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EFEITOS DO INSETICIDA PERMETRINA SOBRE O DESENVOLVIMENTO
DE PEIXES-ZEBRA

Santa Maria, RS
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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Jeferson Luis Franco

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DEDICATÓRIA

Dedico este trabalho aos meus pais, Eugênio e Terezinha Nunes, ao meu irmão Franklin Nunes, que sempre me apoiaram e acreditaram em mim, à minha esposa Renata Neves, que sempre me acompanhou, incentivou e me deu forças para completar esta etapa.

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*"Tudo o que sabemos hoje, aprendemos com os acertos
e erros do passado, e cada vez que desistimos
de alguma coisa por medo de errar estamos nos
privando da possibilidade de descobrir e aprender"*
HAMMED

RESUMO

EFEITOS DO INSETICIDA PERMETRINA SOBRE O DESENVOLVIMENTO DE PEIXES-ZEBRA

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A permetrina (PM) é um inseticida sintético piretróide do tipo I e amplamente utilizada no campo e principalmente em ambientes domésticos no controle de insetos nocivos. Os piretróides são considerados relativamente seguros, com baixa toxicidade em mamíferos. No entanto, estudos tem indicado importante potencial tóxico desse composto sobre organismos em desenvolvimento. O presente trabalho teve como objetivo investigar os mecanismos de toxicidade da PM sobre o comportamento, homeostase redox e respiração celular de peixes-zebra em estágios iniciais do desenvolvimento até a fase adulta. Este trabalho foi dividido em duas etapas: (I) foram determinadas as concentrações letais e não letais de PM em larvas de peixe-zebra expostas de forma aguda, onde foram determinadas a CL50 de 108 µg/L e as concentrações subletais de 25 e 50 µg/L foram utilizadas para as subsequentes análises. Foi observado que ambas as concentrações foram capazes de causar desbalanço redox, levando ao aumento de espécies reativas de oxigênio (EROS). Consequentemente, foram observados danos oxidativos sobre biomoléculas, genotoxicidade e indução de apoptose. Em contrapartida, houve ativação do sistema de defesa antioxidante, os quais não foram suficientes para neutralizar os danos oxidativos, caracterizando o estado de estresse oxidativo em decorrência a exposição à PM. Nesta mesma etapa, observamos significativa diminuição da respiração celular e desvio da via aeróbica, os quais, em conjunto com os danos oxidativos poderiam influenciar as alterações comportamentais não-motoras ligadas a ansiedade observadas na etapa I. Na etapa (II) foram avaliados os efeitos da exposição a PM sobre o desenvolvimento de peixe-zebra por meio da determinação de padrões comportamentais em estágios chave do desenvolvimento. Nesta etapa os embriões de peixe-zebra foram submetidos a uma única exposição ao inseticida durante as primeiras 24h do desenvolvimento e subsequentemente, os comportamentos foram avaliados nas diferentes fases do desenvolvimento até a fase adulta. Observou-se que a exposição foi capaz de alterar comportamentos basais como os movimentos espontâneos ainda durante o estágio embrionário, assim como nos estágios posteriores do desenvolvimento até a fase adulta. Comportamentos ligados à ansiedade e medo foram significativamente alterados durante o período larval e persistiram até a fase adulta, quando também foram observados aumento nos parâmetros de agressividade. Estes resultados indicam que os danos provocados pela a exposição embrionária à PM persistiram durante o desenvolvimento e levaram a alterações comportamentais na fase adulta, os quais podem estar relacionados com distúrbios de ansiedade e depressão. Dados em conjunto, as duas etapas do estudo corroboram para o entendimento dos mecanismos de toxicidade da PM sobre organismos em desenvolvimento e sua relação com distúrbios comportamentais, além de contribuir na validação do modelo de peixe-zebra para estudos de neurodesenvolvimento ligados a contaminantes ambientais.

Palavras-chave: piretróides, neurodesenvolvimento, comportamento, estresse oxidativo, respiração celular, agressividade e ansiedade.

ABSTRACT

EFFECTS OF PERMETRINE INSECTICIDE ON ZEBRAFISH DEVELOPMENT

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Permethrin (PM) is a Type I synthetic pyrethroid (SP) widely used in crops and especially in domestic environments to control harmful insects. Pyrethroids are considered safe, with low mammalian toxicity. However, studies have indicated the potential toxic effects of this compound to developing organisms. The present work aimed to investigate the toxicity mechanisms of PM on behavior, redox homeostasis and cellular respiration from early stages of development until the adulthood. This work was divided into two chapeters: (I) lethal and non-lethal PM concentrations were determined in acutely exposed zebrafish larva, where the LC_{50} was found to be 108 µg/L in this model and the sublethal concentrations of 25 and 50 µg/L were determined and used for subsequent analyzes. It was observed that both concentrations were able to cause redox unbalance, leading to the increased reactive oxygen species (ROS). Consequently, oxidative damage on biomolecules, genotoxicity and apoptosis induction was also observed. On the other hand, there was activation of the antioxidant defense system, which was not sufficient to neutralize oxidative damage, characterizing the oxidative stress state due to exposure to PM. At this same stage itwas observed a significant decrease in cellular respiration and a shift in aerobic energy metabolism, which, alltogether with oxidative damage, could influence the anxiety-related non-motor behavioral changes observed in chapter I. In chapter (II) the effects of PM exposure on zebrafish development were assessed by determining behavioral patterns at key developmental stages. At this stage the zebrafish embryos were exposed only during the first 24h of development. We observed that exposure was able to alter basal behaviors such as spontaneous movements even during the embryonic stage, as well as in the later stages of development until adulthood. Behaviors related to anxiety and fear were altered during the larval period and persisted until adulthood, when increase of aggressiveness parameters were also observeded. These results indicate that damage caused by embryonic exposure to PM persisted during development and led to behavioral changes in adulthood, which may be related to anxiety and depression disorders. Taken together, the two chapters of the study corroborate to the understanding of the toxicity mechanisms of PM on developing organisms and their relationship with behavioral disorders, and contribute to the validation of the zebrafish model for neurodevelopmental studies linked to environmental contaminants.

Keywords: pyrethroids, neurodevelopment, behavior, oxidative stress, cellular respiration, aggression and anxiety.

DISCUSSÃO:

LISTA ABREVIATURAS

˙OH: Radical Hidroxila

3PBA : 3-Fenoxibenzóico

ADP: Adenosina Difosfato

α-GCS: α-Glutamilcisteína Sintetase

ANT: Adenina Nucleotídeo Translocase

ANVISA : Agência Nacional de Vigilância Sanitária

ARE: Elemento de Resposta Antioxidante

ATP: Adenosina Trifosfato

ATSDR: Agency for Toxic Substances and Disease Registry

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CAT: Catalase

CL50: Concentração letal média

CN: Ciano

CYP450: Citocromo P450

EPI: Equipamentos de Proteção Individual

EpRE: Elemento De Resposta Eletrofílica

ERK: Proteína Quinase Regulada por Sinal Extracelular

ERO: Espécies Reativas de Oxigênio

EUA: Estados Unidos da América

EUSES: European Union System for the Evaluation of Substances

GPx: Glutationa Peroxidase

GSH: Glutationa

GST: Glutationa-S-Transferase

H_2O_2: peróxido de hidrogênio

HAPs: Hidrocarbonetos Aromáticos Policíclicos

HO-1: Heme Oxigenase 1

IDA: Ingestão Diária Aceitável

IPS: Inseticidas Piretróides Sintéticos

JNK: C-Jun N-Terminal Quinase

Keap1: Kelch-like ECH-associated protein 1

kg: quilograma

mg: miligrama

NADPH: Nicotinamida Adenina Dinucleotídeo Fosfato

NQO1: NAD(P)H quinona oxidoredutase 1

Nrf2: Fator Nuclear Eritroide Relacionado ao Fator 2

$O_2^{\bullet-}$: ânion superóxido

ºC: Graus Celsios

PI3K: Fosfatidilinositol-3-Quinase

PKC: Proteína Quinase C

PM: Permetrina

ppb: partes por bilhão

SDA: Sistema de Defesa Antioxidante

SMaf: Proteínas pequenas fibrossarcoma musculoaponeurótica

SOD: Superóxido Dismutase

STE: Sistema De Transporte De Elétrons

Trx: Tioredoxina

VSSC: Canais De Sódio Voltagem Dependentes

SUMÁRIO

1. APRESENTAÇÃO

Esta dissertação está descrita na seguinte forma: primeiramente são apresentados a introdução e os objetivos. A seguir, os resultados e a discussão são apresentados nos itens artigo e manucrito. O item conclusão encontrado no final desta dissertação, apresenta interpretações gerais sobre o artigo e manuscrito contido nesta dissertação. As referências bibliográficas apresentadas no final da dissertação referem-se somente as citações que aparecem nos itens introdução e discussão.

2. **INTRODUÇÃO**

2.1 AGROQUÍMICOS

A palavra "agroquímico" é um dos termos utilizados para designar os produtos e os agentes de processos físicos, químicos ou biológicos, destinados ao uso nos setores de produção agrícola. Entre os termos utilizados na literatura, encontramos os termos: agrotóxicos, pesticidas, praguicidas, defensivos agrícolas e, recentemente, fitossanitários (ALMEIDA et al., 2017). A discussão terminológica tem origem na década de 50 e ainda é pauta de discussões entre pesquisadores, agricultores e políticos. No entanto, em junho de 1989 foi promulgada a Lei Nº 7.802 que regulamentou o uso do termo "agrotóxico" como a nomenclatura correta a ser utilizada para os produtos e seus componentes voltados ao uso nos setores de produção, no armazenamento e beneficiamento de produtos agrícolas, nas pastagens, na proteção de florestas, nativas ou implantadas, e de outros ecossistemas e também de ambientes urbanos, hídricos e industriais, cuja finalidade seja alterar a composição da flora ou da fauna, a fim de preservá-las da ação danosa de seres vivos considerados nocivos (ALMEIDA et al., 2017).

Nesse trabalho, adotou-se o termo "agroquímico" para designar os produtos supracitados, uma vez que remete um termo neutro em caráter pré-discriminatório e que, também, remete mais fielmente as características de um produto (químico) aplicado na agricultura (agro), logo agroquímico.

2.2 PROBLEMÁTICA AMBIENTAL DOS AGROQUÍMICOS

A produtividade agrícola brasileira tem crescido para atender a demanda mundial por alimentos e produtos, devido ao aumento da população em escala global. Para garantir a produtividade é necessária a aplicação de agroquímicos voltados ao combate de fitopatologias. No entanto, resíduos desses compostos podem se acumular na superfície de alimentos levando a exposição indireta dos consumidores (ARIAS-ESTÉVEZ et al., 2008).

Adicionalmente, agroquímicos tendem a ser depositados nos recursos hídricos de forma não pontual, sendo lançados de forma difusa ou indireta nos corpos d'água devido a processos de escoamento, drenagem e lixiviação (CEREJEIRA et al., 2003). A contaminação também pode ocorrer pelo transporte atmosférico devido à volatilização dos compostos presentes nos agroquímicos e pela formação de poeira do solo contaminado (COOPER, 1993). Estes tipos de

contaminações são difíceis de serem avaliadas, pois o grau de contaminação pode variar conforme o clima, uma vez que as chuvas podem tanto diluir a concentração de um contaminante presente na água como também transportar mais contaminantes para os recursos hídricos através da lixiviação. Estas características acompanhadas do uso indiscriminado de agroquímicos ao longo do tempo têm desencadeado a acumulação e bioacumulação de compostos químicos nocivos nos ecossistemas aquáticos, afetando a biota por várias gerações e prejudicando a recuperação do ambiente (BITTENCOURT, 2004).

A exposição direta a agroquímicos pode levar a absorção por ingestão ou inalação, principalmente durante o uso no interior de domicílios ou durante a aplicação do produto nas lavouras (DING et al., 2010). No campo, a exposição por inalação ocorre devido à ausência ou uso inadequado de equipamentos de proteção individual (EPI). Os trabalhadores do campo despreparados estão também expostos à contaminação tópica durante o manuseio negligente do produto, além dos riscos de contaminações acidentais (SANTOS; AREAS; REYES, 2007).

Dentre os agroquímicos, a classe dos inseticidas ocupa um terço de todos os compostos utilizados no campo (Figura 1) e, também, chama atenção por sua grande aplicação no ambiente doméstico no combate de insetos e parasitas (*Agency for Toxic Substances and Disease Registry* [ATSDR], 2003). Muitos inseticidas tiveram seu uso proibido devido a sua alta toxicidade e persistência no ambiente. Dentre os inseticidas proibidos, a classe dos organoclorados está proibida no Brasil desde 1985, exceto em campanhas de saúde pública (Portaria no. 329 de 02/09/85 do Ministério da Agricultura). Outras classes de inseticidas, como os piretróides, considerados menos agressivos ao meio ambiente e a saúde, têm ganhado espaço no campo e em áreas urbanas, substituindo também outras classes de agroquímicos, como carbamatos e organofosforados, considerados menos seguros devido a sua alta toxicidade (SANTOS; AREAS; REYES, 2007).

2.3 PIRETRÓIDES

Inseticidas piretróides sintéticos (IPS) são amplamente utilizados em ambos ambientes, compreendendo um quarto do mercado mundial já em 1995 (CASIDA; QUISTAD, 1998). A utilização de IPS aumentou consideravelmente devido a sua baixa persistência e toxicidade ambiental, além de grande eficiência contra uma grande diversidade de insetos e também por apresentar baixa toxicidade em mamíferos, quando comparados a outros inseticidas (KANEKO, 2011). Os efeitos tóxicos de piretróides em organismos não-

alvo foram revistos e relatados como sendo em partes por bilhão (ppb) de valores de toxicidade (WANG et al., 2016a).
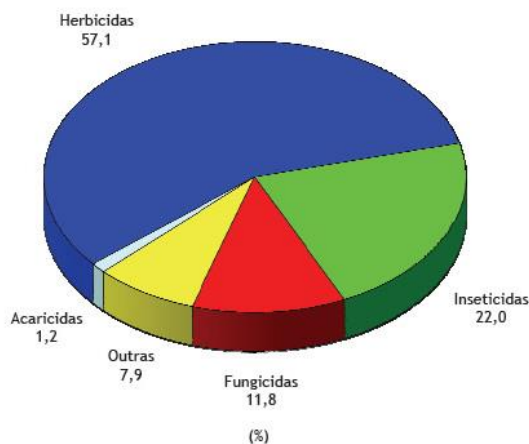
Figura 1. Percentagem das classes de agroquímicos vendidos em 2012 no Brasil.

Fonte: Sindicato Nacional da Industria de Produtos para Defesa Agrícola (SINDAG 2013).

Os piretróides são compostos sintéticos derivados das piretrinas, ésteres isolados das flores de espécies como *Chrysanthemum cinerariaefolium.* No entanto, na década de 70 as piretrinas foram substituídas pelos IPS devido a sua baixa estabilidade no ambiente, o que diminui a sua eficácia no controle de pragas em relação aos IPS (NASUTI et al., 2008b). A mudança estrutural introduzida nas piretrinas conferiu maior estabilidade e potencial inseticida, por meio da inclusão de átomos de nitrogênio, enxofre e de halogênio, mantendo relativa baixa toxicidade aguda em mamíferos (SANTOS; AREAS; REYES, 2007). Em geral, os IPS são praticamente insolúveis na água (cipermetrina = 10 µg/L; Permetrina = l µg/L) e bastante solúveis em solventes orgânicos (LARINI, 1999). Os IPS, também, são pouco persistentes no meio ambiente terrestre, podendo ser classificados, de uma maneira geral, como não persistentes. Em estudos de campo, os IPS apresentam uma larga escala de meia-vida (3 dias a 228 dias). Conforme a *European Union System for the Evaluation of Substances* (EUSES, 2004) esta variação pode ser devido às circunstâncias ambientais diferentes, que afetam os mecanismos de dissipação ou da degradação (tais como: temperatura, intensidade luminosa, aeração do solo, intensidade da chuva, umidade do solo e pH). No entanto, a relativa, baixa persistência dos piretróides no ambiente (SINGH; SINGH, 2008) não é acompanhada por, também, uma baixa tendência de biomagnificação através da cadeia alimentar (SANTOS et al., 2007).

2.4 PERMETRINA

Permetrina (PM), nomeada segundo a lUPAC como 3-fenoxibenzil *(lRS,3RS;lRS,3SR)-3-(2-*diclorovinil)-2,2-dimetilcicloproanocarboxilato. (Figura 2) e com o peso molecular de 391,28 g/mol é o segundo IPS mais utilizado e o primeiro IPS do tipo I sinterizado (REGUEIRO et al., 2007). É usado frequentemente em cultivo de algodão, arroz, café, soja, milho, trigo, tomate, couve, couve-flor, repolho e fumo, e na medicina veterinária, na eliminação de ectoparasitas de animais de pequeno porte e bovinos. Em campanhas de saúde pública, são empregados na erradicação de mosquitos, no armazenamento de grãos (arroz, milho e trigo) e, em uso doméstico, na eliminação de insetos em geral e no controle de formigas (PLATA OVIEDO, MARIA TERESA; FIGUEREIDO TOLEDO, MARIA CECILIA, VICENTE, 2003).
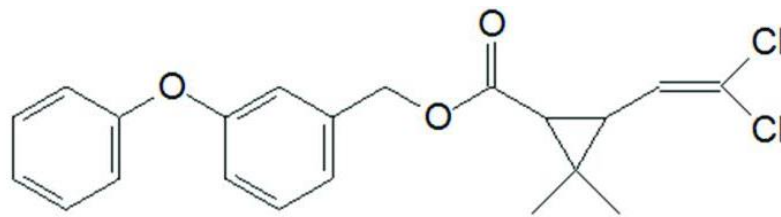
Figura 2: Estrutura química do inseticida piretróide permetrina (Fonte: Google adaptada pelo autor).

A permetrinapertence a classificação toxicológica classe III e apresentando uma ingestão diária aceitável (IDA) de 0,05 mg/kg (Agência Nacional de Vigilância Sanitária -ANVISA), um dos mais comumente utilizados. Segundo o Ministério da Saúde o limite máximo permitido de PM para o consumo humano é de 20 µg/L. No ambiente apresentam concentrações nos recursos hídricos que variam entre 0,05 – 811 µg/L (YANG et al., 2014). Análises quantitativas de sedimentos agrícolas e urbanos demonstraram uma detecção frequente de PM, além da sua prevalência em relação a outros IPS em concentrações que variam de 1,0 – 459 ng/g (HLADIK; KUIVILA, 2009). No Brasil, foram encontrados níveis residuais de PM (8,6 –18,8 mg/Kg) em alimentos acima do limite máximo permitido pela (ANVISA).

2.5 NEUROTOXICIDADE DA PERMETRINA E DISTÚRBIOS NO DESENVOLVIMENTO

O principal alvo molecular da PMé os canais de sódio voltagem-dependente (VSSC), tanto em insetos como em vertebrados (SHAFER; MEYER; CROFTON, 2005b). No sistema nervoso central e periférico as PM liga-se à subunidade α dos canais de sódio dos axônios

promovendo o aumento da permeabilidade da membrana e, consecutivamente, levando a despolarização prolongada, a qual leva a um estado de hiperexcitabilidade (SODERLUND, 2013a). Esse mecanismo atua de maneira imediata sobre os insetos causando paralisia e mortalidade (MEACHAM et al., 2008).

A toxicologia dos IPS, em geral, é relacionada com sua estrutura química, sendo divididos em dois tipos (tipo I e tipo II). Cada tipo é diferenciado pela ausência (tipo I) ou presença (tipo II) de um grupo ciano (CN) na porção fenoxibenzil formando a estrutura alfa-cianofenoxibenzil, a qual é responsável por conferir maior eficiência do inseticida (FIGU; INTE, 2014). As subclasses de piretróides também são divididas com base na caracterização dos sintomas de intoxicação aguda. Piretróides do tipo I, como a PM, atuam no sistema periférico causando tremores, comportamento agressivo, ataxia e convulsões. IPS do tipo II agem no sistema nervoso central causando hipersensibilidade, salivação abundante, agitação nos membros superiores e inferiores e tremores periódicos (SODERLUND, 2013a).

A atividade biológica da PM, também, é dependente da estrutura química e configuração estérica. A toxicidade da mistura racêmica varia com a razão *cis/trans* e com as características do solvente usado (solventes não polares aumentam a toxicidade). Isômeros *cis* demonstram maior toxicidade em relação aos isômeros *trans* (KANEKO, 2011).

Em relação à toxicocinética, a PM é considerada de rápida absorção por diferentes vias (oral, cutânea e respiratória). Em modelos mamíferos, a PMapresenta, relativamente, rápida biotransformação e excreção (6 dias em ratos) (SHAFER; MEYER; CROFTON, 2005a). A biotransformação de piretróides em mamíferos, incluindo humanos, consiste em oxidação, hidrólise de ésteres (ambos são chamados de reações de Fase I) e conjugação com moléculas endógenas (reações de Fase II). As reações de oxidação são catalisadas pelas isoformas do citocromo P_{450} (CYP_{450}), e as ligações éster são hidrolisadas pelas carboxilesterases, favorecendo a excreção dos metabólitos pela urina (WANG et al., 2016a). Peixes são os vertebrados mais sensíveis aos efeitos neurotóxicos de IPS, manifestando efeitos da exposição em concentrações de dez a mil vezes menores em relação aos mamíferos (MONTANHA, 2012). Sua maior sensibilidade é devida, principalmente, ao caráter lipofílico dos IPS, o qual facilita a sua absorção através das brânquias. Adicionalmente, peixes também apresentam uma deficiência no sistema enzimático de detoxificação de fase I, o qual atua na hidrolise de piretróides e outros xenobióticos (NUNES et al., 2018a). Apesar dos IPS serem pouco polares e não se acumularem nos tecidos, peixes em estágios de desenvolvimento demonstram maior taxa de bioacumulação de PM em relação a outros IPS em condições laboratoriais (TU et al., 2014).

O caráter lipofílico dos IPS, em relação aos outros agroquímicos, os torna capazes de atravessar facilmente a barreira hematoencefálica e exercer seu efeito tóxico diretamente no sistema nervoso central (BARR et al., 2010). Além disso, a detecção do biomarcador de exposição à IPS, 3-fenoxibenzóico (3PBA; metabólico piretróide secretado na urina), em 70% das amostras humanas analisadas, segundo a Pesquisa Nacional de Saúde e Nutrição durante 1999-2002 nos Estados Unidos da América (EUA), tem chamado a atenção para os riscos da exposição aos IPS, principalmente para crianças e gestantes, os quais apresentam níveis significantemente elevados desse biomarcador (BARR et al. 2010; BABINA et al. 2012; QI et al. 2012). Esses dados têm levantado preocupações sobre o efeito dos IPS sobre o desenvolvimento neuronal durante os primeiros estágios do desenvolvimento.

Distúrbios do desenvolvimento neuronal têm afetado 10-15% de todos os nascimentos, sendo prevalentes os transtornos comportamentais como autismo, déficit de atenção e hiperatividade, os quais têm crescido significativamente nos últimos quarenta anos (GRANDJEAN; LANDRIGAN, 2014). As causas relacionadas com o aumento dessas patologias ainda permanecem desconhecidas. No entanto, estudos têm apontando a exposição a agroquímicos como fator de risco, uma vez que cérebro humano em desenvolvimento é excepcionalmente vulnerável a exposições a substâncias químicas tóxicas. Os efeitos de exposição durante os períodos iniciais de desenvolvimento podem causar lesões cerebrais permanentes que poderiam apresentar pouco ou nenhum efeito adverso em um indivíduo durante a fase adulta (GRANDJEAN; LANDRIGAN, 2014).

O desenvolvimento embrionário é caracterizado por uma elevada taxa de diferenciação e apoptose celular, a qual podem ser afetadas pelo desequilíbrio redox entre espécies reativas de oxigênio (ERO) e o sistema de defesa antioxidante (SDA) no meio intracelular (GUO, 2009). Flutuações nos níveis de ERO podem desempenhar importantes funções de regulação, mas em concentrações elevadas podem ocasionar danos à biomoléculas, como ácidos nucléicos, proteínas e lipídios, os quais podem alterar fatores epigenéticos (BALABAN; NEMOTO; FINKEL, 2005).

