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João Gabriel dos Santos da Rosa

EXPOSIÇÃO A AGROQUÍMICOS ALTERA O FUNCIONAMENTO DO EIXO HIPOTÁLAMO-HIPÓFISE-INTERRENAL EM ZEBRAFISH.

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Orientador: Profº. Dr Leonardo José Gil Barcellos

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Tese apresentada ao Curso de Pós-Graduação em Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do Título de **Doutor em Farmacologia.**

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DEDICATÓRIA

Dedico esse trabalho aos meus pais, João e Fátima.
Que possam sentir orgulho de mim tanto quanto eu sinto orgulho por ser seu filho.

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RESUMO

EXPOSIÇÃO A AGROQUÍMICOS ALTERA O FUNCIONAMENTO DO EIXO HIPOTÁLAMO-HIPÓFISE-INTERRENAL EM ZEBRAFISH.

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ORIENTADOR: Leonardo José Gil Barcellos

O aumento da ocupação humana em áreas rurais ea intensificação das práticas agrícolas, produz cada vez mais resíduos de agroquímicos que podem atingir ecossistemas aquáticos. Baseados nisso, muitos estudos são necessários para que sejam esclarecidos os mecanismos pelos quais essas substâncias agem nos organismos não-alvo. O presente trabalho objetiva demonstrar os experimentos realizados visando responder algumas questões sobre os mecanismos de ação de xenobióticos em organismos aquáticos. Para tanto, foi utilizado como modelo animal o peixe zebrafish (*Danio rerio*). Foram realizados experimentos com os seguintes objetivos: 1) avaliar se a exposição a agroquímicos tem o efeito de modular o eixo hipotálamo – hipófise – inter-renal (HHI), e para isso foi realizado um experimento de exposição aguda a um inseticida baseado no organosfosforado Metil Paration. Os peixes foram expostos por 96h e foram avaliados genes codificadores de componentes do eixo HHI, e também nível de cortisol tecidual; 2) Verificar se a exposição a agroquímicos causa alterações moleculares no eixo HHI e por quais mecanismos isso ocorre; 3) Verificar se resíduos de agroquímicos no ambiente alteram a percepção química no ambiente, gerando alterações comportamentais; então foi realizado um experimento em câmara quimiotáxica para verificar se a presença de agroquímicos possui efeito atrativo ou aversivo; e 4) Padronizar um novo modelo de estressor que possa ser utilizado para detectar alterações comportamentais, endócrinas e metabólicas frente a exposição a substâncias químicas. Conclusões: 1) Pôde-se verificar que a exposição à agroquímicos organofosforados possui efeito de alterar o eixo HHI inibindo expressão de genes chave para sua ativação, e 2) essa alteração é causada por uma hiperatividade colinérgica. 3) A presença de agroquímicos na água causa alterações comportamentais nos peixes expostos. 4) O novo modelo de estressor foi eficaz para ativar e modular o eixo HHI.

Palavras chave: *Danio rerio***,** agroquímicos, estresse, sistema colinérgico, comportamento.

ABSTRACT

EXPOSURE TO AGROCHEMICALS CHANGES THE FUNCTION OF THE HYPOTHALAMIC-PITUITARY-INTERRENAL AXIS IN ZEBRAFISH.

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The increase of human occupation in rural areas and the intensification of agricultural practices produces increasingly agrochemical waste up to aquatic ecosystems. Based on this, many studies are needed to clarify the mechanisms by which these substances act on non-target organisms. The present work aims to demonstrate experiments conducted to answering a few questions about the mechanisms of action of xenobiotics on aquatic organisms. Zebrafish (*Danio rerio*) was used as an animal model. Experiments were carried out with the following objectives: 1) evaluate whether exposure to agrichemicals has the effect to modulate the hypothalamic-pituitary-inter-renal (HPI) axis, and to answer this question was carried out an experiment of acute exposure to the organophosphorous-based insecticide methyl parathion. The fish were exposed for 96 h and genes of HPI axis components were evaluated, as well as tissue cortisol level. 2) Check whether exposure to agrochemicals cause molecular alterations in the HPI axis and by which mechanisms that occurs. 3) Check whether residues of agrichemicals in the environment interfere on environmental perception, generating behavioural changes; so an experiment was conducted in a chemotaxic chamber to verify if the presence of agrochemicals has attractive or aversive effect; and 4) Standardizing a new stressor that can be used to detect behavioral, endocrine and metabolic changes front exposure to chemical substances. Conclusions: 1) it was verified that exposure to organophosphates agrichemicals has the effect to changing the HPI axis by inhibiting expression of key genes for its activation, and 2) this change is caused by a cholinergic hyperactivity. 3) The presence of agrochemicals in water causes behavioral changes in fish exposed. 4) The new stressor model was effective to activate and modulate the HPI axis.

Keywords: Danior rerio, agrichemicals, stress, cholinergic system, behavior.

SUMÁRIO

CAPÍTULO I

INTRODUÇÃO

Atualmente grande parte dos ecossistemas aquáticos encontram-se contaminados com diversos tipos de substâncias, e consequentemente os organismos habitantes desses ecossistemas estão expostos à essas substâncias. Mesmo em regiões remotas, os ambientes aquáticos podem apresentar traços de contaminantes como pesticidas, e em regiões de alta ocupação humana a água pode conter complexas misturas de contaminantes (Harris et al, 2008; Ross e Birnbaum, 2003). Em ambiente aquático, a cinética de compostos químicos, como agroquímicos e fármacos não é totalmente conhecida, assim como a de seus metabólitos.

As fontes de água, tanto as utilizadas para consumo humano, quanto para utilização na agropecuária estão localizadas geralmente longe dos centros urbanos, e em proximidade com áreas agrícolas, onde o uso de agroquímicos é intenso (Fioreze et al., 2006). Essa proximidade oferece um grande risco de contaminação dessas fontes de água, devido a resíduos de agroquímicos - ou mesmo grande quantidade, que podem atingir esses recursos hídricos de diversas formas, como através de lixiviação do solo, ou contato direto. Além disso, todos o sistema hídrico é interligado, existindo um grande potencial dessas substancias atingirem ecossistemas aquáticos maiores e mais complexos. Com a ocorrência dessa contaminação de ambientes naturais, muitas espécies que habitam esses ecossistemas são atingidas e sofrem os efeitos deletérios dessa exposição; dessa forma, essa contaminação gera efeitos persistentes no meio ambiente (Jiraungkoorskul et al., 2002; Hori et al., 2008).

As concentrações prescritas para um agroquímico geralmente são menores que aquelas que causam mortalidade em animais terrestres ou aquáticos, sendo a contaminação ambiental geralmente acidental e com produtos altamente tóxicos. No entanto, concentrações menores podem causar efeitos discretos nos animais não alvo, porém fisiologicamente importantes. Entretanto, a contaminação da água por baixas concentrações de agroquímicos é de difícil avaliação; no contexto ecológico, parâmetros

fisiológicos como desenvolvimento, reprodução e taxa de crescimento são muito representativos. (Scott and Sloman, 2004). Esses efeitos incluem uma série de alterações orgânicas que acabam colocando em risco a sobrevivência tanto de indivíduos como de uma população (Kreutz et al., 2008). Parâmetros endócrinos podem servir como indicadores de contaminação ambiental quando são verificados em espécies não-alvo, pois quando essas são expostas a concentrações sub-letais, a ocorrência de alterações fisiológicas é muito comum (Kreutz et al. 2008, Fioreze et al. 2006). Berenzem et al (2005) encontraram em rios da Alemanha, em dois locais diferentes, metil-paration em concentrações que variaram de 0,2 a 0,3 μg/L, além do fungicida tebuconazole em concentrações de 0,5 a 1,7 μg/L.

Um dos grandes desafios, tanto da aquicultura, quanto da conservação dos estoques e populações de peixes na natureza, é a exacerbação ou a inibição da resposta ao estresse. A resposta ao estresse é uma reação altamente adaptativa, indispensável à restauração do estado de homeostase após algum fator estressante, biótico ou abiótico. Ou seja, é uma resposta que, se dentro dos padrões fisiológicos (nem exacerbada, nem inibida) dos peixes, é essencial à manutenção da vida (Fuzzen, 2010).

A inibição dessa resposta significa um grave desafio fisiológico ao indivíduo. Sendo essa resposta altamente adaptativa, peixes com a capacidade comprometida de responder ao estresse não conseguem montar uma reação adequada, não reestabelecem sua homeostase e, se essa situação perdurar, vêm a óbito. Por isso, o bloqueio endócrino na resposta ao estresse vem sendo apontado como um dos principais fatores de redução e até extinção das populações de peixes. Esse bloqueio se dá devido às substâncias chamadas bloqueadoras endócrinas que afetam o eixo hipotálamo-hipófise-inter-renal (HHI) das mais diversas formas.

O eixo neuroendócrino HHI é o eixo que coordena a resposta ao estresse. Primeiramente, ao perceber um fator estressante o hipotálamo libera o hormônio liberador de corticotrofina (CRF) estimulando a hipófise a liberar da adrenocorticotrofina (ACTH) que, no tecido inter-renal, homólogo da glândula adrenal dos mamíferos, estimula a síntese e liberação do cortisol, hormônio glicocorticoide, ponto final do eixo HHI (Fuzzen et al, 2010). A síntese do cortisol e seu controle envolvem vários passos, receptores e substâncias. Os genes codificadores desses receptores e substâncias se expressam em maior ou menor intensidade, resultando numa resposta mais ou menos intensa.

Umas das principais substâncias envolvidas na síntese do cortisol é a proteína

regulatória aguda (StAR), que controla e promove a entrada do precursor pregnenolona na mitocôndria, para que ai siga os passos de conversão a cortisol (Stocco et al., 2005).

Outras importantes substâncias são as chamadas proteínas de choque térmico. Todos os organismos respondem ao calor induzindo a síntese de um grupo de proteínas chamadas proteínas do choque térmico (HSP), que é o sistema genético mais conservado de que se tem conhecimento. As HSPs são indicadores de estresse em peixes e representam uma resposta celular aos estressores (Iwama et al., 2004). Sua função é de estabilização de proteínas estruturais sobre condições de estresse (Willer et al., 2000). Assim, a inibição da HSP70, por exemplo, pode causar estresse celular com consequente perda de função celular. A HSP70 ocorre também em regiões do cérebro que coordenam as respostas neuroendócrinas ao estresse (Blake et al., 1990).

Agroquímicos

A contaminação do ambiente natural por agroquímicos é muito comum, e frequentemente são relatados casos de mortalidade de animais logo após o uso desses compostos. Sendo assim, estudos toxicológicos clássicos são baseados principalmente nos efeitos de mortalidade causados por contaminantes, o que se constitui em uma intoxicação aguda com concentrações do agroquímico acima da sua concentração letal (CL50-96h). Esse tipo de contaminação geralmente ocorre de forma acidental, com produtos altamente tóxicos. Entretanto, a contaminação da água por baixas concentrações de agroquímicos é de difícil avaliação. No ambiente, as concentrações de contaminantes encontradas geralmente são mais baixas que as concentrações letais, o que indica que uma exposição aguda ou crônica pode causar efeitos subletais (Tierney et al, 2010). Por não causar a morte imediata dos peixes, na maioria das vezes, intoxicações por concentrações subletais passam desapercebidas, ocasionando um aumento dos efeitos deletérios por permitir uma exposição prolongada do organismo a esses compostos. A exposição prolongada e em baixas concentrações pode ocasionar tanto acumulo de tóxicos nos tecidos, quanto exercer efeitos mais específicos em determinados alvos biológicos, como processos endócrinos. Uma das alternativas para o estudo da contaminação de águas com baixas concentrações de agroquímicos, é a determinação de parâmetros bioquímicos que poderiam ser utilizados como indicadores de uma agressão; entre os parâmetros avaliados estão diversos marcadores moleculares envolvidos na síntese e controle da secreção de

substancias produzidas em resposta a uma agressão, como hormônios relacionados a respostas de estresse. Atualmente uma quantidade cada vez maior de agroquímicos são utilizados com o objetivo de garantir uma produção elevada de alimentos de origem vegetal. A consequência disso é um maior risco de contaminação ambiental, visto que as quantidades utilizadas são muito altas, e em muitos casos a fiscalização é precária.

Organosfosforados são uma classe muito utilizada para controle de insetos e pragas na agricultura, por apresentar baixo custo e alta eficácia. A eficácia está baseada na toxicidade dos compostos, que significam uma ameaça a espécies não alvo, incluindo os seres humanos. Inseticidas organofosforados provocam o acúmulo de neurotransmissores, levando a efeitos neurotóxicos como estresse oxidativo.

Agroquímicos e eixo hipotálamo-hipófise-interrenal

Os efeitos causados por exposição a contaminantes em baixas concentrações se dá pela interação da substância com um sitio ativo receptor, podendo ser um tecido, ou ainda em nível molecular uma enzima ou gene. Organofosforados são inseticidas de contato com efeito sistêmico utilizados para controle de uma grande variedade de insetos. Como são utilizados em grande quantidade, é extremamente difícil evitar que resíduos cheguem até os recursos hídricos. Sendo assim, os organismos que habitam ecossistemas aquáticos próximos a áreas onde ocorre o uso dessas substancias poderão ser expostos de forma acidental.

Apesar de ser um fato cada vez mais frequente, ainda são poucos os estudos que visam esclarecer os efeitos desses resíduos em organismos não alvo, além de estudos que clarifiquem a dinâmica ecológica desses resíduos no meio ambiente. Parte dessas substâncias permanecem na forma inalterada, e os efeitos biológicos causados pela exposição pode produzir efeitos fisiológicos que interferem nas condições de manutenção das espécies aquáticas, podendo influenciar o equilíbrio populacional. Peixes e outros organismos aquáticos são especialmente afetados, já que permanecem expostos durante todas as fases da vida. Estudos que elucidem esses efeitos são necessários, porque sendo esse um fato relativamente recente, as agências regulatórias ainda não possuem dados suficientes para assegurar a inocuidade desses resíduos, tanto para população humana quanto para ecossistemas aquáticos. Embora seja mais preocupante atualmente, desde a

década de 70 existem relatos de resíduos medicamentosos e seus metabólitos na água. Esse potencial perigo de contaminação do ambiente se estende de forma muito preocupante a todas espécies não alvo, inclusive o ser humano.

O descarte inadequado de embalagens, tanto de fármacos quanto agroquímicos, assim como o próprio ato de administrar agroquímicos oferece um grande risco à população e ao trabalhador rural. Muitas vezes essa contaminação é exacerbada justamente pelo alto consumo de medicamentos, onde muitas vezes o trabalhador rural é exposto a agroquímicos e ao efeito de fármacos. Além disso, essas misturas complexas de substancias químicas pode chegar a população através da agua de consumo; e no aspecto ecológico, as populações que habitam ecossistemas aquáticos estão susceptíveis a essa mistura de contaminantes.

O metil paration (O,O-dimethyl O-4-nitro-phenylphosphorothioate, MP) é um dos inseticidas mais utilizados na agricultura (Kramer et al, 2003), sendo utilizado em programas de controle de pestes em silos de armazenagem de grãos, principalmente por sua eficácia e baixo custo (Garcia et al, 2003). No entanto, esse uso intenso causa a geração de resíduos, que significam uma ameaça às espécies não-alvo, incluindo seres humanos. Nos peixes, o MP é prontamente absorvido nas brânquias e transportado aos demais tecidos, como fígado, intestinos e cérebro, podendo ocasionar bioacumulação da substância nesses tecidos (De La Vega Salazar et al., 1997). O mecanismo de ação do MP é a inibição irreversível da enzima acetilcolinesterase (AChE), levando ao acúmulo do neurotransmissor acetilcolina na fenda sináptica, gerando vários efeitos neurotóxicos (Muttraya et al, 2005). Um efeito neurotóxico do MP é a ocorrência de estresse oxidativo, que pode ocorrer tanto pela inibição dos sistemas antioxidantes, quanto pela produção excessiva de espécies reativas ao oxigênio (Monteiro et al, 2006).

