Goymann, 2015). The response of an individual to its everchanging environment and its adaptability play a crucial role in its ability to survive and reproduce (i.e. its fitness; Kanda, 2019; Marn et al., 2017). The fitness of an individual not only depends on its morphological traits, but also on its behavioural plasticity, especially during development (Moczek, 2015), which is often overlooked. Animal fitness strongly depends on the phenotypic plasticity of their behaviour, predicting how they will react under certain circumstances, which therefore impacts the outcome of crucial situations like escaping a predator or competing for resources(Snell-Rood, 2013). Aggressiveness for example is usually beneficial during contest with conspecifics competing for limited resources available in their environment (e.g. food, mate, territory), because it can help the competitor to be the winner of the fight (Pedetta et al., 2010; Sanches et al., 2012). On the other hand, aggressiveness can be costly when an animal has less fear in the presence of a predator or when their behaviour perturbates (allostatic overload) the balance of energy allocation, using too much of the stored energy reserves or other resources critical to lifetime reproductive success (Snell-Rood, 2013). Allostatic loads can be adaptive responses to seasonal and situational demands, like in this example a fight over a mate or territory; these allostatic loads are mostly within limits, even necessary at times. However, if one adds on this an additional load of unpredictable events such as diseases or social interactions (overconsumption of energy due to excessive aggressiveness), then allostatic load can increase dramatically and become allostatic overload, which leads to adverse effects (McEwen, 2017). Thus, Investigating the origins and the extent of behavioural plasticity is crucial to better understand the overall mechanisms regulating and influencing such plasticity.

Hormonal mediation may be one mechanism underlying behavioural plasticity and may therefore be important topics to research, since the rate of change in environmental conditions has increased over the past decades, leading species to respond with appropriate changes in hormonally mediated behaviours either through plasticity or evolutionary changes of hormonal mechanisms. (Pedetta et al., 2010) Exploring the relationships between hormones and behaviour would therefore provide important information allowing to answer these questions (Hau & Goymann, 2015). In vertebrates, hormones are known to be potent mediators of behavioural changes. Studies have shown a direct linear relationship between testosterone (T) and cortisol (F) concentrations and the expression of certain behavioural traits. T is known to promote the expression of courtship behaviour and aggression (Snell-Rood, 2013), whereas high F levels during development in fish have shown to trigger a fearful behaviour later in life (Colson et al., 2015). Glucocorticoids like F are also mostly produced during aggressive interactions and may inhibit or increase aggressive behaviour depending on the context. Chronically increased glucocorticoids generally inhibit aggressive behaviour in submissive individuals in a population which can be explained as an evolutionary adaptive strategy (Ketterson et al., 2009). Sudden spikes in glucocorticoid levels on the other hand actively trigger or enhance aggressive behaviour by the means of specialized regions of the brain like the hypothalamus (Summers et al., 2005). High T levels are associated with

increased activity and aggressive behaviour and in most species, therefore more aggressive individuals are more successful in resource competition or mating. Some behaviours can also be enhanced by a change in the environment which triggers hormonal perturbation (Hau & Goymann, 2015) that is not always reversible. Once a certain threshold passed, the phenotype would have the potential to be fully expressed, irrespective of any further increases in hormone concentrations (Hau & Goymann, 2015). In the past years it has become more and more obvious that certain types of environmental changes have the potential of disrupting the endocrine system of wildlife (Colborn et al., 1994). Moreover, organisms that endure these perturbations during development either in prenatal or early postnatal life can suffer permanent and irreversible adverse effects (Colborn et al., 1994) due to a disruption of the crucial role of hormones during development. This can even lead to transgenerational effects lasting over multiple generations(Brehm & Flaws, 2019). Information is lacking on the link between environmental factors, hormones and their effects on behavioural variations, especially in fish species which are often affected by chemical pollution.

While genetic mechanisms as the origin of phenotypic variations are better researched and documented in some species, epigenetic mechanisms such as microRNAs, histone modifications, DNA methylation and hydroxy methylation might be other important sources of phenotypic plasticity, including behavioural plasticity. Epigenetic changes are molecular modifications or factors that modify gene expression without changing the actual DNA sequence (Ledón-Rettig et al., 2013). Environmental conditions have been shown to affect epigenetic patterning and functions, which affect behaviour and induce adverse effects (Lester et al., 2011).

Because phenotypic traits of animals can be strongly influenced by abiotic changes of the environment, the rise of chemicals released into the environment by agriculture, manufacturing, or medicine, can have potent effects on the behavioural plasticity. For example, PM, a pyrethroid neurotoxic compound that is widely used as an insecticide in agriculture and for domestic use, has been documented to affect animal phenotype, especially in species from water ecosystem (Günal et al., 2021; Wurzel et al., 2020). In fish, Zebrafish that were exposed during early development until 28 days post-fertilization (dph) and monitored until F3 in unexposed environments were significantly affected and displayed behavioural variations, with F1 and F2 larvae being significantly less active than controls. The evidence suggests transgenerational alterations of the neural or neuromuscular functions and influences on neurodevelopment in zebrafish (Blanc et al., 2020). Another study shows F0 fish exposed to PM were hypoactive at adulthood, and offspring males from F1 and F2 generations showed a decrease in anxiety-like behaviour (Blanc et al., 2019). PM has also been shown to induce epigenetic changes in various species. Genome wide DNA-methylation analysis in rats for example showed a genome-wide decline in DNA-methylation levels following early life exposure to PM (Bordoni et al., 2015). Another study on zebrafish proved that PM leads to transcriptional changes in epigenetic factors meaning that neurotoxins like permethrin can in fact impact early epigenome reprogramming in fish influencing their development (Goikoetxea et al., 2021). The fact that a neurotoxin like PM can alter the transcriptome of an individual specifically in behaviour relevant regions, means that the underlying effects may result in persistent modifications of hormone levels or signalling pathways, and ultimately

cause behavioural alterations. For now, it seems that regarding fish species, only effects on zebrafish have been explored for PM toxicity, its effects on morphology, behaviour and hormones (Blanc et al., 2020).

In the present study, we aimed to better understand how PM affects life history traits, behaviour variability and how hormonal mediation may underly behavioural plasticity in response to environmental changes in fish. We investigated the immediate and delayed effects of PM on behavioural plasticity, as well as other phenotypic traits, in juveniles and adults of the mangrove rivulus *Kryptolebias marmoratus.* The mangrove rivulus is the only self-fertilizing hermaphroditic vertebrate, therefore, naturally presents minimal genetic variability inside of the different lineages but naturally manifests an important phenotypic plasticity. Its unique characteristics make it possible to specifically investigate phenotypic differences and without the genetic variations (Soto et al., 2011). The goal of this study is not solely to study the effects and toxicity of PM, but to use PM as an example of a neurotoxin to study how they can have short and/or long-term effects on hormone levels, phenotype and important behavioural traits. Steroid hormones, especially glucocorticoids, androgens and oestrogens, have already been shown to be associated with behaviours such as aggressiveness and dominance during contests in *K. marmoratus* (Earley et al., 2013). High T levels have proven to be associated with a more aggressive behaviour: individuals with higher levels of endogenous T are more prone to attack and have a higher chance of winning (Earley & Hsu, 2008). They are also more likely to escalate contests into mutual attacks. Moreover, previous studies show that contestants with higher pre-contest F, were more likely to lose size matched contests (Li et al., 2020). Because PM can potentially alter hormone levels as previously found for other species (Tu et al., 2016),it may affect hormonal mediation of rivulus behaviour.

We designed a first experiment to investigate the immediate effects of PM on behaviour (locomotion, thigmotaxis, foraging and predation) but also on morphology (growth), metabolic rate (oxygen consumption rate) as well as on hormones levels (F) , gene expression and DNA-methylation in mangrove rivulus larvae. We hypothesized that PM would alter phenotypic traits. We therefore expect juveniles to move less, to be less anxious, to be less successful in foraging and predation and to have a higher metabolic rate than control animals. We also expect that larvae would have lower F levels, reflecting a lower anxiety-like behaviour. We designed a second experiment to explore the delayed effects of PM on morphology (growth), hormone levels (T and F) and aggressive behaviour during contest in adult mangrove rivulus. We hypothesized that PM affects pre-fight F and T levels in adults due to its endocrine disruptive properties observed in Zebrafish(Blanc et al., 2020). Furthermore this may impact fight outcome (the probability of winning) and aggressive behaviour during contests, the link between hormone levels (F and T) on such behaviour has been previously documented but contradictions still exist in literature(Earley & Hsu, 2008).

A reduced activity in *K. marmoratus* has been previously observed, especially for the higher concentration of permethrin in other fish species like *Danio rerio*.(Blanc et al., 2020) Because resulting adults of exposed fish larvae have shown to display less fearful behaviour at low

concentrations of exposure but not in the F0 generations, epigenetic changes was proposed(Tran & Miyake, 2017), influencing endocrine signalling. Even if previous studies have not been able to report these behaviour variations in F0 adults, the highest concentration exposure could reveal a different result. Lastly, the less fearful (therefore also less cautious) behaviour in addition to the hypoactivity may lead to disadvantages during adult contests leading to a higher probability of losing for exposed fish against the control individuals.