2.6 PERMETRINA E DESBALANÇO REDOX

Estudos têm demonstrado efeitos pró-oxidantes da PM em modelos experimentais(GABBIANELLI et al., 2009a; NASUTI et al., 2007; NUNES et al., 2018; RADHAIAH; RAO, 1990; WOLANSKY; HARRILL, 2008; YANG et al., 2014). Danos oxidativos sobre moléculas e estruturas celulares são associados à fisiopatologia de doenças neurodegenerativas. Desse modo, a exposição crônica a PM pode ser considerado um fator de

risco para desenvolvimento dessas doenças (CARLONI et al., 2012). Flutuações nos níveis de EROS podem desempenhar importantes funções de regulação, mas quando presentes em altas quantidades podem causar graves danos a ácidos nucléicos, proteínas e lipídios (BALABAN; NEMOTO; FINKEL, 2005). O estresse ocasionado pela desregulação redox celular é provocado pela produção excessiva de ERO, tais como ânion superóxido ($O_2^{\cdot-}$), peróxido de hidrogênio (H_2O_2) e radical hidroxila ($^{\cdot}OH$) (HALLIWELL, 1992).

O aumento nos níveis de ERO juntamente com a ineficiência nos sistemas de defesa antioxidante, representado por enzimas e componentes não-enzimáticos configuram o estado de estresse oxidativo. O sistema de defesa antioxidante inclue enzimas como superóxido dismutase (SOD), que dismuta o $O2^{\cdot-}$ em H_2O_2, e também enzimas como a catalase (CAT) e glutationa peroxidase (GPx), que se neutralizam o H_2O_2, impedindo a formação de $^{\cdot}OH$ pela reação de Fenton (MIRIM, 2007). Além dessas enzimas, as diferentes isoformas da enzima glutationa-S-transferase (GST) são importantes na detoxificação de xenobióticos e também atuam junto com o sistema de defesa antioxidante (FONSECA et al., 2010). As isoformas de GSTs são uma família de enzimas de fase-II que conjugam compostos electrofílicos (hidrocarbonetos aromáticos policíclicos - HAPs) com glutationa (GSH) e participam na proteção celular contra os efeitos tóxicos de uma variedade de xenobióticos e subprodutos metabólicos oxidados (HAYES et al., 2005). Outro fator que contribui para o estado de estresse oxidativo é a ocorrência de baixos níveis de cofatores dessas enzimas como GSH (BERRY et al., 2010).

As defesas antioxidantes celulares descritas acima são expressas pela ativação do fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2). Em condições fisiológicas, as concentrações de Nrf2 permanecem relativamente baixas, no entanto em condições de estresse oxidativo os resíduos de cisteína que mantem a ligação entre Nrf2 e Keap1 (Kelch-like ECH-associated protein 1) são oxidados no citoplasma resultando na liberação do Nrf2. O Nrf2 livre migra para o núcleo e conjuga-se com proteínas pequenas fibrossarcoma musculoaponeurótica (sMaf) formando um heterodímero, o qual liga-se ao elemento de resposta antioxidante (ARE) ou ao elemento de resposta eletrofílica (EpRE) localizado nas regiões promotoras de genes alvo. A ativação do Nrf2 também ocorre por meio da ação de proteínas quinases, tais como a proteína quinase C (PKC), proteína quinase regulada por sinal extracelular (ERK), c-Jun N-terminal quinase (JNK) e fosfatidilinositol-3-quinase (PI3K), que agem fosforilando o Nrf2 em resíduos de serina ou treonina, facilitando a sua dissociação do complexo com Keap1 e posterior translocação nuclear. Outras defesas antioxidantes também são alvo da expressão regulada pelo Nrf2, tais como: heme oxigenase 1 (HO-1), NAD(P)H quinona oxidoredutase 1

(NQO1), tioredoxina (Trx) e γ-glutamilcisteína sintetase (γGCS)(AHMAD et al., 2014; HAHN et al., 2015). Além disso, a Nrf2 é responsável por modular a expressão de diversos genes ligados à resposta inflamatória, regulação metabólica, proliferação celular, senescência e função mitocondrial (HAHN et al., 2015).

A maior produção celular de EROS ocorre nas mitocôndrias como um produto secundário da respiração aeróbica, onde $O_2^{\bullet-}$, $^\bullet OH$ e H_2O_2 são produzidos constantemente durante os processos do sistema de transporte de elétrons (STE). Em condições fisiológicas cerca de 2% do oxigênio consumido é convertido em $O_2^{\bullet-}$, considerado a primeira etapa da formação das EROs, os quais são neutralizados por uma bateria inteira de sistemas antioxidantes mitocondriais, citosólicos e peroxisomal, conforme citado anteriormente (KOPPERS et al., 2008). Na maioria das células eucarióticas os complexos I e III do STE contribuem mais significativamente para a formação de EROS, mesmo em condições basais (BALABAN et al., 2005).

Como consequência do estresse oxidativo as mitocôndrias sofrem várias adaptações funcionais. Por exemplo, em resposta a EROS, os tióis mitocondriais tornam-se rapidamente oxidados, uma modificação que influencia a atividade de várias proteínas mitocondriais, como a adenina nucleotídeo translocase (ANT), a qual medeia a troca de ADP e ATP entre o citosol e a matriz mitocondrial (BLESA et al., 2015). Esta atividade enzimática fica prejudicada na oxidação, resultando em escassez de ADP intramitocondrial e inibe as subunidades F_1 e F_0 ATPsintase. Além disso, várias mudanças proteômicas são rapidamente desencadeadas nas mitocôndrias pelo estresse oxidativo, incluindo a regulação positiva de sistemas antioxidantes listados acima, bem como de várias proteínas chaperonas (THOMAS et al., 2009). Em conjunto, essas adaptações maximizam a capacidade de tamponamento de EROS, permitindo assim o restabelecimento da homeostase em resposta ao estresse oxidativo moderado. No entanto, se o estresse oxidativo persistir e os danos moleculares associados (peroxidação lipídica, desdobramento da proteína, mutações no DNA) estiverem além da capacidade de recuperação, então a mitocôndria pode induzir essa resposta adaptativa na ativação da morte celular (TEMKIN et al., 2006). Além das mitocôndrias, há outras fontes de EROS, tais como: xantina oxidase, que catalisa a oxidação de hipoxantina a xantina (1º etapa no catabolismo de purinas), oxidases de nicotinamida adenina dinucleotídeo fosfato (NADPH) multicomponente, como NOX1, bem como enzimas da família do citocromo P_{450} (GALLUZZI et al., 2012).

A PM é capaz de causar diversas alterações fisiológicas em concentrações significativamente menores que a concentração letal média (CL_{50}). Nasuti et al. (2008)

demonstrou que a administração oral de PM 1/10 da CL$_{50}$ (340 mg/kg) levam ao desiquilíbrio redox, a alterações comportamentais e à depleção nos níveis de dopamina em ratos neonatais. A sensibilidade frente à exposição a PM pode depender de cada estágio de desenvolvimento, Delorenzo et al. (2006)observou que o estágio larval de *Palaemonetes pugio* foi o mais sensível a exposição a PM, demostrando uma CL$_{50}$ (0,05 μg/L) em concentrações significativamente menores quando comparado aos embriões (6,4 μg/L) e adultos (0,25 μg/L). Em embriões de peixe-zebra, Demicco et al. (2009) e Yang et al. (2014) demonstraram que a exposição à PM exerce, relativamente, baixa letalidade (CL$_{50}$ = 300 μg/L) quando comparado à outros IPS. No entanto, foi possível observar o seu efeito teratogênico em concentrações acima de 200 μg/L e espasmos em concentrações maiores que 100 μg/L. Yang et al. (2014) também demonstrou que a exposição a PM pode levar a diminuição na expressão de importantes genes envolvidos na diferenciação neuronal durante os primeiros estágios do desenvolvimento. Desse modo, estudos que avaliem os efeitos da exposição à PM em concentrações subletais sobre organismos em desenvolvimento tornam-se necessários, assim como a persistência dos seus efeitos toxicológicos até a fase adulta.

2.7 PEIXE-ZEBRA COMO MODELO ANIMAL

A crescente necessidade por pesquisas na área da biologia experimental para o entendimento de mecanismos toxicológicos de xenobióticos (ZON; PETERSON, 2005), tem sido contraposta com uma tendência à diminuição no uso de animais experimentais, especialmente mamíferos (RICHENDRFER; CRÉTON, 2013). Recentemente, peixes como *Danio rerio* (Hamilton, 1822), também conhecido por peixe-zebra, zebrafish ou paulistinha, têm surgido como um modelo animal complementar aos modelos animais de roedores (RICHENDRFER; CRÉTON, 2013). Este pequeno teleósteo de água doce oriundo do sudeste asiático e medindo 3 – 4 centímetros (cm) pertencente à família *Cyprinidae* (Figura 3) (DAMMSKI; MÜLLER, 2011).

Figura 3**:** *Danio rerio* tipo selvagem adulto macho (A) e fêmea (B) (AVDESH et al., 2012).

O estudo dessa espécie começou no final da década de 60 por George Streisinger através de técnicas de mutagênese (GRUNWALD; EISEN, 2002). Este espécie foi de grande valia para o avanço no conhecimento da embriogênese e ciclo de vida dos vertebrados, devido à reprodução externa, presença de ovos translúcidos, abundante prole e rápido desenvolvimento (Figura 4) (BELANGER; RAWLINGS; CARR, 2013). Além disso, o instituto Sanger através de técnicas de sequenciamento do genoma do peixe zebra identificou genes evolutivamente conservados entre esta espécie com os genes de mamíferos, apresentado 70% de genes homólogos aos humanos (STERN, 2003). Outras características, como anatomia básica do encéfalo, sistemas de neurotransmissores similares aos de mamíferos (PANULA et al., 2010)e atributos práticos como fácil manejo, pouco espaço requerido, baixo custo de manutenção, alta taxa de reprodução, pequeno porte e baixo custo para triagens em larga escala (LITTLETON; HOVE, 2013), fazem com que o peixe zebra seja atraente para estudos de laboratório. As vantagens no uso desse modelo animal assemelham-se às oferecidas pelos modelos de culturas de células, mosca da fruta (*Drosophila melanogaster*) e *Caenorhabditis elegans*. No entanto, proporcionam maior complexidade nas interações bioquímicas e maior similaridade com mamíferos, se aproximando do modelo de roedores, mas oferecendo vantagens na manutenção e nos custos (AVDESH et al., 2012).
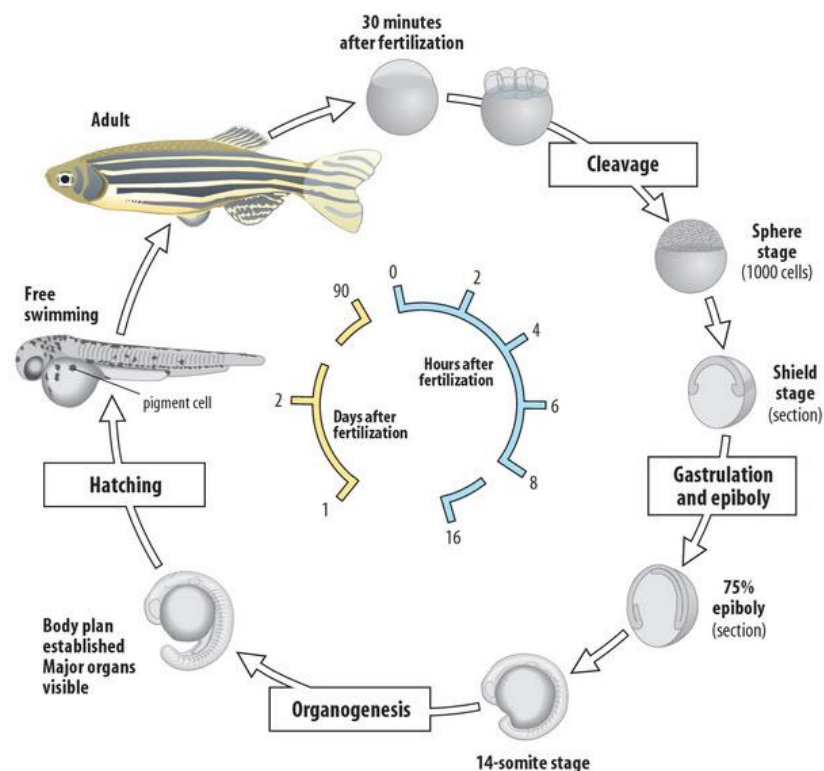
Figura 4: Desenvolvimento embrio-larval de *Danio rerio* (D'COSTA; SHEPHERD, 2009)

A utilização do peixe zebra como modelo animal vem se expandido para várias áreas do conhecimento, como bioquímica, neurociência, farmacologia e biologia do comportamento (KALUEFF et al., 2013; MAXIMINO et al., 2015; RICO et al., 2011). O peixe-zebra tem se mostrado útil em pesquisas biomédicas relacionadas a doenças humanas e em estudos relacionados com as bases moleculares da neurobiologia (GUO, 2009). Nesse sentido, a identificação dos principais sistemas de neurotransmissão existentes em humanos já foi realizada em peixe-zebra (GUO, 2009). Na última década, foram desenvolvidos estudos avaliando características comportamentais do peixe-zebra (BLASER; GERLAI, 2006; CACHAT et al., 2010; GERLAI, 2011; MAXIMINO et al., 2010). O peixe-zebra adulto apresenta um repertório comportamental bastante complexo, onde a exposição a agentes estressores pode evocar medo ou comportamento do tipo ansiedade facilmente quantificáveis através de exploração reduzida, aumento da escototaxia (aversão a ambientes claros), geotaxia (resposta de mergulho), tigmotaxia (preferência pela periferia do tanque), avaliação de risco (entrada parcial no compartimento claro e rápido retorno para o compartimento escuro), preferência por coespecíficos e/ou agressividade (BLASER; GERLAI, 2006; EGAN et al., 2009; KALUEFF et al., 2013; MAXIMINO et al., 2015). Estes *endpoints* comportamentais, em conjunto, podem predizer efeitos neurotóxicos de contaminantes ambientais e efeitos psicofarmacológicos (KALUEFF et al., 2013).Diferentes estágios do desenvolvimento do peixe-zebra podem ser usados como ferramentas de avaliação toxicológica. Durante os estágios iniciais do desenvolvimento é possível avaliar "endpoints" morfológicos, fisiológicos e comportamentais, devido ao rápido desenvolvimento e transparência do córion (membrana que reveste o embrião) (BRAUNBECK; LAMMER, 2006). Durante o desenvolvimento, também é possível a acompanhar o desenvolvimento neurocomportamental, através do repertório comportamental, o qual vai aumentando o grau de complexidade conforme o estágio de desenvolvimento (KALUEFF et al., 2013). Durante a fase da gastrulação (6 hpf), inicia-se a diferenciação celular do tecido que dará origem ao sistema nervoso, movendo-se para regiões distintas do embrião (KIMMEL et al., 1995). No final do perído de gastrulação (9 – 10 hpf) ocorre à formação do tubo neural e subdivisão do mesmo em regiões que darão origem às divisões cerebrais. No período de faríngula, 24 – 48 horas pós-fertilização (hpf), a morfogênese do encéfalo encontra-se em um estágio avançado e dividido em prosencéfalo incluindo o diencéfalo e telencéfalo, mesencéfalo, rombencéfalo e medula espinhal, enquanto os primeiros aglomerados de neurônios estão interconectados por axônios (DE ESCH et al., 2012), nesta fase é possível observar os primeiros movimentos espontâneos ainda dentro do córion (membrana que reveste o embrião). Durante o período de eclosão (48 – 72h), os

ventrículos cerebrais foram formados e o embrião começa a responder a estímulos, como o toque (KiMMEL et al., 1995). Os subtipos de células gliais, oligodendrócitos, células de Schwann e astrócitos são encontrados em larvas de peixe-zebra a partir de 4 dias pós-fertilização (dpf) (COLWILL; CRETON, 2011). O rápido desenvolvimento do sistema nervoso central do zebrafish permite o primeiro teste funcional em 48 hpf, enquanto que testes mais avançados são possíveis a partir de 5 dpf, quando a morfogênese se com a abertura da boca e comportamento exploratório ativo (DE ESCH et al., 2012).

Durante o estágio embrio-larval, as larvas de peixe-zebra apresentam um tamanho relativamente pequeno (4 – 5 mm) e fácil absorção de compostos adicionados diretamente à água, a quantidade dos reagentes a serem testados passa a ser significativamente menor. Desse modo, otimiza-se o uso das drogas de estudo associando uma menor produção de resíduos. Pode-se destacar, com isso, que o peixe-zebra é um animal que combina a relevância de ser um vertebrado, apresentando maior complexidade nas interações neurocomportamentais e maior homologia genética, e também, apresentando facilidades de manejo e baixo custo de manutenção, assemelhando-se a vantagens um modelo animal invertebrado(GOLDSMITH, 2004).

3. JUSTIFICATIVA:

A permetrina é um inseticida presente no ambiente rural e doméstico. Seus efeitos neurotóxicos não são seletivos para os organismos alvo, apresentando risco, principalmente, para organismos em estágios iniciais de desenvolvimento, os quais podem sofrer alterações permanentes no desenvolvimento neuronal. Além disso, diversos estudos têm relacionado o aumento a incidência de casos de distúrbios no neurodesenvolvimento com a exposição a contaminantes ambientais, como a permetrina. Além disso, os mecanismos de toxicidade da permetrina e seus efeitos secundários ainda permanecem não totalmente esclarecidos.

4. HIPÓTESES:

- Concentrações ambientais de permetrina são capazes de causar mortalidade de larvas de peixe-zebra durante 24h de exposição?

- Concentrações subletais de permetrina são capazes de prejudicar o comportamento motor e não-motor de larvas de peixe-zebra?
- Concentrações subletais de permetrina são capazes de induzir desbalanço redox, induzir danos oxidativos e levar a processos apoptóticos em larvas de permetrina?

- A exposição à concentrações subletais de permetrina podem alterar a homeostase energética em larvas de permetrina?

- Os efeitos de exposição a permetrina durante o período de desenvolvimento embrionário podem alterar o comportamento durante os estágios iniciais de desenvolvimento e persistirem até a fase adulta em peixe-zebra?

5. OBJETIVOS:

5.1 OBJETIVO GERAL:

Avaliar os efeitos farmacológicos, toxicológicos e fisopatólogicos induzidos pela permetrina em diferentes fases do desenvolvimento de peixe-zebra.

5.2 OBJETIVOS ESPECÍFICOS:

Capítulo I:
- Identificar a concentração letal aguda de permetrina em larvas de peixe-zebra e determinar as concentrações subletais;

- Avaliar o efeito das concentrações subletais sobre o comportamento motor e não-motor de larvas de peixe-zebra agudamente expostas;

- Avaliar o efeito da exposição aguda a permetrina sobre a homeostase redox em larvas de peixe-zebra;

- Avaliar a genotoxicidade da permetrina e indução de apoptose em larvas de peixe-zebra agudamente expostas;

- Investigar os efeitos da exposição aguda à permetrina sobre a respiração mitocondrial e metabolismo energético em larvas de peixe-zebra.

Capítulo II:

- Investigar os efeitos neurodesenvolvimentais da exposição embrionária a permetrina em larvas de peixe-zebra através de análise de padrões comportamentais;

- Avaliar a persistência dos efeitos de exposição à permetrina em diferentes estágios de desenvolvimento de peixes-zebra por meio de avaliação comportamental;

6. DESENVOLVIMENTO

6.1 CAPÍTULO I**:**

Artigo:

Acute exposure to permethrin modulates behavioral functions, redox and bioenergetics parameters, and induces DNA damage and cell death in larval zebra fish

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ABSTRACT

Permethrin (PM) is a synthetic pyrethroid insecticide widely used as domestic repellent. Damage effects to non-target organisms have been reported, particularly in the development early stages. Studies indicate redox unbalance as secondary PM effect. Therefore, our goal was investigate the acute PM effects about larval zebrafish. Larvae (6 days post fertilization) were exposed to PM (25 – 600 µg/L) during 24 hours, and 50% lethal concentration was estimated. For subsequent assays, the sublethal PM concentrations of 25 and 50 µg/L were used. PM increased anxiety-like behaviors according to the novel tank and light-dark tests. At the molecular level, PM induced increased ROS, which may be related to the increased lipid peroxidation, DNA damage and apoptosis detected in PM-exposed organisms. In parallel, upregulation of the antioxidant system was detected after PM exposure, with increased superoxide dismutase, glutathione S-transferase and glutathione reductase activities, and thiol levels. The increased of Nrf2-target genes and the activation of an electrophile response element-driven reporter (Tg *EPRE*:LUC-EGFP) suggest that Nrf2 pathway can mediate a fast response to PM, leading to antioxidant amplification. By using high-resolution respirometry, we found that exposure to PM decreased the oxygen consumption in all respiratory stages, disruptioning of oxidative phosphorylation and inhibit the electron transfer system, leading to decrease in bioenergetics capacity. In addition, PM led to increases of residual oxygen consumption and changes in substrate control ration Glucose metabolism seems to be affected by PM, with increased lactate dehydrogenase and decreased citrate synthase activities. Taken together, our results demonstrated the adverse effects of acute sublethal PM concentrations during larval development in zebrafish, causing apparent mitochondrial dysfunction, indicating a potential mechanism to redox unbalance and oxidative stress, which may be linked to the detected cell death and alterations in normal behavior patterns caused by acute PM exposure.

Keywords: pyrethroid; anxiety; oxidative stress; apoptosis; mitochondrial bioenergetics; development, *Danio rerio*.

1. INTRODUCTION

Permethrin (PM; 3-phenoxybenzyl-(1R,S)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) is one of the most frequently used Type I synthetic pyrethroids (SP) (REGUEIRO et al., 2007). The main molecular targets of SP are the voltage-dependent sodium channels (VDSC) to which they bind and delay the inactivation of sodium channels, resulting in neurotoxicity effects and ultimately death (MEACHAM et al., 2008b). This mode of action is common to all organisms exposed to SP (MEACHAM et al., 2008b; SHEETS, [s.d.]).

PM is used in a range of insecticide formulas to control pests in residential and agricultural areas due to its high efficiency and low mammalian toxicity (RAY; FRY, 2006). However, several studies have shown that PM can cause a variety of side effects in non-target organisms, including teratogenicity, cardiotoxicity, endocrine dysfunction, hepatotoxicity, and cytotoxicity both in vertebrates and invertebrates (for a review see Wang et al., 2016(WANG et al., 2016a)). Besides that, studies have found detectable levels of the SP metabolite 3-phenoxybenzoic acid in children and pregnant women (BERKOWITZ et al., 2003; LU et al., 2006, 2009; MORGAN et al., 2007; WHYATT et al., 2002), though little is known on the mechanisms by which SP induce toxicity during the early stages of life.

In general, developing organisms are most vulnerable to toxic effects of SPs (PATEL; PATIL, 2016) and epidemiologic studies demonstrated that exposure to pesticides during pregnancy and/or early childhood is associated with neurodevelopmental outcomes in children (BURNS et al., 2013), which can change functional neurodevelopmental endpoints such as behavior (PARRÓN et al., 2011). This higher sensitivity of developing organisms has been associated with cellular redox unbalance, which can impair normal development (DENNERY, 2007; HAHN et al., 2015). Additionally, others evidences have indicated oxidative stress as a secondary mode of action of PM due to changes in redox biomarkers in different animal models, including DNA, lipid and protein damage, modulation of the antioxidant system (SELLAMI et al., 2014, 2015; WANG et al., 2016b; YANG et al., 2014), and activation or repression of transcription factors regulating pro-inflammatory, anti-inflammatory and apoptotic responses (CARLONI et al., 2013a; DHIVYA VADHANA et al., 2013a).

Oxidative phosphorylation (OXPHOS) is one of the metabolic pathways sensitive to environmental toxicants (IGLESIAS et al., 2018). Mitochondrial dysfunction can lead to

increase in the partial reduction of oxygen by electrons leaking from mitochondrial electron transport chain (ETC), generating an increase in reactive oxygen species (ROS) steady-state levels and, potentially, oxidative stress (KOWALTOWSKI et al., 2009). Besides that, the mitochondrial ATP production exerts several functions in cell metabolism, such as energy metabolism, ROS production and elimination, and cell death (OTT et al., 2007; SASTRE, FEDERICO V . PALLARDO, JOS&; PALLARDÓ; VIÑA, 2000). Thereby, the mitochondrial OXPHOS and the ETC can be the possible targets of PM toxicity and influence cellular fate and function through redox unbalance.

Alternative experimental models have arisen as important tools in toxicology, providing valuable information about the mechanisms of toxicity of several chemical molecules (GARCIA; NOYES; TANGUAY, 2016). Among them, zebrafish (*Danio rerio*) appears as an important vertebrate model (MCCOLLUM et al., 2011; PANULA et al., 2006), with substantial genetic and physiological homology in relation to humans and easily genetically manipulated (BRANNEN et al., 2010). These advantages have led to the use of the zebrafish model in drug discovery, toxicological screening and developmental toxicology (PETERSON et al., 2008).