Contaminação ambiental e processos ecológicos

Algumas características, como baixa volatilidade e relativa solubilidade, permitem que a distribuição dessas substâncias no ambiente ocorra principalmente através da água. Parte dessas substâncias permanece na forma inalterada, e os efeitos biológicos causados pela exposição pode produzir efeitos fisiológicos que interferem nas condições de manutenção das espécies aquáticas, podendo influenciar o equilíbrio populacional. Peixes e outros organismos aquáticos são especialmente afetados, já que

permanecem expostos durante todas as fases da vida nesse ambiente.

A contaminação dos ecossistemas, no contexto ecológico, interfere em parâmetros fisiológicos como desenvolvimento, reprodução e taxa de crescimento (Scott and Sloman, 2004). Além disso, exposição a substancias químicas pode aumentar a mortalidade por interferir em processos ecológicos como falha na detecção de um potencial predador, inabilidade de se alimentar e baixa resistência a doenças. Vários estudos já relataram que certos contaminantes podem causar esse efeito, alterando a atividade locomotora (Beauvais et al, 2000) ou inibindo a habilidade de detectar um predador (Moore et al, 2007).

Mesmo quando expostos a baixas concentrações de contaminantes, os organismos estão sujeitos a agressões fisiológicas mais sutis, que, ao invés de serem detectadas facilmente (alta mortalidade), essas agressões interferem em processos ecológicos importantes como predação, comportamento social, comportamento de fuga, habilidade olfatória e migração. A impossibilidade dos peixes de realizar esses comportamentos representa uma importante ameaça à manutenção das espécies em ambiente natural (Scott et al. 2003).

Devido à facilidade de medir e avaliar comportamentos importantes do ponto de vista ecológico, os peixes são animais considerados ideais para analisar o impacto de contaminantes na natureza (Scott e Sloman 2004). Dentre as espécies utilizadas para monitoração dos efeitos de contaminantes, o zebrafish apresenta um repertório comportamental extremamente interessante. Comportamentos como velocidade de deslocamento, distância percorrida, permanência em determinada área do ambiente e padrão de deslocamento são relativamente fáceis de observar e quantificar, e servem como indicadores (Amorim et al, 2016; Blaser e Gerlai 2006; Kane et al. 2004).

Fungicidas, como o fungicida baseado em tebuconazole (FBT) são uma classe amplamente utilizada na agricultura para proteção dos produtos. Seu mecanismo de ação é a inibição enzimática, bloqueando a conversão de intermediários metabólicos e provocando a decomposição da parede celular dos fungos (Di Renzo et al, 2007). Porém, esse mecanismo de ação pode se estender a outras espécies que possuem atividades celulares mediadas por essas mesmas enzimas, o que pode resultar em vários efeitos adversos da aplicação desse fungicida (Robinson et al., 2012). Vários estudos (Liu et al., 2011; Goetz and Dix, 2009; Hester and Nesnow, 2008) relacionaram a exposição a fungicidas triazóis com alterações na concentração ou transcrição de genes envolvidos na

homeostase esteroidal. Outra classe de agroquímicos, que já são utilizados há muito tempo, são os herbicidas. Essa classe é intensamente utilizada, de forma global, sendo o mais usado na Europa (European Commission, 2007) e Estados Unidos (EPA, 2011). Além da aplicação na agricultura, herbicidas são amplamente utilizados em áreas urbanas para tratamento de jardins públicos (EPA, 2011).

A maioria dos compostos com atividade herbicida como o herbicida baseado em glifosato (HBG) ou em atrazina (HBA), tem a característica de se ligar ao substrato, e, por isso, podem ocorrer pulsos de contaminação quando ocorrem chuvas e o sedimento pode ser carregado para rios e corpos d'água (Giesy et al, 2000), além de poder haver contaminação ambiental através de efluentes urbanos (Botta et al, 2009). Alguns estudos (Mast et al. 2007) afirmam que herbicidas podem ser carregados por longas distâncias a partir do ponto de aplicação, através da água da chuva, existindo assim a possibilidade de contaminar uma extensa área ambiental. Os herbicidas impedem o metabolismo de proteínas essenciais as plantas, interferindo na síntese de aminoácidos aromáticos. Outro alvo comum dos herbicidas são os processos bioquímicos de obtenção de energia pelas células. Portanto, herbicidas podem agir em espécies não-alvo e induzir uma série de efeitos biológicos. Em peixes podem induzir estresse oxidativo pela produção de EROs ou interferindo nos mecanismos antioxidantes, causando danos ao DNA, lipídeos e proteínas (Cavalcante et al, 2008; Ferreirra et al, 2010).

Herbicidas e seus metabolitos também são considerados interruptores endócrinos por causar toxicidade neuroendócrina (Foradori et al, 2014), agindo no sistema nervoso central (Foradori et al, 2013; Goldman et l, 2013). Inseticidas são outra classe de agroquímicos utilizados amplamente, e com real potencial de contaminar o meio ambiente.

Zebrafish como modelo experimental

O zebrafish (*Danio rerio*) é um pequeno ciprinideo originário do oeste da Índia. Constitui um valioso modelo animal em experimentação científica devido a várias características, como rápido desenvolvimento, facilidade de manutenção e disponibilidade de aquisição. Além disso, o tamanho pequeno (5-6 cm), a transparência óptica durante a embriogênese (que proporciona facilidade na detecção de anormalidades morfológicas) e a relativa facilidade de traçar perfis genéticos e o completo conhecimento

de seu eixo HHI favoreceram a utilização dessa espécie como modelo experimental (Westerfield, 2007). O zebrafish, demonstra sistemas fisiológicos suficientemente semelhantes a vertebrados superiores, como primatas e humanos (cerca de 70% de homologia genética) e por isso é considerado uma espécie ideal para o estudo da fisiologia do estresse por ter seu eixo hipotálamo-hipófise-inter-renal muito bem caracterizado (Alsop and Vijayan, 2009; Fuzzen et al., 2010). Ainda, o zebrafish apresenta uma resposta muito robusta e confiável quando manipulado geneticamente, através de uma série de técnicas com o objetivo de elucidar a função de genes, assim como patologias diversas. Também apresenta respostas muito satisfatórias quando utilizado como modelo comportamental e simulando diversos etogramas.

OBJETIVOS

O **objetivo geral** foi verificar o mecanismo de ação que provoca o efeito disruptor dos agroquímicos sobre o eixo hipotálamo-hipófise-inter-renal e, consequentemente, sobre a resposta ao estresse, e sua relação com os componentes que fazem a ativação e manutenção desse eixo, bem como seus possíveis efeitos ambientais.

Os **objetivos específicos** foram (1) avaliar se a exposição aguda a uma concentração subletal do princípio ativo metil paration causa bloqueio do eixo hipotálamo-hipófise-interrenal (HHI) e, consequentemente, na resposta fisiológica ao estresse; (2) identificar o mecanismo pelo qual uma concentração subletal do princípio ativo metil paration provoca uma desregulação endócrina; (3) avaliar se a exposição aguda a concentrações subletais de agroquímicos interferem em processos ecológicos de espécies aquáticas e (4) padronizar um novo modelo de estressor, que possa ser utilizado para detectar alterações comportamentais, endócrinas e metabólicas frente a exposição a substâncias químicas.

CAPÍTULO II

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Impaired brain StAR and HSP70 gene expression in zebrafish exposed to Methyl-Parathion based insecticide

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Impaired brain StAR and *HSP*70 gene expression in zebrafish exposed to Methyl-Parathion based insecticide

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ABSTRACT

Fish production ponds and natural water body areas located in close proximity to agricultural fields receive water with variable amounts of agrochemicals, and consequently, compounds that produce adverse effects may reach nontarget organisms. The aim of this study was to investigate whether waterborne methyl-parathion-based insecticide (MPBI) affected gene expression patterns of brain glucocorticoid receptor (*GR*), steroidogenic acute regulatory protein (*StAR*), and heat shock protein 70 (*hsp70*) in adult zebrafish (*Danio rerio*) exposed to this chemical for 96 h. Treated fish exposed to MPBI-contaminated water showed an inhibition of brain *StAR* and *hsp70* gene expression. Data demonstrated that MPBI produced a decrease brain StAR and *hsp*70 gene expression.

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Zebrafish are a reliable model system to examine stress responses (Carlsson et al., 2014) and the role of the hypothalamic-pituitary-interrenal (HPI) axis in the stress response is well established (Alsop and Vijayan, 2009; Fuzzen et al., 2010; Wendelaar Bonga, 1997). HPI and its peripheral product, cortisol, play a key role in the metabolic, ionic, and physiologic adjustments necessary for coping with stress. Cortisol is secreted and binds to glucocorticoid receptor (GR), a ligand-activated nuclear transcription factor. GR regulates transcription of target genes related to glucose metabolism, immune function, and behavior (Mommsen et al., 1999). Consequently, any adverse effect on the functioning of the HPI axis may compromise the ability of the animal to mount an adequate response to stressors (Hontela, 1998, 2005).

In cortisol synthesis, the steroidogenic acute regulatory protein (StAR), which shuttles cholesterol from the outer to the inner mitochondrial membrane, is a key rate-limiting protein in steroid synthesis (Stocco et al., 2005). The brain is not only a target but also a steroid-producing organ, and steroid concentrations in brain fluctuate independently of plasma cortisol concentrations (Sierra, 2004). The co-localization of StAR in brain cells that also express P450scc demonstrates a similar importance in production of neurosteroids (King et al., 2002; Sierra, 2004). These neurosteroids are thought to play important roles in neuroprotection, modulation of brain function, and neuronal development (King et al., 2004).

Heat shock proteins (*hsps*), other indicators of stress response in fish, represent a cellular response to stressors (Iwama et al., 2004), and their function is to stabilize protein structures under stress conditions (Willer et al., 2000). Thus, the inhibition of hsp70 might initiate cellular stress with a consequent loss of function. The *hsp*70 occurs particularly in the brain regions that coordinate the neuroendocrine response to stress (Blake et al., 1990).

Interrenal StAR protein and *hsp*70 might serve as targets for toxicants and possible mechanisms producing disruption in steroid production (Aluru

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et al., 2005; Aluru and Vijayan, 2006; Gravel and Vijayan, 2006). However, apparently few data are available regarding the involvement of brain StAR and *hsp*70 as targets for xenobiotics.

Methyl-parathion-based insecticides (MPBI) are a type of organophosphorous (OP) pesticide largely used to prevent agricultural losses due to insects, as well as being employed in food storage. In addition, MPBI has also been used in fish culture ponds for eliminating aquatic larvae of predatory insects (Szarek et al., 2000). Thus, based upon current uses and practices, MPBI is environmentally widespread and able to reach various nontarget organisms including fish (Cericato et al., 2008). MPBI, commercially named Folidol 600® (600 g/L of MP), is a less persistent OP insecticide, moderately soluble in water and acutely toxic to fish (Walton et al., 1997).

Investigators previously reported impairment of the hypothalamus–pituitary–interrenal axis (HPI) provoked by MPBI in zebrafish (Rosa et al., 2013) and in jundiá, *Rhamdia quelen* (Koakoski et al., 2014). MPBI also produced several changes in expression of genes encoding some fish brain proteins (Huang and Huang, 2011; Ling et al., 2012), indicating an action of MPBI in the central nervous system (CNS).

Thus, the aim of this study was to determine the influence of MPBI, acute stress, or a combination of stress and chemical exposure on the expression of brain StAR protein, *hsp*70, and GR.

Materials and methods

Animals

Two hundred and forty adult male wild-type zebrafish (*Danio rerio*) weighing approximately 0.7 g each were obtained from a commercial supplier (Delphis, Porto Alegre, RS, Brazil). Fish were acclimated for 3 d before the tests in aquaria (40 L, with constantly aerated dechlorinated tap water), housed in groups of 20 fish, kept under a 14/10-h day/night cycle, and fed 3 times per day with commercial flakes (TetraMin). Throughout the experiment, water temperature was 28 ± 2°C, pH ranged from 6.6 to 7.2, and dissolved oxygen ranged from 5.2 to 7.1 mg/L. Total ammonia was lower than 0.5 mg/L.

Experimental protocol

After acclimation, fish were randomly distributed into four experimental groups, each housed in six aquaria in a static test design. The first group consisted of fish without any stressor or contaminant (C group). The second group consisted of fish exposed for 96 h to 5.2 mg/L of MPBI (MPBI group; concentration based on previous findings from Bellavere and Gorbi [1984], Huang and Huang [2011], and Ling et al. [2012]). Fish exposed to a stressor formed the third group (S group; 60 s of chasing with a net), while the fourth group consisted of fish exposed to both stressor and contaminant (MPBI + S group).

The stressor was applied after 96 h of exposure, and fish were sampled at hour 97, cryoanesthetized, and euthanized (Wilson et al., 2009) for brain extraction and gene expression analysis (n = 10). Previously, Rosa et al. (2013) reported that this acute stress protocol increased whole-body cortisol in zebrafish, where the stressed group showed approximately sevenfold increase in cortisol levels compared to control. A schematic illustration of experimental design is presented in Figure 1. The expression of GR, StAR, and *hsp*70 genes was assessed by a semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) assay. Zebrafish DNA sequences encoding for GR, StAR, and *hsp*70 were retrieved from the GenBank database and used for searching specific primers, which were designed using the Oligos 9.6 program (Table 1). The β-actin primers were designed as described previously (Chen et al., 2004). TRIzol reagent (Invitrogen) was employed for isolating total zebrafish brain RNA and the total gDNA was eliminated by DNase I treatment, and its purity was quantified by spectrophotometry. Subsequently, all samples were adjusted to 160 ng/µl, and cDNA were synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen). RT-PCR conditions were optimized in order to determine the number of cycles that would enable product detection within the linear phase of mRNA transcript amplification (Table 1). The PCR reactions were performed using 0.1 µM primers, 0.2 µM dNTP, 2 mM $MgCl_2$, and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen). PCR products were submitted to electrophoresis using a 1% agarose gel. The fragment length of the PCR reactions was confirmed with a

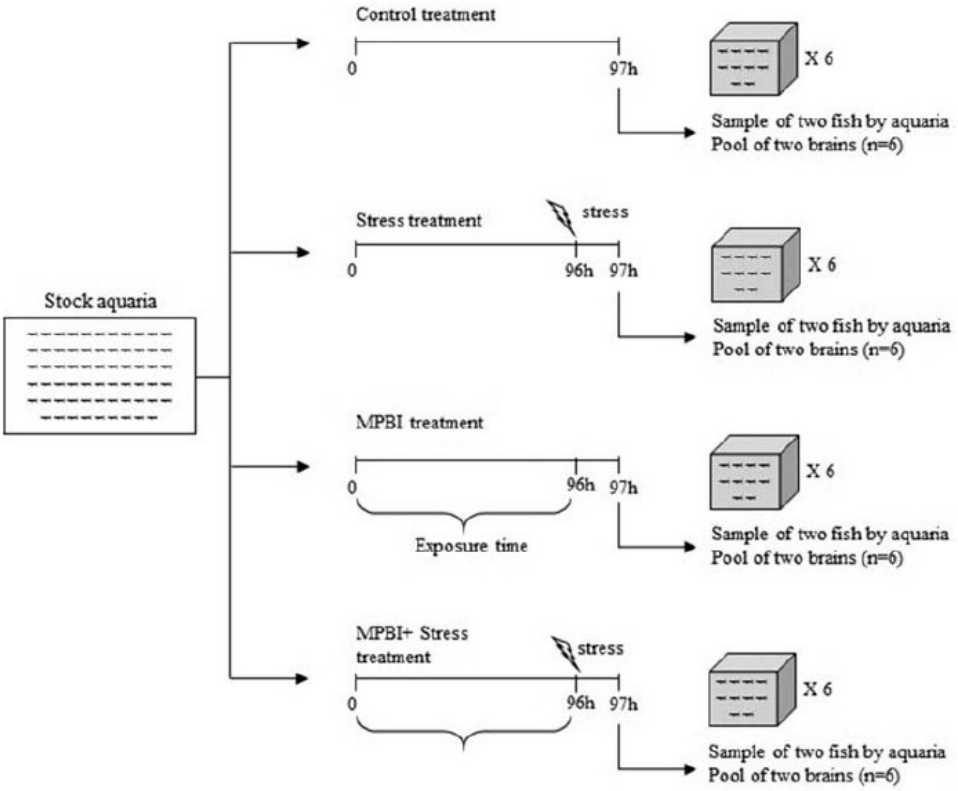

Figure 1. Schematic view of experimental design.