2. Methods and Materials

2.1 Housing and PM exposure

2.1.1 *Laboratory breeding*

The individuals used in this study were offspring from adult hermaphrodites of the "DC4 lineage "of *Kryptolebias marmoratus* located at the University of Namur (UNamur). The DC4 stock population was obtained from a F2 generation which originates from specimens sampled by Ryan L. Earley and D. Scott Taylor in 2010 in Dove Creek (Tavernier, Florida; N25°01'45.64", W080°29'49.24"). Stock fish are kept individually in tanks under controlled conditions: a water salinity of 12 ± 1 ppt (Instant Ocean Sea Salt), a temperature of 27 ± 1°C and a photoperiod of 12h:12h. Adults are fed daily ad libitum with nauplii of *Artemia salina*.

2.1.2 Egg collection and development

On May 2021, eggs were collected from hermaphrodite individuals (N=60) and placed individually in 24-well plates made for cell culture (Cellstar®) filled with 2ml of 12ppt saltwater (26 ± 1°C). Every 3-4 days, water was renewed to ensure constant conditions until hatching. In order to ensure that future resulting fish would have the same age during experiment, multiple synchronized hatchings were performed 4 weeks after egg collection (i.e. when embryos reached their final stage of development and were in diapause)(Mesak et al., 2015). To do so, we used a technique developed in our laboratory by I. Blanco (LEAP, university of Namur, BE) that consists in placing microplates in incubation Ziplock bags already containing an Anaerocult™ P sachet (purchased from Merck, Inc.). This sachet contains chemical components which, combined with water bind oxygen, create an anaerobic environment that triggers hatching within hours (Wells et al. 2015; pers. obs.).

2.1.3 Permethrin exposure

To investigate the influence exposure to PM on rivulus larvae, newly hatched larvae were placed individually inside 12 well-plates (4mL) and exposed to 3 different concentrations of PM during 7 days. Larvae were therefore exposed either to 0 µg/L ("control" dose: C ; N=185), 5µg/L ("environmental" dose: E; N=83) or 200µg/L ("sub-lethal" dose, S; N=89) of PM (Fig. 1). The E treatment was chosen based on PM concentration that can be found in the living area of the mangrove rivulus in the Florida Keys (i.e. range 5.1 to 9.4µg/L; Pierce et al., 2005).

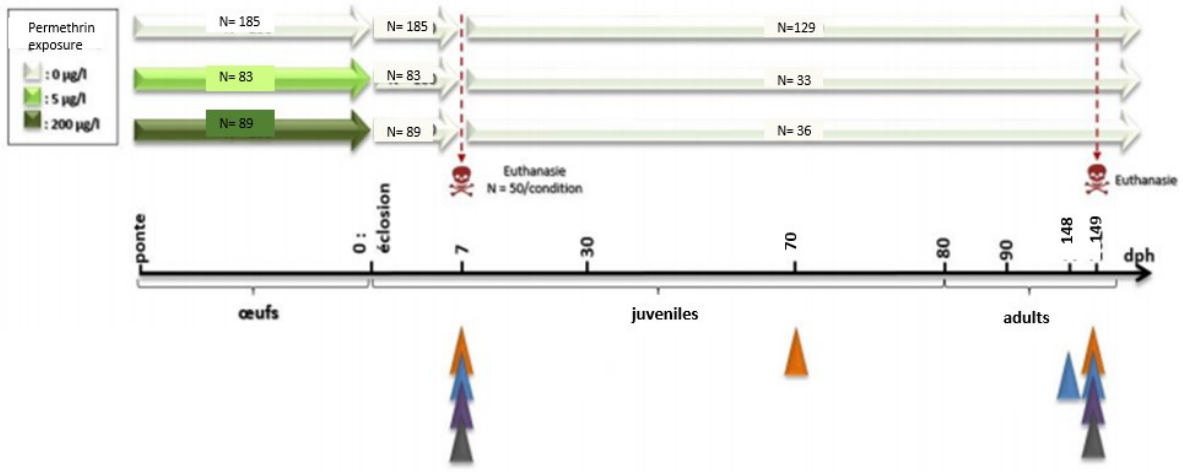
The S treatment was chosen to observe PM effects at much higher concentration than dose found in the environment while remaining under the lethal dose. For reference, the LC50 (96h) of permethrin was found to be 300 µg/L in zebrafish (DeMicco et al., 2009). Because PM is known to be relatively water insoluble (ref1), PM stock solutions were prepared in 99% Dimethyl Sulfoxide (DMSO). To avoid solvent interferences, PM working solutions were obtained by diluting stock solutions in saltwater (12 ± 1 ppt); the final concentration of DMSO in each treatment, including the control group, was 0.1% (v/v).

PM working solution were prepared daily by diluting PM stock solution with saltwater before use and stored in amber glass bottles to ensure the most precise concentrations and avoid degradation. In addition, 100% of the solution was renewed daily. Finally, to determine the exact exposure concentrations, samples (1mL) from working solution were collected and immediately frozen at -80°C in order to further assess the actual PM content using High-performance liquid chromatography

2.1.4 PM quantification HPLC

Real PM concentrations of working solutions (nominal 200 µg/L and 5µg/L) were measured using a HPLC method. 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 were 147±4µg/L (N=10), 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, we therefore chose to keep nominal concentration throughout the text.

Figure 1: Summary of data collection at different timepoints. (Orange=Size measures;Blue: Behaviour observations; Purple:Homrone extractions; Grey: DNA/RNA extractions)



2.2 Experiment 1: Immediate effects of early exposure to PM

Here, we aimed to test the early effects of exposure to PM on life history traits, behaviour, hormone titers and O2 consumption in juvenile of *K. marmoratus*.

In order to test the immediate effect of PM exposure on morphological traits, pictures of larvae were taken at 1- and 7-dph, using a Nikon Digital Camera USB3 ½.5 15 IM/SEC mounted on a Nikon SMZ1270 stereomicroscope and the NIS Elements® program. These pictures were then analysed via ImageJ software to measure the standard length of individuals (i.e. snout-to-tail length).

At 7dph, a series of behavioural tests were conducted. Larvae were transferred individually in 6-well plates filled with 8mL of saltwater (12ppt). After 5min of acclimation, individuals were recorded, and locomotion behaviours were measured during 10 min using the DanioVision™ system and the Ethovision XT™ program (Noldus). Total distance travelled, swimming speed, total swimming time and mobility were determined. Thigmotaxis (frequency and time spent in the centre of arena) was also recorded in order to characterize their anxiety-like behaviour levels; an animal that displays thigmotaxic behaviour ("wall hugging") strongly avoids the centre of an enclosure and stays or moves close to the walls (Schnörr et al., 2012). After locomotion and thigmotaxis assays, larvae were transferred in new 6-well plates that already contained 10 nauplii of *Artemia salina* in order to count foraging effort (i.e. number of capture trials) and prey capture ability (i.e. number of captures failure and success) during 5 min. Previous studies have shown that prey capture serves as a good model system to study natural behaviour in vertebrates and can be successfully measured in larvae from 4 dph (Muto & Kawakami, 2013). 8 individuals (5,3 from "C" and "E" groups respectively) were excluded from the predation success results as 0 capture attempts were performed.

Finally, a respirometry system that is adapted to fish larvae (Loligo™) was used to measure their oxygen consumption rate, which is an important physiological parameter characterizing the floor and ceiling in aerobic energy metabolism(Yang et al., 2019). This system is based on a 24-channel optical fluorescence oxygen reading device: individual organisms are introduced inside a 500 µl glass chambers fitted with an oxygen sensor spot on a 24-well microplate footprint. The device measures (in real-time) the rate of decline in oxygen content over time in each chamber during the measurement phase. Each fish was therefore placed during 10 min in the microplate chambers filled with 500µL saltwater (12 ± 1 ppt, 26 ± 1 °C). At the end of the tests, larvae were put back to their 6-wells plates.

The next day, part of the individuals was sacrificed and stored at -80°C for subsequent basal F levels quantification (N=40, 41 and 43 respectively for C, E and S treatment) and molecular analyses (N=11, 7 and 8 respectively for C, E and S treatment), while the rest of the individuals were kept in 318 mL glass jars filled with 200mL saltwater until adulthood (between 60 and 80 dph) in lab rearing conditions. Previous studies on fishes have shown that F levels go back to control levels 60 min after stress induced by handling in laboratory experimental procedures (e.g. see Ramsay et al., 2009).

2.3 Experiment 2: Delayed effects of early exposure to PM:

Here, we aimed to test the delayed effects of early exposure to PM on the behaviour of sexually mature mangrove rivulus during contests, in relation to the role of hormones and the expression of certain genes in the brain.

First, we chose to perform morphometric measurements at 70 dph to test delayed effects of PM on fish growth. At the same time, sexually mature rivulus were transferred from their glass jar into 1L polypropylene adult tanks filled with 400mL of saltwater. All but one mature individual were hermaphrodites. The unique male was discarded from the data. We also added a piece of cotton wool to provide cover for the fish to hide in and lay their eggs.

Adult basal hormone levels (T and F) were assessed at day 147dph, i.e. 1 day before the behavioural observations (N= 112, 27 and F respectively for C, E and S treatment). At the end of this procedure (described below), fish were anesthetized using a solution of 400mg/L of MS-222 (with sodium bicarbonate as buffer) and were marked under binocular microscope by cutting one spine either at the top or at the bottom of the caudal fin, to ensure tracking during behavioural experiment. Individuals were then placed back into their original tank. 3 individuals were excluded from data at this step: two due to physical malformations and one developed secondary male characteristics (Rhee et al., 2017). The following day (148 dph), fish were transferred in contest arenas. Two individuals were randomly placed in one of the two compartments of a standard aquarium (12 x 8 x 20 cm; 1.4 L saltwater at 12 ± 1 ppt), separated by an opaque partition to allow acclimation during 10 min. The partition was then removed to allow the fish to swim freely. We observed contests between i) an “S”-exposed fish versus a control fish (N = 32), ii) an “E”-exposed fish versus a control fish (N = 27) or iii) two control fish (N = 29). Contests were recorded using Sony HDR-CX625 cameras.