The present study sought to determine toxicological effects of acute PM exposure and the relationship between changes in behavior patterns, redox parameters, mitochondrial function and cell death in zebrafish larvae acutely exposed to sublethal concentrations of PM.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Except for Lactate dehydrogenase activity kit (Labtest – Lagoa Santa, MG, Brazil), SYBR Safe DNA gel stain (Life Technologies – Carlsbad, CA, US) and anti γ-GCS, HO-1 and NQO-1 primary antibodies (Santa Cruz Biotechnology, Inc. - Dallas, TX, US), all other chemicals were purchased from Sigma – Aldrich (São Paulo, SP, Brazil).

2.2 Zebrafish maintenance and reproduction

Adult *Danio rerio* (wild-type) were obtained from a local commercial supplier and maintained in a recirculating aquatic system (Zebtec®) under appropriate water conditions (pH 7.2 ± 0.5, 400 ± 50 µS conductivity, 28 ± 1 °C temperature and dissolved oxygen equal or above 95% saturation) with a 14h:10h (light:dark) photoperiod. Ammonia, nitrite, and nitrate values were kept lower than 0.2 ppm, 0.05 ppm, and 0.05 ppm, respectively. This water was used in the preparation of test solutions of all assays performed. The fish were fed on commercial flocked fish food and supplemented with brine shrimp (*Artemia salina*). The experimental protocols used in this work were approved by the local Ethics committee (CEUA – Unipampa: protocol 003-2016).

Experiments also used the transgenic lines Tg(*EPRE*:LUC-EGFP) (a generous gift from Dr. Michael Carvan, University of Wisconsin, WI, US) (KUSIK; CARVAN III; UDVADIA, 2008) and Tg [(*HSP70*:EGFP)_*unspecified*] (a generous gift from Dr. Michael Hahn, Woods Hole Oceanographic, MA, US and Dr. John Y. Kuwada, University of Michigan, MI, US) (HAHN et al., 2014; HALLORAN et al., 2000). These animals were maintained in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL, USA) on a 14:10 h light/dark cycle. Water quality was maintained at 27 - 29°C, pH 7.0-8.0, using 60 ppm artificial seawater (Instant Ocean, Foster & Smith, Rhinelander, WI, USA). The fish were fed on Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats, Inc.) and supplemented with brine shrimp. All procedures were approved by the Institutional Animal Care and Use Committee of the Duke University (IACUC protocol: A139-16-06).

Male and female adult fish (6 – 12 month) with an optimal ratio of 2:1 were placed in pairs overnight. The reproduction was induced by the light irritation on the next morning. Fertilized eggs were collected, washed with fish system water for several times and incubated in system water in a BOD (Biochemical Oxygen Demand) incubator at 28 °C.

2.3 PM exposure and survival rate

The stock solution of permethrin (PM, 98.3%, a mixture of isomers) was prepared in ethanol and stored at -20 ºC in nominal concentrations of 25, 50, 75, 100, 200, 300 and 600 mg/L. Toxicological assays were based on the OECD guidelines for the testing of chemicals 210 - Fish, Early-life Stage Toxicity Test (OCDE, 2014), with some modifications. Briefly, a number of 50 larvae zebrafish (per group) with 6 days post-fertilization (dpf) were selected by simple randomization for acute exposure by immersion to PM during 24h in 50 ml plastic tubes (falcon tubes, which not contain concentrations of bioactive substances), in a total of 6

replicates for each independent experiments (totalizing 300 larvae/concentration/experiment). For LC$_{50}$ (defined as the concentration causing 50% of mortality in the exposed animals) and others experiments, larvae were exposed to nominal concentrations of 0, 25, 50, 75, 100, 200, 300 and 600 µg/L of PM during 24h. The exposure solution was diluted in water of maintenance system (WMS) and the final concentration of ethanol in each treatment, including the control group (CTL), was 0.01% (v/v). The sublethal concentrations and sample size were based in dose-effects demonstrate in experiments performed by Demicco et al. (2009)(DEMICCO et al., 2009) and Yang et al. (2015)(YANG et al., 2014). For the consequent analyzes will be chosen the concentrations that will not increase mortality and that did not present teratogenicity effects according to the literature (DEMICCO et al., 2009; YANG et al., 2014).

2.4 Behavior assessment

The larval behavior assays were adapted to larval developmental stage followed the adults methods described in the previous article, published in Molecular Neurobiology (NUNES et al., 2016). Briefly, the behavioral tests were performed after the time exposure between 1:00 – 5:00 pm. A number of 35 – 50 larvae were selected by simple randomization and individually placed in each well of 24-wells cell culture plate (hereafter called apparatus), filled with WMS (2 mL, 27 ± 1 °C), and the behavioral activities of zebrafish were recorded for a single session of 300 seconds. The experimental procedures were performed on a stable surface with all environmental distractions kept to a minimum. For swimming location and determination of behavioral parameters we followed the same methods described by Nunes et al. 2017(NUNES et al., 2016).

2.4.1 Open Field test

Locomotors and exploratory activities were analyzed in the Open Field test. The swimming pattern behavior was analyzed as described elsewhere (ALTENHOFEN et al., 2017). The behavioral activities were recorded after 300 seconds of habituation. The apparatus was virtually divided into two circular sections (central and periphery) to assess the spatial exploration by the following endpoints: total time and average time spent per visit in the central zone (s), which were used to measuring of fear/anxiety-related behaviors. Total

distance traveled (m), absolute turn angle (°) and total immobility time (s) were used to measure locomotors and motor patterns.

2.4.2 Novel Tank test

The exploratory behavior followed established protocols using zebrafish larvae (ALTENHOFEN et al., 2017; COLWILL; CRETON, 2011b), which were originally adapted from adult behavior tests (CACHAT et al., 2010; NUNES et al., 2016; ROSEMBERG et al., 2011). The behavioral activities in the Novel Tank test were recorded without habituation time, which may reflect a direct response to novelty stress in contrast to the Open Field test. The apparatus was virtually divided into two circular sections (central and periphery areas) to assess the spatial exploration by the following endpoints: total time and average time spent per visit in the periphery (s), which were used to estimate fear/anxiety-related behaviors. Total distance traveled (m) and total time immobility (s) were used to measure locomotors and motor patterns.

2.4.3 Light–Dark preference test

This test was adapted from light/dark preference behavioral assays carried out with adult (MAXIMINO et al., 2010a; NUNES et al., 2017) and larval zebrafish (STEENBERGEN; RICHARDSON; CHAMPAGNE, 2011a). The surface of the apparatus was physically divided into two areas (black and white) of equal size, using black or white opaque tapes and no physical barrier between them. Each animal was placed initially in the lit (white) area, and the number of entries into the dark area, total time spent (s) in the lit area (s), latency to enter the dark area (s), and the number of risk assessments episodes were measured. Risk assessments were defined as a partial entry in the dark area followed by a fast return to the lit area.

2.5 Measurement of ROS steady-stead levels

The ROS steady-stead levels were measured using the fluorescent dye 2,7-dichlorofluorescein-diacetate (DCFDA) (ALI; LEBEL; BONDY, 1992), following methods described in the previous article, published in Molecular Neurobiology [35]. At the end of the exposure, twenty-five larvae were pooled per sample (n = 6 per group).

2.6 Lipid Peroxidation Estimation Assay

Lipid peroxidation was estimated by thiobarbituric acid reactive substance (TBARS) assay (DRAPER; HADLEY, 1990), following methods described in the previous article, published in Molecular Neurobiology [35]. At the end of the exposure, twenty-five larvae were pooled per sample (n = 6 per group).

2.7 Antioxidant Enzymes activity

Antioxidant enzyme measurements were performed using six independent experiments per group (n= 6) and twenty-five larvae were pooled per sample, following methods described in the previous article, published in Molecular Neurobiology [35]

Catalase (CAT) activity was assessed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm (AEBI, 1984). The specific activity was determined in a cuvette reader using the extinction coefficient of 40 M/cm and expressed as µmol/min/mg of protein.

Superoxide dismutase (SOD) activity was analyzed through the inhibition capacity of quercetin oxidation (406 nm) by superoxide radical in the presence of N,N,N,N-Tetramethylethane-1,2-diamine (TEMED) at pH 10 (KOSTYUK; POTAPOVICH, 1989). The SOD activity is expressed in units SOD/mg of total protein, where 1 unit is the amount of SOD required to give 50 % maximal inhibition of the initial rate of quercetin reduction.

Glutathione peroxidase activity (GPx, selenium-dependent) was measured following the rate of NADPH oxidation at 340 nm, in the presence of GSH, glutathione reductase (GR) and H_2O_2 (PAGLIA; VALENTINE, 1967).

The GR activity was measured following the rate of NADPH oxidation at 340 nm by reduction GSSG to GSH. The assay mixture consisted of potassium phosphate buffer (100 mM, pH 7.0), 0.15 mM NADPH and 1 mM GSSG. The specific activity was determined using the extinction coefficient of 6.22 mM/cm and expressed as nmol/min/mg of protein.

Glutathione S-transferase (GST) activity was measured following conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with GSH at 340 nm (HABIG; PABST; JAKOBY, 1974).

2.8 Determination of thiols levels

Ten larvae were pooled per each sample (n=6 per group), following methods described in the previous article, published in Molecular Neurobiology [35]. The fluorescence related to the thiol levels (non-protein thiols) was read at 350 nm (ex) and 420 (em) (HISSIN; HILF, 1976).

2.9 Western Blotting Analysis

Western blotting was performed according to a previous protocol from our group using zebrafish (COSTA-SILVA et al., 2015), with minor modifications. Fifty larvae were homogenized per sample (n = 4 per group) in Tris NaF buffer (50 mM Tris pH 7.0 containing 1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride, and protease inhibitor cocktail), and 10 µL of sample was taken out for protein analysis prior incubation with 4% SDS stop solution (4% SDS, 50 mM Tris, 100 mM EDTA, pH 6.8). Samples were further mixed with 25% glycerol and 8% β-mercaptoethanol. Proteins were separated by SDS–PAGE using 12% gels and then electrotransferred to nitrocellulose membranes for approximately 3 hours. The membranes were blocked with 5% skimmed milk for 1 hour, washed in Tris-buffered saline with Tween-20 (100 mM Tris–HCl, pH 7.5, 0.9% NaCl and 0.1% Tween-20), and incubated overnight at 4º C with the following rabbit primary antibodies: γ-GCS polyclonal antibody (rabbit anti-human; 1:1,000 dilution; cat. no. sc-22755; Santa Cruz Biotechnology); HO-1 (rabbit anti-human; 1:1,000 dilution; cat. no. sc-10789, Santa Cruz Biotechnology); NQO-1 (rabbit anti-human; 1:1,000 dilution; cat. no. sc-16464, Santa Cruz Biotechnology); and anti- β-actin polyclonal antibody (rabbit anti-human; 1:1,000 dilution; cat. no. A5060; Sigma - Aldrich). Subsequently, membranes were washed in Tris-buffered saline with Tween-20 and incubated for 1 hour at room temperature with horseradish peroxidase-linked anti-IgG (1:10000) secondary specific antibodies. The immunoblots were visualized on the IS4000MM Pro Bruker imaging system using ECL-detection reagent and the band density was quantified using the Scion Image® software.

2.10 Assessment of antioxidant response *in vivo* using zebrafish transgenic lines

For these assays, Tg(*EPRE:*EGFP) and Tg(*HSP70:*EGFP) zebrafish larvae were used for the exposures. The Tg(*EPRE:*EGFP) line express a fused luciferase (LUC) - green fluorescence protein (EGFP) fusion protein under the regulation of the electrophile response element (EPRE). This EPRE sequence consisted of the mouse *Gsta1* EPRE fused to the

minimal promoter from the mouse *mt1* gene. The Tg(*HSP70:*EGFP) line express EGFP under the regulation of the *hsp70-4* promoter region. Both transgenic lines have been shown to respond to oxidative stress by increasing the expression of EGFP via the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) pathway in zebrafish (HAHN et al., 2014; KUSIK; CARVAN III; UDVADIA, 2008).

The exposures were carried out as described in section 2.3, with the addition of 0.2 mM phenylthiourea (PTU) at 24 hpf to prevent the interference of pigmentation on fluorescence imaging. Each experiment consisted of 6 – 9 embryos per exposure group, and the experiments were repeated twice (n = 15 – 17). At the end of the exposure, animals were individually transferred to a 96-well zebrafish imaging plate (ZF plate, Hashimoto Electronic Industry, Takasucho, Japan) with the addition of 100 µL tricaine (200 mg/L). Images were acquired using a BZ-X700 automated fluorescence microscope (Keyence Corporation of America, Itasca, IL, USA) with 470/40 nm (em) and 525/50 nm (ex) filters. Fluorescence was quantified using ImageJ software and expressed as fold change normalized to the control group.

2.10 Determination of Cellular Death

2.10.1 Comet Assay

Genotoxicity was assessed by DNA breaks through the comet assay (KOSMEHL et al., 2008). After the exposure, twenty larvae per group were selected by simple randomization and carefully homogenized manually with a microtube pestle in 1 ml of Dulbecco's Modified Eagle Medium (DMEN) and centrifuged for 10 minutes at 200 x g. The pellet containing the isolated cells was collected and diluted in PBS buffer pH 7.4. An aliquot of 10 µL was added to 0.75% low melting agarose and transferred to the slides. The slides were placed at 4°C for 15 minutes and then added in a lysis solution containing 100 mM EDTA, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO (pH 13.0) in the dark at 4 °C overnight. Slides were further immersed in a neutralizing solution containing 400 mM Tris at pH 7.4 for 30 minutes. For the unwinding of the DNA, the slides were soaked for 30 minutes in a horizontal electrophoresis tank containing an alkaline buffer (12 g/L NaOH and 0.37 g/L EDTA, pH 13) at 25 V and 300 mA. After the electrophoresis run, the slides were washed in distilled water, fixed with 70% ethanol for 5 minutes, and placed in the refrigerator (4ºC) for 1 hour. Finally, slides were stained with Sybr Green for 5 minutes and analyzed by fluorescence microscopy (Olympus 1x71) at 100x magnification, with an exposure of 1044.7 ms, fluorescence at emission (500 nm) and excitation (530 nm) (Olympus U-RFL-T UV light), and an image analysis system (Q- Capture). The images were analyzed by ImageJ/Open Comet image analysis software.

2.10.2 Apoptosis analysis *in vivo* with Acridine Orange staining

The fluorescent dye Acridine Orange (AO) was used to detect apoptotic cells. After the exposure, fifteen larvae were selected by simple randomization and incubated in 2 mL of 5 µg/mL AO diluted in WMS for 30 min in the dark (TILTON; LADU; TANGUAY, 2008). Larvae were washed three times in WMS and mounted in slides using 1.5% methylcellulose. Images were obtained by fluorescence microscopy (Olympus 1x71) at 40x magnification, and 500 milliseconds of exposure using the filter fluorescein isothiocyanate at emission (530 nm)

and excitation (490 nm). Images were analyzed by ImageJ software, and the overall fluorescence in the head area was quantified as a frequency estimate of apoptotic cells.

2.11 Mitochondrial respiration assays

Mitochondrial bioenergetics were measured *in vitro* by high-resolution respirometry (HRR) using an Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria). Briefly, one hundred whole-body of larval zebrafish were pooled per sample (n = 6 per group) and gently homogenized in 70 µL of 5 mM Tris-HCl (pH 7.4) containing 250 mM sucrose and 2 mM EGTA. The homogenate (50 µL) was immediately transferred to 2 mL respiration buffer (3 mM HEPES pH 7.2 containing 115 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, 1mM EGTA and 0.2% essentially fatty acid-free BSA 0.2%). All experiments were performed at 28 °C using DatLab 4.0 software (Oroboros Inc., Austria), with continuous stirring at 750 rpm.

Using titration protocols (AW et al., 2016; CARVALHO et al., 2013), we assayed the abilities of a series of substrates and inhibitors to influence mitochondrial function as reflected in differences in respiration states. Succinate or a mixture of glutamate + pyruvate + malate were used as oxidizable substrates in all experiments, as described below. Changes in mitochondrial respiratory chain complexes and respiratory rates were determined.

After signal stabilization, the (i) routine state supported by endogenous substrates was assayed, and the (ii) complex I (CI)-mediated leak (LEAK) respiration was determined using 5 mM pyruvate, 5 mM glutamate and 1 mM malate in the absence of ADP. (iii) CI-mediated OXPHOS (OXPHOS) was determined with the same substrates but in the presence of ADP (2.5 mM). Based on these values, the respiratory control ratios ($RCR = CI_{OXPHOS}/CI_{LEAK}$) can be determined as an indicator of the state of mitochondrial coupling. Next, (iv) the convergent electron flow during the maximal OXPHOS respiration was determined by simultaneously using substrates of the CI (glutamate, pyruvate and malate) and the complex II (CII, 10 mM succinate), and was named $CI + CII_{OXPHOS}$. The electron transport system (ETS) respiration represents the uncoupled respiration using carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) as an uncoupler (optimum concentration reached between 0.5 and 1.5 µM), and the (v) CI + CII-mediated ETS respiration ($CI + CII_{ETS}$) was determined using FCCP. (vi) CII-mediated ETS respiration (CII_{ETS}) was determined using the CI inhibitor

rotenone (0.5 µM). Lastly, (vii) the residual oxygen consumption (ROX) with small contributions from electron leak in the uncoupled state was assayed in the presence of antimycin (2.5 µM), with the inhibition of complex III (CIII) and resulting in no mitochondrial respiration.

2.12 Citrate synthase and Lactate dehydrogenase activity

Fifty larvae were pooled per each sample (n=6 per group) and homogenized in 100 µL of 20 mM potassium phosphate buffer, pH 7.5. Citrate synthase (CS) activity was determined spectrophotometrically in the homogenates according to the method previously described (SRERE, 1969). The enzyme activity in the presence of the acetyl-CoA and oxaloacetate, and the resulting CoA-SH was determined at 412 nm and 37 °C. The lactate dehydrogenase (LDH) activity was determined in the homogenized sample using a commercial kit (Labtest) following the manufacturer's instructions.

2.13 Total Protein Quantification

Protein content was determined using bovine serum albumin (BSA) as standard, according to Bradford (1976) (BRADFORD, 1976).

2.14 Statistical Analysis

The mean lethal concentration (LC50) for 24 h was calculated using the Nonlin fit test. Normality (Kolmogorov Smirnov) and homogeneity (Bartlett's) tests were applied. The parametric dates are expressed as mean ± standard error (SEM) and were analyzed by one-way ANOVA followed by Tukey post-hoc; and nonparametric dates expressed as median interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. HRR assays were analyzed by unpaired Students t test. The significance was set at $p \leq 0.05$.

3. RESULTS

3.1 Acute PM exposure impairs larval survival

The potential toxicity of PM to zebrafish larvae was initially determined with a 24 hours survival curve. The LC_{50} obtained was 108 µg/L (range, 64.12 – 182) (Fig. 1). The significant effects ($F_{7,104}$ = 150.1, $p < 0.0001$) on survivor were observed on concentrations higher than 50 µg/L (NOEC). For further experiments, the sublethal concentrations of 25 and 50 µg/L were used.

3.2 PM exposure modulates behavioral functions in zebrafish larvae

In the Open Field test (which has a habituation step), no differences were observed in all evaluated endpoints (Fig 2). In the Novel Tank test (which does not have a habituation step), a significant decrease in distance traveled ($F_{(2, 25)} = 4.828, p = 0.0115$) was observed in larvae exposed to 50 µg/L PM (Fig. 3A). This same exposure group also increased the time spent in the central zone ($F_{(2, 59)} = 3.502, p = 0.0365$) (Fig. 3C), and average time per visit ($F_{(2, 55)} = 15.69$, $p < 0.0001$) (Fig. 3D). No differences were observed in immobility time (Fig. 3B).

In the Light-Dark test, it was observed that both PM concentrations decresed the total time spend in lit area ($F_{(2, 105)} = 5.930, p = 0.0036$) (Fig.4A) and unchanged the latency to first enter in dark area (Fig. 4B). However, the number of entries in dark area ($F_{(2, 115)} = 7.877, p = 0.0006$) (Fig.4C) and episodes of risk assessments ($F_{(2, 115)} = 3.314, p = 0.0406$) (Fig. 4 D) were increased in both PM concentration.

3.3 PM exposure induces oxidative stress

Both concentrations of PM caused a significant increase in ROS steady-state levels ($F_{(2, 30)} = 8.324, p = 0.0013$) (Fig. 5A), TBARS levels ($F_{(2, 27)} = 13.70, p <0.0001$) (Fig. 5B) and SOD activity ($F_{(2, 20)} = 12.18, p = 0.0003$) (Fig. 5C). The CAT activity (Fig. 5D) ($F_{(2, 13)} = 6.503, p = 0.0110$) decreased only after exposure to 50 µg/L PM.

3.4 PM modulates the response of the glutathione defense system and proteins encoded by Nrf2-target genes

Acute exposure to 50 µg/L PM significantly increased GST activity ($F_{(2, 33)} = 11.55$, $p = 0.0002$) (Fig. 6A) and the levels of low molecular weight thiols ($F_{(2, 12)} = 5.911$, $p = 0.0163$) (Fig. 6B). No differences were observed between groups in GPx activity (Fig. 6C). In addition, GR activity increased in response to both 25 and 50 µg/L PM ($F_{(2, 15)} = 23.95$, $p < 0.0001$) (Fig. 6D). It was also detected a significant increase in γ-GCS ($F_{(2, 15)} = 18.91$, $p < 0.0001$) (Fig. 7B) and NQO-1 ($F_{(2, 9)} = 20.63$, $p = 0.0004$) (Fig. 7D) protein levels in larvae exposed to 50 µg/L PM, while a decrease in HO-1 protein levels ($F_{(2, 9)} = 29.02$, $p = 0.0001$) was detected in this same exposure group (Fig. 7B). This upregulation of the glutathione defense system and Nrf2 targets was further investigated using two transgenic lines with reporter genes responsive to oxidative stress via the Nrf2 pathway. EGFP fluorescence levels were not altered in Tg HSP70:EGFP zebrafish (Fig. 8A), but were increased in Tg EPRE:EGFP zebrafish exposed to 25 µg/L PM (Fig. 8B), particularly in the tail region.

3.5 DNA fragmentation and cell death induced by PM

PM exposure induced DNA damage and apoptosis as evaluated by the comet assay and AO stain, respectively. Both PM exposure groups had a significant increase in the lengths of the whole comet ($F_{(2, 101)} = 14.95$, $p < 0.0001$) (Fig. 9B) and the tail ($F_{(2, 99)} = 28.73$, $p < 0.0001$) (Fig. 9C). This same pattern was observed in the AO test, with a substantial increase in the relative fluorescence in the head of the larvae ($F_{(2, 31)} = 20.31$, $p < 0.0001$) (Fig. 10B), demonstrating increased in the apoptotic cell death induced by acute exposure to both PM concentrations.

3.6 PM exposure decrease all states of mithocondrial repiration

To investigate the effects of PM at the mitochondrial level, we verified the mitochondrial bioenergetics function using HRR (Fig. 11A). Only the control and PM 50 µg/L groups were evaluated in this assay. All states of mithocondrial respiration were significantly decreased after PM exposure: Basal ($t0.05;6 = 4.082$, $p = 0.0065$), CI_{LEAK} ($t0.05;6 = 3.452$, $p = 0.0136$), CI_{OXPHOS} ($t0.05;6 = 4.042$, $p = 0.0068$), $CI+CII_{OXPHOS}$ ($t0.05;6$

= 6.674, p = 0.0104), CI+CII$_{EST}$ (*t0.05;6 = 3.865, p = 0.0083*), CII$_{ETS}$ (*t0.05;6 = 2.821, p = 0.0303*) and Ama (*t0.05;6 = 3.383, p = 0.0148*). Thereby, PM-related effects were detected in the mitochondrial CI and CII activity both in the dissipative component due to proton leak, proton slip, cation cycling and electron leak states (LEAK)or the ADP-activated state (OXPHOS). Analysis of the total maximum oxygen flux consumption (CI+CII$_{ETS}$) or the CII-dependent maximum oxygen flux consumption (CII$_{ETS}$) by FCCP-dependent decoupling of the ETS also showed significant decrease in oxygen flux consumption by PM exposure. Addition of antimycin A resulting in residual oxygen consumption (ROX) returned the oxygen flux consumption to values close to basal.

3.7 PM exposure changes other mitochondrial parameters, including mitochondrial flux control ratio, bioenergetics capacity and substrate control ratio.