Table 1. Primer Sequences and PCR Amplification Conditions.

Gene	GenBank accession number	Primer sequences (5–3′)	PCR fragment (bp)	Anneling T_m (°C)	Cycles
GR	EF_436284	AACATGCTGTGTTTCGCTCC (forward) CTGCAAGCATTTCGGGAAAC (reverse)	401	62	35
StAR	BC_075967	TGCTATGTGCAACAAGGGCAAGAAGC (forward) GGACATTTACAAAGTCTCTTGGGC (reverse)	304	62	35
Hsp70	NM_131397	CCACCTGCGCCACGTGGCGTC (forward) CCTCCTCGCTGATCTTGCCTTTCAGG (reverse)	343	62	30
β-Actin*	AAC13314	GTCCCTGTACGCCTCTGGTCG (forward) GCCGGACTCATCGTACTCCTG (reverse)	678	54	35

*PCR primer sequences previously described (Chen et al., 2004).

low DNA mass ladder (Invitrogen, USA), and β-actin was utilized as an internal standard. The relative abundance of each mRNA versus β-actin was determined in the organs studied by densitometry using the freeware ImageJ 1.37 for Windows.

The concentration of MPBI in water was monitored daily from the moment of introduction to until 96 h of exposure. MPBI was analyzed by high-pressure liquid chromatography (HPLC) using the methodology described by Sabharwal and Belsare (1986), which was developed by Getz and Watts (1964) and modified by Jain et al. (1974). Water samples were extracted with chloroform, washed with *n*-hexane and acetonitrile, and evaporated to dryness at 50°C. The remaining precipitant was dissolved in acetone, filtered, and extracted with chloroform. After washing with sodium sulfate and adding 1% diethylene glycol, the solution was evaporated to dryness. The color was developed by adding cyclohexylamine and *p*-nitrobenzyl pyridine in equal proportion and heating at 175°C. The absorbance was read at 540 nm using a spectrophotometer. The methyl parathion content was determined from a previously prepared calibration curve. Immediately after

introduction, the concentration of MPBI measured in the water was 5.044 mg/L, and after 96 h, MPBI was detected at a concentration 45% lower than initially (2.808 mg/L) (Rosa et al., 2013).

Data are expressed as mean ± SEM and analyzed with the Graph Pad InStat 3.00 statistical package (GraphPad Software, San Diego, CA) by an analysis of variance (ANOVA), followed by Tukey's multiple range tests. Statistical significance was set at $p < .05$.

Results

No apparent mortality was observed in all groups. The expression levels of brain StAR, *hsp70*, and GR genes are depicted in Figure 2. Brain StAR (Figure 2A) and *hsp*70 (Figure 2B) expression levels in MPBI + S treated zebrafish were significantly lower than in all other treatments. Concerning brain GR (Figure 2C), no marked differences were found between the MPBI, S, and MPBI + S groups, but these treatments showed significantly high expression ratios when compared to C.

Discussion

Data demonstrated that exposure to combined MPBI and stress inhibited expression levels of brain StAR protein gene in zebrafish. The role of StAR protein in brain is not fully understood, but the significant decrease in its expression in the MPBI + S fish suggests a possible involvement of HPI axis activation or signaling, suggesting that brain StAR as a possible mechanism underlying impairment of steroid synthesis (Arukwe et al., 2008).

Since brain is a steroid-producing organ (Sierra, 2004) and since neurosteroids are postulated to play important roles in brain function and neuronal development (King et al., 2002, 2004; Sierra, 2004), the noted inhibition of brain StAR gene expression in fish exposed to MPBI is an interesting finding. Brain StAR expression was also altered in Atlantic salmon exposed to ethynylestradiol (Lyssimachou and Arukwe, 2007) and nonylphenol (Arukwe, 2005). Indeed, studies on steroidogenesis disruption focused mainly on levels of steroids and

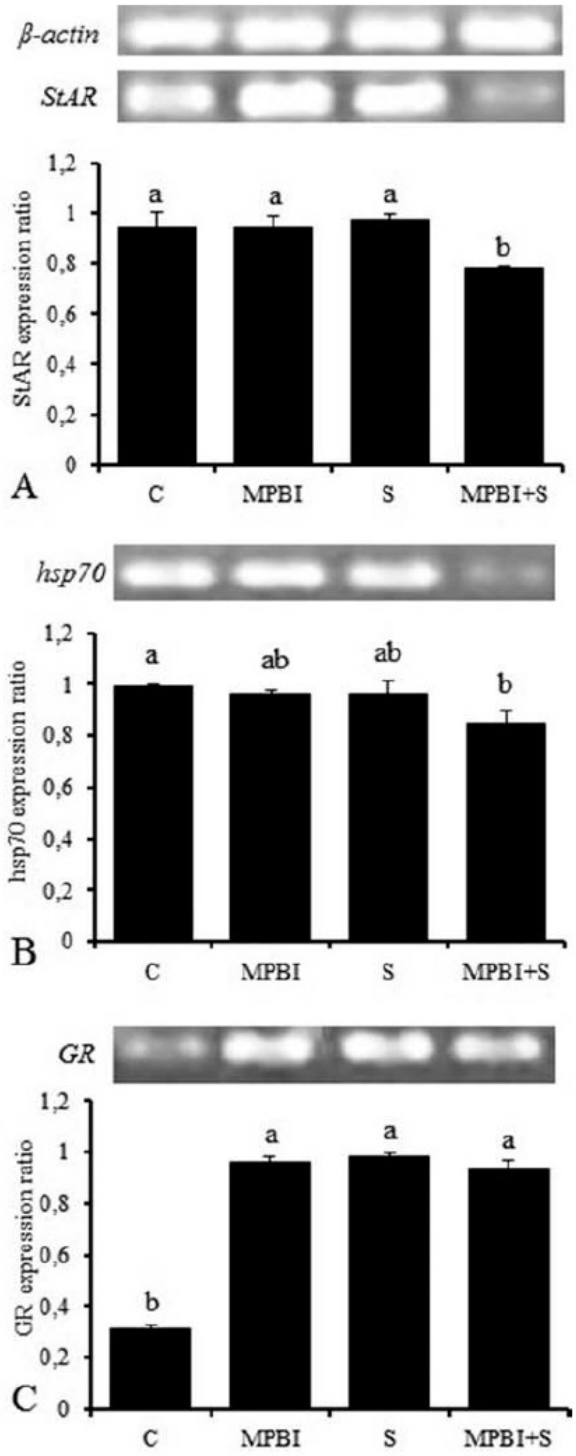

Figure 2. Effects of stress (S), acute exposition to methyl-parathion-based insecticide (MPBI), or stress plus acute exposition to MPBI (MPBI + S) on StAR (A), hsp70 (B), and GR (C) expression in zebrafish brain. Data are expressed as mean ± SEM. Different letters above the standard error bars indicate a significant difference between treatment groups (ANOVA followed by Tukey's multiple range test, $p < .01$; $n = 6$).

receptor-mediated effects (Armiliato et al., 2014) but few data are apparently available on the effects and mechanisms of endocrine modulators

on neural steroid-mediated effects (LaPage et al., 2011). The current research on chemical-induced endocrine disruption still needs to examine involvement of neural steroids and the process of steroidogenesis (Lyssimachou et al., 2006).

The diminished *hsp*70 expression in zebrafish in the MPBI + S group might be an indicator of cellular stress in the brain, which takes place when stress occurs concomitantly with contaminant exposure. As noted in PCB-exposed Arctic char (Aluru et al., 2004), *hsp*70 inhibition suggests a loss of neurons, especially those involved in the HPI axis function, in response to MPBI exposure in zebrafish. Reinforcing this argument, *hsp*70 is expressed in the brain, particularly in regions that coordinate neuroendocrine responses to stress (Blake et al., 1990). *Hsp*70 levels usually increase in fish of different species in response to stress and/or contaminant exposure, including in *Oreochromis niloticus* (Piner and Üner, 2013), *Cyprinus carpio*, (Jiang et al., 2012) and *Oncorhynchus mykiss* (Ceyhun et al., 2010). However, in the present study a decrease in the *hsp*70 expression levels was found. Although this may seem contradictory, expression levels of *hsp*70 were also reported to be lower in other studies, including in *Salvelinus alpinus* (Aluru et al., 2004), *Cyprinus carpio* (Xing et al., 2013), and *Salmo salar* (Olsvik et al., 2014). Differences in the species, exposure protocol, concentration, sampling timing, and methodology might all account for the disparate results both for *hsp*70 and brain StAR protein expression levels.

Brain *GR* expression in fish from the MPBI, S, and MPBI + S groups was elevated compared to the control. Similar results were noted by Gravel and Vijayan (2006), who exposed rainbow trout (*Oncorhynchus mykiss*) to salicylate and reported that brain GR mRNA levels were markedly increased. In contrast, inhibition of GR expression levels occurred in *Salvelinus alpinus* exposed to polychlorinated biphenyls (PCB) (Aluru et al., 2004). The difference between these results may be related to different exposure intervals, chemical differences between compounds, and/or variations in protocols employed.

Finally, since the expression of StAR and *hsp*70 was not determined in interrenal tissue, our data did not shed light on whether a relationship exists between altered cortisol levels and diminished brain StAR and *hsp*70 expression levels. However, inhibition of StAR and *hsp*70 gene expression levels were significantly reduced in stressed fish exposed to MPBI, a finding that requires further investigation. It would also be interesting to evaluate these parameters if animals were initially exposed to stress followed by MPBI treatment. Our overall results suggest that environmental manipulations affect GR expression to a greater extent than expression of StAR or *hsp*70. It is also possible that these genes respond in a different time manner, which might account for different observations.

A major limitation in our study was the use of only one concentration of MPBI, and of only one gender, males. However, this does not lessen the importance of our findings, as at present data regarding the effects of agrochemicals on gene expression in fish brain are scarce. Taken together, the results presented herein showed that MPBI produced a downregulation in brain StAR and *hsp*70 gene expression. However, the biological relevance of these observations requires further investigations. Based upon our data, it would appear that the expression of these proteins in the brain might serve as sensitive diagnostic tools for acute exposure of fish to methyl parathion and as an initial point for studying the effects of MPBI on neurosteroid production.

Ethics note

This study was approved by the Ethics Commission for Animal Use (CEUA) at Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol 3/2011-CEUA), and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

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CAPÍTULO III

ARTIGO

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RESEARCH ARTICLE

WILEY ENVIRONMENTAL TOXICOLOGY

Muscarinic receptors mediate the endocrine-disrupting effects of an organophosphorus insecticide in zebrafish

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Abstract

The glucocorticoid cortisol, the end product of hypothalamus-pituitary-interrenal axis in zebrafish (*Danio rerio*), is synthesized via steroidogenesis and promotes important physiological regulations in response to a stressor. The failure of this axis leads to inability to cope with environmental challenges preventing adaptive processes in order to restore homeostasis. Pesticides and agrichemicals are widely used, and may constitute an important class of environmental pollutants when reach aquatic ecosystems and nontarget species. These chemical compounds may disrupt hypothalamus-pituitary-interrenal axis by altering synthesis, structure or function of its constituents. We present evidence that organophosphorus exposure disrupts stress response by altering the expression of key genes of the neural steroidogenesis, causing downregulation of *star*, *hsp70*, and *pomc* genes. This appears to be mediated via muscarinic receptors, since the muscarinic antagonist scopolamine blocked these effects.

KEYWORDS

acetylcholine receptors, *Danio rerio*, methyl-parathion, scopolamine, steroidogenesis

1 | INTRODUCTION

Steroidogenesis is an essential process for several biological reactions in order to maintain homeostasis. A key step for steroid synthesis is the transport of cholesterol to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR).[1] The cleavage of cholesterol into pregnenolone is the first step to the synthesis of all steroid hormones, including cortisol, the corticosteroid produced in response to activation of an important neuroendocrine axis in teleost fish and humans.[2,3]

In zebrafish, cortisol is the final product of the hypothalamus-pituitary-interrenal (HPI) axis, and promotes important adaptive processes involved in metabolism and immune function.[4,5] Stressor stimuli excite the parvocellular neurons in the hypothalamus, activating the HPI axis and promoting the synthesis and release of corticotropin-releasing factor (CRF), which leads to synthesis and release of proopiomelanocortin (POMC). The adrenocorticotropic hormone (ACTH), a trophic hormone derived from POMC stimulates the interrenal cells to synthesize and release corticosteroids.[6–8]

As a neuronal axis, the HPI is susceptible to interference from several factors that may interrupt, attenuate, or simulate its biological effects. Several components are involved in the functioning and maintenance of the HPI axis. Proteins like StAR and HSP70 are essential to

adequate steroidogenesis and may be altered by chemical compounds, as well the mRNA expression of trophic hormones. Substances that block the endocrine systems and neuroendocrine axis are called endocrine disruptors, and many chemical compounds are classified as such. Organophosphate (OP) insecticides are still widely used pesticides and constitute a group of chemicals with known endocrine disrupting properties.[9–11] Methyl-parathion based insecticides (MPBI) are OP substances that inhibit cholinesterase widely used on agriculture to control insect plagues. The crescent use of agrichemical pesticides is proportional to the demand for food quality and volume production. They are known to reach and contaminate water sources, affecting both marine and freshwater.[12] The exposure of nontarget organisms may occur directly, when the pesticide is in the water, or indirectly, when the runoff of agrichemical causes contamination pulses.

Here we utilized adult zebrafish to investigate the biological consequences of exposure to MPBI, in a static test of acute exposure. The subjects were exposed to MPBI with concomitant stress challenge and, since MPBI is an organophosphorus insecticide that inhibits cholinesterase activity, were also administered the muscarinic antagonist scopolamine, in order to evaluate its possible reverting effects along with MPBI exposure. Our study focuses the molecular effects in key genes of steroidogenic pathway, and proteins and receptors that maintain the correct functioning of HPI axis were studied. In addition, we explored the toxicity mechanisms for its possible modulation.

2 | METHODS

2.1 | Ethical note

All methods were executed with the accomplishment of directives of Animal Use Ethics Committee (CEUA-UPF, protocol 006-2012).

2.2 | Drugs

We used scopolamine hydrobromide trihydrate (CAS: 6533-68-2). Methyl-parathion based insecticide (MPBI), commercially known as Folisuper600™ (O,O-dimethyl O-4-nitrophenyl phosphorothioate, 600 g/L CAS: 298-00-0).

The drugs scopolamine (200 μM) and MPBI (5.2 mg/L) were administrated directly on water. The MPBI concentration used was corresponding to 10% of LC50 for zebrafish. This concentration was based on previous studies.[13–15] Although may be considered a high concentration, it can easily be found on environment due to phenomena like biomagnification.[16] The concentration of MPBI in the water was monitored immediately, 48 and 96 h after application, by high-pressure liquid chromatography (HPLC) using the general methodology described by Zanella et al.,[17] along with specific methodology for MPBI.

2.3 | Subjects

The experimental population consisted in 200 mixed-sex 6-month-old adult wild-type zebrafish (*Danio rerio*) held on adaptation aquaria (40 L, with constantly aerated dechlorinated tap water) for 7 days. The zebrafish weighted approximately 0.7 g each and were obtained from a commercial supplier (Delphis, Porto Alegre, RS, Brazil). Fish were housed in groups of 25, kept under a 14/10-h day/night cycle, and fed with commercial food (TetraMin). Because cortisol is a glucocorticoid that might be influenced by starvation,[18] the fish were fed daily during the 96 h exposure (24, 48, and 72 h after the beginning of exposure) at a rate of 0.75% of their biomass. Throughout the experiment, water temperature was 28 ± 2°C, and water pH, dissolved oxygen and total ammonia was held in adequate levels for the species.