Contest behaviours of *K. marmoratus* have been previously described in the literature (Hsu & Wolf, 2001). Usually, the two opponents orientate and swim towards each other.

After a few intimidation-behaviour displays, one of the fish may retreat. Otherwise, one of them - the initiator - displays a first aggressive behaviour by swimming rapidly towards its opponent in order to push or bite it. Sometimes the attacked fish retreats while its opponent chases it; otherwise, it can also responds with attacks. In this study we chose to stop the fight after 30 min; the individuals that display most of the retreat-behaviours were considered the losers of the fights, and its opponent the winner. A contest was considered "without escalation" if only one of the two opponents performed attacks during the encounter; and considered "with escalation" if both individuals attacked at least once. Several interactions were measured during the combats, aggressive behaviour such as biting, chasing, and attacking was regrouped under total aggressiveness. In order to avoid confusing effects on the outcome of contests (e.g. see Li et al. 2018), we matched the opponents of a dyad by size and age (difference in age ≤ 24 hours). We performed *a posteriori* measurement to confirm the similarity in size (paired-t test; t = 1.9006, df = 82, p= 0.06087) and body mass (paired-t test; t = 1.5303, df = 82, p-value = 0.1298) between the opponents of a same dyad. Since our interest lies in the behaviour during the combat, all fights where no interaction between subjects was observed were excluded from the rest of the results.

Finally, individuals were sacrificed the next day (149 dph) and weighed using an electronic balance, additionally, pictures were taken for subsequent standard-length measurements.

2.4 Hormones collection, extraction and quantification:

2.4.1 Hormones collection and extraction

Experiment 1: Each larva was placed in a 1.5 mL conical microcentrifuge tube with 0.5 mL of methanol (99% HPLC grade) and was crushed. After 1h at room temperature, samples were centrifuged (5min, 10 000g) and 400mL of the supernatant were put into new 1.5mL Eppendorf tubes and dried in a heated centrifuge (Thermo/Jouan RC10-10; 45°C). Samples were then stored at -20°C until resuspension in 200µL of Enzyme Immuno-Assay (EIA) buffer.

Experiment 2: Collection and extraction of water-borne hormones (T and F) were performed according to the protocol developed by Earley et al. (Earley et al., 2013). This technique allows measurement of hormones in water rather than plasma, based on hormone diffusion through gills, urine and faeces (Ellis et al. 2013; Félix et al. 2013). It is thus relatively non-invasive and well suited for small fish species for which sufficient plasma cannot be obtained non-lethally. Each adult was transferred from their tank to 400 mL glass jars (one fish/jar) filled with 200 mL clean 12 ppt saltwater where they remained for 4h. In order to reduce disturbances, jars were housed inside opaque containers such that fish were visually isolated from the environment. Water samples were stored in a −20 °C freezer for hormone extraction. Hormones were extracted from water-samples with a vacuum pump and passed through a C18 solid-phase column (Waters Sep-Pak 500 mg 3 cc) fitted to a 20-port manifold. Before use, the columns were primed with 2 x 2 mL HPLC grade methanol washes followed by 2 x 2 mL distilled water washes. After use, the columns were purged of salt with 2 x 2 mL distilled water washes. Hormones were eluted from the columns by 2 × 2 mL ethyl acetate

washes and the eluted solvent was evaporated using vacuum centrifuge (Thermo/Jouan RC10-10; 45°C). The resulting hormone residues were resuspended in 600 µL of EIA buffer supplied with the Cayman Chemicals Inc. EIA kits, and the samples were stored at -20°C until assay.

2.4.2 Hormones quantification

Cayman Chemicals Inc. EIA kits were used for each hormone following the procedures recommended by manufacturers. Each hormone was assayed for each individual in duplicate on five 96-well plates. The standard curve was run in duplicate on each plate as well as a pooled sample (N = 21 and 30 for larvae and adults, respectively) in duplicate in each plate to determine intra and inter-assay coefficient of variation (CV). Absorbance was read on a BMG LABTECH microplate reader (FLUOstar® Omega) at 405 nm. All the data on hormone levels are presented as pg/mL.

Experiment 1: One larva from C treatment and one larva from S treatment were discarded from F levels data as samples were felt out of the linear range of the standard curve (final sample sizes: N=39, 41 and 42 respectively for C, E and S treatment). Intra-assay CV ranged from 0.4-8.8% (mean: 4.4%; N = 6 plates) and the inter-assay CV was 6.6%.

Experiment 2: two larvae from C treatment, one larva from E treatment and three larvae from S treatment were discarded from F levels data as samples appeared out of the linear range of the standard curve (final sample sizes: N=110, 26 and 25 respectively for C, E and S treatment). On the same way, five larvae from C treatment, one larva from E treatment and one larva from S treatment were discarded from T levels data as samples were felt out of the linear range of the standard curve (final sample sizes: N=107, 26 and 27 respectively for C, E and S treatment). Intra-assay CV ranged from 2.0-7.7% for F (mean: 4.5%; N = 6 plates) and from 0.42-4.0% for T (mean: 1.9%; N = 6 plates) and the inter-assay CV was 12.1% for F and 7.2% for T.

2.5 DNA/RNA extraction and analysis:

Molecular analyzes including relative gene expression(qPCR) and DNA methylation (bisulfite pyrosequencing) will not be covered in this article, as not all results were obtained at this point, a subsequent publication will cover the obtained results. The choice of candidate genes was based on previous studies, or the genes' involvement in mechanisms effected by PM(see supplementary data S3).

2.6 Statistical analysis:

Statistical analyses were performed using R and RStudio Software, using a significance level $\alpha = 0.05$. When possible, parametric analyses in which the assumed distribution of residuals was matched to the non-continuous data were used rather than transforming data to fit standard assumptions (Wilson & Hardy 2002; Hardy & Briffa 2013). Continuous data were in some cases transformed as follows to meet normality distribution. However, non-

parametric tests were performed when parametric conditions were not satisfied. Shapiro-Wilk normality test were performed and residual plots were analysed to confirm data normality. When a significant effect of PM treatment was found, Tukey post hoc tests were run for 1-way ANOVA, and Dunn's post hoc multiple comparison tests were run for Generalized linear models (GLM) and Kruskal-Wallis (Dunn 1961), in order to establish which modalities significantly differed from each other.

Experiment 1: First of all, we performed a 1-way ANOVA to investigate the effect of PM exposure on larvae size at 7 dph. After sqrt-transformation, the same test was used to explore the effect of PM exposure on the larval growth between 1 and 7dph. One-way ANOVAs were also run to test the effect of PM exposure on larvae total relative swimming distance (calculated as "total swimming distance / standard length of individual"; sqrt-transformed) and mobility (sqrt-transformed), as well as velocity (exp-transformed). Kruskal-Wallis tests were used to explore if duration of swimming, frequency and the time spent in the centre of the experimental significantly with the PM exposure. Moreover, a Kruskal-Wallis test was performed to determine if PM exposure affected significantly larvae foraging effort (calculated as the 'number of capture trials'), while a GLM with quasi-binomial distribution of errors (link function = 'logit') was run to determine if the predation success of larvae (calculated as the 'number of successful captures / number of the capture trials) significantly differed between PM treatment. Data from larvae that did not try to capture prey were not considered for this analysis . Then we calculated animal MO^2 (y) as derived from the slope (K) of the linear regression of the oxygen content (kPa) over time (h) according to the equation:

$$y = KV\beta L^{-1}$$

where y (MO_2) is the oxygen consumption rate ($mgO_2\ cm^{-1}\ h^{-1}$), K is the rate of decline (kPa h^{-1}) in oxygen content over time in the respirometer during the measurement phase, V is
the volume of the respirometer (L), β is the solubility of oxygen in water at the experimental water temperature and salinity ($mgO_2\ L^{-1}\ kPa^{-1}$) and L is the total length of the animal (cm) (Svendsen *et al.* 2015; Rosewarne et al. 2016). As larvae were not weighed during experiment 1, their theorical body mass were determined from a formula based on data that were measured in a previous experiment on 59 DC4 adults *K. marmoratus* (see Supplementary data S1). Previous study also stated that the coefficient of determination (r^2) associated with the declining oxygen curve (i.e. oxygen content (kPa) over time (h)) should be > 0.9 (Genz et al. 2013). Seventeen individuals (N = 8, 7 and 2 for control, ED-exposed and SD-exposed fish, respectively) were therefore discarded from our analysis (Table 1). A Kruskal-Wallis test was performed in order to test whether larvae oxygen consumption rate varied significantly with exposure to PM. The same test was used to investigate the influence of PM exposure on F basal levels.

Experiment 2:

Delayed effects of PM exposure on growth were determined by performing a 1-way Anova test on sqrt transformed growth factor (standard length at day 7/standard length at

day 70) between 7 and 70 dph and the same procedure was followed to test the effects on final size at 149 dph . After logb transforming, a one-way ANOVA was also performed to investigate effects of PM exposure on growth between 70 and 149 dph. A one-way Anova was also performed to on sqrt transformed size at 149 dph.

All combat related data of adults was stored in pairs of individuals as focal individual and opponent (Control fish), therefore following tests are performed on focal individuals and/or relative variables.