Additional analyses of mitochondrial metabolism were carried out to further investigate the effects of PM at the mitochondrial level. The respiratory control ratio (RCR) is indicator of mitochondrial coupling and was used to evaluate mitochondrial functionality, the PM exposure not change the RCR values (Fig. 11B). The PM exposure was able to significant increases the magnitude of residual oxygen consumption (non-mitochondrial respiration) relative to the maximum oxygen consumption capacity (FMAX) (*t0.05;6 = 6.208, p = 0.0008*), which was determined ROX/ETS ratio (Fig. 11C). In addition, the exposure to PM was decreased the mitochondrial bioenergetics capacity (t0.05;6 = 4.374, p = 0.0047) (Fig. 11D), which was quantified by subtracting the ADP-induced CI$_{OXPHOS}$ values from the CI$_{LEAK}$. Lastly, PM exposure not alters the OXPHOS coupling efficiency (Fig. 11E), which was estimated as a result of CI$_{LEAK}$/CI$_{OXPHOS}$ ratio.

In order to determine the PM exposure effects on mitochondrial respiratory control, the quantified the substrate control ratio (SCR) was determined. The PM exposure not was able to alters the CI$_{OXPHOS}$/CII$_{ETS}$ ratio (Fig. 12A). However, when the contribution of CII in the convergent electron flow through the Q-junction was evaluated in mitochondrial respiratory control we noticed a no differences in CII$_{EST}$/CI+CII$_{OXPHOS}$ (Fig. 12B) and significant increase of CII$_{EST}$/CI+CII$_{ETS}$ (t0.05;6 = 18.58, p < 0.0001) (Fig. 12C) induced by exposure to PM.

3.10 PM exposure alters the enzymatic metabolism of glucose

Zebrafish larvae exposed to 50 µg/L of PM had significantly increased LDH activity ($F_{(2, 17)} = 3.555, p = 0.0423$) (Fig. 13A) and decreased CS activity ($F_{(2, 18)} = 7.816, p = 0.0036$) (Fig. 13B) when compared to the control group.

4. DISCUSSION

Pyrethroids are considered safer in relation to other pesticides, mostly due to their low toxicity to mammals and low environmental impact. Approximately one out of ten pesticides commonly used at homes and gardens has pyrethroids as active ingredients (ROSITA et al., 2015). As a result, preschool children have been found to be potentially exposed to PM from several sources and through several routes in their daily environments (MORGAN et al., 2007; NAEHER et al., 2009; TULVE et al., 2006). These facts have attracted attention to the effects of pesticides in developing organisms, which are under intense and controlled cell proliferation, differentiation and apoptosis (SHEETS, [s.d.]). These events are closely associated with strict regulation of cellular redox homeostasis (COLE; ROSS, 2001; DENNERY, 2007; HAHN et al., 2015), which can be potentially altered by the redox alterations triggered in response to SP exposures, including PM (WANG et al., 2016a). In this study, we analyzed the toxic effects of acute PM in a developmental animal model and demonstrated significant toxic effects at the behavioral, mitochondrial and molecular level, what appears to be related to redox alterations and consecutive cell death.

Our results demonstrated a significant decrease in larval survival (6 dpf) after exposure for 24 hours to PM (≥ 75 µg/L). The estimated LC_{50} was 108 µg/L, one-third of the LC_{50} showed by others researchers (DEMICCO et al., 2009; YANG et al., 2014) in zebrafish exposure to PM from 3 – 144 hpf (100 – 800 µg/L of PM). Yang et al. (2014) (YANG et al., 2014) reported a great increase in mortality rate, morphological alterations and behavior changes in 6 dpf larvae exposed to higher levels of PM (≥ 300 µg/L), a similar developmental stage to the one used in the current study. Therefore, it is possible that these later developmental stages can be more susceptible to PM toxicity, once that the gill innervation and respiration start between 5 to 7 dpf (JONZ; NURSE, 2005). Fish are the most sensitive vertebrate organisms to SP due to their chemical lipophilic property while having a high absorption rate through the gills (RADHAIAH; RAO, 1990).

Changes in normal behavioral patterns in zebrafish larvae were the first signs of toxicity to sublethal PM exposure. This behavioral profile is an effective method to characterize the effects of different compounds on swimming activity of fish, predicting the potential action of a compound in the central nervous system (EGAN et al., 2009). Such types of neurodevelopmental deficits usually include a broad spectrum of disorders and dysfunctions, namely developmental delays, behavioral problems, and deficits in gross or fine motor skills (BURNS et al., 2013). In the present study, no differences were observed in all endpoints analyzed in the Open Field test, demonstrating that sublethal PM exposure does not impair overall larval locomotor activity. The mode of action of pyrethroids, generally, involves binding to votagegated sodium channels causing prolonged opening and disruption of the channel (Soderlund, 2012), imparing motor activity (wolansky e al 2009). This mechanism is conserved among vertebrates; however, the sublethal PM concentrations tested in this work unable to impairs the larvae motor activity. Similar behavior results was demonstrated by Awoyemi et al. (2019), where larvae exposed (5 hpf – 5 dpf) to PM concentrations of 0.1 – 1000 µg/L not showed changes in morphological and locomotor behaviors.

We detected, for the first time, that PM exposure caused alterations in non-motor behavioral patterns, such as decreased defensive behaviors in the Novel Tank and Light-Dark tests linked to thigmotaxis and scototaxis. Despite the exploratory patterns not been altered in the Open Field test, PM-exposed larvae submitted to the stress of a novel environment (Novel Tank test) had decreased distance traveled. This result may be attributed to increase anxiety, which in this test is accompanied by decreased exploration (EGAN et al., 2009; GERLAI, 2003). However, in the Novel Tank test adapted to zebrafish larvae is possible to evaluate the thigmotaxis, where animals that are engaged in thigmotaxic behavior strongly avoid the central area, presenting a preference to stay or to move in the peripheric area of a novel environment (SCHNÖRR et al., 2012a). This behavior is evolutionarily conserved in a wide range of fish species (CHAMPAGNE et al., 2010; COLWILL; CRETON, 2011b; LÓPEZ-PATIÑO et al., 2008; PEITSARO et al., 2003; SCHNÖRR et al., 2012a; SHARMA et al., 2009) and can be modulated by anxiogenic drugs (SCHNÖRR et al., 2012b; SHARMA et al., 2009). The exposure to PM may have decreased this anxiety-like behavior in zebrafish larvae, altering the normal preference of the periphery in favor of the central area, based on the increased total time spent and the average time spent per visit in the central area. The results from the Light-Dark test corroborate with this hypothesis. PM exposure changed the normal preference of the lit area, observed by an increase in the number of entries into the dark area,

which was linked to increase in the number of risk assessment episodes. This test assesses the natural behavior of zebrafish larvae to avoid dark zones (scototaxis) (STEENBERGEN; RICHARDSON; CHAMPAGNE, 2011a, 2011b), and also suggest to a modulation in the anxiety-like phenotype in zebrafish larvae exposed to PM.

The concentrations tested in this work may be considered environmentally relevant, once the concentrations found in the environmental water column (0.05 – 811 µg/L)(OLUSHOLA M. AWOYEMI, NAVEEN KUMAR, CASSANDRA SCHMITT, SEENIVASAN SUBBIAH, 2019; YANG et al., 2014). Thereby, it is possible that behavioral changes observed in our laboratorial conditions can reflect environmental exposure conditions to PM. In the natural environmental, fear and anxiety function to avert situations that may be harmful to health or overall well-being, such as avoid predators. The molecular mechanisms involved in physiological responses to danger are conserved among vertebrates and are essential for the survival and maintenance of the species [38]. Thereby, the environmental exposure to PM may impairs the normal behavior and survival of non-target species, and, consequently, reduce environmental quality and influence essential ecosystem functioning by reducing species diversity.

The brain can be particularly susceptible to oxidative stress due to its high oxygen consumption and lower antioxidant activity (HALLIWELL, 1992; STEINBRENNER; SIES, 2009). Excessive production and/or insufficient degradation of ROS can cause oxidative damage in astrocytes and/or neurons, followed by acute brain injury, neurodegenerative diseases, and altered motor and non-motor functions (BEHL; MOOSMANN, 2002). Besides that, the increase in steady-state ROS levels in response to toxins can cause adverse effects through the further production of peroxides and other free radicals. In this study, we demonstrated the increase of steady-stead ROS levels as well as the increase in MDA in larvae exposed to 50 µg/L PM, possibly as a result of such elevated ROS levels. MDA is a marker of lipid peroxidation, the most abundant individual aldehyde resulting from this oxidation event, which can be easily quantified (KHOUBNASABJAFARI; ANSARIN; JOUYBAN, 2015; ZHOU; SUN; ZHAO, 2015). Lipid peroxidation is commonly used as a biomarker of oxidative stress in fish and contributes to impair cellular function under oxidative conditions (MONTEIRO et al., 2006).

To evaluate the response to the possible oxidative effects mediated by the increased ROS, enzymes of the antioxidant system were analyzed. CAT and SOD are important antioxidant enzymes which play pivotal roles in the first antioxidative defense line of organisms (YANG; LEE, 2015). In the review published by Wang et al., (2016) (WANG et

al., 2016b) it is discussed that the antioxidant enzymes affected by PM exposure present gender-, dose-, time-, tissue- or enantioselective-dependent manner. We observed an inhibitory effect of PM in CAT activity in whole larvae exposed to 50 µg/L PM. In the other hand, SOD activity increased in both exposure groups. Moderate oxidative stress may be upregulated the antioxidant enzymes; however, under high ROS levels, their activities can be decreased (RODRIGUEZ et al., 2004). SOD dismutates $O_2^{\bullet-}$ to H_2O_2, which is further metabolized by GPx and CAT (ASAGBA; ERIYAMREMU; IGBERAESE, 2008). The $O_2^{\bullet-}$ can also react with nitric oxide ($^{\bullet}NO$) leading to the formation of peroxynitrite ($ONOO^-$), while H_2O_2 can react with myeloperoxidases to produce hypochlorous acid or be reduced to hydroxyl radical ($OH^{\bullet-}$) through Fenton reactions (FRANCO et al., 2010). Therefore, the proper regulation of both $O_2^{\bullet-}$ and H_2O_2 needs to be accurately regulated by the cell.

Upregulation of glutathione-related defenses was also detected after PM exposure, probably in response to increasing ROS formation. Increased thiol levels (including GSH) and GST activity can help to mediate the phase II biotransformation of unsaturated aldehydes, which are generated during lipid peroxidation and can then be excreted through a GS-X pump transporter (HAYES; FLANAGAN; JOWSEY, 2005; KEPPLER, 1999). While the biotransformation of pyrethroids in fish involves oxidation and hydrolysis reactions, and conjugation with glucuronic acid or sulfate (GAN et al., 2010), GSH has been associated with protection against PM-induced DNA damage in rats (FALCIONI et al., 2010), and GST knockdown increases the susceptibility of ticks to PM (DUSCHER et al., 2014). It remains unclear if such GSH/GST response has a direct effect in the metabolism of PM, but its role as an effective cellular protection mechanism against ROS oxidative reactions is well known, and could also benefit from the increased GR activity after PM exposure.

Awoyemi et al. (2019) showed that larvae zebrafish exposed to environmental relevant concentrations of PM upregulated Nrf2 expression (OLUSHOLA M. AWOYEMI, NAVEEN KUMAR, CASSANDRA SCHMITT, SEENIVASAN SUBBIAH, 2019). This pathway is activated under oxidative and electrophilic stress conditions, when Nrf2 is released from its sequester (Kelch-like ECH-associated protein 1 - Keap1) and translocated to the nucleus to increase the expression of antioxidant and phase II biotransformation genes (JARAMILLO; ZHANG, 2013; RUSHMORE; KONG, 2002). The increased SOD, GR, GST activities in parallel to the high thiol levels and increased γ-GCS and NQO-1 protein levels after PM exposure indicate the possible activation of this pathway. This was further investigated using two zebrafish transgenic lines with reporter genes regulated under the Nrf2-related antioxidant response element/electrophile response element (ARE/EPRE): Tg (*EPRE*:LUC-EGFP) and

Tg (*HSP70*:EGFP). Both of them have been used before to characterize the response of the Nrf2 pathway, and the data from the Tg (*EPRE*:LUC-EGFP) indicate an overall increase in the activity of the Nrf2 pathway after PM exposure. Apparently, PM exposure causes an increase in ROS levels in zebrafish larvae, which is followed by the upregulation of key antioxidant molecules via a classic regulator of the antioxidant and phase II biotransformation system.

Our results also demonstrated that treatment with acute sublethal doses of PM caused genotoxicity, as well as an increase in apoptotic cells in the head of zebrafish larvae. It is known that cells exposed to PM have increased oxidation rates of pyrimidine and purine bases, which can be associated with cell death (GABBIANELLI et al., 2004, 2009b). Besides that, the increase in ROS levels and lipid peroxidation could be linked to the activation of cell death pathways (FRANCO; CIDLOWSKI, 2012). Thereby, following oxidative stress, cell death can occur via apoptotic or necrotic mechanisms (WANG et al., 2010). It is important to note that NQO-1 can also act as a pro-oxidant enzyme, producing intermediates that are capable of alkylating nucleophilic sites including DNA (COLE; ROSS, 2001; ROSS et al., 2000). It is unclear if the higher protein levels of NQO-1 after PM exposure are associated with this mechanism of DNA damage, what could contribute to the cell death detected in zebrafish larvae.

The oxidative stress induced by PM exposure in larvae zebrafish has the potential to alter various bioenergetics parameters, such as oxygen consumption, the activity of the electron transport chain, or mitochondrial membrane potential, and all these effects can impair the rate of mitochondrial ATP production (SONG; SCHARF, 2009). Besides that, the normal mitochondrial function includes an unavoidable leak of proton and electron within mitochondria, which generates $O_2^{\bullet-}$. The unbalance on these side reactions can cause a series of deleterious effects (KOWALTOWSKI et al., 2009). Furthermore, damage to this organelle, for example by environmental contaminants, can play both a direct and indirect role in the ROS generation and can induce a drastic impairment in cellular bioenergetics metabolism (OTT et al., 2007). In this context, considering the critical role of mitochondria in toxicological processes, we investigated whether the mitochondrial function was a target of PM in zebrafish larvae. Our results indicated that PM exposure impair bioenergetics capacity and increase the residual oxygen consumption, however does not impair mitochondrial efficiency and oxidative phosphorylation. However, we demonstrate that exposure to PM impaired also the mitochondrial function through the decrease in all states of mitochondrial respiration. We demonstrated that PM can impair redox homeostasis, which is known to

potentially oxidize mitochondrial protein thiols groups, triggering mitochondrial transition permeability, and increasing ROS production rates (CARVALHO et al., 2013; LE BRAS et al., 2005; LEE; WEI, 2012). In addition, SCR results indicate that PM exposure changes the mitochondrial bioenergetics metabolism of NADH oxidation, indicating a compensatory response of the CII respiration in relation to the convergent pathway, which is able to trigger the accentuated superoxide generation in mitochondria (ALI et al., 2012).

The pro-oxidative condition could trigger the release of iron from the ironsulfur center present in CI and II, which may exacerbate ROS generation via Fenton-like mechanisms(THOMAS et al., 2009). Thereby, the impairment effects of PM on mitochondrial function can be associated with the decrease in HO-1 expression observed in larvae exposed to 50 µg/L PM. The HO-1 is the rate-limiting enzyme of heme degradation, catalyzing heme degradation and generating ferrous iron, carbon monoxide, and biliverdin, the latter two of which have anti-inflammatory and anti-oxidant (LUNDVIG; IMMENSCHUH; WAGENER, 2012; MAINES, 1997). In oxidative stress conditions, HO-1 may translocate to the mitochondria and the generated carbon monoxide may play an important role in mitochondrial biogenesis, suggesting an intimate link between HO-1 and mitochondrial function during stress (CONVERSO et al., 2006; PIANTADOSI; SULIMAN, 2012). Additionally, the toxicity of both hydrogen and lipid peroxides is greatly enhanced by reacting with heme or heme-containing proteins that generate strong pro-oxidant ferryl forms of heme (KUMAR; BANDYOPADHYAY, 2005). Thereby, it is possible the lower protein levels of HO-1 after PM exposure may be affecting mitochondrial biogenesis or decreasing the rate of heme degradation, and both of these scenarios can be toxic to the mitochondria.

Effects on mitochondrial function can shift the cell metabolism towards an inefficient way of producing ATP at the cost high fluxes of anabolic pathways. We showed an increase in LDH (glucose metabolism) and decrease in CS activity (citric acid cycle) in PM-exposed larvae. These alterations can be related to the Warburg Effect (LIBERTI; LOCASALE, 2016), a mechanism that occurs in tumors and other cells under proliferation or development, where glycolysis is the major energetic pathway even in the presence of oxygen. The NADH generated from glucose is re-oxidized to NAD^+, reducing pyruvate to lactate and completing the aerobic glycolysis. Despite this scenario showed a different situation than the one proposed in the Warburg Effect, a similar mechanism can have been adopted as an attempt to maintain ATP levels by glycolysis even in the presence of oxygen. PM exposure decreased mitochondrial oxygen flux consumption, and potentially decreased activity of the citric acid cycle (CS activity) and increased glucose anabolism (LDH activity), thus it is possible that

glycolysis is been used to provide the extra fuel necessary for physiological brain function through the generation of ATP. Glucose is the foundation for neuronal and non-neuronal cellular maintenance, as well as the generation of neurotransmitters (MERGENTHALER et al., 2013). It is unclear if such changes observed in the regulation of glucose metabolism by PM exposure in zebrafish larvae have a side effect, as glucose consumption is expected to be higher and this is a critical molecule for brain physiology, which may lead as changes in anxiety-like behavior observed.

In conclusion, we showed that acute and sublethal PM exposure modulates different anxiety-like behaviors patterns in zebrafish larvae. Additionally, we also demonstrate that PM enhances enzymatic antioxidant defenses, with focus on the glutathione-related system; however, this upregulation was not enough to prevent the increase in ROS levels and oxidative effects such as lipid peroxidation, DNA damage and cell death. It is possible that unbalance in the redox state of the cells can be either a cause or consequence of changes in mitochondrial function and glucose metabolism. We also emphasize that behavioral alterations, as well as the changes in redox parameters and mitochondrial function in larval zebrafish, may serve as important tools to better understand the molecular mechanisms induced by acute exposure to PM in developing organisms.

5. Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

6. Conflicts of Interest

The authors declare that they have no conflicts of interest.

7. Acknowledgments

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9. Figure captions

Fig. 1

Survival rate (%) of zebrafish larvae after acute exposure (24h) to different concentrations of permethrin (25, 50, 75, 100, 200, 300 and 600 µg/L) and control group (CTL). Values are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey as post hoc comparison. Groups not sharing letters are significantly different ($p < 0.05$).

Fig. 2

Behavioral assays using the Open Field test to measure the exploratory and motor behaviors in control (CTL) and permethrin (25 and 50 µg/L) exposed zebrafish larvae. (A) Total distance traveled (average for control group = 0.58 m), (B) absolute turn angle (average for control group = 16,400°), (C) total immobility time (average for control group = 27 s), (D) total time spent in the central zone (average for control group = 92 s), and (E) average time spent in central zone per visit (average for control group = 5 s). The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different ($p < 0.05$).

Fig.3

Behavioral assays using the Novel Tank test to measure non-motor anxiety-like behaviors in control and permethrin (25 and 50 µg/L) exposed zebrafish larvae. (A) Total distance traveled (average for control group = 0.76 m), (B) Total immobility time (average for control group = 114.3 s), total time spent in the central zone (average for control group = 84.8 s), and (D) average time spent in the central zone per visit zone (average for control group = 5 s), were evaluated after exposure time. The parametric dates are expressed as the mean ± SEM and analyzed by one-way ANOVA followed by Tukey as post hoc comparison; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. Groups not sharing letters are significantly different ($p < 0.05$).

Fig. 4

Behavioral assays using the Light-Dark test to measure non-motor anxiety-like behaviors in control and permethrin (25 and 50 µg/L) exposed zebrafish larvae. (A) time spent in the lit area traveled (average for control group = 270 s), (B) latency to enter in the dark area (average for control group = 67 s), (C) Entries to the dark area (average for control group = 1 entry), and (D) the number of risk assessment episodes (average for control group = 2 episodes). The dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. Groups not sharing letters are significantly different ($p < 0.05$).

Fig. 5

Oxidative stress biomarkers in control (CTL) and permethrin (25 and 50 µg/L) exposed zebrafish larvae. (A) ROS steady-stead levels (average for control group = 0.23 µmol DCF/mg of protein), (B) TBARS levels (average for control group = 1.6 nmol MDA/mg of protein), (C) CAT activity (average for control group = 10.6 µmol/min/mg of protein), and (D) SOD activity (average for control group = 177 U/mg of protein). Values are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-hoc. Groups not sharing letters are significantly different ($p < 0.05$).

Fig. 6

Effects of permethrin exposure (25 and 50 µg/L) in the glutathione defense system in zebrafish larvae. (A) GST activity (average for control group = 0.16 µmol GS-DNB/min/mg of protein), (B) thiols levels (average for control group = 330 nmol SH/ mg of protein), (C) GPx activity (average for control group = 16 nmol/min/mg of protein), (D) GR activity (average for control group = 6.85 nmol/min/mg of protein). Values are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-hoc. Groups not sharing letters are significantly different ($p < 0.05$).

Fig. 7

Exposure to permethrin (25 and 50 µg/L) modules the protein levels of Nrf2-target antioxidant enzymes in zebrafish larvae. (A) Representative immunoblots, and densitometric analysis of immunoreactive bands of (B) γ-GCS (average for control group = 0.52 y-GSCs/b-actin ration), (C) HO-1 (average for control group = 1.3 HO-1 /β-actin ration) and (D) NQO-1 (average for control group = 0.63 NQO-1/β-actin ration). The parametric dates are expressed

as the mean ± SEM and analyzed by one-way ANOVA followed by Tukey as post hoc comparison; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. Groups not sharing letters are significantly different (p<0.05).

Fig. 8

Effects of permethrin exposure (25 and 50 µg/L) in the Nrf2-related antioxidant response in zebrafish larvae. Amplification of the antioxidant system was estimated by fluorescence microscopy using two different zebrafish reporter lines. Expression of EGFP in (A) Tg(*HSP70*:EGFP) and (B) Tg(*EPRE*:LUC-EGFP) in zebrafish larvae. Relative fluorescence levels and representative images are shown for each transgenic construct and exposure group. Arrows indicate the presence of cells expressing high fluorescence in the caudal region of zebrafish larvae. Values are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post-hoc comparison. Groups not sharing letters are significantly different (p<0.05).

Fig. 9

Exposure to permethrin (25 and 50 µg/L) causes genotoxicity in zebrafish larvae. (A) Representative images of single cell gel electrophoresis (comet assay), (B) comet length (average for control group = 430 microns), (C) tail length (average for control group = 195 microns). The dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. Different letters indicate statistically significant differences (p <0.05).

Fig. 10

Exposure to permethrin (25 and 50 µg/L) causes cell death by apoptosis in zebrafish larvae. The frequency of apoptotic cells was estimated in the head of zebrafish larvae by measuring the total fluorescence in the area. (A) Representative image of the acridine orange staining and fluorescence imaging, indicating the presence of apoptotic cells (white arrows), and (B) the respective quantification of acridine orange relative fluorescence. Values are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post-hoc comparison. Different letters indicate statistically significant differences (p <0.05).

Fig. 11

Effects of permethrin exposure (50 µg/L) in mitochondrial function of zebrafish larvae. (A) The analyzed mitochondrial function by HRR is presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured: basal metabolic rate (Routine), + glutamate, pyruvate and malate (CI LEAK), + ADP (CI OXPHOS), + succinate (CI + CII CIIOXPHOS), + FCCP (CI + CII ETS), + rotenone (CII ETS), + antimycin A (ROX, residual oxygen consumption). Average for basal control group = 600 pmol O_2/s/mg. (B) Respiratory Control Ratio (RCR) for complex I (RCR = CI_{OXPHOS}/CI_{LEAK}). (C) Residual oxygen consumption relative to the maximum oxygen consumption capacity (ROX/ETS). (D) Analysis of bioenergetics capacity (CI_{LEAK}-CI_{oxphos}). (E) Analysis of oxidative phosphorylation coupling efficiency ($CI_{LEAK}/CI_{OXIPHOS}$). Values are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post-hoc or expressed as median ± interquartile range and analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters indicate statistical differences ($p<0.05$) between the exposure groups at each state of respiration.