2.4 | Experimental design

After the acclimation period, fish were placed on experimental sets (25 fish per aquaria) and subjected to a static test. The experimental design involved four groups: the first group consisted of fish in normal conditions named control group; a second group only submitted to a stressor stimulus; a third group, exposed to methyl-parathion based insecticide (MPBI) for 96 h and then submitted to stressor; and a fourth group exposed to MPBI for 96 h and to scopolamine for 2 h, and finally submitted to a stressor stimuli. The stressor stimulus consisted of 2-min net chase, already described as effective in activate the HPI axis.[19] To perform the exposure to a muscarinic antagonist, it was administrated a solution of scopolamine (diluted in distilled water) directly on test aquaria to a final concentration of 200 μM.[20] The start of scopolamine exposure was determined by its pharmacokinetic properties, to assure that in the moment of stress treatment the drug was already systemically distributed. In addition, the duration of exposure was based on substance half-life (approximately 4 h). The experimental protocol is depicted on Figure 1. Fifteen minutes after the stressor stimulus, fish were transferred to another test aquaria to perform the behavioral tests. The behavior was assessed by novel tank test (NTT).[21–23] In this test, the fish were individually placed in a glass test tank (24 × 8 × 20 cm; width × depth × height) and filmed for 6 min. The videos were analyzed using ANY-maze® software (Stoelting CO, USA) for animal behavior analysis and total distance (m), mean speed (m/s) and absolute turn angle were scored. After behavioral analysis, the fish were captured and euthanized by immersion in ice-water bath (4°C or less). Briefly, the fish were immersed in liquid nitrogen to perform the brain dissection and cortisol measurement. The whole-body cortisol was determined by enzyme-linked immune sorbent assay kit (EIAgen CORTISOL test, Bio Chem Immuno Systems) from tissue extracts resuspended in PBS buffer.[15,24,25] To perform the brain dissection, the animals were removed from nitrogen, and the procedure was executed with the animal frozen, to prevent RNA degradation and diminished the possibility of sample contamination.

2.5 | Sample collection and cDNA synthesis

Each sample consisted in a pool of five brains. The total RNA was purified from approximately 30 mg of brain pooled using RNeasy Mini Kit (Qiagen, Hilden, Germany). The genomic DNA was treated with RNase-Free DNase Set (Qiagen, Hilden, Germany) and the cDNA

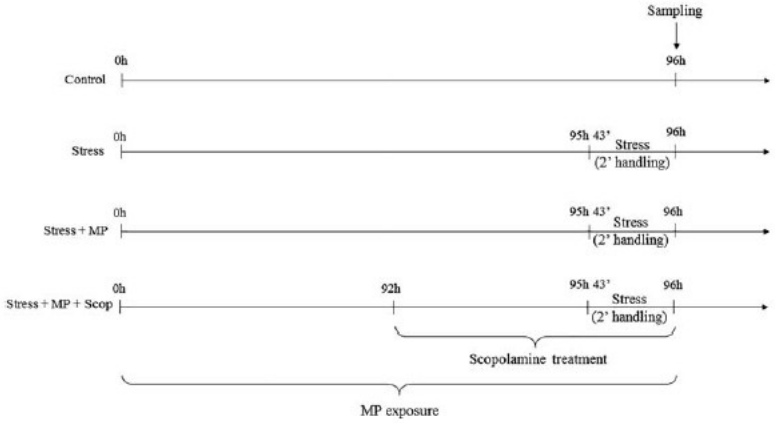

FIGURE 1 Schematic representation of the experimental design

synthesis was performed by reverse transcription using a QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany).

2.6 | Primers and PCR internal control

The set of primers (*star*, *pomc*, *bgr*, *crf*, *hsp70*, *c-fos*, *β-actin*, *elongation factor 1-alpha* and *β-2m*) used to determine gene expression is described in Table 1. To construct the internal controls, cDNA obtained from one sample (stressed fish) was amplified using the primers described in Table 1 and was included a final step of 72°C for 30 min. The PCR-specific product was cloned into the pGEM-T-Easy vector (Promega) according to the manufacturer's instructions.

2.7 | Quantitative real-time PCR (qPCR) analysis

qPCR was carried out in 48-well plates (MicroAmp® Optical 48-Well Reaction Plate, Applied Biosystems) in a total volume of 20 μL containing 500 nM of each primer, 1× SYBR Select Master Mix (Applied Biosystems) and 1 μL of cDNA. PCRs were run on a StepOne™ Real-Time PCR System (Applied Biosystems), using the following thermocycling conditions: an initial denaturation step (95°C, 10 min) followed by 40 cycles of 95°C for 30 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 s (extension). The annealing temperature for *hsp70* was adjusted at 48°C. Calibration curves for all molecules were obtained using tenfold serial dilutions of a plasmid DNA containing the specific nucleotide sequence (PCR internal control) of each gene. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[32] Afterwards, fold change values ($2^{-\Delta\Delta Ct}$) were Log-transformed as recommended by Willems et al.[33]

2.8 | Statistical analysis

The normal distribution of the data was confirmed by Kolmogorov-Smirnov and Levene tests. The data are expressed as mean ± standard error of mean (SEM) and results were analyzed by one-way ANOVA followed by Tukey's post hoc test, except for the locomotor parameters that were analyzed by Kruskal-Wallis test since its distribution were not normal. Differences were considered significant at $P < .05$.

3 | RESULTS

MPBI was persistent in the water after 96 h. Immediately, 48 h and 96 h after the inoculation, MPBI concentration was 5.04, 3.69, and 2.86 mg/L, corresponding to 97%, 71%, and 55% of the initial nominal concentration, respectively.

TABLE 1 Primer sequences used to perform real-time reverse transcriptase-polymerase chain reaction assay

Gene name	Primer sequence (5'-3')	Reference	Efficiency (%)
bactin1	F) CGAGCAGGAGATGGGAACC; R) CAACGGAAACGCTCATTGC	Keegan et al.26	98.1
β-2-microglobulin	F) GCCTTCACCCCAGAGAAAGG; R) CGGTTGGGATTTACATGTTG	McCurley and Callard27	99.2
elongation factor 1-α	F) CTTCTCAGGCTGACTGTGC; R) CCGCTAGCATTACCCTCC	Chen et al.28	100
hsp70	F) GTC TTA CGC CTT CAA CAT; R) TTG GAG ATG ACT GGA TTG	Zhang et al.29	97.2
star	F) CCTGTTTTCTGGCTGGGATG; R) GGGTCCATTCTCAGCCCTTAC	Fuzzen et al.8	98.7
pomc	F) CGCAGACCCATCAAGGTGTA; R) CGTTTCGGCGGATTCCT	Fuzzen et al.8	98.9
crf	F) ACGCACAGATTCTCCTCGCC; R) TCCGCGGCTGGCTGATT	Dhanasiri et al.30	98.5
bgr	F) ACAGCTTCTTCCAGCCTCAG; R) CCGGTGTTCTCCTGTTTGAT	Alsop and Vijayan5	99.2
cfos	F) CAGCTCCACCACAGTGAAGA; R) GCTCCAGGTCAGTGTTAGCC	Moore and Whitmore[31]	98.5

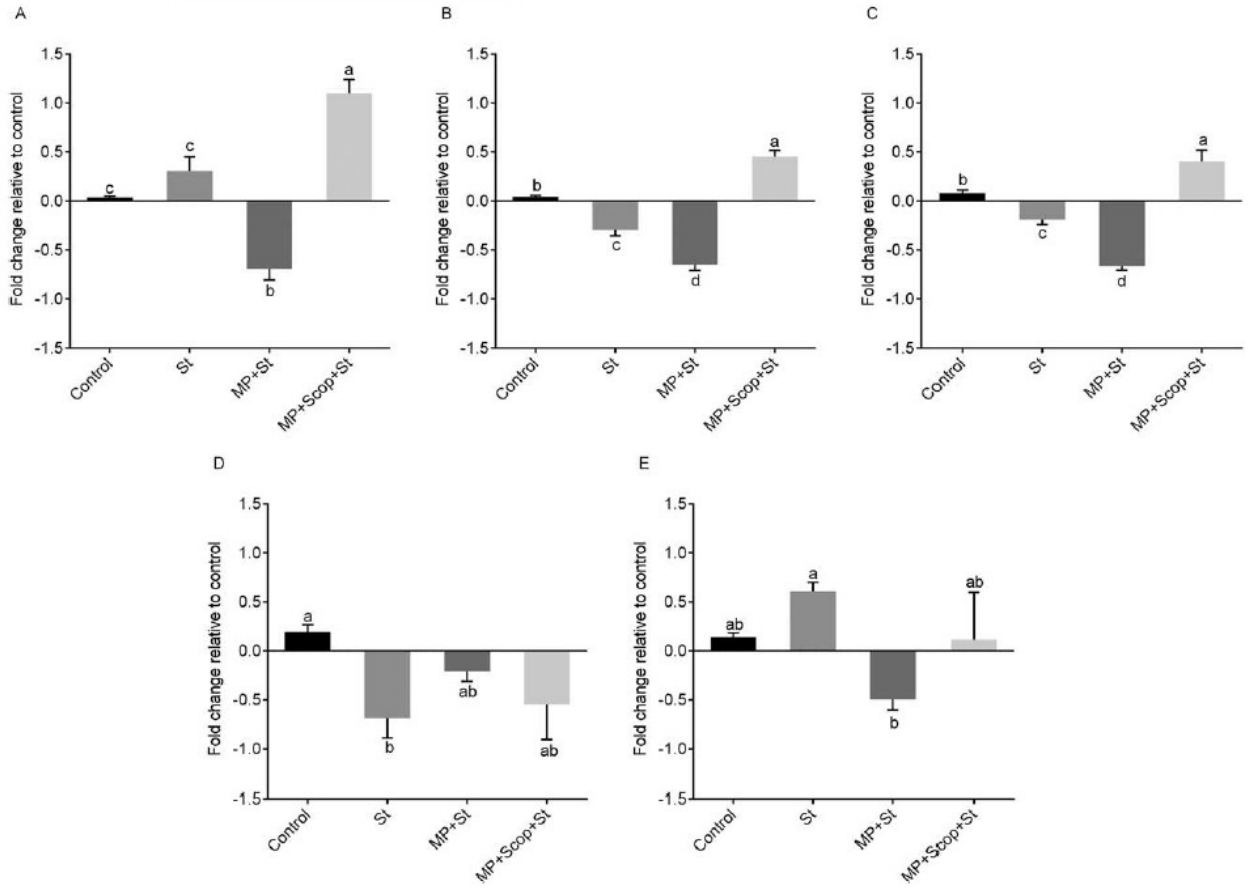

FIGURE 2 Relative mRNA expression of *star* (A), *pomc* (B), *bgr* (C), *crf* (D), and *hsp70* (E) of stressed fish (St), fish exposed to MP + stress (MP + St) and fish exposed to MP, scopolamine and stress (MP + Scop + St). Data expressed by mean ± SEM. Means were compared by one-way ANOVA followed by Tukey's multiple range post hoc test. Different letters indicate statistical differences between treatment means. $N = 5$

3.1 | Stress response related genes

The stressor was able to increase the expression of *star* and *hsp70* mRNA, reaching 0.3-fold (*star*, Figure 2A) and 0.6-fold (hsp70, Figure 2E), which did not happen with concomitant exposure to MPBI (MPBI + St), which treatment decreases the mRNA expression in 0.96-fold (*star*) and 0.49-fold (*hsp70*) (Figure 2A,E, respectively). However, the exposure to scopolamine before stress provoked a 1.10-fold upregulation of *star*, being significantly different from all treatments, and 0.11-fold for *hsp70*.

Regarding *pomc* (Figure 2B), the stress protocol decrease the expression in 0.3-fold, and the combination of stress and MPBI exposure caused a decreased expression of 0.64-fold. However, the administration of scopolamine before stress leaded to an increased expression of 0.45-fold, significantly different from other groups. The same pattern of decreases occurred for *bgr* (Figure 2C), where the treatments St and St + MPBI decreased the expression in 0.18 and 0.66-fold, respectively. As occurred with *pomc*, the administration of scopolamine increased the mRNA expression in 0.40-fold.*crf* mRNA expression (Figure 2D) were only altered on one treatment, where the stressor provoked the decrease of expression in 0.68-fold.

3.2 | Whole-body cortisol

The control group presented usual values for cortisol, consistent with previous results from our group. The stressor stimulus was effective in producing an acute stress response, with marked cortisol elevation ($F_{(3,28)} = 31.15$, $P < .0001$, Figure 3). The fish exposed to MPBI did not respond to the stressor stimulus. However, the exposure to scopolamine seems to block the blunted stress response induced by MPBI.

3.3 | c-fos

The expression of *c-fos* mRNA were evaluated in zebrafish brain. Concerning to *cfos* mRNA expression (Figure 4), the St + MPBI treatment caused a decrease of 0.55-fold, while the administration of scopolamine provoked an up regulation of 0.65-fold.

3.4 | Locomotor parameters

We also evaluated the effects of stress, MPBI and scopolamine, isolated or combined, on locomotor activity in zebrafish. The locomotor parameters of the control group showed expected values. However,

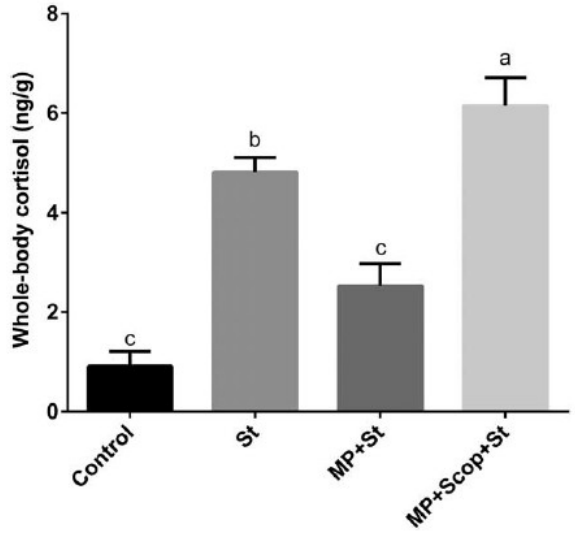

FIGURE 3 Whole-body cortisol concentrations of control fish (C), stressed fish (St), fish exposed to MP + stress (MP + St) and fish exposed to MP, scopolamine and stress (MP + Scop + St). Data were expressed by mean ± SEM of eight fish. Means were compared by one-way ANOVA followed by Tukey's multiple range post hoc test. Different letters indicates statistical differences between treatment means

the stressed group showed abnormal results. This could be due to interval between stress submission and novel tank test, which may lead to a restoration of parameters to basal levels. Distance and mean speed were similar in both control and stressed group (Figure 5A,B). On the other hand, the exposure of stressed animals to MPBI caused a decrease in these parameters. Similarly, the administration of scopolamine on stressed/exposed group reduced distance and mean speed. Regarding to absolute turn angle (Figure 5C), all treatments decreased this parameter when compared to control.

4 | DISCUSSION

Here we show that organophosphorus MPBI blunts the stress response in zebrafish, probably by reducing the expression of some key genes of the steroidogenic pathway. In addition, we provided evidence that these effects were mediated by muscarinic receptors, since the muscarinic antagonist scopolamine reverted the MPBI-induced impairment of HPI axis response. Regarding the impaired cortisol synthesis/release after MPBI exposure, we confirmed our previous results.[11,15] Since the stressed group showed expected values for cortisol, our stress protocol showed to be effective in activating the HPI axis.[15,18,19,34] However, with the exposure of stressed group to MPBI, this pattern did not occur, indicating that MPBI blocked the axis, preventing the synthesis or release of cortisol.[9,11,35]

It is currently accepted that the cortisol stress response is coordinated by HPI axis; thus, all its regulating factors can be targeted by contaminants. Previous studies correlate the acute stress response with the expression of StAR in zebrafish.[8,34] The StAR protein is a key factor for steroids synthesis, and the *star* gene expression is also a target for many environmental toxicants, representing a possible cause for endocrine disruption.[36,37] The steroidogenesis impairment by StAR protein expression inhibition has been described both pre- and post-transcriptionally.[15,38,39] We found that the exposure to an OP compound impaired the expression of brain *star*, *pomc*, *bgr* and *hsp70* mRNA, essential components of HPI axis functioning. The disruption of StAR protein expression by chemical substances can trigger biological reactions that influences all physiological systems.