Non-parametric Kruskal-Wallis tests were used to test if PM exposure significantly affected pre-fight F an T levels followed by Dunn's test for multiple comparisons. Furthermore, relative F and T pre-fight levels (focal hormone level/opponent hormone level) were used as a factor in a two-way ANOVA to evaluate possible effects of PM exposure, hormones(for F and T independently) and their interaction on winning the fight (GLM with binomial distribution of errors), initiating the fight (GLM with binomial distribution of errors) and relative aggressiveness (GLM with quasi-binomial distribution of errors, link function = 'logit') calculated by total focal aggressive behaviours / total opponent aggressive behaviours.

2.7 Ethics statement:

All *Kryptolebias Marmoratus* husbandry and experimental procedures were approved by the University of Namur Local research Ethics Committee (UN KE 20/365) the Belgian animal protection standards were followed. Agreement number of the laboratory for fish experiments: LA1900048.

3. Results

4.1 Experiment 1: Immediate effects of early exposure to PM

4.1.1 Growth and size

We found a significant effect of PM exposure on growth between 1 and 7 dph (1-way ANOVA: F = 76.99, df=2, p<0.001; Fig. 2a). In fact, larvae from "S" treatment grew significantly less (mean ± SE = 1.06 ± 0.003) than the fish from "E" treatment (mean ± SE= 1.12 ± 0.0039; TukeyHSD post hoc test: p < 0.001) and "C" treatments (mean ± SE = 1.10 ± 0.002; TukeyHSD post hoc test: p < 0.001) and "E" exposed subjects grew significantly more (mean ± SE = 1.25 ± 0.001) than the control group (mean ± SE= 1.213 ± 0.0004; TukeyHSD post hoc test: p=0.0019). At 7dph, larvae had significantly different sizes depending on PM treatment they received (1-way ANOVA: $F_{2,354}$=, P < 0.001; Fig.2b). Larvae from "S" treatment were therefore smaller (mean ± SE = 1.13 ± 0.005 cm) than juveniles from both "C "(mean ± SE = 1.23 ± 0.004 cm; TukeyHSD post hoc test: p < 0.001) and "E" treatment (mean ± SE = 1.23 ± 0.006 cm; TukeyHSD post hoc test: p < 0.001) while the latter were not significantly different (TukeyHSD post hoc test: p = 0.4).

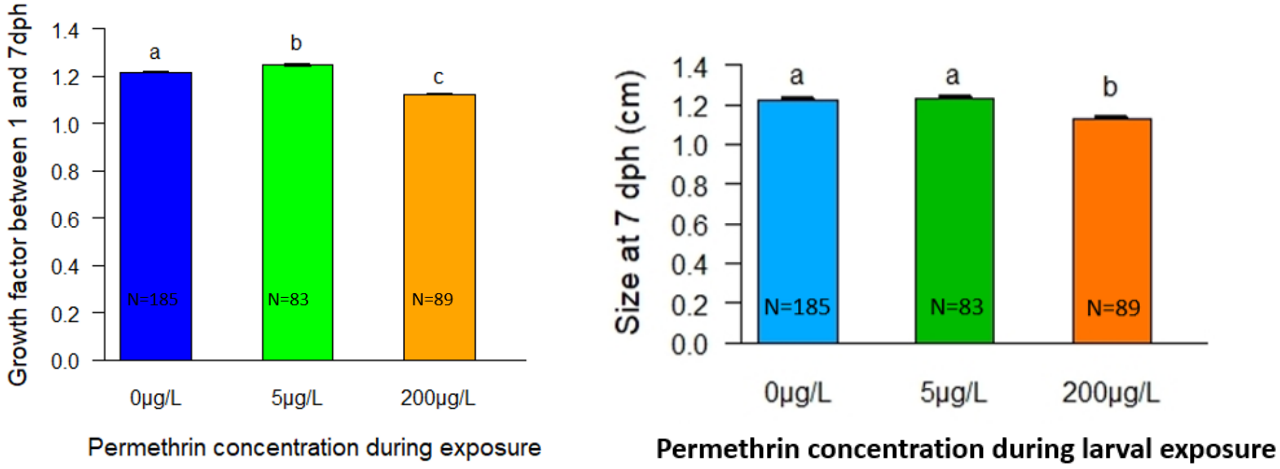


Figure 2: Immediate effects of early exposure on a) mean (± SE) growth rate (1-7 dph), and b) mean (± SE) standard length at 7 dph. Different letters indicate significant effect between conditions.

4.1.2 Locomotion and thigmotaxis

PM had a significant effect on the relative distance (1-way ANOVA: F=7.2182, df=2, p<0.001,Fig. 3a), "S" exposed fish swam a shorter relative distance in total (mean ± SE = 13.20 ± 0.47) than "E" exposed individuals (mean ± SE = 15.3 ± 0.38; TukeyHSD post hoc test: p < 0.001) and control fish (mean ± SE = 14.7± 0.27; TukeyHSD post hoc test: p < 0.01), while the relative distance travelled did not vary significantly between "E" exposed fish and control fish (TukeyHSD post hoc test: p = 0.45). A similar significant effect of PM was observed on the mobility (1-way ANOVA : F= 17.849, df=2, p < 0.001; Fig. 3b). Fish from "S" treatment were significantly less mobile (mean ± SE = 3.77±0.0996) than "E" exposed fish (mean ± SE = 4.45± 0.089, Tukey's test: p=0.0058) and control fish (mean ± SE = 4.39± 0.064, Tukey's test: p= 0.0038). No significant difference was found between "E" and "C" exposed subjects (Tukey's test: p= 0.87). Furthermore, PM had a significant effect on the velocity (Fig4), (exp, One-way Anova: F=8.8292, df=2, p=0.0001812). The "S" exposed fish moved significantly slower (mean ± SE = 1.472166± 0.0345) than the "E" exposed subjects (mean ± SE = 1.687±0.036, Tukey's test: p=0.0003) and slower than "C" fish (mean ± SE = 1.634± 0.028, Tukey's test: p= 0.0015). No significant difference was found between "E" and "C" exposed subjects (Tukey's test: p= 0.511).

Regarding thigmotaxis, no effect of PM exposure on frequency of crossing into the center area was observed (Anova type II, GLM with quasi-Poisson error distribution: F= 0.6227, df=2, P =0.5371), however the treatment had a significant effect on the total duration spent in the center area (Fig6) (Kruskal-Wallis: chi-squared = 25.355, df=2, p= 3.121e-06). "S" exposed subjects spent significantly more time in the center of the novel tank (mean ± SE = 111.93± 8.81 s) than "E" exposed subjects (mean ± SE = 62.32± 7.89s; Dunn's multiple comparison test: p <0.001) and control subjects (mean ± SE = 76.59± 5.77s; Dunn's multiple comparison test: p <0.001).

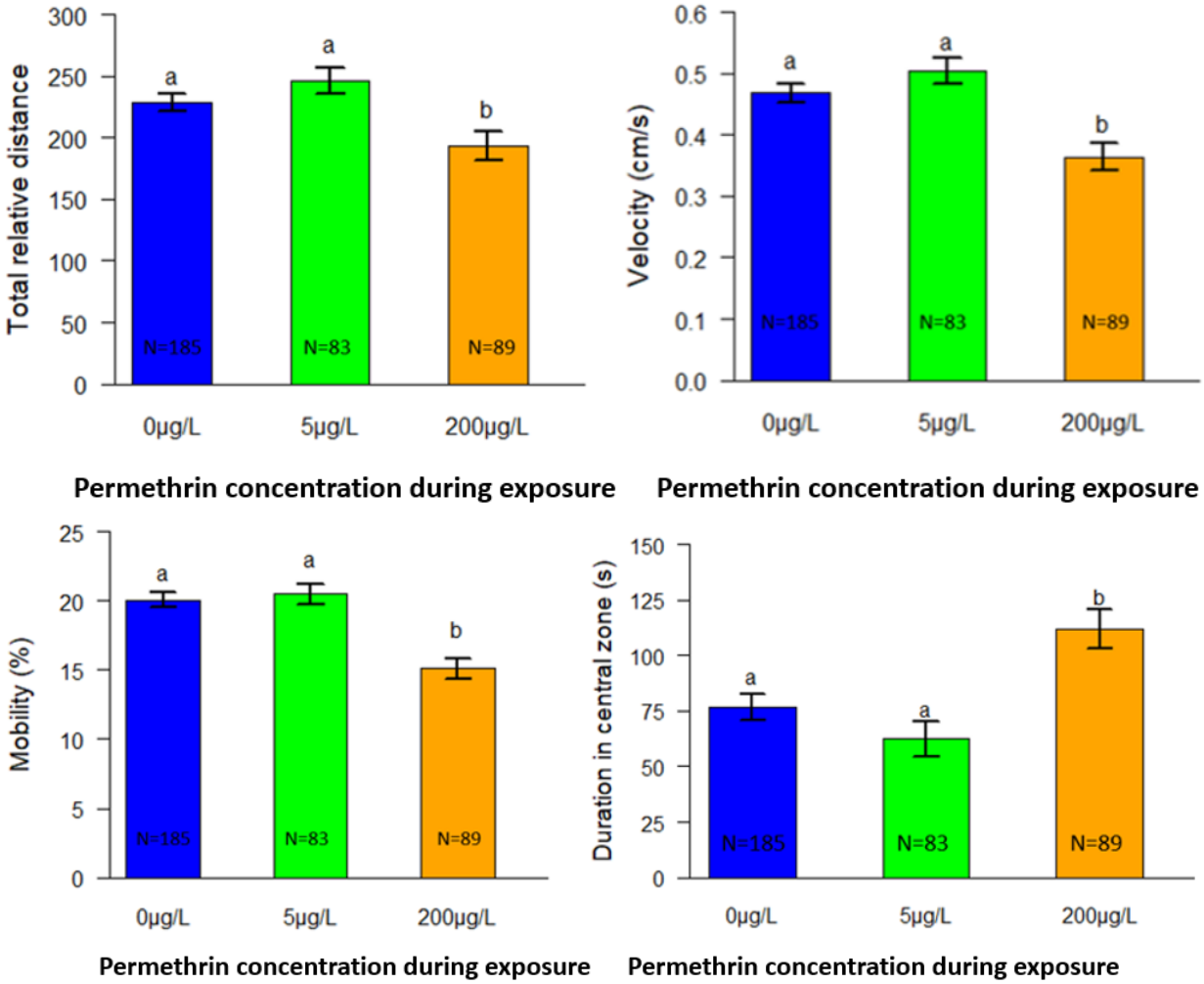


Figure 3: Immediate effect of early exposure to PM on a) mean (± SE) total relative distance travelled during 10min at day 7dph, b) mean (± SE) mobility, c) mean (± SE) velocity, and d) mean (± SE) duration in the center of arena. Different letters indicate significant effect between conditions.