Fig. 12

Analysis of substrate control ratio (SCR) of zebrafish larvae exposed to permethrin 50 µg/L. (A) CI_{OXPHOS}/CII_{ETS} ratio; (B) $CII_{ETS}/CI + CII_{oxphos}$ ratio; and (C) $CII_{ETS}/CI + CII_{ETS}$ ratio. The dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. Different letters indicate statistically significant differences ($p <0.05$).

Fig. 13

Effects of permethrin exposure (25 and 50 µg/L) in biomarkers of glucose metabolism in zebrafish larvae. (A) Lactate dehydrogenase activity (average for control group = 750 U/L) and (B) citrate synthase activity (average for control group = 0.036 nmol/min/mg). Values are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post-hoc comparison. Groups not sharing letters are significantly different ($p<0.05$).

10. Figures

Figure 1:

Figure showing a bar graph. Y-axis: "Survival - 24h (fold change)" ranging from 0.0 to 1.5. X-axis: "Permethrin (µg/L)" with values 0, 25, 50, 75, 100, 200, 300, 600. Bars labeled with letters a, a, a, b, c, d, d, d. Text box: LC$_{(24h)}$=108 µg/L
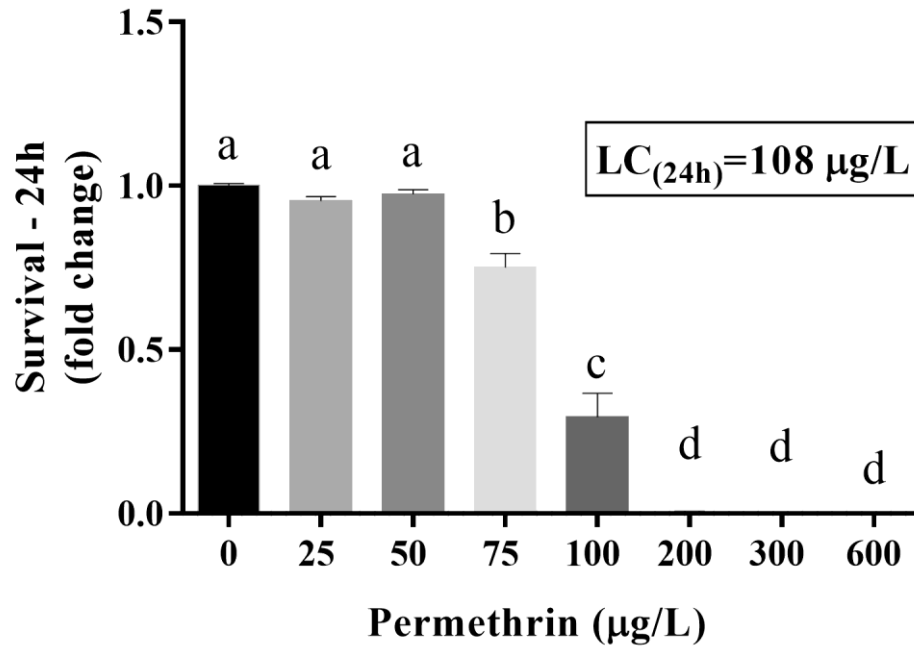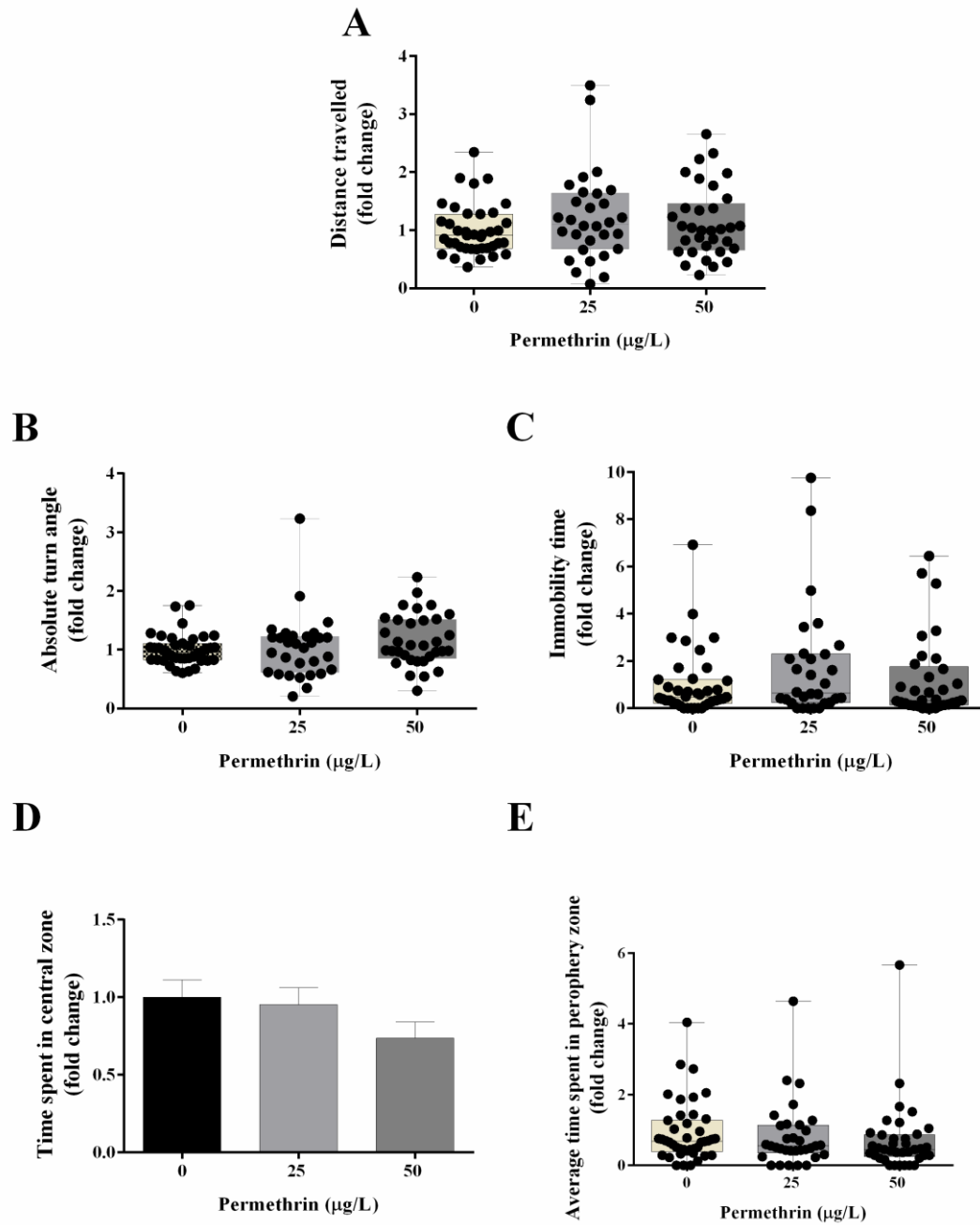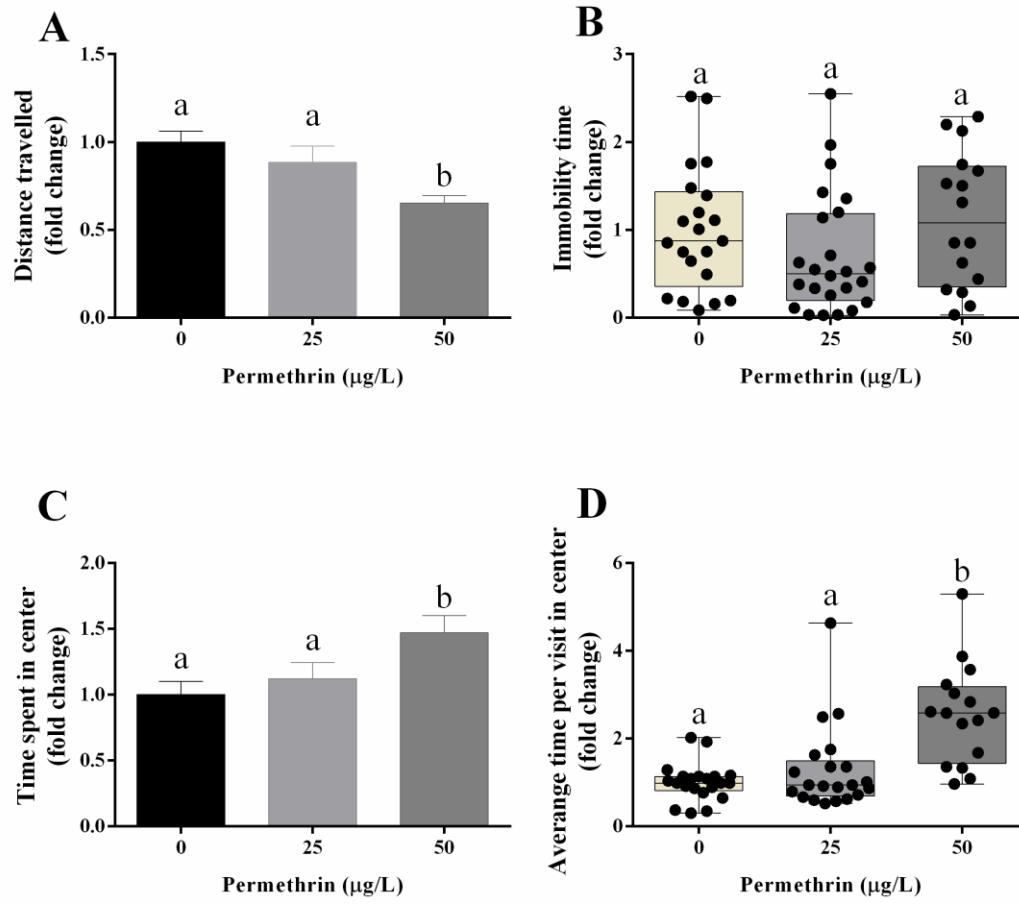
Figure 2:

Figure 3:

Figure 4:
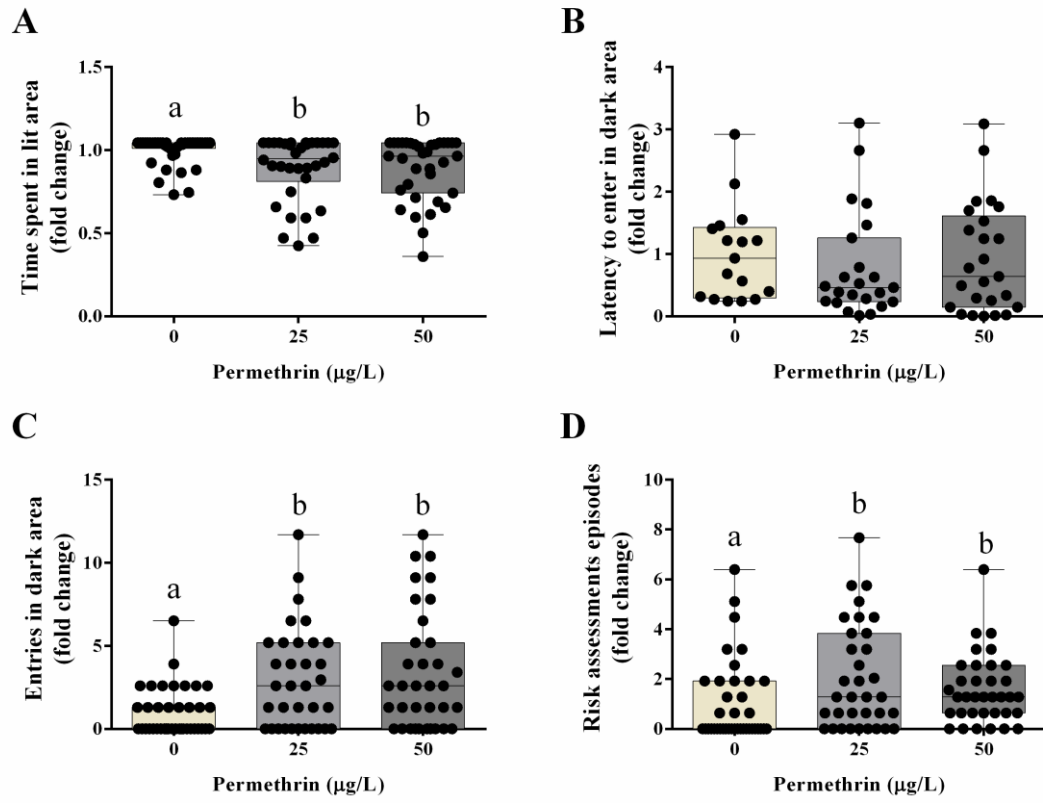
Figure 5:

Figure 6:

Figure 7:

A

B   **C**   **D**
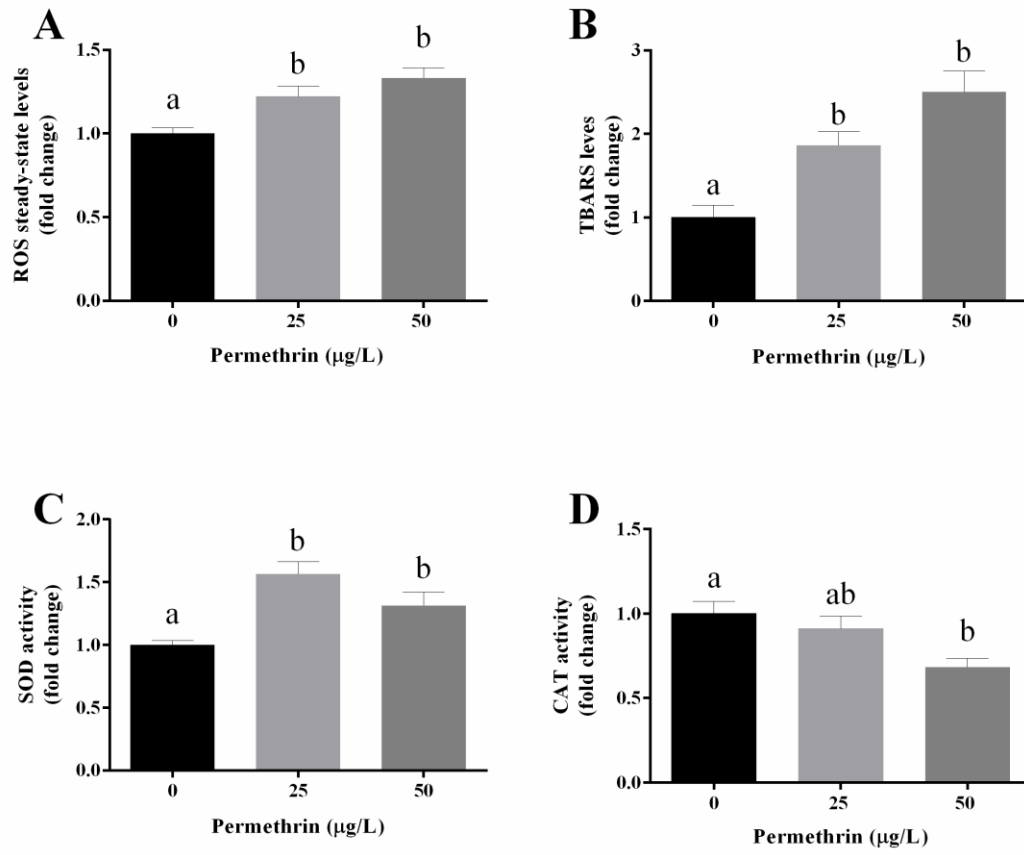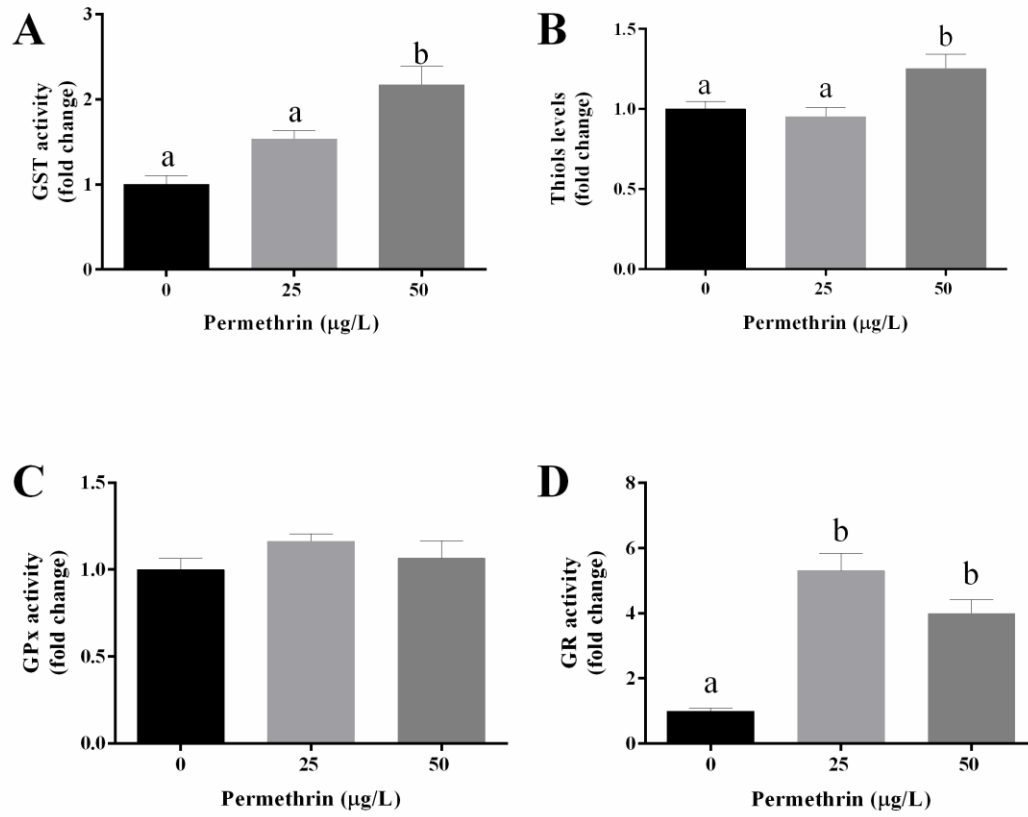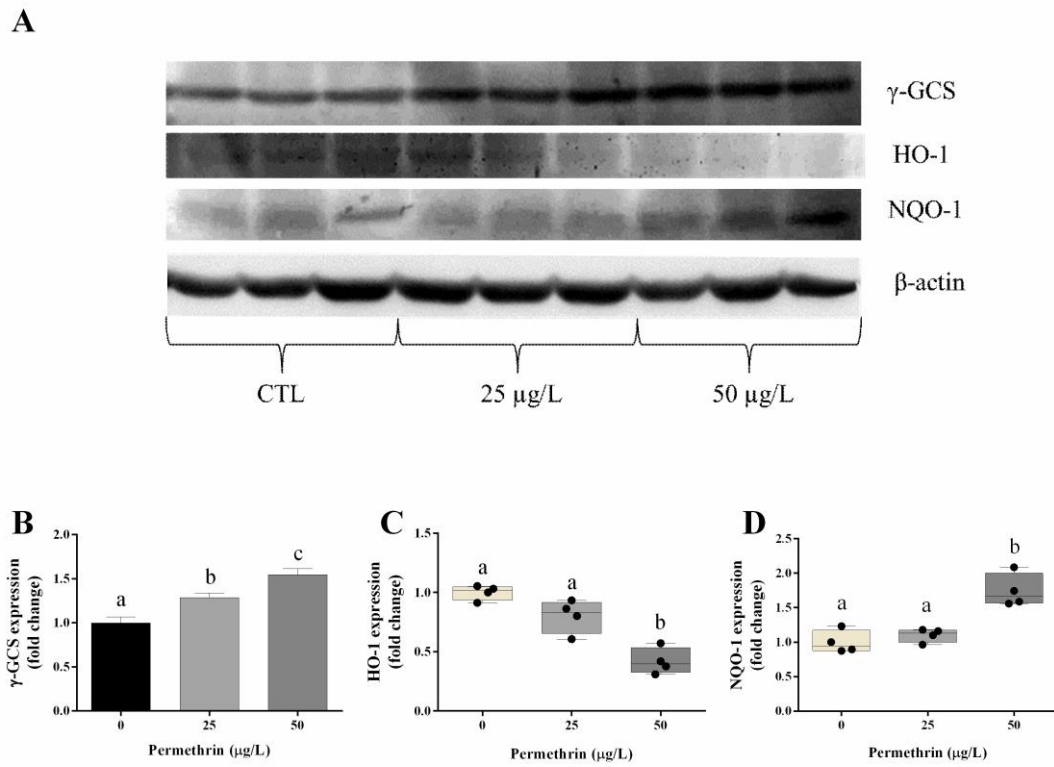
Figure 8:

Figure 9:

Figure 10:

A

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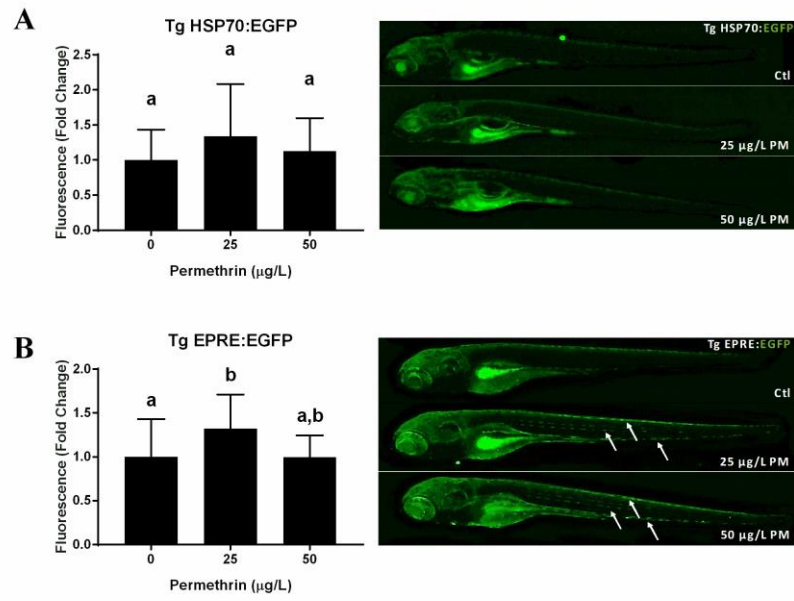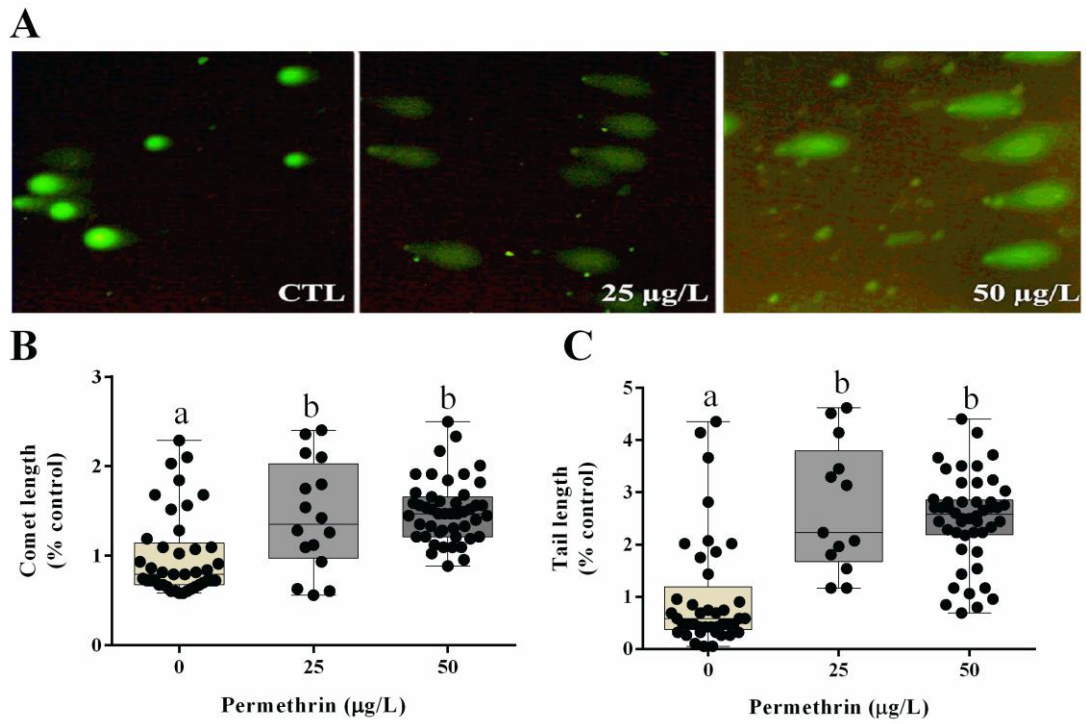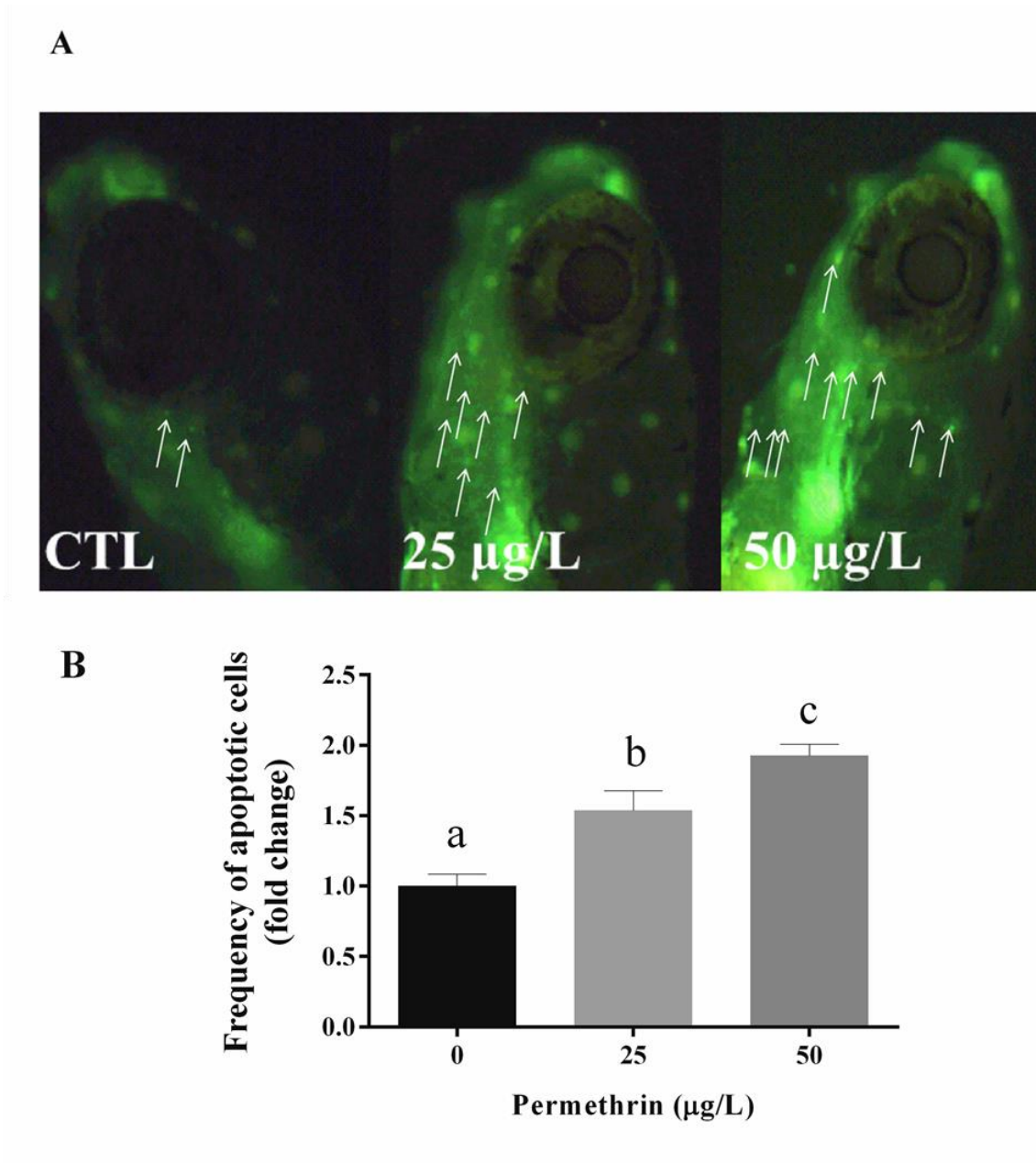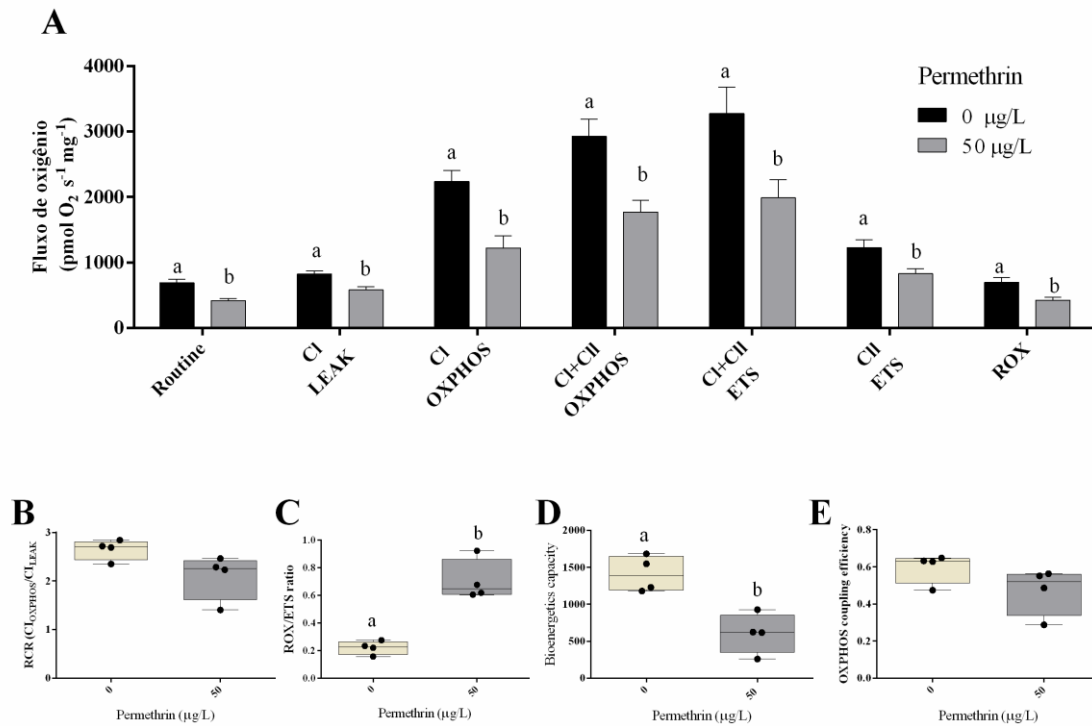
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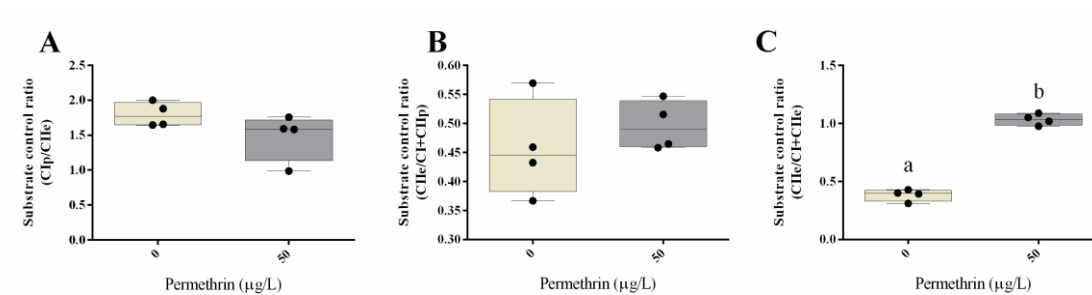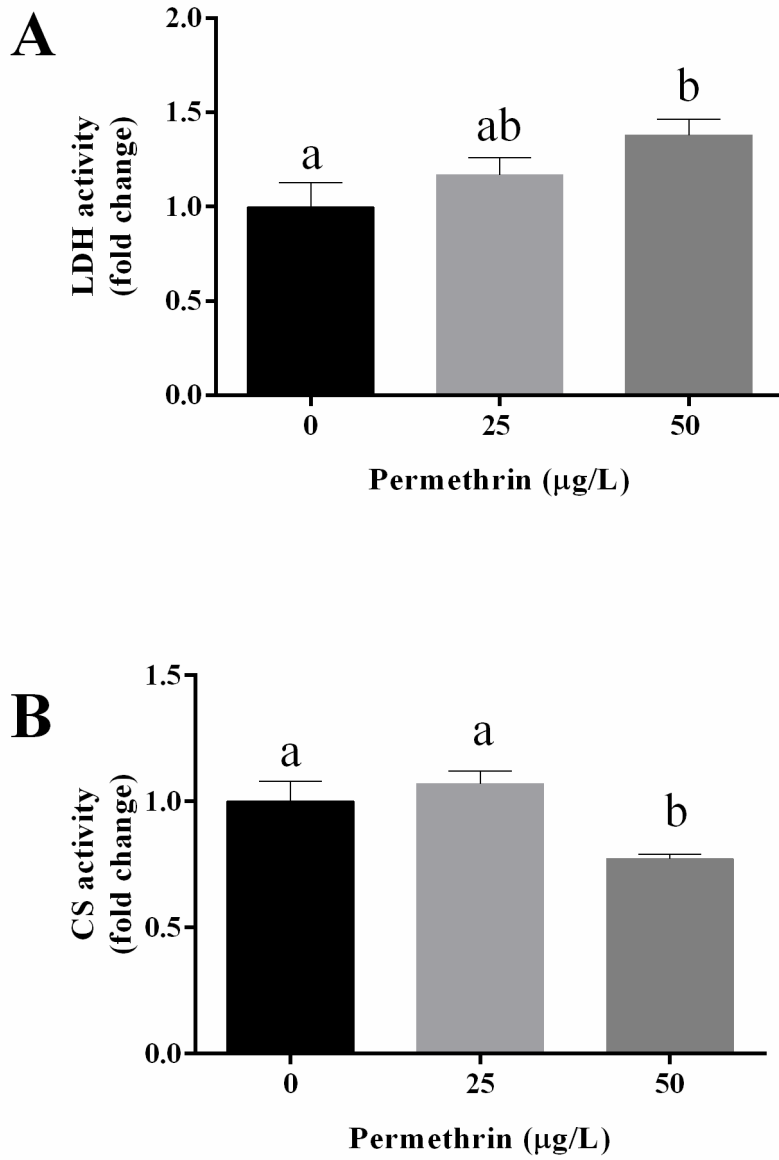
6.2 Capítulo II:

Manuscrito:

Acute zebrafish embryonic exposure to permethrin induces behavioral changes related to anxiety and aggressiveness in the adulthood

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ABSTRACT

Permethrin (PM) is one of the most used type I synthetic pyrethroid worldwide. Exposure to this compound during pregnancy and early childhood has been indicated as a risk factor for neurodevelopmental disorders. We evaluated the long-term effects of embryonic PM exposure in different stages of zebrafish development. Briefly, embryos (3 hpf) were exposed to sublethal concentrations of PM (25 and 50 µg.L^{-1}) during the first 24h of life, and then behavioral parameters were evaluated during embryonic (28 hpf), eleutheroembryonic (3 dpf), larval (7 dpf) and adult stage (90 dpf). PM exposure was able to decrease spontaneous movement at 28 hpf and decreased thigmotaxis in eleutheroembryos. The long-term effects of PM include changes in non-motor behaviors such as fear and anxiety in larva and adults. Adults embryonically exposed to PM also showed significant increase in aggressiveness parameters. These results demonstrated that embryonic exposure to PM induces persistent neurotoxic effects in adulthood, which can impair the cognitive and behavioral fitness of non-target species contributing to the development of neurodevelopmental disorders.

Keywords: neurodevelopmental, fear, anxiety, aggressiveness, pyrethroids.

1. INTRODUCTION

The inset of neurodevelopmental disorders has significantly increased over the last decades (GRANDJEAN; LANDRIGAN, 2014). Evidence indicates that environmental pollutants cause injury to the developing brain either through direct toxicity or interactions with genome (LANDRIGAN; LAMBERTINI; BIRNBAUM, 2012). Among environmental contaminants, pesticides are considered as major risk factors involved to initiation and progression of neurodevelopmental and neurodegenerative diseases (PARRÓN et al., 2011).

Permethrin (PM; 3-phenoxybenzyl-(1R,S)-cis, trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate) is a synthetic Type I pyrethroid insecticide, which have been detected at high levels in domestic environments, being the second most frequently detected pyrethroid (TANG et al., 2018a). PM widely use is commonly associated to low mammalian toxicity; however, evidences suggest that PM might induce neurotoxic effects (NASUTI et al., 2008a). The PM acute neurotoxic effects have been related with impairs in motor and sensorimotor function, such as whole-body tremor and convulsions (WOLANSKY; HARRILL, 2008). However, the chronic effects of PM about non-motor behaviors and the persistence of this effects during the development still need to be clarified.Epidemiological studies have shown that the detection of urinary 3-phenoxybenzoic acid (3-PBA), biomarker of exposure to pyrethroids, indicate that children and pregnant may bioaccumulate higher levels of pyrethroids than adults (WATKINS et al., 2016). Thus, children exposed during early developmental stages to PM have raised concerns mainly due vulnerable of developing brain to neurotoxic pesticides effects (GRANDJEAN; LANDRIGAN, 2014). Additionally, studies in mammals have being related the persistence effects of perinatal exposure to subtoxic doses of pesticides with changes in anxiety, aggressive and abnormal social behaviors during adult stage (BJØRLING-POULSEN; ANDERSEN; GRANDJEAN, 2008). The zebrafish (*Danio* rerio) has become an outstanding tool for biological psychiatry (LEVIN; KALUEFF; GERLAI, 2015) and neurodevelopmental studies (ROPER; TANGUAY, 2018), due to its fast embryonic *ex-utero* development and highly conserved genetic programming during early stages of life (KALUEFF et al., 2013). Thus, the aim of this study was to evaluate the long-term effects of embryonic exposure to low concentrations of PM through behavioral endpoints across different developmental stages.

2. MATERIALS AND METHODS

2.1 **Animals maintenance and reproduction**

Wild-type zebrafish (*Danio rerio*) short fin of both sexes were maintained in a recirculation aquatic system (Zebtec®) under appropriate water conditions and fed thrice daily on commercial flocked fish food as established elsewhere (COSTA-SILVA et al., 2018a).

Adults zebrafish (2/1 male/female; 6–12 months-old) were placed overnight. The reproduction was induced by the light irritation on the next morning. Fertilized eggs were washed with Hank's solution (Costa-Silva et al., 2018) for several times and incubated in system water in a Biochemical Oxygen Demand (BOD) incubator at 28°C.

2.2 Experimental design

PM stock solutions (Sigma-Aldrich; product code 45614) was prepared in ethanol. To prepare working solutions, PM stocks were diluted in HS and the final concentration of ethanol in each treatment, including the control group (CTL), was 0.001% (v/v) to avoid solvent interferences.

Zebrafish embryos at 3 hours post-fertilization (hpf) were exposed during 24h in Petri dishes containing HS (final volume of 30mL) in the presence/absence of PM concentrations (25 and $50\mu g.L^{-1}$). Embryos were kept in BOD throughout the treatment period. After, embryos were removed from the exposure medium and allowed to develop in HS until 7 days post fertilization (dpf). When larva start fed, they were kept in paramecium solution and supplemented with brine shrimp until 30 pf. During the 30 –90dpf the juvenile zebrafish were maintained in 10L aquaria constantly aerated containing water of the Zebtec® system (± 27°C) at a density of three animals *per* liter. Briefly, Fig. 1 shows the experimental timeline of embryonic exposure and subsequent behaviors assays. The experimental protocols used were approved by the local Ethics committee (CEUA – Unipampa: protocol 003-2016) and were based on the OECD GUIDELINES FOR THE TESTING OF CHEMICALS 236 - Fish Embryo Acute Toxicity (FET) Test, (OECD, 2012). Experiments were conducted in triplicates with 30 embryos per dose-group and were repeated at least three times independently.

The sublethal concentrations of PM were chosen based on LC_{50} ($300\mu g.L^{-1}$) (DEMICCO et al., 2009; YANG et al., 2014) and "no observed adverse effect level" (NOAEL) during embryonic exposure to PM determined in unpublished previous studies.

2.3 Behavior assays

Behavior analyses were evaluated in different time-stages of development (embryo-28hpf, *eleuthero*embryo-3dpf, larval-7 and adult-90dpf). The swimming location in the exploratory behaviors (carried out in the 7 and 90dpf) were based on Nunes et al. (2016).

2.3.1 Embryonic behavior

A number of 45 embryos *per* group with 28hpf were randomly selected to determination of Spontaneous Movement. This assay was performed as described by Costa-Silva et al. (2018).

2.3.2 Eleutheroembryos behavior

Eleutheroembryos (3–4 dpf) were submitted to touch stimulation test to assess sensory-motor capacity test, as described by Costa-Silva et al. (2018). The number of eleutheroembryos able to swim to periphery (standard escape) also was counted and used as a thigmotaxis parameter. A number of 45 eleutheroembryos were randomly selected and evaluated by two blind operators.

2.3.3 Larval behavior

Larvae zebrafish (6–7dpf) were submitted to two different behavioral assays: Open Field and Light-Dark preference tests.

Open Field test was used to analyze locomotor and exploratory activities as described by Altenhofen et al. (2017). Each larvae (n=50/group) was placed individually in each well in 24-wells cell culture plate (hereafter called apparatus). The apparatus was virtually divided in central and peripheric areas to assess the exploratory activity by following endpoints: distance travelled, absolute turn angle, time spend in periphery area and average time spent in periphery area.

The light-Dark test was used to analyze the scototaxis and was based in method described by Steenbergen et al. (2011). Briefly, in a well of 24-wells cell culture plate physically and equally divided in light (white) and dark (black) areas, each larva (n=30/group) was placed in light area and was recorded during 5min. The risk assessments episodes, time

spend in the lit area and average time in lit area were measured. Risk assessments were classified as a fast entry in lit area.

2.3.4 Adult behavior

The adult zebrafish (n=30/group) with 90dpf were submitted to different behavioral assays: Novel Tank, Light-Dark, Predator test, Conspecific Preference and Mirror-Induced Aggression (MIA).

The novel tank test was used to analyze the locomotor and exploratory parameters, reflecting the habituation to novelty stress (CACHAT et al., 2010). This assay followed the methods of Nunes et al. (2016). Locomotor parameters were measured by distance travelled, transitions and turn angle. To assess the vertical exploration, the apparatus was virtually divided into two horizontal sections (top and bottom) by the following endpoints: time spend in top, average time in top and latency to top.

The light-dark test was used to analyze the scototaxis and was carried out based in the method described by (MAXIMINO et al., 2010b). Briefly, the apparatus was divided into two equal sizes: light and dark. Each zebrafish was placed in light compartment and was recorded for 5 min. Scototaxis was measured by time spend in lit area, latency to enter the lit area and risk assessments.

Predator exposure test was used to analyze the antipredator behavior, while is a natural adaptive response related to survival and that reflect the aversion to a dangerous situation (GERLAI, 2003). This test was based in the method described by (GERLAI, 2011). Briefly, each zebrafish was placed in the central of apparatus, sharing the same environment with the predator (*Astronotus ocellatus*) being separated by transparent barrier. The apparatus digitally divided into two equal sections (far and close of predator) to determination of time spent, average time spent, latency and transitions to predator area.

Social preference test was performedto analyze the natural tendency of zebrafish to establish an interaction group (MILLER; GERLAI, 2007) and this assay followed the methods of Müller et al. (2018). For determination of group approach, the apparatus was digitally divided into two sections (far and close to stimulus group), and time spent, average time spent, latency and transitions to close area was measured.

MIA test was used to determinate the aggressive rate and was followed the methods of (NUNES et al., 2016). The apparatus was placed front to mirror at 22.5º (image stimulus) to the back wall of the tank so that the left vertical edge of the mirror was touching the side of

the tank and the right edge was further away. Thus, when the experimental fish swam to the left side of the tank, their mirror image appeared closer to them. Each zebrafish was placed in center of apparatus and recorded for 5min to determination of the number, time and average duration of total aggressive episodes. For determination of time spend closer of opponent the apparatus was digitally divided into three areas (A1–close, A2–middle and A3–far of opponent), and time, entries and aggressive episodes in each area were measured. In addition, the amount of time the fish spent with aggressive display, or attack behavioral, was also measured and analyzed as aggression.

2.4 Statistic

Normality (Kolmogorov Smirnov) and homogeneity (Bartlett's) tests were applied. The parametric data were expressed as mean ± standard error (SEM) and subjected to analysis of variance (one-way ANOVA) followed by Tukey post-hoc; and nonparametric data were expressed as median interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. The statistical significance was set at $p \leq 0.05$.

3.   Results

3.1 PM exposure impairs embryonic motor behavior

Embryos at 28hpf exposed to sublethal PM concentrations (25 and 50µg.L^{-1}) during the first hours of development showed a significant decrease of spontaneous movement episodes (Fig. 2).

3.2 Exposure to PM decrease sensory-motor capacity in eleutheroembryos

When the exposed embryos reached the eleutheroembryo developmental was observed a significant increase in the number of stimuli needed to first scape response in embryos exposed to 50µg.L^{-1} as compared to control (Fig. 3A). The percent of eleutheroembryos able to escape to periphery of the apparatus was significantly decreased in both exposed groups (Fig. 3B).

3.3 PM exposure alters non-motor behaviors during larval stage

Embryos were submitted to Novel tank and Light-dark tests. The locomotor parameters measured in Open Field test, distance traveled (Fig. 4A) and absolute turn angle (Fig. 4B), were not altered by PM exposure, however the non-motor parameters linked to thigmotaxis (time and average time spent in periphery area) were significantly decrease in both exposed groups in relation to control (Fig. 4 C and D). When the larvae were submitted to Light-dark test, it was able to observe that embryos exposed to PM 25µg.L^{-1} showed a significant decrease in the number of risk assessment episodes in relation to control (Fig. 5A). The group exposed to PM 50µg.L^{-1} showed significant decreases in time and average time spent in lit area (Fig. 5C and D).

3.4 Exposure to PM decrease exploratory activities in adult stage

The effects of embryonic exposure to PM persisted into adult stage. In the Novel Tank test, the PM-exposed groups showed significant decreases in distance travelled (Fig. 6A) and transitions (Fig. 6B) between top and bottom of apparatus in relation to untreated group. However, the absolute turn angle (Fig. 6C) was not altered among groups. The normal geotaxis patterns of adult zebrafish was also altered by embryonic exposure to PM; the time and average time spent in top area in the Novel Tank test were decreased in both PM-exposed groups in relation to control (Fig. 6C and D). The PM-exposed groups also showed a significant increase in the latency to first entry in top area of the apparatus (Fig. 6E).

3.5 Embryonic exposure to PM decrease defensive behavior in adult stage

Adult zefrafish to PM showed decreases in defensive behavior in the Light dark test. No differences between groups in the time spent in dark area (Fig. 7A) were observed; however the latency to first entry in dark area (Fig. 7B) was significantly increased in both PM-exposed groups. Also, exposed animals presented decreased episodes of risk assessment (Fig. 7C).

3.6 Embryonic exposure to PM decrease the aversion to a life-threatening situation in adult stage

In the predator exposure test, both PM-exposed groups showed significant increase in time spent in the predator area (Fig. 8A) and decrease latency to first entry in predator area (Fig. 8B) in relation to control group. The number of transitions (Fig. 8C) among the two sections was significantly increased in both exposed groups when compared to control group.

3.7 PM exposure during embryonic development did not change the social interaction parameters

In the Social Preference test, the embryonic exposure to PM did not alter the time, average time and transitions to the area close to stimulus group (Fig. 9 A, B and C). Only latency to close section was significantly increased in PM 50µg.L^{-1} when compared to control (Fig. 9 D).

3.8 Embryonic exposure to PM induces increased of aggressiveness in adult stage

Adult zebrafish exposed to PM showed significant increase in total number of aggressive episodes (Fig. 10A), total time (Fig. 10B) and average duration of total aggressive episodes (Fig. 10C). The apparatus was divided in to three section (apparatus representation; Fig. 10H), where was possible observed the significant increase of time (Fig. 10D) and aggressive episodes (Fig. 10E) in A2 and A3 section in exposed groups in relation to control. The transitions to A1 and A2 were decreased in both groups exposed to PM concentrations (Fig. 10E). Only in the A1 area there was significant decrease in the time spent in exposed groups in relation to control group (Fig. 10G).

4. Discussion

The aim of this study was to examine behavioral effects of embryonic exposure of zebrafish to sublethal concentrations of PM during different stages of development. The acute intoxication by PM is characterized by tremors, aggressiveness, hypersensitivity and ataxia (Meacham et al., 2008). However, the long-term effects about development are not yet well understood.