Although being a target for the action of steroids, the brain is also considered a steroidogenic organ, where occurs *de novo* synthesis of steroids, and, the steroid concentration in plasma and brain seems to oscillate independently.[35] Since several brain cell populations, such as neurons and glia,[40,41] are able to synthesize steroids, the presence of StAR protein in these cells is essential to neuro steroidogenesis. It is known that trophic hormones act via second messengers, and its stimulation induces the increase of intracellular cAMP.[42] The regulation of *star* mRNA expression is controlled primarily by activation of cAMP, since several studies demonstrated that *star* expression is responsive through stimulation of the cAMP pathway.[40,43–45]

The classical effects of OP compounds are primarily due to prevention of the acetylcholine hydrolysis by acetylcholinesterase on cholinergic transmission sites. Thus, the accumulation of neurotransmitter potentiates the pharmacologic action of acetylcholine in muscarinic receptors (mAChR). The mAChR subtypes M2 and M4 bound to G_i protein, causing the inhibition of adenylyl cyclase and leading to a decrease of cAMP. Reduced cAMP caused by M2 and M4 activation is a possible mechanism to decrease *star* mRNA.[39,43–45]

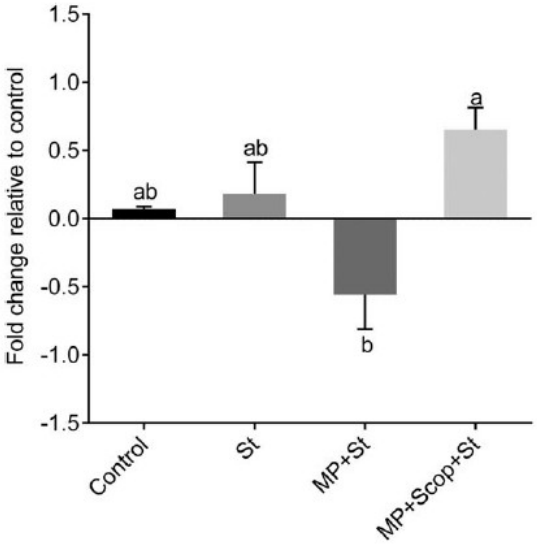

FIGURE 4 Relative mRNA expression of *c-fos* of stressed fish (St), fish exposed to MP + stress (MP + St) and fish exposed to MP, scopolamine and stress (MP + Scop + St). Data were expressed by mean ± SEM of five fish. Means were compared by one-way ANOVA followed by Tukey's multiple range post hoc test. Different letters indicate statistical differences between treatment means

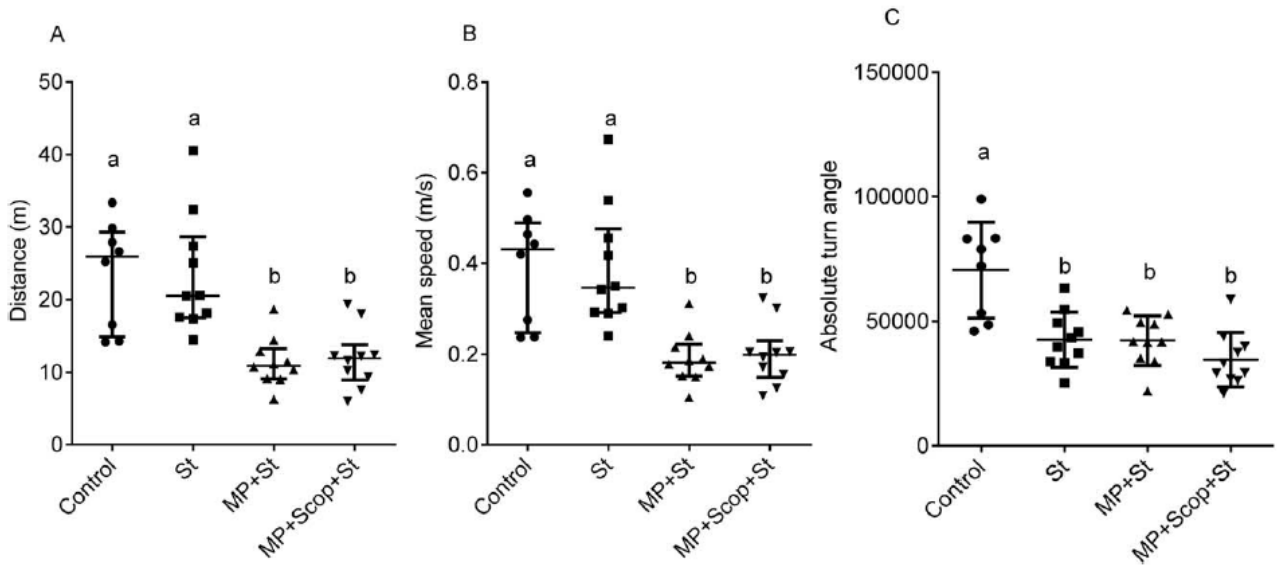

FIGURE 5 Locomotor parameters in control, stressed, stressed fish exposed to MP and stressed fish exposed to MP and treated with scopolamine. (A) total distance; (B) mean speed and (C) absolute turn angle. Data were expressed by median with interquartile range and compared by Kruskal-Wallis. Different letters indicates statistical differences between treatment means. $N = 10$

The cholinergic hyperactivity that may be caused by OP exposure[10] could lead to cAMP inhibition, and consequently affecting *pomc* expression. As a trophic hormone, CRF signalize for *pomc* synthesis via cAMP pathway. In *Rhamdia quelen* exposed to methyl-parathion, the same pattern of response was reported, where the fish could not mount an appropriated cortisol stress response.[46]

Scopolamine is a muscarinic competitive antagonist that acts as a ligand targeting the mAChR. After an initial receptor activation, the stimulus ends and the inhibitory activity of M2 and M4 ceases, resulting in regeneration of cAMP levels, consequently increasing *star* and *pomc* expression and reverting the OP effects. An alternative hypothesis is the decrease of neurosteroids synthesis. In brain, steroids are considered trophic factors, and play an important role in development and survival of neurons.[47,48] It is known that the hypothalamus is sensitive to neurosteroids,[49] and its endocrine function is related to the effects of local steroids. Since treatment with OP caused the decreased expression of *star* and *pomc*, it is plausible to assume that the hypothalamus was not stimulated, thus there was no activation of the HPI axis. In addition, neurosteroids are involved in neuron repair after injuries,[50] indicating another possible cause: the lack of neurosteroids caused by OP exposure. The toxic effects of OP may be responsible for neuronal instability, explained by the reduction of neuron protection, which may be reflected on the activation of the HPI axis. We hypothesized that the reverser effect of scopolamine is mediated by M2 and M4-subtype receptors inhibitory activity. We suggested this event based on both our and literature reports, despite the fact that are no evidence of drugs with mAChR subtype specificity.[51]

HPI axis is primarily activated by CRF and its transcription is promoted as a response to a stressor stimulus.[52] The metabolic adjustments of cortisol, as well as the control of physiologic levels are mediated by glucocorticoid receptors (GR). GR and HSP70 are important components of stress response modulating is activity. In our study, *bgr* mRNA levels were down regulated on St group. This could indicate the beginning of a negative feedback loop, in order to regulate the cortisol levels after a stressor event.[34,53] Interestingly, in MP + St exposed fish, a more intense down regulation occurred. This could be related to the HPI axis disruption, where the pesticide provoked a disturbance on the negative feedback mechanism, leading to an impairment on cortisol synthesis.[54]

The HSPs are proteins of a high conserved system of cell protection and repair against cell damage. These proteins occur in all living beings, from bacteria to humans and can be considered targets of chemical threats of the environment, including serving as biomarkers.[55,56] Particularly HSP70 is a valuable toxicant biomarker and could indicate stress, both for fishes and other aquatic species.[57,58] Since HSP proteins are essential components of biological systems, and our results showed that stress treatment provoked an slightly increase the *hsp70* expression, this indicates that the HPI axis was functionally adequate, however this did not happen when the subjects are exposed to MPBI, which decrease the *hsp70* expression. This suggests that this effect contributed to the cortisol response impairment, preventing a satisfactory response to the stress stimulus. This appears to be reverted by administration of scopolamine, since *hsp70* mRNA was comparable to control values in this group.

It is known that neuropeptides (i.e. CRF) are expressed in certain areas of hypothalamus, as preoptic nuclei,[59] and since our study evaluated the whole brain, *crf* expression values may be underestimate, since the specific areas were not evaluated. In addition, duration and nature of stressor stimulus, differences associated with the species and its genetic background, as well methodologies of analysis could alter mRNA expression patterns.

C-fos is an reliable marker of neuronal activity, and its expression may be induced by stressors.[60] Chen et al.[61] demonstrated that C-fos is, among other areas, expressed in the habenula, a preserved area

amongst vertebrates,[62] associated with limbic system and monoaminergic neurons in the brain.[63,64] The habenula neurons activation can induce several behavioral alterations, including anxiety and depression.[65,66] As our treatment with scopolamine caused and overexpression of *cfos*, the decreased locomotor parameters that we found, which could be an indicative of anxiety, may be explained by these behavioral alterations caused by *cfos* activation. However, the same pattern of locomotor alterations was observed in MPBI + St group. The decrease locomotion could be a direct effect of the MP exposure, being one classic sign of OP mechanism of action

However, since our samples were constituted by homogenates of whole-brain, our results are indicative of this phenomenon, and an analysis of specific brain areas are needed to confirm this hypothesis.

Here we used the zebrafish to evaluate the effects of OP compounds on the HPI axis. In summary, we show that MPBI can modulate the neural steroidogenesis, through the inhibition of *star* and *pomc* gene expression. This reflects systemically causing a cortisol synthesis blockage. However, since the mechanism of action of OP is the inhibition of acetylcholinesterase, we show that this effect can be blocked by administration of a muscarinic antagonist, indicating that these are related to hyperactivity of cholinergic transmission.

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Fish Aversion and Attraction to Selected Agrichemicals

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Abstract In agriculture intensive areas, fishponds and natural water bodies located in close proximity to these fields receive water with variable amounts of agrichemicals. Consequently, toxic compounds reach nontarget organisms. For instance, aquatic organisms can be exposed to tebuconazole-based fungicides (TBF), glyphosate-based herbicides (GBH), and atrazine-based herbicides (ABH) that are potentially dangerous, which motivates the following question: Are these agrichemicals attractant or aversive to fish? To answer this question, adult zebrafish were tested in a chamber that allows fish to escape from or seek a lane of contaminated water. This attraction and aversion paradigm was evaluated with zebrafish in the presence of an acute contamination with these compounds. We showed that only GBH was aversive to fish, whereas ABH and TBF caused neither attraction nor aversion for zebrafish. Thus, these chemicals do not impose an extra toxic risk by being an attractant for fish, although TBF and ABH can be more deleterious, because they induce no aversive response. Because the uptake and bioaccumulation of chemicals in fish seems to be time- and dose-dependent, a fish that remains longer in the presence of these substances tends to absorb higher concentrations than one that escapes from contaminated sites.

Tebuconazole-based fungicides (TBF) and glyphosate-based (GBH) and atrazine-based herbicides (ABH) are largely utilized in agriculture. TBF have been used in several plant cultures or as wood preservatives (Lebokowska et al. 2003). TBF disrupt the endocrine stress response (Cericato et al. 2008, 2009; Koakoski et al. 2014) and provoke severe oxidative stress in fish (Ferreira et al. 2010, 2012, 2013; Toni et al. 2011). GBH and ABH have

been widely used throughout the world in some cultures that have huge cultivated areas, such as soy and bean. GBH and ABH have been shown as moderate endocrine disruptors but also act as oxidative stressors for fish (Cericato et al. 2008, 2009; Koakoski et al. 2014; Ferreira et al. 2010, 2012, 2013; Toni et al. 2011).

Fish depend on chemoreception to deal with many environmental challenges, such as finding food (Moyle and Cech 2000) and mates (Stacey and Sorensen 2005), aggregation or schooling (Sorensen and Stacey 1999), or avoiding predators (Døving et al. 2005). The execution of these behaviors is based on the logic of approaching or avoiding attractant or aversive stimuli. Animals can be attracted (Kessler et al. 2015) or repelled (Tierney et al. 2007) by toxic substances, and the deleterious effects that substances, such as GBH, ABH, and TBF, can impose may be more or less pronounced, acting as attractive or aversive chemical stimuli. Both attraction or aversion reactions have strong biological and toxicological significance. The risk of fish seeking contaminated sites is more direct and easy to perceive. Otherwise, fish that remain for longer periods in the presence of these substances (attractive or not perceived) tend to absorb higher concentrations than ones that escape from contaminated sites (aversive substance). Thus, in an environment contaminated with a substance with repellent properties, the fish will actively avoid the area, which, in turn, may change population dynamics and behavior.

For these reasons, our question is plausible, and to address it, we used adult zebrafish as the animal model for testing in a chamber that allows fish to escape from or seek a lane of TBF, GBH, and ABH contaminated water.

Methods

Ethical Note

This study was approved by the Ethics Commission for Animal Use (CEUA) at Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol 29/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Animals

Adult (180 days) wild-type zebrafish (*Danio rerio*) of the short-fin (SF) strain, mixed-sex (50:50), were used as our stock population. The fish were fed twice a day, at 10:00 and 16:00 h, with commercial flaked food provided to satiation (Alcon® Basic, MEP 200 Complex, Brazil). The mean water temperature in the holding tank was maintained at 24 ± 2 °C, and the dissolved oxygen concentrations varied from 5.6 to 7.2 mg/l. The pH values ranged from 6.2 to 7.4. The total ammonia–nitrogen concentration was less than 0.5 mg/l.

Agrichemicals Tested

All chemicals were obtained from commercial suppliers. The agrichemicals that were used are a tebuconazole-based fungicide (Tebufort DVA, 200 g/l of RS-1-p-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl) pentan-3-ol, CAS# 107534-96-3), glyphosate-based herbicide (Roundup Original™, 360 g/l of *N*-phosphonomethylglycine, CAS# 1071-83-6), and an atrazine-based herbicide (Siptram 500SC, 500 g/l of 6-chloro-N_2-ethyl-N_4-isopropyl-1,3,5-triazine-2,4-diamine, CAS# 1912-24-9).

We chose an environmental concentration already related in the literature and 10 % of a previously determined $LC_{50\text{–}96\ h}$. This percentile of $LC_{50\text{–}96\ h}$ was based on previous works in our laboratory using these specific contaminants (Kreutz et al. 2010). The nominal agrichemical concentrations in the water were confirmed by high-pressure liquid chromatography (HPLC) using the general methodology described by Zanella et al. (2003), along with specific methodologies for TBF (Zhao et al. 2008) and GBH (Hidalgo et al. 2004). The agrichemical concentrations and references are depicted in Table 1.

Experimental Apparatus

The experimental apparatus consisted of a 30-L acrylic tank (Fig. 1a; 50 × 25 × 25 cm, length × width × height) as described in Abreu et al. (2016). Briefly, the apparatus had two chambers leading to two lanes of water with laminar flow running in parallel without mixing. A flow rate of 2 l/min was used for each track, and the manifold for each mixing chamber had a single door to allow for the introduction of the test substance.