Prey-capture test:

Significant effects of PM exposure on foraging effort was determined (Fig7), (Anova type II, GLM with quasi-Poisson error distribution: F=14.25, df=2, p =1.116e-06). "S" exposed fish displayed a significantly higher foraging effort with more prey capture attempts (mean ± SE= 11,9 ± 0.43) than "E" exposed fish (mean ± SE= 11,9 ± 0.43; Dunn's test for multiple comparisons: p= 0.0000) and control fish (mean ± SE= 9.42± 0.26; Dunn's test for multiple comparisons: p= 0.0000).

Significant dose-dependent effects of PM exposure on predation success was observed (Fig8) (Anova type II, GLM with quasibinomial error distribution: F= 19.718; df=2, p <0.001), control fish (mean ± SE = 83.39 ± 1.13 %) were more successful than "E" exposed fish (mean ± SE = 77.42 ± 1.83 %; Dunn's multiple comparison test: p= 0.003) and "S" exposed fish(mean ± SE = 68.76 ± 2.48; Dunn's multiple comparison test: p <0.001). Moreover "E" exposed fish had a higher predation success rate as "S" exposed fish (Dunn's multiple comparison test: P = 0.0149).

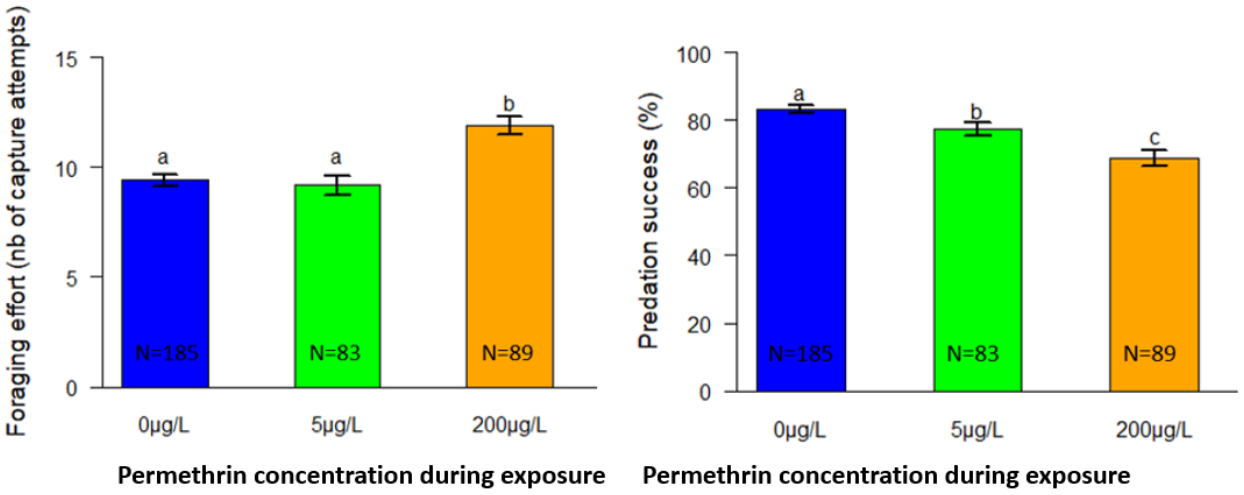


Fig4(left): Foraging effort=total number of prey capture attempts during 5min at day 7dph, after PM exposure at different concentrations. Bars indicate mean ± standard error of mean. Different letters (a,b) mean significant (P < 0.05) effect between conditions. Fig8(right): Predation success=total number of successful prey captures/ total number of prey capture attempts during 5min at day 7dph, after PM exposure at different concentrations. Bars indicate mean ± standard error of mean. Different letters (a,b,c) mean significant (P < 0.05) effect between conditions.

Respirometry and cortisol levels:

Metabolic rate results (MO2) were corrected by their theoretical body mass obtained from a conversion of their individual body length (Cf. supplementary data S1), no significant effect of PM exposure on metabolic rate was observed (Kruskal-Wallis: chi-squared = 8.1203, df=2, p= 0.01725), and no significant effect of PM exposure on F levels at 7dph larvae was found (Kruskal-Wallis chi-2 = 5.4757, p = 0.06471)("C": N= 39 ; "E" : N=41 ; "S" : N= 42)(supplementary data S3).

4.2 Delayed effects on adults:

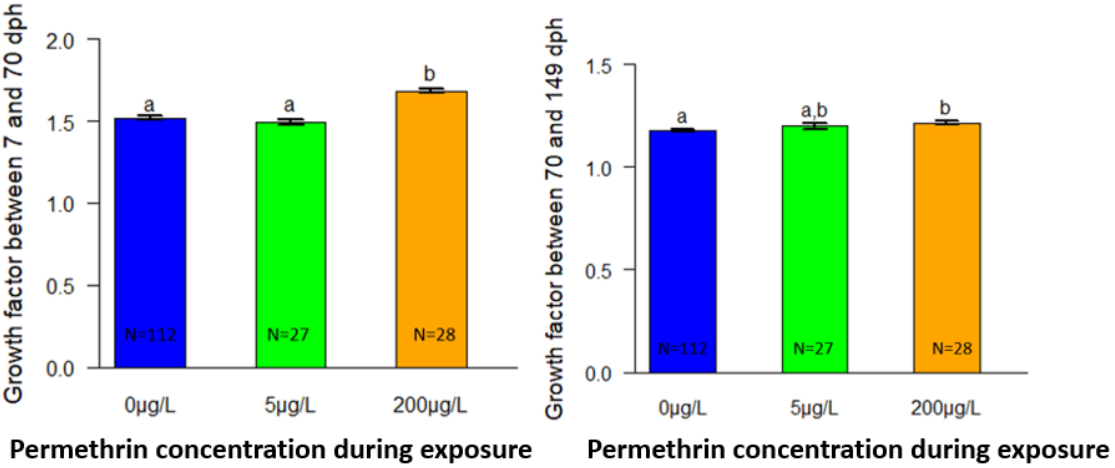
Growth:

We found a significant effect of PM exposure on growth between 7 and 70 dph (1-way ANOVA: F = 45.892, df=2, p<0.001; Fig. 5a). In fact, adults from S treatment grew significantly more (mean ± SE =1.297±0.0056) than fish from "E" (mean ± SE= 1.22± 0.0059; TukeyHSD post hoc test: p < 0.001) and "C" treatments (mean ± SE = 1.232± 0.0036 ; TukeyHSD post hoc

test: p < 0.001) while no difference was found between fish from “C” and “E” treatment (TukeyHSD post hoc test: p=0.39). At 70dph, fish had significant different size depending on PM treatment they received (1-way ANOVA: F=2,354, df=2, P < 0.001; Fig.2b). Larvae from “S” treatment were therefore smaller (mean ± SE = 1.13 ± 0.005 cm) than juveniles from both “C” (mean ± SE = 1.23 ± 0.004 cm; TukeyHSD post hoc test: p < 0.001) and “E” treatment (mean ± SE = 1.23 ± 0.006 cm; TukeyHSD post hoc test: p < 0.001) while the latter were not significantly different (TukeyHSD post hoc test: p = 0.4) from each other.

We also found a significant effect of PM exposure on growth between 70 and 149 dph (1-way ANOVA: F = 4.0453, df=2, p=0.019; Fig. 5b). In fact, adults from “S” treatment grew significantly more (mean ± SE= 0.194± 0.0087) than fish from “C” treatment (mean ± SE= 0.163± 0.0056; TukeyHSD post hoc test: p=0.01997) while no difference was found between fish from “C” and “E” treatment (TukeyHSD post hoc test: p=0.32) or “S” and “E” treatment (TukeyHSD post hoc test: p=0.6).

PM had a significant effect on final standard length at 149 dph (1-way ANOVA: F= 17.727, df=2, p<0.001, df=2, Fig. 5c). In fact, “S” exposed fish were bigger (mean ± SE = 1.51± 0.005) than “E” exposed individuals (mean ± SE = 1.48 ± 0.0069; TukeyHSD post hoc test: p= 0.0053) and control fish (mean ± SE = 1.479± 0.0024; TukeyHSD post hoc test: p < 0.001), while the body size did not vary significantly between “E” exposed fish and control fish (TukeyHSD post hoc test: p = 0.15).