Neurotoxic induced by PM is not dependent on the administration route (WEINER et al., 2009). However, the range of neurobehavioral alterations depends on the dose and time as well as on the brain developmental stage (DUBOVICKÝ, 2010). Besides that, low PM concentrations can impair fitness and non-motor behaviors, leading to abnormal brain function and consecutive behavioral, emotional and/or cognitive dysfunctions (GRANDJEAN; LANDRIGAN, 2014)

In our experimental conditions PM decreased spontaneous movements just a few hours after treatments were finished. This behavior is an endpoint of early development, being the first movements realized by embryos and is originated from the spinal cord triggered by the development of motor neurons innervating muscles (Kimmel et al., 1995). Yang et al. (2014) showed that zebrafish embryos exposed to concentrations $>200\mu g.L^{-1}$ resulted in deleterious effects on zebrafish morphological development. Accordingly, the PM concentrations tested in our work were unable to induce marked morphological changes during development. However, the observed effect might be explained due to a potential impairment of developmental neuronal system by PM, which may be related to prolonged opening and disruptions of the voltage-gated sodium channels in neuronal membranes (SODERLUND, 2013b)

During zebrafish eleutheroembryos developmental stage, the animal remains inert in the environment, responding only to aversive stimuli (BRUSTEIN et al., 2003). Thereby, when embryos reached 3dpf, was observed that PM $50\mu g.L^{-1}$ treated animals needed significantly more stimuli to induce a first scape, demonstrating less sensory-motor capacity in the touch stimulation assay. Similar effects were also observed in rodents exposed acutely to PM showing decreases in motor activity and grip strength, and increased click response (Soderlund et al., 2002). The decrease of responsiveness to adverse stimuli may be relate with paresthesia in the sensory nervous system as previously reported in mammals (Soderlund et al., 2002). The same behavior assay enable to observe that exposed eleutheroembryos decreased the thigmotaxis in relation to control group, decreasing the escape to periphery of apparatus (illustrate in Fig. 2C), demonstrating less active avoidance from an imminent threat (touch stimulus). Aversive stimuli may also trigger the emotions of fear and anxiety (KALUEFF et al., 2013). The touch is characterized as aversive stimuli, because simulates a predatory action towards eleutheroembryos. Escape behaviors are fear emotions and are evolutionary conserved among vertebrates (COLWILL; CRETON, 2011b).

The PM treated larva showed increase preference to periphery (thigmotaxis) without changes in swimming patterns (motor behavior) in the open field test. This assay assesses

avoidance behaviors, which may be used as an anxiety indicator (COLWILL; CRETON, 2011a). To a more comprehensive evaluation of behavioral domains linked to anxiety, the larvae were submitted to light-dark test, where changes related to non-motor behaviors also were observed. Exposed group showed decrease in scototaxis and decreased number of risk assessment, a defensive behavior which may be used as an anxiety indicator (MAXIMINO et al., 2010b). These results demonstrate persistent effects of PM exposure on non-motor behaviors during larval stage. Tu et al. (2014) showed that eleutheroembryos exposed to similar PM concentration during 48h eliminated ~65% of PM bioaccumulated after depuration (24h), which reduced the impact linked to body accumulation. However, pyrethroids are able to easily cross the blood–brain barrier and exert their toxic effect directly on the central nervous system (CNS) (NAEHER et al., 2009). Therefore, persistent effects of PM may not be related to bioaccumulation but potentially due to damaging effects to the CNS developing.

Experimental studies have demonstrated that PM impairs locomotor behavior in animal model (CARLONI et al., 2013a; DHIVYA VADHANA et al., 2013b; JIN et al., 2009; NASUTI et al., 2008c). However, locomotor behavior changes were no longer when exposed embryos reached 7dpf. Similar results were observed when exposed embryos become adults (90dpf), indicating that long-term effects of PM in zebrafish involves the expression of more complex behavioral phenotypes other than mere locomotor impairments.

Indeed, adult presents a highly complex behavioral repertoire as compared to larva (OLIVEIRA; SILVA; SIMÕES, 2011a). The exposure to stressors can evoke fear or anxiety-like phenotype that can be easily quantified by reduced vertical exploration, increased scototaxis, geotaxis and risk assessment episodes (CACHAT et al., 2011). We analyzed non-motor patterns associated with anxiety-like behaviors in the novel tank and in the light–dark tests. Novel tank test showed decreases in the exploratory parameters without changes in locomotor activity in exposed groups, observed by decrease in distance travelled and transitions number between two sections, unchanging absolute turn angle. Exposed groups also decreased vertical exploration, since time spent in the upper half decreased, and latency to enter the top area significantly increased. This behavior patterns may be related with an anxiogenic effect (CACHAT et al., 2011). However, in the light-dark test we observed changes in the scototaxis, increased latency to first entry in the dark and decrease risk assessments episodes. Scototaxis is the preference for dark environments in detriment of bright ones and considered as an index of anxiety in adult zebrafish (MAXIMINO et al., 2015). Thereby, in the light-dark test, PM exposed zebrafish adopted less anxious behavior,

similar to anxiolytic effects (MAXIMINO et al., 2010b). Since anxiety is an emotion associated with risk assessment-like behavior toward potential threats in a new environment (MAXIMINO et al., 2014), which were significantly decreased, the two assays brought to light contradictory effects. The opposing effects observed between novel tank and light-dark tests in relation to anxiety can be associated to impaired exploration and habituation, which may be related with depression-like behaviors (Abreu et al., 2018). However, dark preference is controversial, once a dark environment can simulate a cave that may harbor predators that remain difficult to detect for the diurnal zebrafish that uses vision as one of its primary senses (GERLAI, 2011). On the other hand, zebrafish neurophenotyping is insufficient for correct behavior interpretation of data; more specific tests are needed to assess motor, anxiety and depression-related domains in parallel.

Regarding the fear-like responses, the embryonic exposure to PM showed a prominent decrease in fear when fish reached adult stage in the predator exposure test. The abnormal fear responses may be likely the result of the malfunction of neurobiological mechanisms during early developmental stage (neurotransmitters and neuromodulators) that were evolutionarily conserved among vertebrates to support avoidance of predators or other harms (GERLAI, 2011). Besides, the complex behaviors observed in zebrafish suggest conserved regulatory mechanisms with mammals, including shared modulatory neurotransmitter systems and homologous brain areas (MUELLER; WULLIMANN, 2003). Results observed in this work demonstrate for the first time long-term effects of embryonic exposure to PM in anxiety-fear disorders in the adulthood.

The preference for conspecifics reflects, evaluated by social preference test, a natural tendency of zebrafish to establish a group of interaction (MILLER; GERLAI, 2007). Fish aggregation may be modified by different compounds; however these behavioral parameters were not altered by exposure to PM, except for the latency to conspecific area in the PM $50\mu g.L^{-1}$. Preference for conspecifics belongs to the social interaction behaviors that include: fighting/aggression, shoaling/schooling, courtship and spawning. It can also be manifested in approach/boldness and social recognition (KALUEFF et al., 2013). Thereby, the decrease in time to first entry in close conspecific area may be related with other social behaviors cited above.

Aggression serves various adaptive functions, such as the establishment of dominance relationships hierarchies and competition for key resources, playing a major role in Darwinian fitness (OLIVEIRA; SILVA; SIMÕES, 2011a). A previous study in humans showed that long-term exposure of children to PM caused increased aggressiveness (CHRUSTEK et al.,

2018). Our results demonstrated that embryonic exposure to sublethal and environmental relevant concentrations of PM was able to induce increases in aggressiveness in the adult stage. The aggression is often comorbid with stress, anxiety and depression in humans, being associated with psychiatric disorders (KOLESNIKOVA et al., 2018).

Taken together, the long-term effects of PM showed disruption in anxiety, fear and aggressive behaviors in adulthood, which are modulated by neuroendocrine and monoamine neurotransmitters (KANEKO, 2011). Carloni et al. (2013b) suggest that post-natal PM treatment induces long-lasting dopaminergic neuronal disorders, monitored by Nurr1 alteration in adults rats exposed to NOAEL concentrations during early days of development. Besides, redox unbalance resulted of PM exposure during early stages of development (Awoyemi et al., 2019) may lead to epigenetic changes, influencing gene expression throughout the zebrafish life, and leading to alterations of certain parameters related to behavior function. The early developing organisms, generally, are more sensitive to oxidative damage, due intense and controlled cell proliferation, differentiation and apoptosis (SHI et al., 2011). Thereby, the functions of several macromolecules are programmed during embryonic period and, for this reason, the exposure to chemicals during embryonic period can influence the epigenetic in adulthood (OLIVEIRA; SILVA; SIMÕES, 2011a).

In conclusion, we demonstrated that a single acute embryonic exposure to environmentally relevant concentrations of PM was able to induce long-lasting effects in the adulthood. These neurotoxic effects may impair the cognitive capacity and fitness of non-target species, and could also modify interspecific interactions leading to decrease in survival and maintenance of the species. The exact mechanisms involved in the detrimental effects of PM in zebrafish still lacks of elucidation, however, the dopaminergic system and redox balance might be potential targets. These epigenetic mechanisms will be object of further investigations by our group. Nevertheless, our results reinforce the concept that early exposure to pyrethroids may induce deleterious neurodevelopmental effects, which might contribute to a better understanding of the etiology of psychiatric and mental disorders in youth and adulthood.

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Figure Captions:

Fig. 1:

Timeline of embryonic exposure and subsequent behavior assays during development.

Fig. 2:

Analyses of spontaneous movement in the embryos with 28 hpf, expressed by episodes number per minute. The dates were expressed as median ± interquartile range and analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different ($p < 0.05$).

Fig. 3:

Analyses of sensory-motor capacity in the eleutheroembryos (3 dpf). (A) number of stimulus (touch) needed to first scape response, expressed by stimulus number; (B) percentage of group that able to swim to periphery area after stimulus, expressed by % group; (C) behavioral assay design of touch response test. The dates were expressed as median ± interquartile range and analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 4:

Locomotor and exploratory activities of larvae (7 dpf) assess by Open Field test. (A) distance travelled, expressed by meters; (B) absolute turn angle, expressed by total sum of all angles formed during the swimming trajectory; (C) time spent in periphery area, expressed in seconds; (D) average time spent in periphery area, expressed in seconds. . The dates were expressed as median ± interquartile range and analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 5:

Scototaxis in larval stage (7 dpf) assess by light-dark test. (A) risk assessment, expressed by number of episodes; (B) time spent in lit area, expressed in seconds; (C) average time spend in lit area, expressed in seconds. The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 6:

Locomotor and exploratory activities of adult stage (90 dpf) assess by Novel Tank test. (A) distance travelled, expressed by meters; (B) transitions, expressed by number of transitions among top and botton section; (C) absolute turn angle, expressed by total sum of all angles formed during the swimming trajectory; (D) time spent in top area, expressed in seconds; (E) average time spent in top area, expressed in seconds; (F) latency to first entry in top area, expressed in seconds. The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 7:

Scototaxis in adult stage (90 dpf) assess by light-dark test. (A) time spent in lit area, expressed in seconds; (B) latency to first entry in dark area, expressed in seconds; (C) risk assessment, expressed by number of episodes. The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 8:

Aversion to a dangerous situation assess by predator exposure test in adult stage (90 dpf). (A) time spent in predator area, expressed in seconds; (B) latency to first entry in predator area, expressed in seconds; (C) transitions among two areas, expressed in number of transitions. The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 9

Interaction group assess by social preference test in adult stage (90 dpf). (A) time spend in the close area of group, expressed in seconds; (B) average time spent in close area of group; expressed in seconds; (C) transitions, expressed in number of transitions among two areas; (D) latency to first entry in close area of group, expressed in seconds. The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).

Fig. 10

Aggressive behavior assess by Mirror-Induced Aggression (MIA) test in adult stage (90 dpf). (A) total number of aggressive episodes, expressed in number of attacks; (B) total time of aggressive episodes, expressed in seconds; (C) average duration of aggressive episodes, expressed seconds; (D) aggressive episodes in each area, expressed in number of attacks; (E) time of aggressive episodes in each area, expressed seconds; (F) transitions to each area, expressed in number of entries in each area; (G) time spent in each area, expressed in seconds; (H) representative apparatus used to MIA test, the A1 is the closets from to

opponent image in the mirror, A2 is the transition area and A3 is the farthest area from the opponent. The parametric dates are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey post hoc; and nonparametric dates expressed as median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's post-hoc. Groups not sharing letters are significantly different (p<0.05).
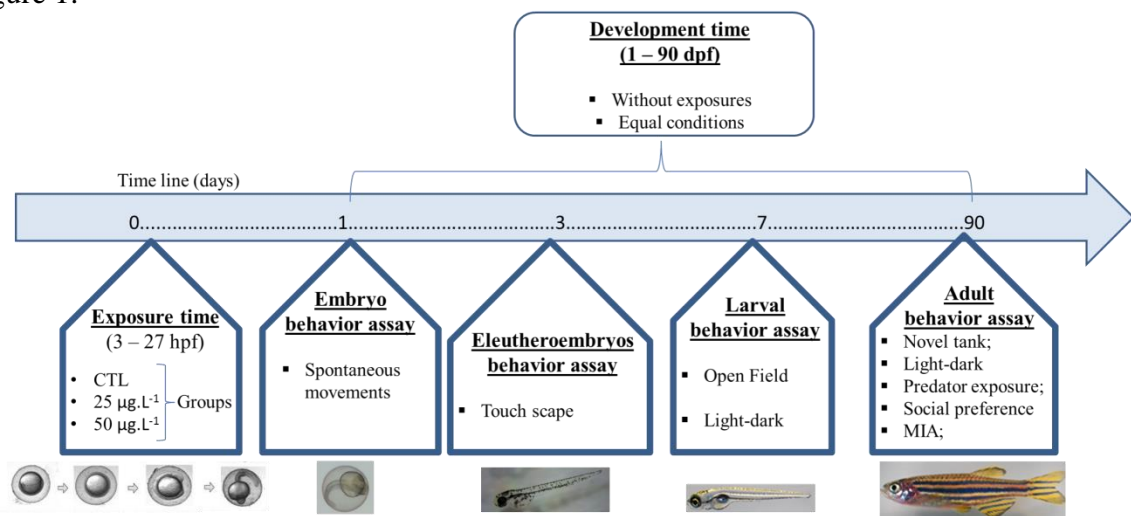
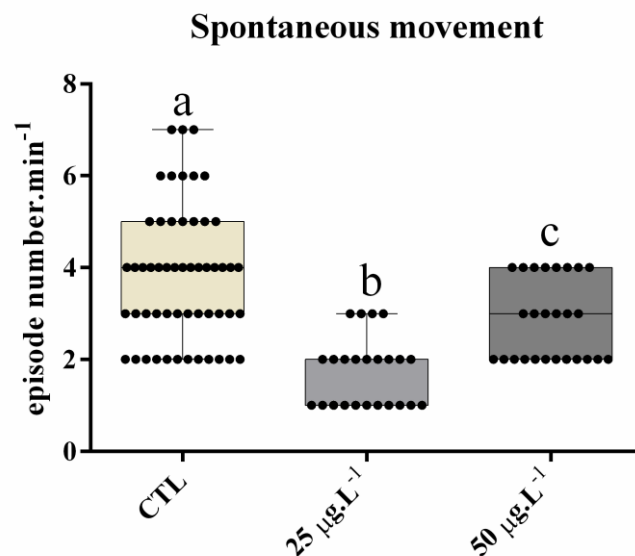**Figures:**

Figure 1:

Figure 2:

Figure 3:

A Touch response

B Thigmotaxis

C

Petri dish

Femto Laser Spatula

Gentle touch on the tail
(touch stimulus)

Periphery area
(5 mm)

Figure 4:

A

Distance travelled

B

Absolute turn angle

C

Time spent in perephery area

D

Average time spent in perephery area

Figure 5:

A

Risk assessments
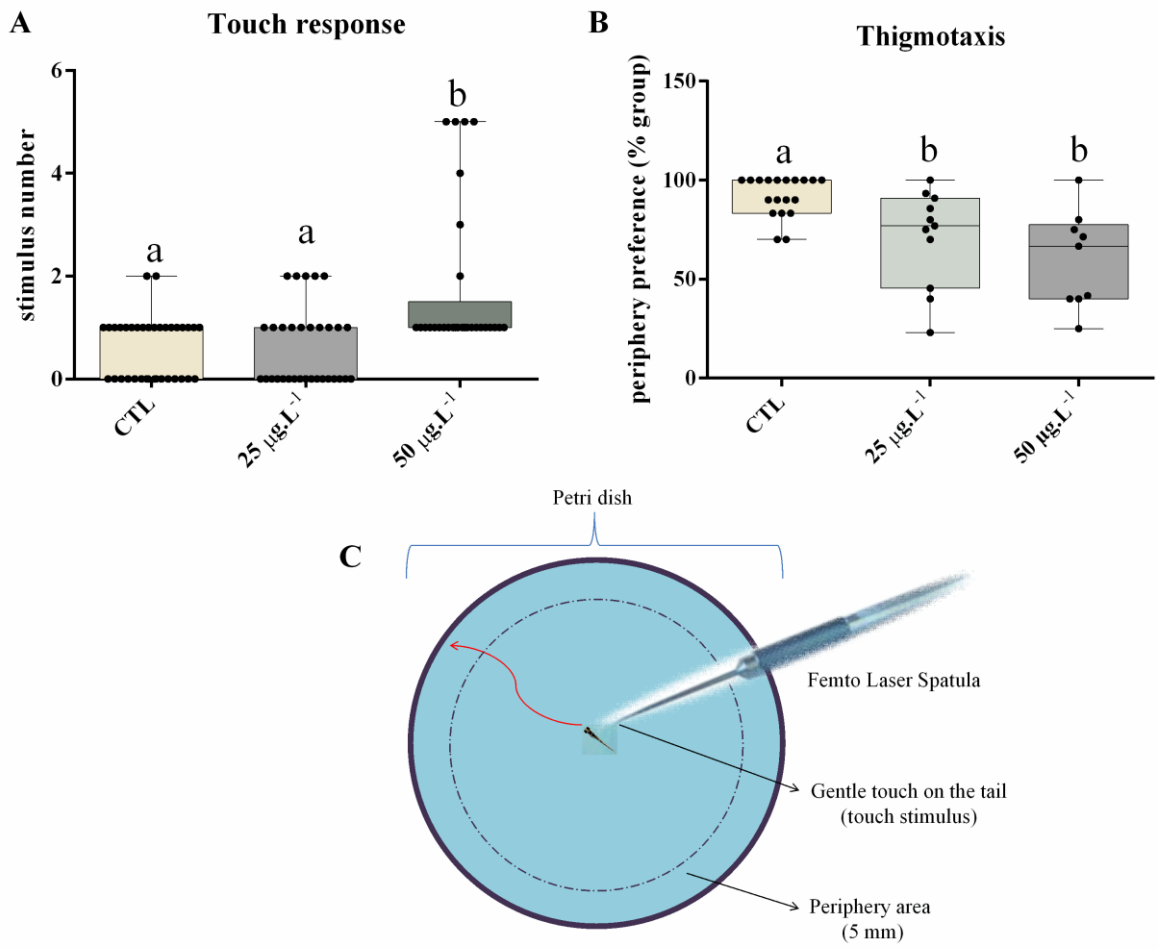

B

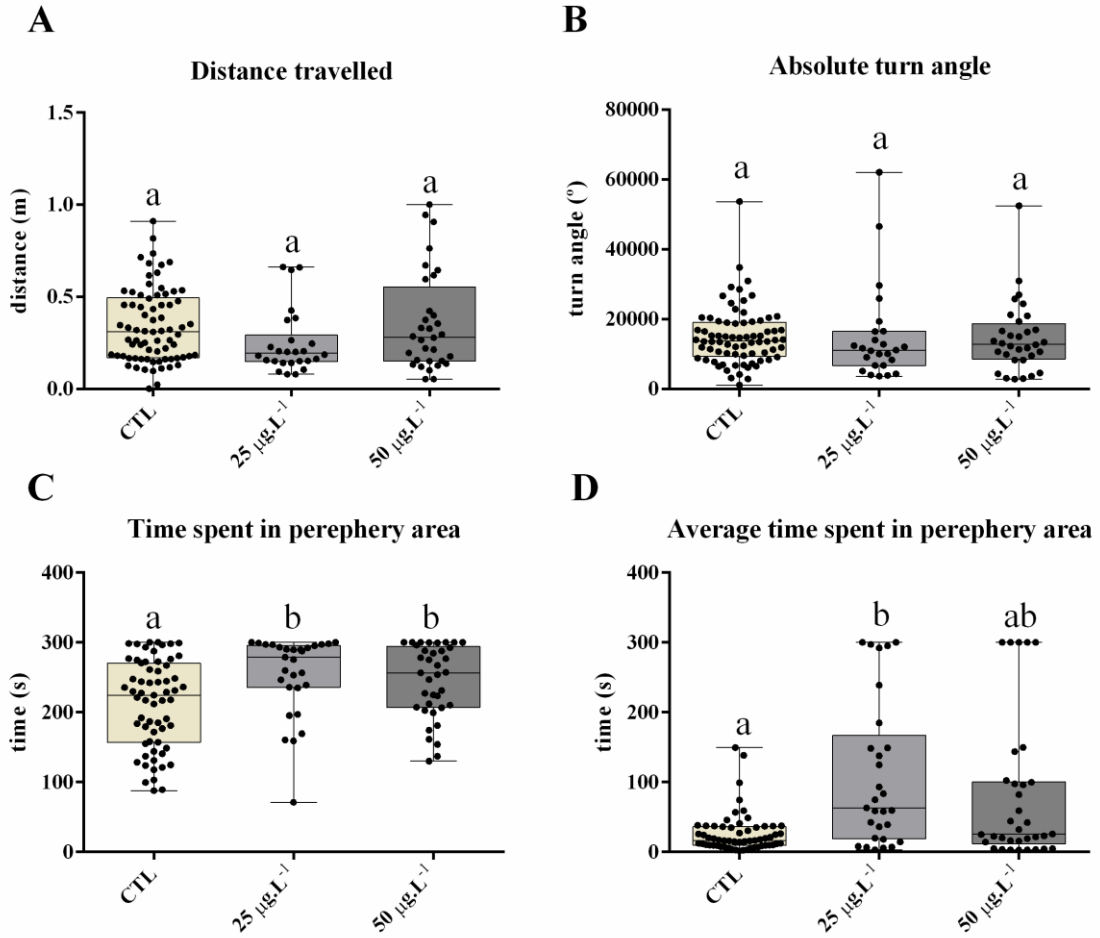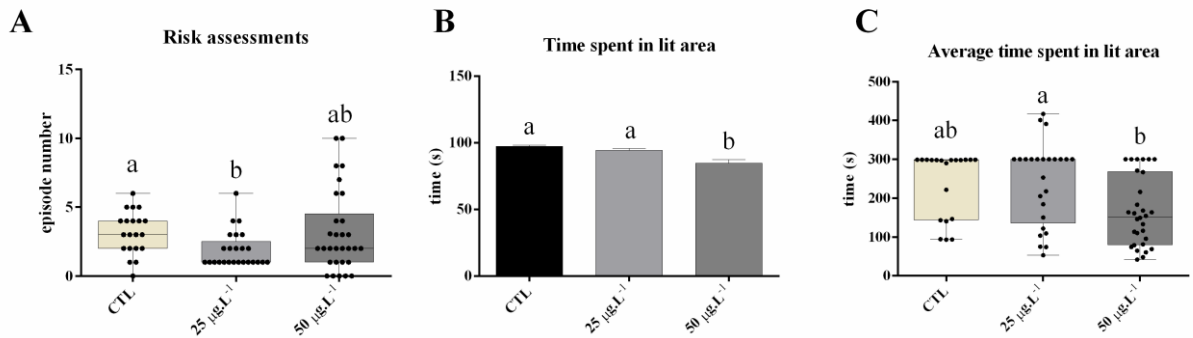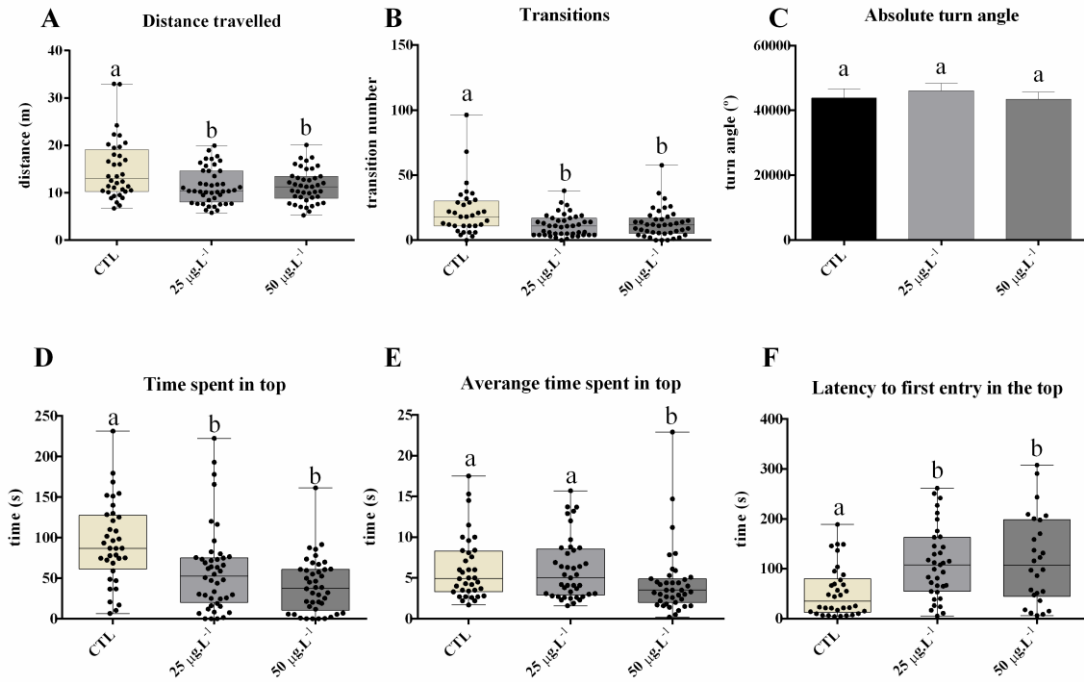**Time spent in lit area**