Experimental Protocol

The basic strategy of the present study consisted of a two-choice test first used by Korver and Sprague (1989), then adapted to test anesthetics (Readman et al. 2013) and psychoactive drugs (Abreu et al. 2016), in which we quantified, in individually reared zebrafish, the choice for paths having contaminated or clean water flows. Choice was operationally set as the time spent in each path and the shuttle frequency between them. To conduct this choice test, fish from our stock tank were transferred to the experimental tank. After the transfer, fish were allowed to acclimate for 150 s, and subsequently a dose of the test compound at a predetermined concentration was introduced into one of the mixing chambers for a period of

Table 1 Nominal and measured concentrations of agrichemicals used

Substance (reference to environmental and LC50 concentrations)	Concentrations (mg/l)	
	Nominal	Measured (%)
GBH 10 % CL50 (own data)	5.2	5.148 (99 %)
GBH env. (Tierney et al. 2009)	0.00659	0.006853 (104 %)
TBF 10 % CL50 (Sánchez et al. 2012)	26.8	26.07 (97.3 %)
TBF env. (Elsaesser and Schulz 2008)	0.2	0.202 (101 %)
ABH 10 % CL50 (Al-Sawafi and Yan 2013)	1	1.03 (103 %)
ABH env. (Pratt et al. 1997)	9.567	9.37566 (98 %)

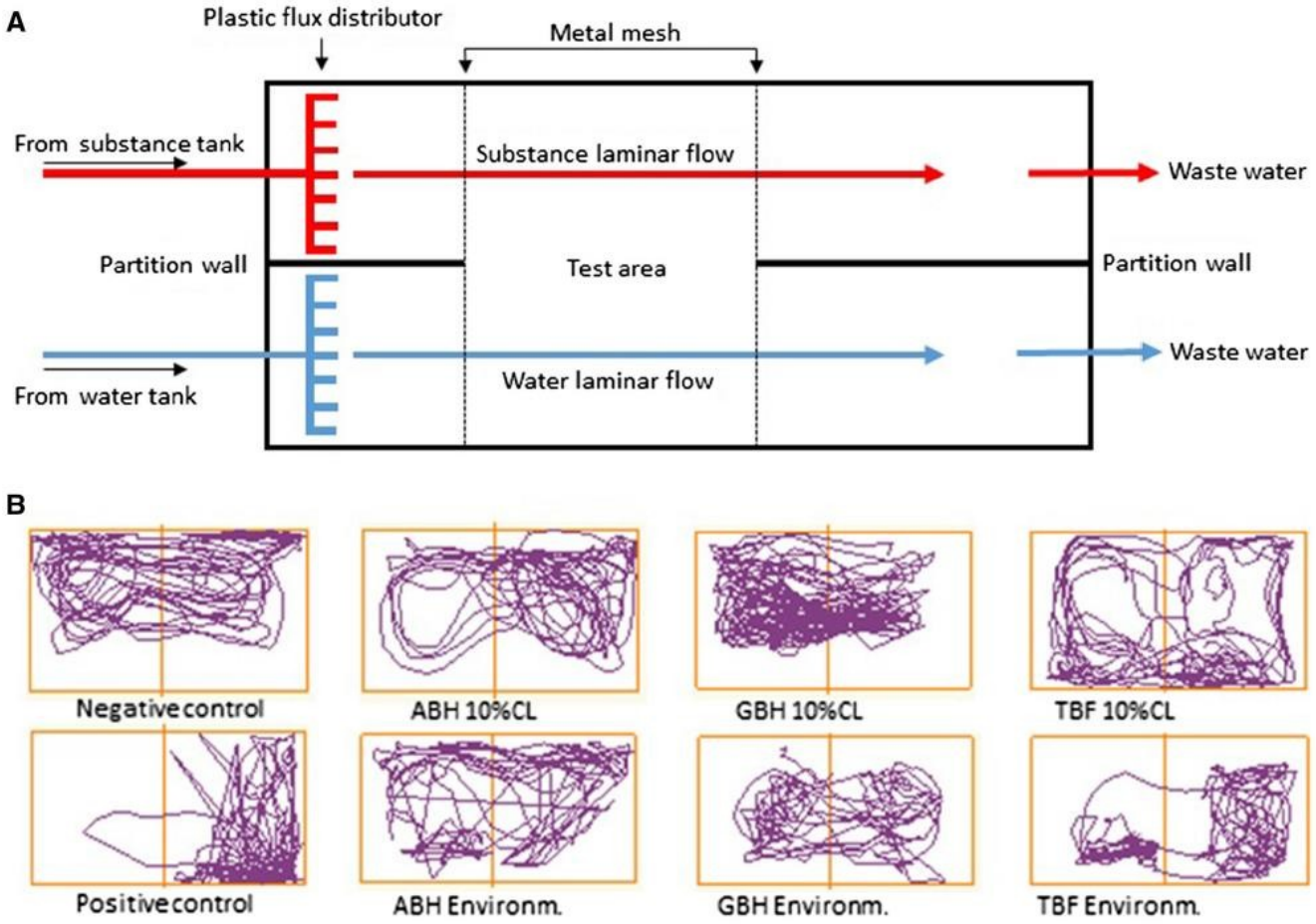

Fig. 1 **a** Schematic representation of the test chamber test. **b** The most representative video tracked movements of zebrafish

150 s of exposure. This time was based on our previous work with psychoactive drugs residues (Abreu et al. 2016).

During the tests, fish were not fed. The position (left or right) of the clean and contaminated water lanes was switched between each of the trials to prevent a possible bias caused by a fish preference for the left or right lane. The horizontal gradient created by the laminar flow within the tank allowed the untreated lane to remain uncontaminated, creating two lanes between which the fish could move freely. Following each experiment with one fish, the system was flushed to remove any test substance residues. The fish behavior with access to both contaminated or clean water lanes was recorded via video camera for the whole experimental period. Ten zebrafish were evaluated

for each contaminant. The video camera was positioned directly above the tank. The analyses of the video recordings were performed using AnyMaze® (Stoelting CO, USA) and quantification lasted over the 150-s exposure period. Because the contaminated water flux rapidly filled all the test area, we did not consider this time in the 150 s of analysis.

Control observations were conducted to evaluate potential bias of the experimental tank. When fish were exposed to flows of clean water in both lanes, undistinguishable choice was observed for any lane of the apparatus. A positive control test was conducted by providing a lane with an acid water flow (hydrochloric acid solution at pH 3.0). In this case, zebrafish showed a clear aversion to acid water flow. They chose to stay in the clean water (pH 7) lane instead of the acid one: time spent in clean water was 118 ± 6.2 s and in the acid water was 32 ± 6.2 s ($n = 10$; $P = 0.0039$; Wilcoxon matched-pairs signed-rank test).

In each substance trial, the following locomotor parameters were evaluated: total distance traveled, mean speed, absolute turn angle, and the number of body rotations. All the parameters were evaluated during entire 150 s, and additionally in five times fragments of 30 s to estimate choice preferences and locomotor activity across time.

Statistics

Homogeneity of variance was determined using Hartley's test, and normality was tested using the Bartlet test. The time spent in treated and control lanes were compared by paired Student's t test or Wilcoxon matched-pairs signed-rank test depending on data normality. Locomotor parameters were compared by Student's t test or Mann–Whitney test (depending of normality and homogeneity tests) by contrasting the treatment lane values against the clean water control lane. Differences were considered statistically significant at P values <0.05.

Results

The tested compounds (GBH, ABH, and TBF) did not alter water pH and DO levels (Table 2). Zebrafish spent significantly less time in the GBH-treated lane at the concentrations of 10 % of LC_{50} (Wilcoxon matched-pairs signed-rank test; $P = 0.0020$), suggesting an aversion response to this compound at this concentration (Fig. 2a). The same pattern of aversion was observed in the across-time analysis, except for the first 30 s of exposure. Regarding TBF environmental concentration, an aversion pattern was observed during a brief period of exposure (between 0–30 s and 30–60 s; Fig. 2b). No differences on time spent in untreated and contaminated lanes were found for GBH environmental concentration and both environmental and 10 % LC_{50} concentrations of TBF and ABH (Fig. 2a).

The results and the statistics of the tested locomotor parameters are depicted respectively in Fig. 3a, b. Briefly, GBH environmental concentration did not demonstrate diminished mean speed; however, in the analysis throughout the time, a decrease of speed was observed in the segments 0–30, 30–60, and 90–120 s. Regarding the number of body rotations, GBH environmental concentration also presented diminished values in the segments 30–60, 60–90, and 120–150 s. TBF showed decrease in mean speed in the first stage of exposure (0–30 s), and TBF environmental concentration showed elevated level of absolute turn angle in the segment 90–120 s. The remaining treatments did not show differences in any locomotor parameter.

Discussion

We showed that glyphosate-based herbicide was aversive to zebrafish except for the first 30 s of exposure. This suggests that the fish does not immediately avoid the contaminated water, but this avoidance is caused by a perception after a brief exposure to the substance. This

Table 2 pH and dissolved oxygen (mg/l) levels in clean and contaminated water

Substance	pH		Dissolved oxygen	
	Water	Substance	Water	Substance
Water (control)	6.8 ± 0.10	6.8 ± 0.07	6.2 ± 0.05	6.1 ± 0.1
pH3	6.9 ± 0.10	3.0 ± 0.10	5.7 ± 0.10	5.8 ± 0.05
GBH 10 % CL50	6.7 ± 0.15	7.0 ± 0.06	5.9 ± 0.10	5.7 ± 0.15
GBH env.	6.7 ± 0.10	6.5 ± 0.08	5.7 ± 0.05	5.6 ± 0.10
TBF 10 % CL50	6.7 ± 0.20	7.0 ± 0.05	5.6 ± 0.10	5.7 ± 0.07
TBF env.	7.4 ± 0.10	7.2 ± 0.08	6.0 ± 0.09	6.2 ± 0.14
ABH 10 % CL50	7.0 ± 0.10	6.9 ± 0.05	6.2 ± 0.20	6.0 ± 0.08
ABH env.	7.1 ± 0.20	7.3 ± 0.08	6.1 ± 0.09	6.4 ± 0.14

Data are expressed as mean ± SEM of four water samples

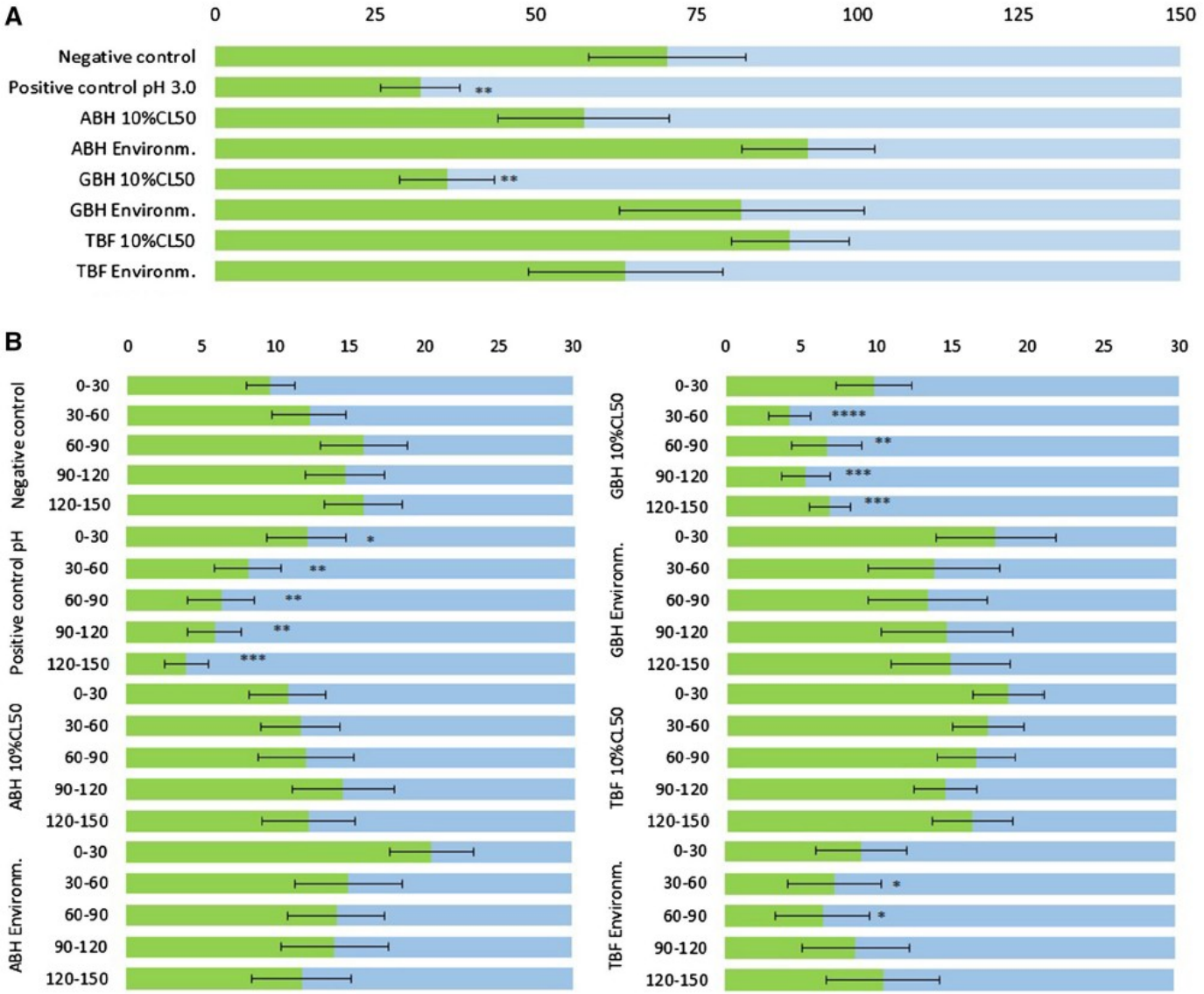

Fig. 2 **a** Time spent (s) in the substance or water lanes in a 150-s test. Data are expressed as the mean ± SEM in each lane ($n = 10$). **b** Time spent (s) in the substance or water lanes in five period fragments of 30 s. Means were compared by the paired t test or Wilcoxon matched-pairs signed-rank test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$)

latency to avoid the substance actively may be due to the chemical properties of GBH, which can be an irritant to fish, taking a longer period to cause disturbance. Tebuconazole-based fungicide provoked an aversion in the first segments, suggesting that the fish is desensitized throughout the exposure. Tebuconazole-based fungicide environmental concentration and atrazine-based herbicide neither attract nor repel zebrafish.

Thus, these chemical do not impose an extra toxic risk by being attractant to fish, although TBF and ABH can be deleterious, because they induce no aversive response. Because the uptake and bioaccumulation of chemicals in fish seems to be time- and dose-dependent (Hamelink and Spacie 1977; Geyer et al. 2000; Paterson and Metcalfe 2008), a fish that remains for an extended time in the presence of these drugs (attractive or not perceived drugs) tend to absorb higher concentrations than ones that escape from contaminated sites (aversive drugs).

The protocol and apparatus of this chemotaxic preference test was previously validated to evaluate aversion of fish anesthetics (Readman et al. 2013) as well psychotropic drug residues (Abreu et al. 2016). In this and former studies, the positive control test with hydrochloric acid was clearly aversive to zebrafish and that they display strong avoidance behavior, showing the ability of zebrafish to detect the acid pH by its chemosensory traits. In the water-water control, no choice between apparatus lane was observed. These evidences show that zebrafish respond to chemical stimulus displaying place choice and the test apparatus has no bias. Thus, any behavioral change can be

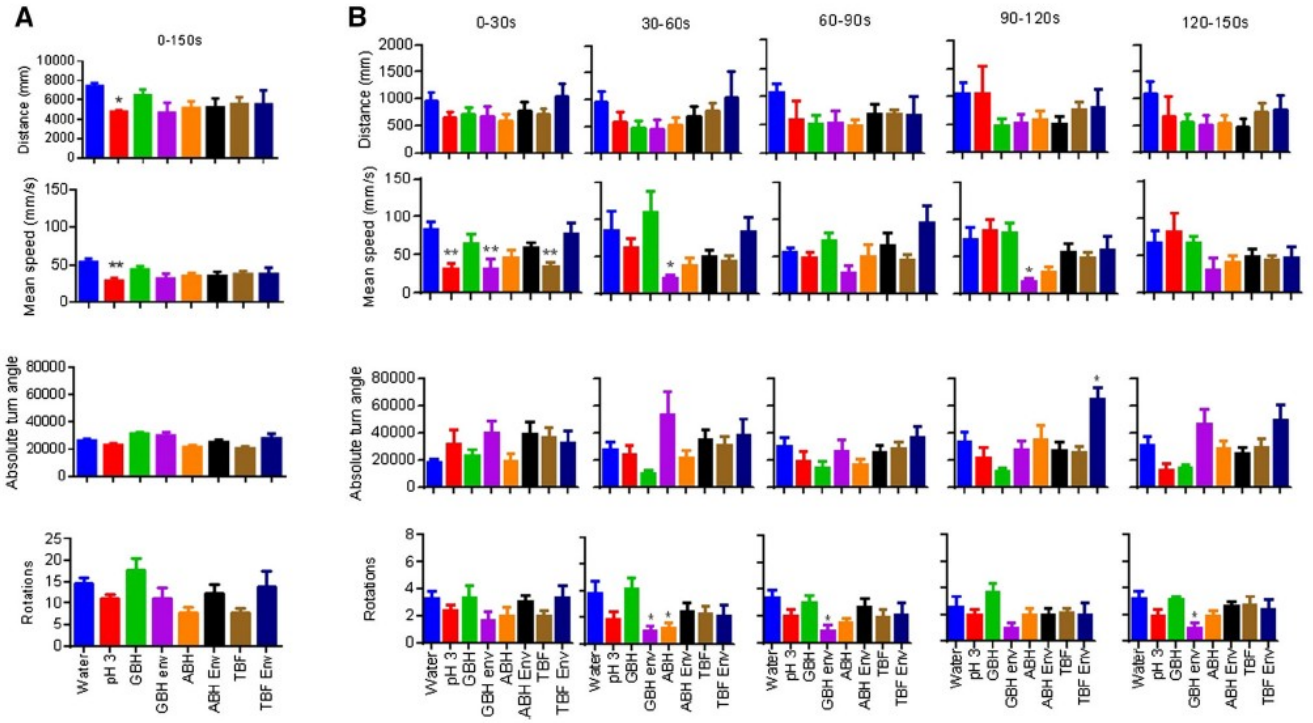

Fig. 3 **a** Locomotor activity of zebrafish during the 150-s test, and **b** locomotor activity of zebrafish in five period fragments of 30 s. Data are expressed as mean ± SEM ($n = 10$). *GBH* glyphosate-based herbicide, *TBF* tebuconazole-based fungicide, *ABH* atrazine-based herbicide. *Asterisks* indicate differences between each substance/concentration against the water control (*clean line*). One-way Anova followed by Dunnet test or Kruskal–Wallis test depending on the normality (Bartlet) and homogeneity of variance (Hartley) tests, $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 7$–10

interpreted clearly as an effect of these chemicals than any interference variable.