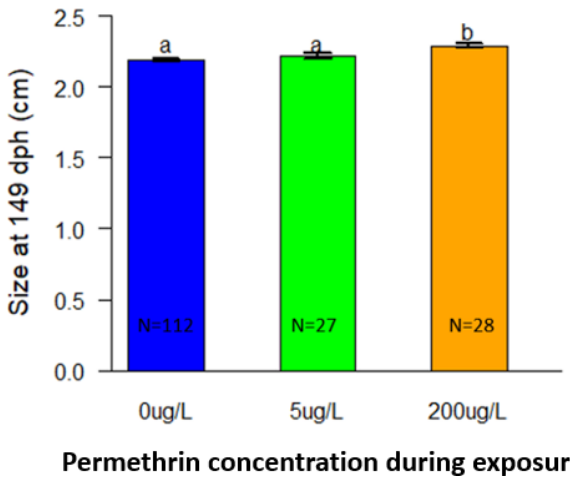


Fig5: a) Growth factor(day7-70), after PM exposure; b): Growth factor(day70-149), after PM exposure c) standard length at 149 dph. Different letters (a,b,c) mean significant ($P < 0.05$) effect between conditions, bars indicate mean ± standard error of mean.

Basal hormone levels and contest behaviours:

No significant effect of PM exposure was observed on adults F basal levels (Kruskal-Wallis: $\chi^2_2 = 0.04$, df=2, p=0.98). However, we found that T basal levels differed significantly between PM treatment (Kruskal-Wallis: $\chi^2_2 = 7.81$, df=2, p=0.02; Fig. 6a). Adults from the "S" treatment had significantly lower T levels (mean ± SE = 21.0 ± 3.0 pg) than fish from the "C" treatment (mean ± SE = 35.8 ± 3.2 pg; Dunn's post hoc test: $p < 0.01$) but no difference was observed between fish from "S" and "E" treatment groups (mean ± SE = 22.8 ± 2.3 pg; Dunn's post hoc test: p = 0.11) or between "C" and "E" treatment (Dunn's post hoc test: p=0.13).

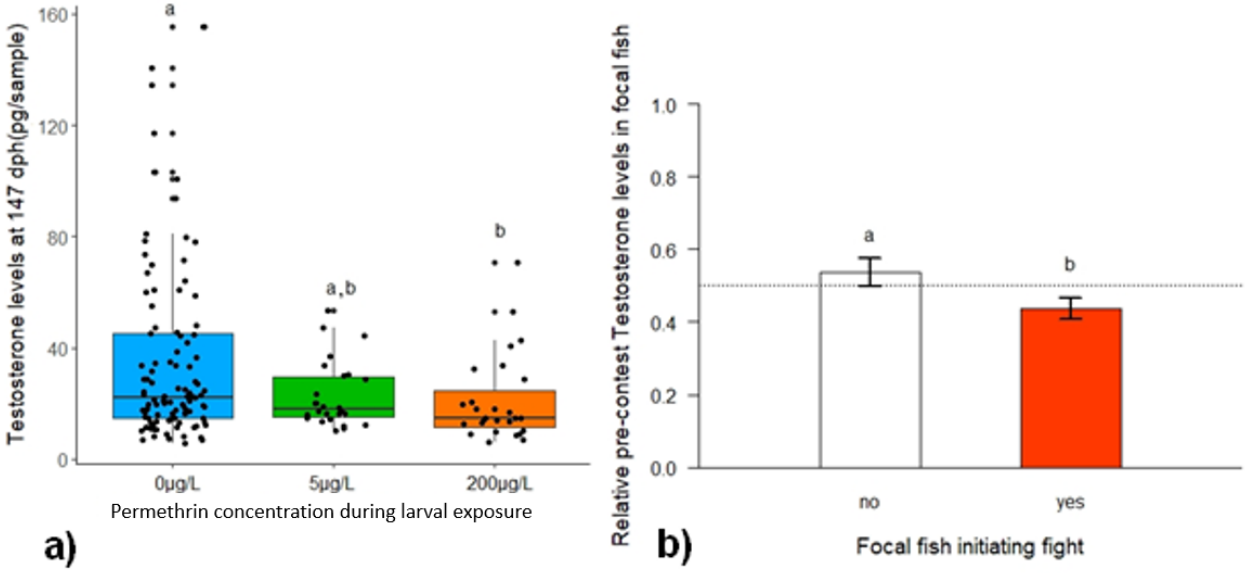


Figure 6: a) Delayed effects of early exposure to PM on T basal levels in adults (N=107, 26 and 27 for C, E and S treatment, respectively). b) Mean (± SE) relative pre-contest T levels in focal fish that initiated fight (N= 29) or did not (N=48).

We determined no significant effect of PM treatment (GLM with binomial distribution of residuals: $\chi^2 = 5.99$, df =2, p= 0.05), relative pre-contest F levels ($\chi^2 = 0.97$, df =1, p= 0.323), or their interaction ($\chi^2 = 3.89$, df =2, p= 0.143) on probability of focal fish winning the fight. Furthermore, we found no significant effect of treatment (GLM with binomial distribution of residuals: $\chi^2 = 0.438$, df = 2, p= 0.80), relative F levels ($\chi^2 = 1.088$ df =1, p= 0.30) or their interaction ($\chi^2 = 0.83$, df =2, p= 0.66) on probability of focal fish initiating the fight. Lastly no significant effect of treatment (GLM with quasi-binomial distribution of residuals: F= 1.9638;

df=2, p= 0.15), F levels (F= 0.716; df=1, p= 0.716) or their interaction (F= 1.07; df=2, p= 0.35) on relative aggressiveness of focal fish.

No significant correlation was found between PM treatment (GLM with binomial distribution of residuals: $\chi^2 = 5.07$, df = 2, p = 0.08), relative pre-contest T levels ($\chi^2 = 1.95$, df =1, p = 0.16), or their interaction ($\chi^2 = 0.36$, df = 2, p = 0.83) on probability of focal fish winning the fight. In addition, while we found no significant effect of interaction between PM treatment and relative T levels (GLM with binomial distribution of residuals: $\chi^2 = 3.44$, df = 2, p = 0.18) or PM treatment alone ($\chi^2 = 0.17$, df = 2, p = 0.92) on probability of focal fish initiating the fight, we highlighted a significant effect of relative pre-contest T levels ($\chi^2 = 4.42$, df = 1, p = 0.36). Fish with higher relative T levels (mean ± SE = 0.44 ± 0.03) were therefore less likely to initiate a fight than fish with lower relative T levels (mean ± SE = 0.54 ± 0.04; Fig. 6b). Finally no significant effect of treatment (GLM with quasi-binomial distribution of residuals: $F_{2,45}$= 1.38,df=2, p= 0.26), T levels ($F_{1,45} = 1.26$, df=1, p = 0.27) or their interaction ($F_{2,45} = 1.60$, df=2, p = 0.21) on relative aggressiveness of focal fish was found.

4. Discussion

In this study, we investigated the potential early and delayed effects of PM in hermaphroditic mangrove rivulus *Kryptolebias marmoratus* on developmental plasticity, by studying growth, locomotion, thigmotaxis behaviour, ability to catch prey, metabolic rate, and F levels. Furthermore, in the second part of this study we investigated possible effects of PM exposure on resulting adults' growth, F and T levels and conspecific fight characteristics between adult hermaphrodites.

PM possesses endocrine disrupting properties in some species and in zebrafish, it was previously documented that it can affect endocrine signaling and cortisol levels at similar concentrations as used in this study (Zhang et al., 2017) (at least at the highest tested concentrations). Our results revealed no significant correlation between PM exposure and cortisol level. However, the referenced study performed PM exposure on zebrafish subjects at embryo level while the present study exposed newly hatched individuals, suggesting that the early-life development is a critical window for exposure to permethrin, thus differences in life stages and duration of exposure lead to very distinct effects on resulting individuals. One should add that that the cortisol levels were corrected using a theoretical body mass conversion calculated from adult *K. marmoratus* data. Using a theoretical body mass conversion calculated from larvae size and body weight should be used, or the experimental individuals in future studies should be weighed if handling the fish does not interfere with the results. The cortisol levels per sample (larvae) showed elevated standard errors, especially for the "E" exposed larvae, which may indicate problems with the experiment itself, a manipulation error during extraction or quantification could be the origin of this abnormally high variation in measurements inside the "E" group.