C

Average time spent in lit area

Figure 6:

Figure 7:

Figure 8:

Figure 9:

A

Time in the close area

B

Average time spent in close area

C

Latency to first entry close area

D

Transitions
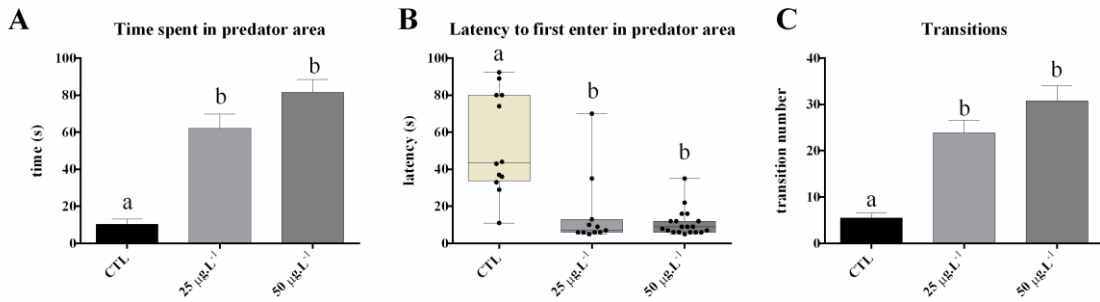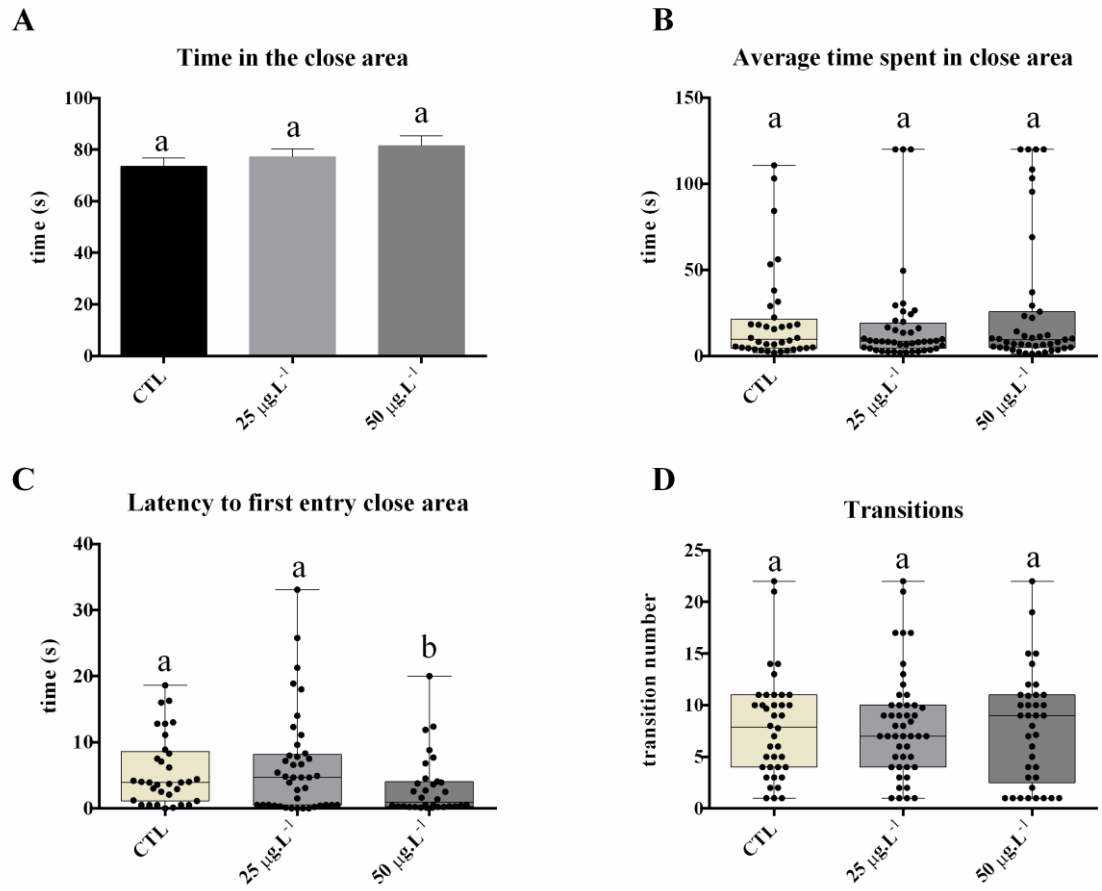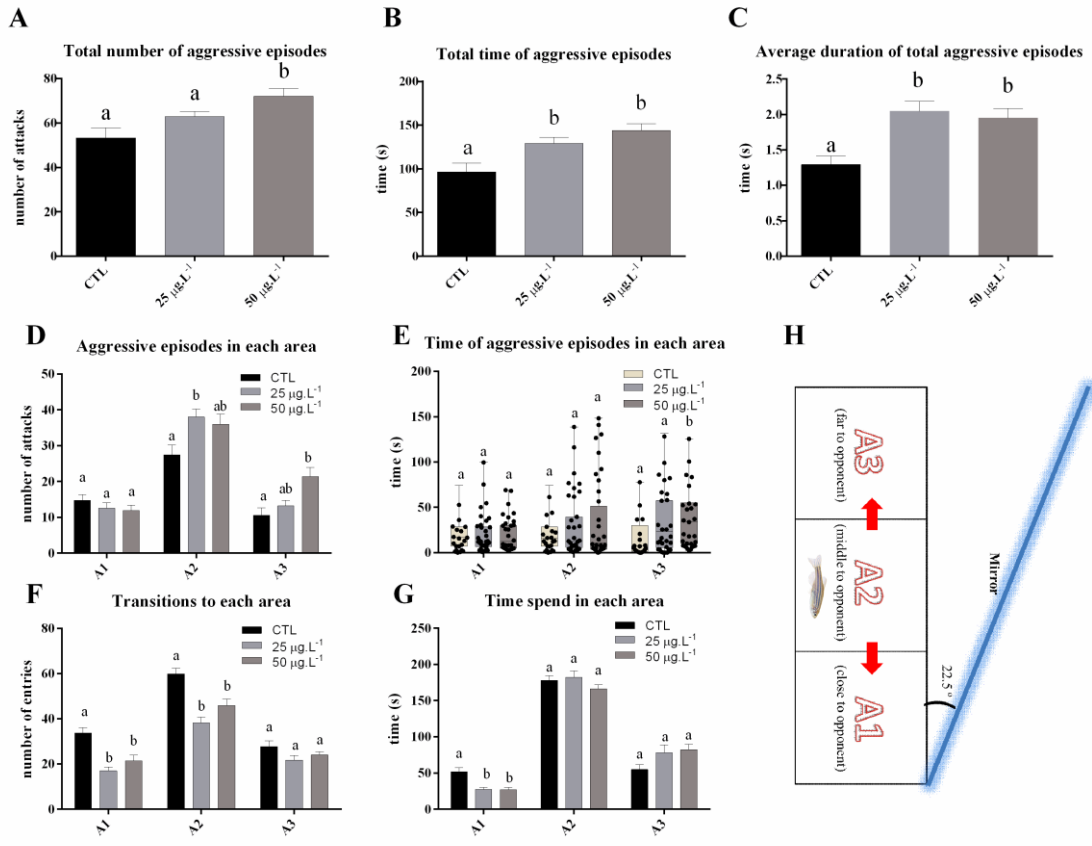
Figure10:

7. DISCUSSÃO

A crescente demanda por alimentos e demais produtos agrícolas no Brasil e no mundo tem levado ao aumento no uso de implementos agrícolas, principalmente agroquímicos. Além do uso no campo, a classe dos pesticidas é amplamente utilizada nos ambientes domésticos no controle de insetos nocivos (ALMEIDA et al., 2017). Inseticidas piretróides são comumente utilizado como composto ativo de produtos utilizados no controle de mosquitos e ectoparasitas, como piolhos (FIGU; INTE, 2014). Estudos epidemiológicos em diferentes países têm demonstrado a prevalência de biomarcadores de exposição à piretróides em até 70% das amostras (WATKINS et al., 2016). Esses estudos têm levanto preocupações sobre a presença em níveis de IPS consideravelmente mais elevados, em crianças e gestantes, os quais se encontram em estágio de desenvolvimento e, consecutivamente, são mais sensíveis aos efeitos de exposição à contaminantes ambientais (SHAFER; MEYER; CROFTON, 2005a). Dentre os pitróides, a permetrina (PM) representa um dos mais frequentes piretróides utilizados (TANG et al., 2018b). Em vista disso, este estudo teve como objetivo principal avaliar os efeitos da exposição à PM em concentrações ambientalmente relevantes sobre o desenvolvimento, utilizando o modelo de peixe-zebra.

No artigo foi observado o efeito da exposição a PM em larvas de peixe-zebra, a qual se encontra em estágio intermediário de desenvolvimento. Nesse estágio, os principais órgãos e tecidos se encontram, relativamente, desenvolvidos e diferenciados. O foco do artigo foi determinar as concentrações subletais de exposição aguda para o estágio larval de peixe-zebra e investigar os efeitos secundários da PM sobre o comportamento, homeostase redox e os possíveis subsequentes efeitos sobre a viabilidade e respiração celular. Larvas com 6 dias pós fertilização (dpf) foram expostas a concentrações de PM (0 – 600 µg/L), sendo determinada a concentração de 108 µg/L como CL_{50}. Com base nesse resultado foram estipuladas as concentrações de 25 e 50 µg/L como subletais e utilizadas para as determinações posteriores.

Nas análises comportamentais, observamos que a exposição a PM não foi capaz de alterar a capacidade motora. Desse modo, foi possível observar que as concentrações testadas não foram capazes de exercer os efeitos de intoxicação clássico dos inseticidas piretróides como tremores e paralisia, conhecida como síndrome T (WEINER et al., 2009). No entanto, quando parâmetros não-motores foram analisados, foi observado que a exposição PM foi capaz de modular comportamentos ligados a ansiedade, determinados pela frequência de comportamentos defensivos (GERLAI, 2011). Inseticidas piretróides, em geral, apresentam

caráter lipofílico o que permite transpor membranas biológicas como a barreira hematoencefálica e exercer sua toxicidade sobre o sistema nervoso central (SNC) (HAYA, 1989). Carloni et al. (2012) demonstrou que ratos expostos durantes os primeiros dias de desenvolvimento, à concentrações subletais de PM, apresentaram modulação do sistema dopaminérgico, o qual está diretamente ligado a disfunções psiquiátricas e processos neurodegenerativos. Os mecanismos patológicos que levam aos distúrbios dos neurônios dopaminérgicos ainda não são totalmente compreendidos, no entanto evidências apontam a disfunção mitocondrial, alterações na homeostase do cálcio e estresse oxidativo como os principais fatores (SCHÜLE et al., 2010).

Em nossas condições experimentais foi demonstrado que a exposição aguda à concentrações subletais de PM foi capaz de aumentar os níveis de EROS e modular o sistema de defesa antioxidante endógeno com aumento na atividade e expressão de enzimas do sistema glutationa. No entanto, a aumento das defesas não foi suficiente para impedir os danos oxidativos observados pelo aumento da peroxidação lipídica e de genotoxicidade, o que pode levar a indução de apoptose (SIES, 1999).

Ainda no artigo, foi possível observar que o estresse oxidativo induzido pela exposição à PM pode estar relacionado com a diminuição dos parâmetros bioenergéticos, observado pela diminuição do O_2 consumido durante o sistema de transporte de elétrons (STE) na mitocôndria. Além disso, a exposição à PM diminuiu a atividade de enzimas chaves do ciclo do ácido cítrico (citrato sintase) e aumentou a atividade da enzima lactato desidrogenase (via anaeróbica). Estes resultados apontam para um desvio da oxidação da glicose da via aeróbia para a via anaeróbica, o que pode prejudicar a manutenção celular neuronal, a qual é dependente dos elétrons fornecidos pelos processos de oxidação da glicose [107]. Em conjunto, os resultados obtidos no artigo, representados na figura 5, podem indicar os mecanismos de toxicidade relacionados aos efeitos comportamentais de peixes-zebra expostos durante o período embrionário à PM e a persistência desses efeitos ao longo da vida (manuscrito). No manuscrito, foi observado que a exposição à PM foi capaz de alterar padrões comportamentais em diferentes estágios do desenvolvimento. Neste trabalho, o foco foi avaliar a persistência dos efeitos toxicológicos gerados pela exposição à PM somente durante as primeiras 24 horas de desenvolvimento. É importante ressaltar que as concentrações utilizadas não apresentam efeito morfológico, conforme descrito na literatura (DEMICCO et al., 2009; YANG et al., 2014).
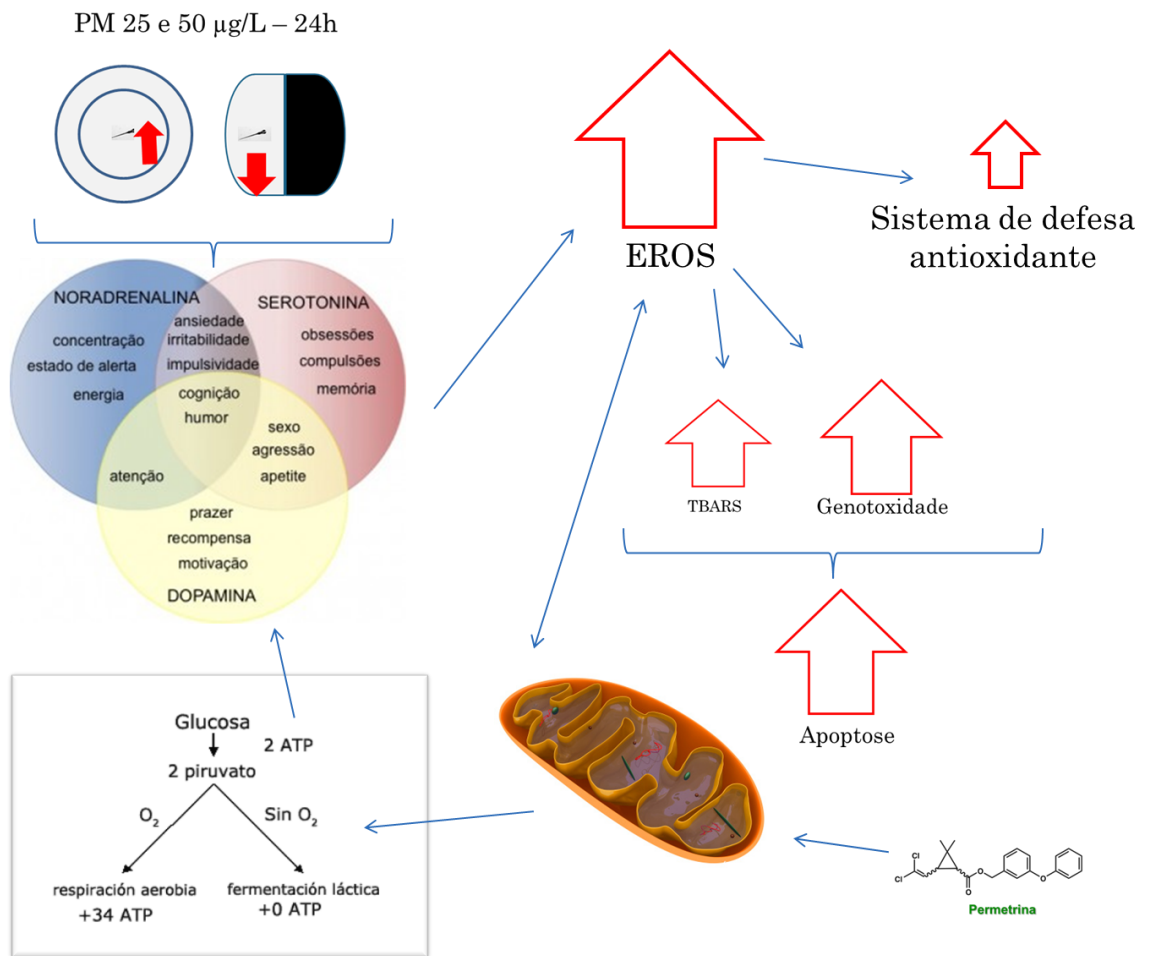
Figura 5. Mecanismos de toxicidade aguida permetrina sobre paramêtros da homeostase redox e enértica, e sua influência sobre parâmetros comportamentais não motores em larvas de peixe-zebra.

No segundo manuscrito, foi possível observar que exposição embrionária à PM exerceu efeitos danosos sobre neurodesenvolvimento de embriões de peixe-zebra, uma vez que os efeitos comportamentais foram persistentes em diferentes estágios do desenvolvimento, modulando comportamentos ligados ansiedade, medo e agressividade. Alterações nesses comportamentos estão relacionados com distúrbios psiquiátricos como depressão e transtorno de ansiedade (VIEL et al., 2017), que podem comprometer o fitness e, em consequência, a sobrevivência e manutenção da espécie no ambiente (OLIVEIRA; SILVA; SIMÕES, 2011b).

Os compostos do tipo I prolongam a abertura VSSC apenas o tempo suficiente para causar o disparo repetitivo de potenciais de ação (descarga repetitiva) (SHAFER; MEYER; CROFTON, 2005a). Evidências têm apontado que a pertubações na funcionalidade dos VSSC durante o desenvolvimento pode prejudicar a estrutura e a função do sistema nervoso (ref) e

estudos tem apontanto o sistema dopaminérgico e colinérgico muscarínico como possíveis alvos secundários da PM No entanto, estudos que examinaram esses sistemas relataram resultados inconsistentes até o momento (SHAFER; MEYER; CROFTON, 2005b). Disfunções no sistema monoaminérgico são relacionados a alterações comportamentais não-motoras ligadas perturbações psiquiátricas e cognitivas, como comprometimento do sono, ansiedade, depressão, cognição prejudicada e demência (XI; NOBLE; EKKER, 2011). Os mecanismos patológicos que levam a disfunção nos mecanismos de neurotransmissão ainda não são totalmente compreendidos, no entanto evidências apontam a disfunção mitocondrial, disfunção ubiquitina-proteassoma, alterações na homeostase do cálcio e estresse oxidativo como os principais fatores (SCHÜLE et al., 2010).

8. CONCLUSÕES

A partir dos resultados obtidos podemos concluir que:

1. Exposição aguda às concentrações subletais de permetrina exercem toxicidade significativa sobre larvas de peixe-zebra.

- A exposição à permetrina induziu aumento de EROS e subsequente dano oxidativo sobre biomoléculas.

- A resposta do sistema de defesa antioxidante glutationa frente ao aumento de EROS, no entanto não foi o suficiente para evitar os danos oxidativos e genotóxicos observados.

- A exposição a PM exerceu efeito sobre o sistema bioenérgitico, diminuindo a respiração mitocondrial e promovendo a via anaeróbica em detrimento da via aeróbica.

2. A exposição a PM modula comportamentos não-motores.

- A exposição aguda à PM foi capaz de induzir alterações comportamentais ligadas à ansiedade e medo em larvas de peixe-zebra, sem alterar parâmetros locomotores.

- A exposição durante o período embrionário à PM foi capaz de alterar os primeiros comportamentos ainda durante a fase embrionária e persistir durante estágios do desenvolvimento subsequentes.

- Os efeitos da exposição embrionária a PM persistiram até a fase adulta promovendo aumento da agressividade e modulação dos comportamentos ligados ao medo e ansiedade.

- As concentrações testadas nesse trabalho indicam o pontecial toxicológico da permetrina para organismos não-alvo e em estágio de desenvolvimento expostos a concentrações ambientais desse composto.

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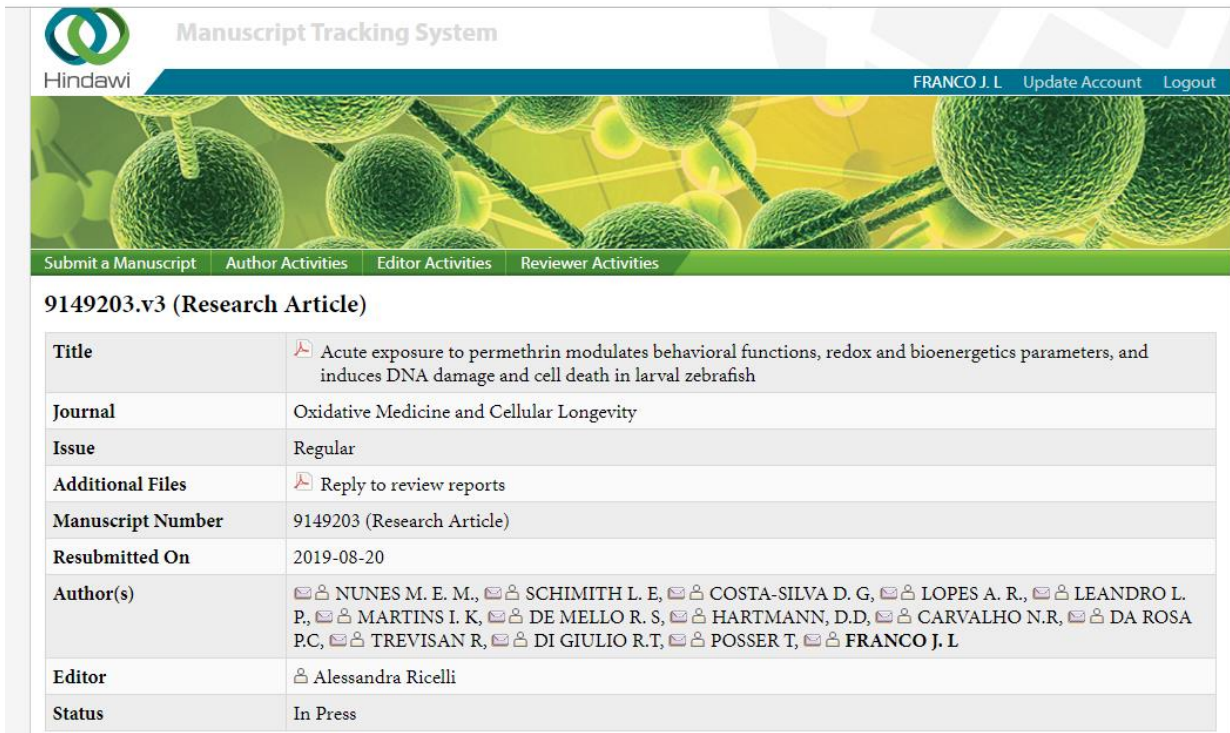
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10. ANEXOS:

10.1 ANEXO I – COMPROVANTE DE ACEITE DO ARTIGO

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9149203.v3 (Research Article)

Title	Acute exposure to permethrin modulates behavioral functions, redox and bioenergetics parameters, and induces DNA damage and cell death in larval zebrafish
Journal	Oxidative Medicine and Cellular Longevity
Issue	Regular
Additional Files	Reply to review reports
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Editor	Alessandra Ricelli
Status	In Press

8.2 ANEXO II – COMPROVANTE DE SUBMISSÃO DO MANUSCRITO:

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