Considering the nature of test used (chemo sensorial) and the acuteness of the exposure (150 s), we hypothesized that the fish aversion to GBH is a result of different sensorial stimulation. Although it was not the goal of our study, GBH may cause pain (tactile stimulation), because glyphosate was referred as acid causing several histopathological changes in fish (Jiraungkoorskul et al. 2002), which might be another reason for zebrafish GBH avoidance. Previous studies revealed that the acid pH was detected (and avoided) by taste (Strieck 1924) and olfaction (Hidaka and Tatsukawa 1989). We discard these pH-related sensory cues, as responsible for zebrafish GBH aversion, because GBH did not alter water pH. In addition, Vera et al. (2010) also verify that different GBH concentrations did not alter water pH.

Rainbow trout fry did not avoid GBH at concentrations of 0.1, 1, and 10 mg/l (Folmar et al. 1979), whereas in concentrations of 2–8 times the 96 h LC_{50} rainbow trout fingerlings actively avoided glyphosate-based compounds (Morgan et al. 1991). The same pattern of avoidance by a GBH (Roundup) was found by Tierney et al. (2007), in which trout avoided the active substance. In contrast, environmental concentrations of pesticide mixture containing glyphosate provoked attraction for zebrafish (Tierney et al. 2009). In our study, this same concentration of glyphosate found in the pesticide mixture used by Tierney et al. (2009) did not provoke aversion neither attraction. It is important to take in account that, in these studies (Tierney et al. 2009; Strieck 1924; Hidaka and Tatsukawa 1989; Folmar et al. 1979; Morgan et al. 1991), the test methodology are very different from the one proceed in the present study. These methodology differences might affect the results.

The speed was significantly affected by GBH in the first minute of exposure, presenting diminished values. Tierney et al. (2007) also demonstrated that glyphosate exposure can induce a reduction of fish locomotion. Regarding the number of rotations, this parameter also was reduced, suggesting that fish become desensitized to the GBH locomotor effects over the exposure period. Only the higher concentration of TBF provoked an increase in the absolute turn angle for a brief moment. Those high turn angles would suggest possible neuromuscular effects, because the absolute turn angle is a sensitive measure of motor coordination (Blazina et al. 2013). The altered locomotor coordination might be a possible causation factor of the not-aversive response of zebrafish against this agrichemical, where the fish is not capable of escaping the affected area.

We found that fish did not avoid ABH and TBF contaminated sites probably by absence of perception of these compounds in the specific concentrations and/or changes in locomotor activity (Fig. 3a, b). This absence of detection/reaction might be dangerous, because fish did not move away from contaminated sites. The changes in locomotor parameters provoked by GBH and TBF reinforce our conclusion that these compounds are dangerous, because fish did not avoid contaminated areas by not perceiving the drugs or due to locomotor and/or neuromuscular impairments.

Regarding GBH, despite the well-related negative effects (Cericato et al. 2009, 2008; Koakoski et al. 2014; Ferreira et al. 2010, 2012, 2013; Toni et al. 2011; Jiraungkoorskul et al. 2002; Langiano and Martinez 2008; Glusczak et al. 2007; Armiliato et al. 2014) fish actively avoided contaminated sites if concentrations were higher than 10 % of LC50. This fact represents a defensive behavior that can protect fish from the deleterious effects of GBH. However, avoidance/aversive behavior in a location where fish have no escape to avoid this chemical might be an unavoidable stress source with potentially harmful effects.

The concentrations used were very plausible in terms of contamination of natural water bodies and fishponds. In addition, the GBH is used directly in water bodies to control aquatic macrophytes. Even TBF and ABH, which are not used directly in water, can easily reach the water bodies in small concentrations as a result of leaching by rain or as a result of accidents (Soumis et al. 2003) causing harmful effects to fish. In addition, aquatic organisms may be exposed to accidental spills of pollutants, incorrect discharges of substances or contaminants already present in the water. Such contamination may cause biomagnification, in which concentrations much higher than those found in the environment may be observed. This phenomenon has been reported for the presence of pesticides (Niethammer et al. 1984; Kelly et al. 2007; Goerke et al. 2004).

Finally, a limitation of this study is that we cannot directly extrapolate these results to the aquatic environment, where fish are chronically exposed to xenobiotics since early development, and in this study, fish were briefly exposed to the agrichemicals. However, this does not lessen the importance of our results, because data about the attraction/aversion paradigm related to agrichemical exposures are very scarce. In addition, the attraction/aversion paradigm might be an innovative and interesting approach in toxicological studies.

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Author Contributions The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. L.J.G.B., M.S.A, R.E.B., and J.G.S.R. conceptualized the study, interpreted the data, and wrote the paper. M.S.A., A.C.V.G., G.K., F.K., T.A.O., and H.H.A.B. collected and analyzed the data.

Compliance with Ethical Standards

Conflict of interest The authors declare no competing financial interests.

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Original Article

Just Keep Swimming: Neuroendocrine, Metabolic, and Behavioral Changes After a Forced Swimming Test in Zebrafish

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Abstract

In this study, we show that an adaptation of the spinning test can be used as a model to study the exercise–exhaustion–recovery paradigm in fish. This forced swimming test promotes a wide range of changes in the hypothalamus–pituitary–interrenal axis functioning, intermediary metabolism, as well in fish behavior at both exercise and recovery periods. Our results pointed that this adapted spinning test can be considered a valuable tool for evaluating drugs and contaminant effects on exercised fish. This can be a suitable protocol both to environmental—to evaluate contaminants that act in fish energy mobilization and recovery after stressors—and translational perspectives—effects of drugs on exercised or stressed humans.

Keywords: *Danio rerio*, exercise-exhaustion-recovery paradigm, cortisol, novel tank test, anxiety-like behavior, exercise levels

Introduction

Several behaviors as schooling, prey–predator relationship, reproduction, and migration depend on the ability of fish to move through all over the ecological niches and their capacity of swimming.[1–3] The success of these behaviors are related to swimming performance, in other words, if the fish cannot swim adequately, the search for food or mates is unproductive, leading to an imbalance on population and individual levels.

Swimming activity could represent an exercise of mild, moderate, or high intensity, depending on the stimulus strength. Swimming exercise is primarily maintained by aerobic metabolism.[4] Metabolic parameters, such as carbohydrate reserves (glycogen, glucose, and lactate), are frequently used as physiological stress indicators in fish and could be altered on blood and tissues after exercise.[5] In some situations (e.g., predator–prey interactions), burst-type exercise is supported by anaerobic glycolysis.[4,6] According to Wood,[7] the aptitude of decomposing the accumulation of lactate in muscles is classified as exercise capacity.

Exercise can cause osmotic, fluid, and electrolyte disturbance,[8] consequently leading to stress and increase of corticosteroids that last until 6 h after exercise,[9] aiming to mobilize energy reserves[10] to cope with these changes. In fish, increased plasma cortisol in conditions of exhaustive exercise seems to delay the restoration of metabolic status to pre-exercise levels inhibiting the glycogenesis.[11,12] In addition, exhaustive exercise can induce behavioral alterations related to locomotor adjustments, which directly influence on the ability to cope with environmental threats.[13]

The forced swimming task (called spinning test) was previously proposed by Blazina *et al.*[14] who exposed zebrafish to three rotational velocities, evaluating locomotor parameters (total distance traveled, time in the upper half, and absolute turn angle) and a light–dark task comparing with effects of psychotropic substances. However, neuroendocrine and metabolic parameters were not assessed, and we

hypothesize that such a condition influences energy metabolism and the normal functioning of the hypothalamus–pituitary–interrenal (HPI) axis. In this study, we evaluate the neuroendocrine, metabolic, and behavioral alterations in zebrafish during the exercise and restoration periods, using an adaptation of the spinning test previously reported.

Methods

Ethical note

This experimental setup was approved by the Ethics Commission for Animal Use of the Universidade de Passo Fundo, Brazil (Protocol #29/2014), and followed the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Animals

A stock population of 432 male and female adult zebrafish (*Danio rerio*), wild-type short-fin strain, weighing 0.5 ± 0.2 g was housed (one fish per liter) in 12-L tanks equipped with biological filters, under constant aeration and a natural (14-h light–10 h dark) photoperiod (lights on at 20:00 pm). Water temperature was maintained at 27 ± 1°C, with pH = 7.3 ± 0.1. Fish were fed twice a day, with commercial flake fish food (Alcon® Basic, MEP 200 Complex, Brazil).

Experimental design

Our strategy was to submit adult zebrafish to an adapted spinning test, using three rotational velocities in three different periods (Fig. 1). Considering the recipient test diameter, we used three different speeds: 410 rpm corresponding to 0.283 m/s, 480 rpm corresponding to 0.333 m/s, and 570 rpm corresponding to 0.383 m/s. The recipient water volume was 1.2 L, and all water quality parameters were similar to maintenance tanks, within the desired range to zebrafish.

Since the fish position in relation to the center of the recipient may interfere in the force that fish needs to maintain his position, we have filmed the assays to confirm that all fish maintained always at the edge of recipient and occasionally cross the center. The video camera (Logitech HD Webcam C525 camera, Romanel-surMorges, Switzerland) was positioned directly above the recipient. Thus, we believed that this variation source was eliminated between treatments.

In the first study, we evaluated the cortisol profile, metabolic and behavioral parameters during the forced exercise period, to determine which velocity is able to cause an activation of HPI axis and consistent metabolic changes. After, in the study 2, taking into consideration the results of the study 1, we evaluated the metabolic, endocrine, and behavioral changes in the velocity/time that showed major changes. We also assessed the recovery period 30, 60, and 120 min after the forced swimming test. The protocol is schematized in Figure 1.

Experimental procedures

Parameters evaluated.

Whole-body cortisol. For the whole-body cortisol quantification, we used the method described by Oliveira *et al.*[15] After capture, fish were immediately frozen in liquid nitrogen for 10–30 s and maintained at −20°C until cortisol extraction. We measured the cortisol levels in duplicate samples using enzyme-linked immune assay kit (EIAgen CORTISOL kit, BioChem ImmunoSystems). The kit validation is fully described by Sink *et al.*[16] Briefly, the specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions of the tissue extracts in phosphate buffered saline.

The standard curve constructed with the human standards ran in parallel to that obtained using serial dilutions of zebrafish tissue extracts. In the linear regression test, a high positive correlation was found between the curves. The intra-assay coefficient of variation was 3.33%–3.65%. Measurement accuracy was evaluated by calculating the levels recovered from samples spiked with known amounts of cortisol (50, 25, and 12.5 ng/mL). The mean detection of spiked samples was 94.3%. All cortisol values were adjusted for recovery using the following equation: cortisol value = measured value × 1.0604.

Metabolism. In the first study, we evaluated whole-body glucose, glycogen, and lactate. In the main study, we evaluated these parameters and the enzyme activity of creatine kinase (CK). The whole-body glucose concentration was determined by glucose oxidase kit (Labtest, MG, Brazil). Glycogen content was determined by Van Handel[17] method while the determination of lactate concentration followed the method previously reported (Kit Vis Interteck/Katal). The CK activity was determined using the UV Interteck/Katal Kit (SP, Brazil).

Behavioral activity. The behavior of zebrafish was evaluated using the novel tank test (NTT).[18,19] Briefly, fish were transferred individually to a glass transparent test tank (24 × 8 × 20 cm; width × depth × height) and filmed for 6 min. The test tank was divided into three virtual zones (upper,

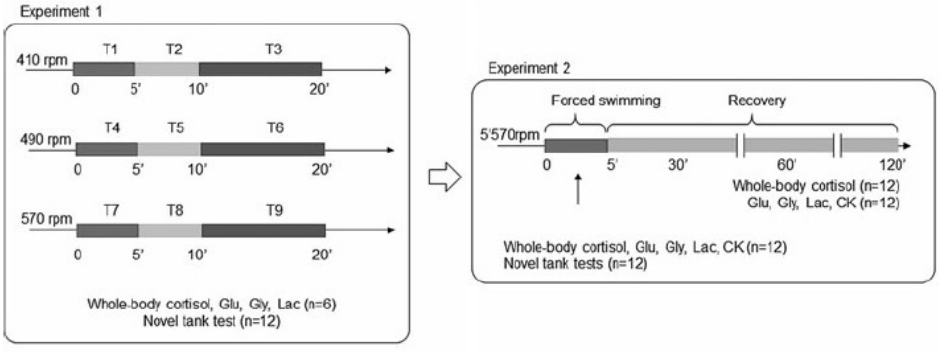

FIG. 1. Schematic representation of the experimental design and strategy. To expose the animals to defined velocities, a beaker containing a stir bar was set at the top of a magnetic stirrer settled at different levels, corresponding to desired velocities.

middle, and bottom zones). The videos were then analyzed using ANY-maze® software (Stoelting CO), and the following behaviors were scored: total distance traveled (m), number of crossings between the tank zones, and absolute turn angle. The following were also scored—relative time at upper zone, entries in upper zone, and latency to first entry in upper zone.

Statistics

In the first study, whole-body cortisol levels were compared by the Kruskal–Wallis test followed by Dunn's multiple comparisons test, while metabolic and behavioral parameters were compared by ANOVA (two and one way, respectively) followed by Tukey's test. In the second study, whole-body cortisol, metabolism, and behavioral data were compared by the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Data normality was assessed by the Bartlett's test for significant effects. In all experiments, p was set at <0.05.

Results

Study 1

Whole-body cortisol. The forced swim induced a significant increase in cortisol levels typically related to an acute stress response. All groups, except the times 5 and 20 min at the velocity of 410 rpm, presented increased cortisol levels (Fig. 2).

Metabolic parameters

Whole-body glucose. There was a significant difference between the control group and fish exposed to 410 rpm for 5 and 10 min, with this group presenting higher whole-body glucose level ($p < 0.05$). Fish exposed to 570 rpm for 5 min presented similar levels of glucose when compared to control animals. After 10 min, fish exposed to both 480 and 570 rpm presented decreased glucose levels contrasting with fish exposed to 410 rpm (Fig. 3A).