Regarding effects of PM on growth in previous studies, growth differences in F0 zebrafish were observed (Blanc et al., 2020) (10 µg/L (PM), 1 µg/L), and "none of the treatments

induced a significant change in larval locomotor activity". In contrast, PM exposure in our study showed significant effects on locomotor activity and growth even at the lower concentration of 5 µg/L (growth). "S" exposed larvae were significantly hypoactive regarding percentage of mobility, total distance travelled and mean speed of movement suggesting general inhibitory effects on the locomotor activity. Reduced locomotor activity has previously been documented for other neurotoxic compound and is often linked with neuronal degeneration (Chen et al., 2011). Similar results were found in the previously referenced study(Blanc et al., 2020) for F1 and F2 generation Zebrafish larvae, likely the extended exposure window of 7 days in contrast to their 4 day exposure made adverse effects more visible and pronounced at F0. The observed adverse effects on efficient hunting behaviour and locomotion suggests that PM mostly affects brain functions during development. (Carion et al., 2018)

Statistically significant body size differences of 7 dph larvae revealed phenotypic variation due to PM exposure inducing slower growth at the highest tested concentration, however, size-differences of subjects at 7 dph were minimal (1mm± 0,005) and therefore considered biologically irrelevant as the fitness of a fish is determined by a complex interaction of variables in the wild, the difference of 1mm would quickly be overshadowed by other influencing factors, our size differences were obtained in laboratory conditions with minimal environmental variation (T°, food, salinity etc.), it is easy to imagine several other natural biotic or abiotic variations would result in greater size differences of larvae, for example salinity and food availability have a great impact in this species on life history traits such as size (Earley et al., 2012). Surprisingly morphological significant size differences were also determined for 70 and 149 dph old adults contrary to other studies that mostly show no significant effects on morphology on resulting F0 adults.(Blanc et al., 2020) The "S" exposed individuals grew surprisingly more than other groups and thereby overcompensated the stunting experienced in early development stages. However, noticeable differences were mostly present in "S" exposed individuals probably due to higher PM exposure used in this study and once again the differences in size were considered minor and therefore not deemed relevant regarding fitness or social impact. The fact that no major differences in growth were observed at environmental concentrations suggests that these effects do not occur in natural populations of *K. marmoratus*. The overall instant and delayed effects of PM treatment on fish growth may in fact be a consequence of endocrine disruption as proposed by recent studies (Blanc et al., 2020; Wang et al., 2018).

Regarding metabolism, PM exposure in other species has been shown to induce a decrease of oxygen consumption, by disrupting the oxidative phosphorylation and inhibiting electron transfer, leading to lower bioenergetics capacity (Nunes et al., 2019). While oxidative stress was recorded in multiple species (Wang et al., 2016) , our finding suggest no effect on O2 consumption in 7-day-old mangrove rivulus larvae. Their adaptive response and vide range of oxidative stress tolerance could be connected to the stabilized energy consumption and O2 consumption. This species is known to be particularly adaptive to oxidative stress; they live in different microhabitats varying in oxygen levels(Mackiewicz et al., 2006; Taylor, 2000, 2012) and do not undergo metabolic depression even during emersion (Ong et al., 2007).

Thigmotaxis results showed exposed 7-day-old *K. marmoratus* larvae spent significantly more time in the central zone of the novel tank experiment, displaying a less fearful behaviour at the highest concentration of PM exposure than fish exposed to lower concentrations and control animals. Although chemical exposure can affect fish anxiety-like behaviour both positively or negatively, depending on the chemical and exposure, cortisol levels generally parallel anxiety behaviours (Cachat et al., 2010). As no significant F level differences were found between treatment groups, the anxiolytic effect of PM doesn't seem to stem from hormone level disturbance. From a different perspective, reduced exploration may also be caused by reduced swimming activity as the mobility and speed of the PM exposed individuals has been affected by PM exposure, some anxiety-like behaviour differences could simply be another consequence of locomotor impairment as one locomotor phenotype can affect another. In our case however this doesn't account for the greater time spent specifically near the outer area of the novel tank. Thigmotaxis, and especially "wall-hugging" behaviour is a valid index of anxiety for which the involvement of D1 and D2 dopamine receptors has been documented in mice, evidence shows the anxiogenic-effects are associated with a rise of dopaminergic transmissions.(Colwill & Creton, 2011; Simon et al., 1994). The results of our behaviour assay represent a correlation of PM and anxiety-like behavior, to validate the effects of such essays, it is advised to use known anxiolytic or anxiogenic drugs for comparative results(Colwill & Creton, 2011).

The less successful prey capture results of PM exposed 7 dph larvae show dose-dependent effects of PM. Moreover, it is not simply an additional result of hypoactive behaviour, in fact S exposed fish had the highest foraging effort with most attempts of prey capture. In this case the less successful PM exposed subjects appear to have impaired cognitive functions. Prey capture behaviours involve important neural processes including recognition, decision-making, and motor skills, thus, the prey capture test serves as a good model system to evaluate cognitive functions underlying natural behaviours in vertebrates.(Muto & Kawakami, 2013) As previous research on fish larvae behaviour shows, larvae mostly feed visually and in close proximity of their prey to successfully capture prey (Houde, 2019). Perturbation of the visual system would lower the success rate of captures and thus would be interesting to consider, although no evidence in past studies suggests such interaction. However, the observations of our study reinforce the hypothesis of movement impairment by PM exposure which is consistent with literature(Blanc et al., 2020). The higher number of failed prey captures indicates that larvae exposed to PM need more capture trials to ensure similar food intake and thereby have more energy expenditure. The lower food intake would consequently impact fish growth as observed, and is proposed to negatively impact reproduction. (Carion et al., 2018)

One could also consider adaptation of energy allocation to explain the differences in growth, movement, and prey capture. Differences in growth rate and reproductive investment have been observed between isogenic strains of mangrove rivulus (Turko et al., 2011). Compensatory growth is defined as accelerated growth, once favorable environmental conditions return after a period of growth depression and is common in fish species. (Ali et al., 2003) Compensatory growth has a significant metabolic cost and can lead to changes in general activity and mobility (Gerrard & Grant, 2003) suggesting developmental constraints

(Frommen et al., 2011). Although compensatory growth has negative impacts on energy costs and mobility, one can assume growth plays a role in the animal's survival capabilities, as morphology and behaviour are closely related in *K. marmoratus* (Turko et al., 2011) and greater size ensures better ability to jump out of the water, and escape predators or other stressors (Ferrari et al., 2010; Frommen et al., 2011; Turko et al., 2011). When raised in uncontaminated waters compensatory growth can be observed in *K. marmoratus* (Voisin et al., 2016) as a response to different chemical exposures. One should also suspect a potential trade-off between somatic and reproductive investment, but in the mangrove rivulus positive relationship between growth and reproductive effort has been documented (Grageda et al., 2005) which explains the advantage of favoring growth over other energy expenses. Growth compensation due to plasticity could therefore be a development strategy used by the mangrove rivulus after PM exposure stress.

Larvae behaviour was influenced by PM exposure, but no significant adverse effects on behaviour were observed in adult life stages contrary to some other studies of early-life exposure to pesticides (Vaiserman, 2014). The evidence here suggests that the subjects have been able to recover from behaviour alterations while developing in PM-free conditions after 7 dph until adulthood. In some cases, behaviour variations are hidden in adult fish exposed to neurotoxins (during their early life stages) which does not always equal to no harm done. Adult Zebrafish exposed to PM for example (Blanc et al., 2020) have not displayed differences in behaviour but transgenerational behaviour effects on resulting descendance were observed. Possible behaviour variations might be hidden in F0 generation, transgenerational studies of PM exposure on *K. marmoratus* could lead to conclusive results. Neurogenesis can be another explanation of missing neurological effects in adults. Neurological damage experienced during development in fish can be repaired by specialized mechanisms that direct migration of new cells to the injured regions, ensure the differentiation of cells, and their integration into the neural network (Zupanc, 2009). Analyzes suggested that adult neurogenesis is a primitive vertebrate trait, which ensures proper interaction of muscle fibers/sensory receptor cells and central elements involved in motor control/processing of sensory information. (Zupanc, 2009)

Furthermore, significant differences in pre-contest F or T levels due to PM exposure were not observed on 149 dph old adults and no effect of F or T levels on contest outcome or aggressive behaviour during contests was determined, however negative correlation of T levels and the probability of initiating the fight was observed which can be interpreted as lower aggressive behaviour. There appears to be contradictory information in literature regarding pre contest F and T levels and their effect on contest outcome. While no link has been determined in this study, some studies show T correlated positively and F negatively with winning contests and initiating contests (Earley & Hsu, 2008) and both pre-experience T and F correlated positively with the fish's agonistic behaviour towards its mirror image (Chang et al., 2012) which contradicts our findings. But also no overall mean difference in pre- or post -contest T or F between winner or losers was found in the same referenced study. The hormone levels alone could not account for the observed effects. (Earley & Hsu, 2008) It was therefore proposed that winner and loser cannot easily be revealed by the complex associations of endocrine systems and social behaviour (Earley & Hsu, 2008). The results of another study show that

individuals with higher levels of T were more likely to attack their opponent, while individuals with higher F levels were more likely to lose. (Li et al., 2020) There appears to be a link between hormone levels and certain contest behaviours of *K. marmoratus* but the nature of the mechanisms driving this divergence in different studies is unclear at this point. As a perspective for future studies, epigenetic changes (DNA methylation) and gene expression differences) resulting in behaviour changes in subsequent generations should be further examined as they could explain certain unresolved questions in literature.

5) Conclusion

Our experiments demonstrate that permethrin affects phenotypic variation of a new vertebrate model species *Kryptolebias Marmoratus.* Juvenile *K. marmoratus* fish's locomotion behaviour and phenotype i.e., their growth were altered as the result of a 7-day exposure to the neurotoxic compound. The resulting smaller, slower, and less mobile fish presented lesser predation success than control subjects which could in turn affect their survival chances during the critical period of development and growth in a natural environment. The findings in laboratory-controlled conditions, however, cannot be simply extrapolated to the complex conditions of their natural habitat but correlation of these variables with PM exposure was found. The evidence that PM affected their locomotion and successful hunting behaviour, seems to indicate that PM mainly affects brain functions in fish. The adverse effects were more noticeable at the highest concentration as "E" exposed individuals did not differ significantly from control fish in locomotion behaviour or growth but did present significant lesser predation success. Similarly PM effects reducing mobility and prey capture success have also been previously documented in other species like *Danio rerio* (Blanc et al., 2020) indicating adverse effects of PM on a large number of fish species could be expected. Compensatory growth was observed for individuals whose growth was affected by PM exposure during larval stages, this seems to indicate a development strategy used by the mangrove rivulus after PM exposure to increase their predator avoidance and reproductive capabilities. No noticeable effects of PM treatment on adult behaviour during conspecific contest were observed and the link between pre-fight cortisol or testosterone levels and fight outcome or PM treatment was not proven, however, fish with higher relative T levels were less likely to initiate fights than fish with lower relative T levels. Both conventional and unconventional concentration-response patterns were taken from these experiments, and we encourage further exploration of *Kryptolebias marmoratus* phenotypic variability and behaviour responses to environmental stressors.

6) ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Anthony Mathiron for all the help, patience, and support during my master's. Also thank you to Dr. Frédéric Silvestre and the rest of the LEAP team for the valuable feedback and assistance along the way and for making this study possible. Thanks to everybody for welcoming me into the team, I wish you all he best.

7) Supplementary data

S1) Theoretical body mass conversion

In order to calculate the theoretical body mass of larvae in our study, we performed the linear regression between the Log (body mass (g)) and the Log (standard length cm)) that were measured in a previous experiment (unpublished data) on 59 adults *K. marmoratus* from the DC4 lineage. With the coefficient of the regression line (Fig. S1), we then were able to calculate the theoretical body mass of larvae such as:

$$\textbf{Body mass}_{\textbf{theo}} = \textbf{Exp (-3.615919) * (standard length}_{\textbf{obs}})^{2.301246}$$

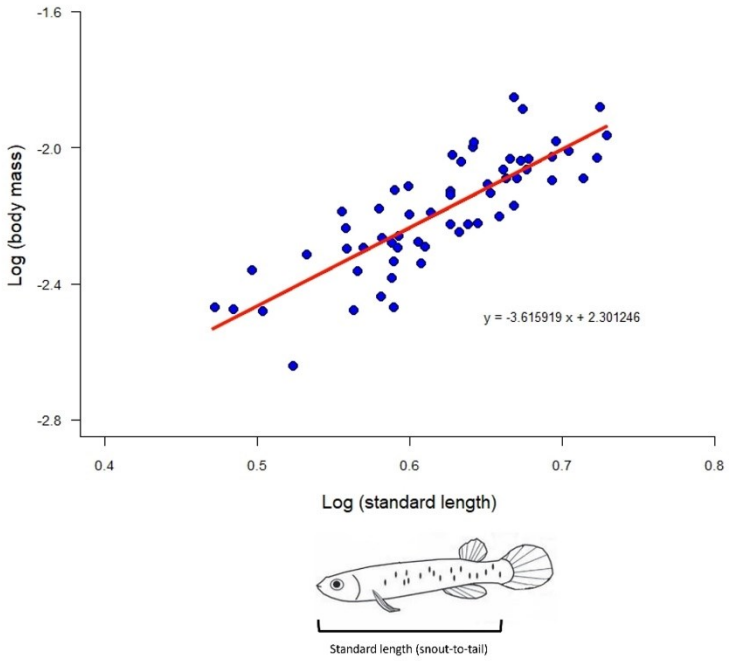


Figure S1: Linear regression between Log (body mass (g)) and Log (standard length (cm)) that was established from 59 *Kryptolebias marmoratus* of the DC4 isogenic lineage.

S2) Model species

S2.1) General information & habitat

The mangrove Kilifish *Kryptolebias marmoratus* (Poey, 1880), is a small killifish of the family Rivulidae in the order of Cyprinodontiformes.

It resides mostly in brackish water of red and black mangrove forests and is able to survive and tolerate a wide array of environmental conditions often inhabitable by other fish species (hypoxic, high variations of salinity, temperature, and water levels) (Marson et al., 2019; Taylor, 2012). Its occurrence has been documented from central Florida (USA), through the Yucatan Peninsula and Bay of Campeche (Mexico), along most of the Central American and northern South American coast (Taylor, 2012).

K. marmoratus has been found to live in different micro-habitats, such as intermittently dry shallow, stagnant pools; crab burrows and any other small pockets of water that are at least temporarily flooded. (Taylor 2000; Taylor et al. 2003, 2008)

The mangrove killifish is known to survive substantial periods arial exposure, thanks to its newly documented ability of gill remodeling (Nilsson, 2017). The process is reversible and increases the ability to absorb oxygen from air by stabilizing the lamellae. It is therefore capable of venturing into the terrestrial environment for several days. Like the cyprinids, *K. marmoratus* retains the lamellae while it changes the volume of the ILCM(lamellar length and interlamellar cell mass)(Nilsson, 2017)

S2.2) Reproduction and genetics

K. marmoratus and is one of the two only known self-fertilizing hermaphroditic vertebrates, with the second one being its sister species *K. hermaphroditus* (Berbel-filho et al., 2016).

Image sourced from F. Silvestre, Laboratory of Evolutionary and Adaptive Physiology (LEAP).

Top: *Kryptolebias marmoratus* adult hermaphrodite; Bottom: male.

This unique reproductive strategy, results in heterozygosity being halved with every generation, which can lead to fully homozygous individuals producing isogenic lineages.(Marson et al., 2019) Most individuals develop as hermaphrodites with ovotestes (gonads made of ovarian tissue and small pockets of testicular tissue

(Marson et al., 2019), simultaneously producing eggs and sperm. Pure males however rare, still occur in *K marmoratus* populations (primary males; Ellison et al., 2015), they are reported to make up between 2% (Florida) and 25% (Belize) (Lins et al., 2018; Turner et al., 2006) of populations and develop if the eggs are incubated in lower temperatures (18-20°C)(Ellison et al., 2015). Occasionally hermaphrodites can undergo sex change and develop into secondary males (Harrington, 1971; Turner et al., 2006). In the tropics, it is safe to assume that reproduction occurs year-round, except for prolonged droughts and/or low tides (Taylor, 2000). Males perform outcross events with hermaphrodites, which leads to extreme recombinational potential in the resulting descendants. *K. marmoratus* therefore displays a "mixed-mating" system where continued self-fertilization leads to a quasi-clonal population structure that occasional outcrossing then tends to dismantle in the wild.(Avise, 2016) This species has recognized advantages as a model species especially in ecotoxicology for its reproduction strategy. The minimal interindividual genetic variability within the same lineage of *Kryptolebias marmoratus*, allows to observe the variability of evolutionary molecular mechanisms directly related to the environment.

S3) Larvae cortisol levels

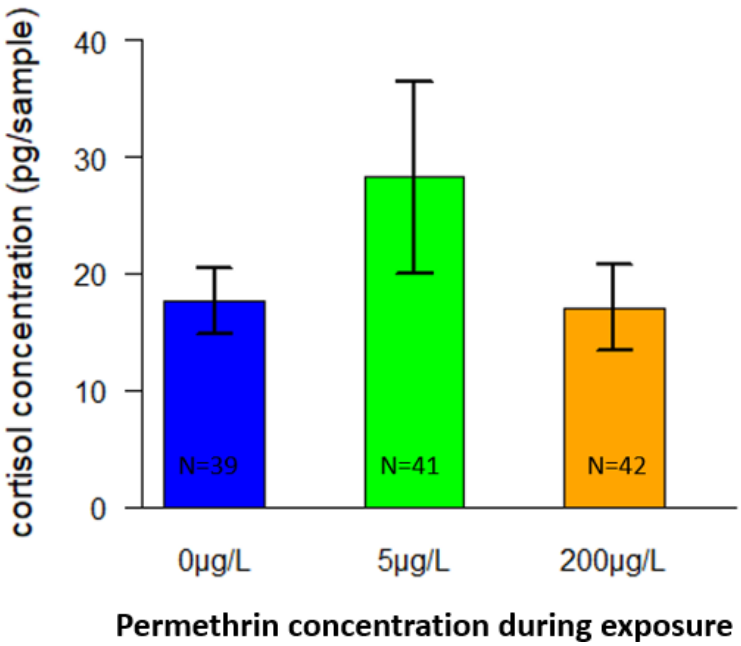


Fig7: cortisol concentration in pg/larvae, after PM exposure at different concentrations. Bars indicate mean ± standard error of mean.

S4) Candidate genes/genes of interest

NR3C1: A link between F levels as a stress response and differential expression of some key stress genes including NR3C1 were observed for different stress conditions in the European sea bass *Dicentrarchus labrax* (Goikoetxea et al., 2021).

Nr3C2: As the endocrine system may also be disturbed by altering receptors or their gene expression, Nr3C2, a gene coding for F receptors, might be interesting to investigate.

DRD4: A significant decrease of expression for the dopamine receptor D4 has previously been observed following an early life exposure to a different neurotoxin suggesting possible intracellular dopamine depletion in the brain(Carion et al., 2020).

Previous results on zebrafish showed that NR4A2 has a function in the DA differentiation pathway, and DA dysregulation can effect locomotor activity (Blin et al., 2008). The receptor NR4A2A/Nurr1 therefore seems to have a developmental control of locomotor activity.

Toll-like receptors (TLRs) are the subject of numerous recent studies as not all of their functions have yet been successfully described. Considering their known important role in innate immune systems, (Rebl et al., 2010) Toll-like receptors (TLRs) can also be of interest while studying the effects of toxins and thereby the impact on expression of receptors like TLRs. One can also add that a correlation of permethrin exposure with lysoPC content has been found (Blanc et al., 2020). LysoPC are molecules linked to inflammatory responses, therefore the encounter of epigenetic changes in toll-like receptors might be possible.

Additional genes of interest: GP9, IGF2b, plek2, mecp2

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