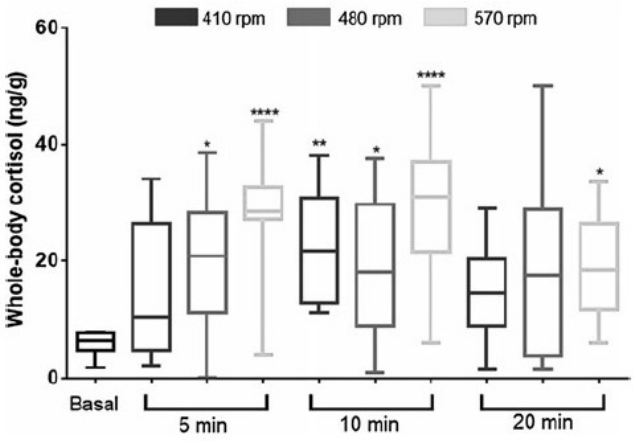

FIG. 2. Whole-body cortisol concentrations in fish exposed to three rotational velocities during three periods. Data are expressed as mean ± standard error of the mean of 12 fish and compared by the Kruskal–Wallis test followed by Dunn's multiple comparisons test. p Values in the figure (*$p < 0.05$; **$p < 0.01$; and ****$p < 0.0001$).

Whole-body glycogen. Fish exposed to 570 rpm for 5 and 10 min presented decreased glycogen levels ($p < 0.05$) when compared to the control group. Animals subjected to 410 and 480 rpm (Fig. 3B).

Whole-body lactate. Fish exposed to 570 rpm for 10 and 20 min presented decreased levels of lactate, similar to control. In the 5-min group, fish exposed to 480 presented increased lactate levels contrasting with ones exposed to 410 and 480 rpm (Fig. 3C).

Behavioral parameters

NTT. All treatments decreased the distance traveled (Fig. 4A). The major change occurred at 5 min at lower velocity ($p < 0.0001$). The relative time spent in the upper zone increased after 20 min in 410 and 570 rpm (Fig. 4C). Regarding the number of entries in the upper zone, animals exposed to 570 rpm for 5 min increased the frequency ($p < 0.05$) (Fig. 4D). The frequency of line crossings and the latency to first entry in the upper zone (Fig. 4B, E, respectively) did not alter after exposure to the different rotational velocities and periods.

Study 2

Whole-body cortisol. Fish immediately after the spinning test and after 30 and 60 min of recovery presented increased cortisol levels than control fish. After 120 min, cortisol levels are statistically indistinguishable than basal level. Overall, the decrease in whole-body cortisol levels during recovery period was apparently time dependent (Fig. 5).

Metabolic parameters

During all recovery times, glucose and glycogen levels were lower than those detected in the control groups (Fig. 6A, B). Lactate levels were increased at the three recovery times when compared to the control and in comparison to fish tested immediately after the spinning task (Fig. 6C). In addition, fish analyzed immediately after the spinning test and after 30 and 60 min of recovery presented increased CK levels than control fish (Fig. 6D).

Behavioral parameters

NTT. General locomotor parameters were altered after exposure to the spinning test. Total distance increased by forced exercise, returning to the control level after at least 60 min (Fig. 7A). The frequency of line crossings (Fig. 7B) increased after forced exercise, but not significantly in relation to control. However, this frequency diminished significantly after 60 and 120 min of recovery. The absolute turn angle decreased in the recovery period, presenting significant differences related to control and forced exercise (Fig. 7C).

The treatment increased the time spent in the upper zone as well as the number of entries in that zone, and those parameters reduced to control levels during the recovery period (Fig. 7D, E). Regarding latency to first entry in the upper zone (Fig. 7F), despite the difference between treatments, there was no significance, probably due to a high standard error.

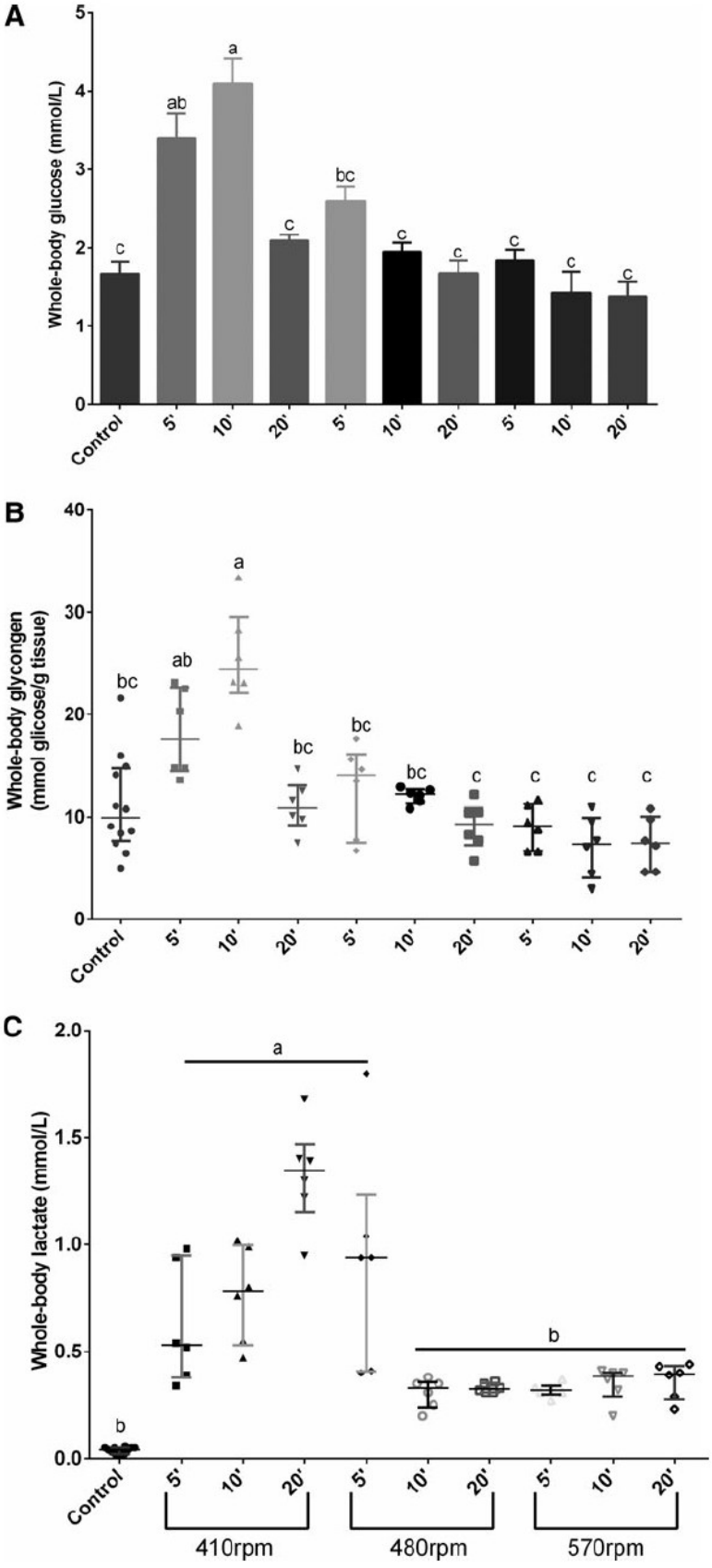

FIG. 3. Metabolic changes in zebrafish exposed to a spinning test. **(A)** Whole-body glucose concentrations; **(B)** whole-body glycogen content; and **(C)** whole-body lactate. Data are expressed as mean ± SEM of 12 fish and compared by one-way ANOVA followed by Tukey's multiple range test **(A)** and by the Kruskal–Wallis test followed by Dunn's multiple comparisons test **(B, C)**. *Different small letters* indicates statistically significant diferences between means.

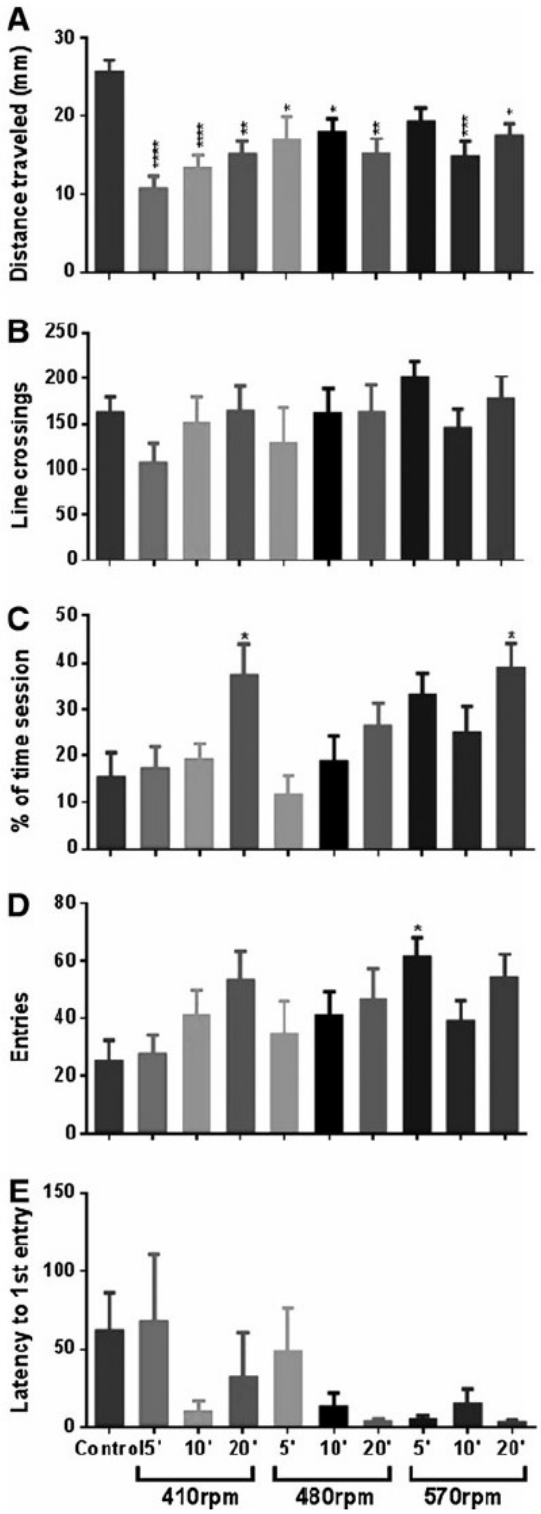

FIG. 4. Locomotor parameters in zebrafish exposed to a spinning test. **(A)** Total distance traveled; **(B)** *line crossings*, **(C)** relative time at *upper zone*; **(D)** entries in *upper zone*; **(E)** and latency to the first entry in *upper zone*. Data are expressed as mean ± SEM of 12 fish and compared by one-way ANOVA followed by Dunnet's multiple range test. *p* Values in the figure (*$p<0.05$; **$p<0.01$; ***$p<0.01$; and ****$p<0.0001$).

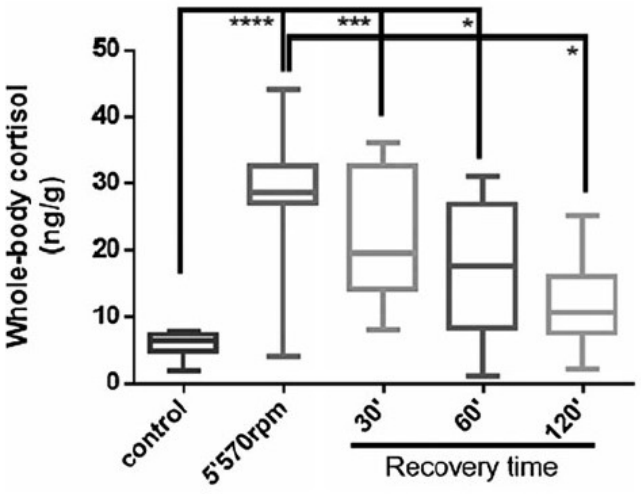

FIG. 5. Whole-body cortisol concentrations in fish exposed to three rotational velocities during three periods. After 120 min of recovery, cortisol levels are statistically indistinguishable from the control level. Data are expressed as median ± interquartile interval of 12 fish and compared by the Kruskal–Wallis test followed by Dunn's multiple comparisons test. *p* Values in the figure (*$p<0.05$; ***$p<0.01$; and ****$p<0.0001$).

Discussion

In this study, we show that the spinning test, first validated by Blazina *et al.*,[14] can be used as a model to study the exercise–exhaustion–recovery paradigm. This forced swimming test was capable of promoting a wide range of changes in the HPI axis functioning, in the intermediary metabolism, as well as in fish behavior.

Regarding whole-body cortisol, the adapted-spinning test protocol was effective to promote endocrine alterations. In the first study, the group exposed to 570 rpm for 5 and 10 min showed significant increase in cortisol levels, showing that this velocity can promote the activation of the HPI axis. We can assume that this response occurs due to forced exercise, since our protocol eliminates the ''fright'' that can occur in other protocols.[9] This increase appears to be stimulated by the forced exercise, since cortisol plays a key role in energy substrates recruitment.[20,21]

Since the 5 and 10 min of exercise at 570 rpm showed similar results, we chose to use 5 min of exercise to optimize the laboratory procedures. In the main study, we tested the higher velocity (570 rpm) for 5 min, as well as three times of recovery. The cortisol profile confirmed the results of the pilot study, where 5 min of exercise at 570 rpm can increase cortisol levels. Concerning the recovery time, only at 120 min after exercise, the fish could restore the cortisol at similar levels as control.

The intermediary metabolism was also modulated by forced exercise. At the velocity of 410 and 480 rpm, depletions on glycogen and glucose levels did not occur, indicating that the swimming activity for this time (5 and 10 min) does not decrease the carbohydrate reserves. As the exercise intensifies (480 and 570 rpm), glycogen and glucose reduce, indicating consumption of carbohydrate reserves.[22] The decrease of glycogen and glucose is proportional to the increase in exercise intensity. According to Navarro and Gutiérrez,[22] the glycogen mobilization seems to be more related to

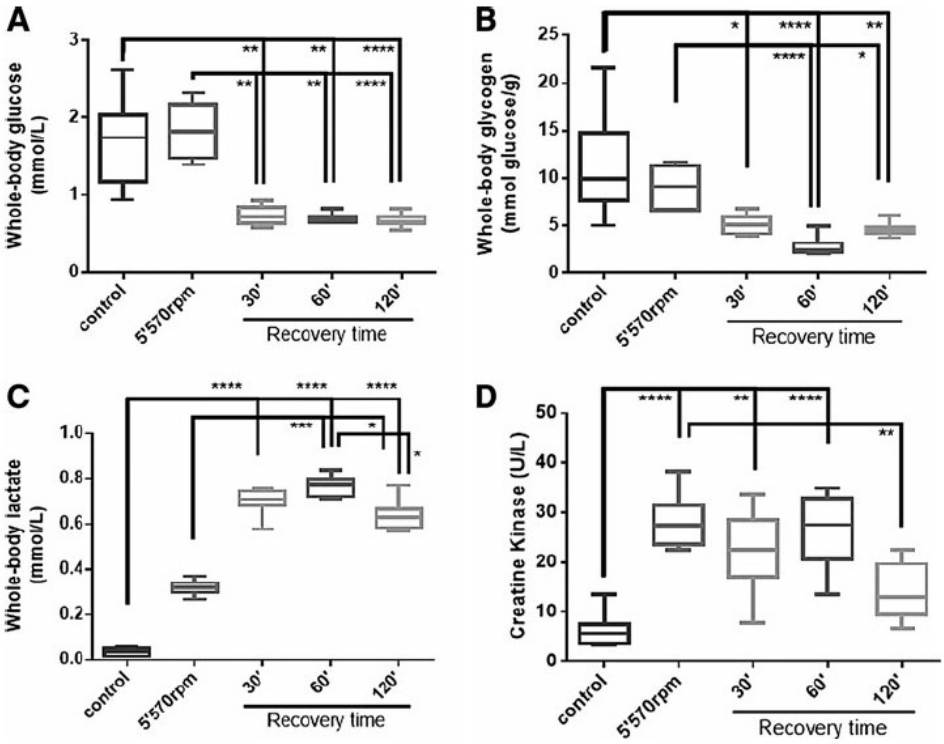

FIG. 6. Metabolic changes in zebrafish exposed to a spinning test. **(A)** Whole-body glucose concentrations; **(B)** whole-body glycogen content; **(C)** whole-body lactate; and **(D)** creatine kinase. Data are expressed as mean ± interquartile range of 12 fish and compared by the Kruskal–Wallis test followed by Dunn's multiple comparisons. *p* Values in the figure (*$p < 0.05$; **$p < 0.01$; ***$p < 0.01$; and ****$p < 0.0001$).

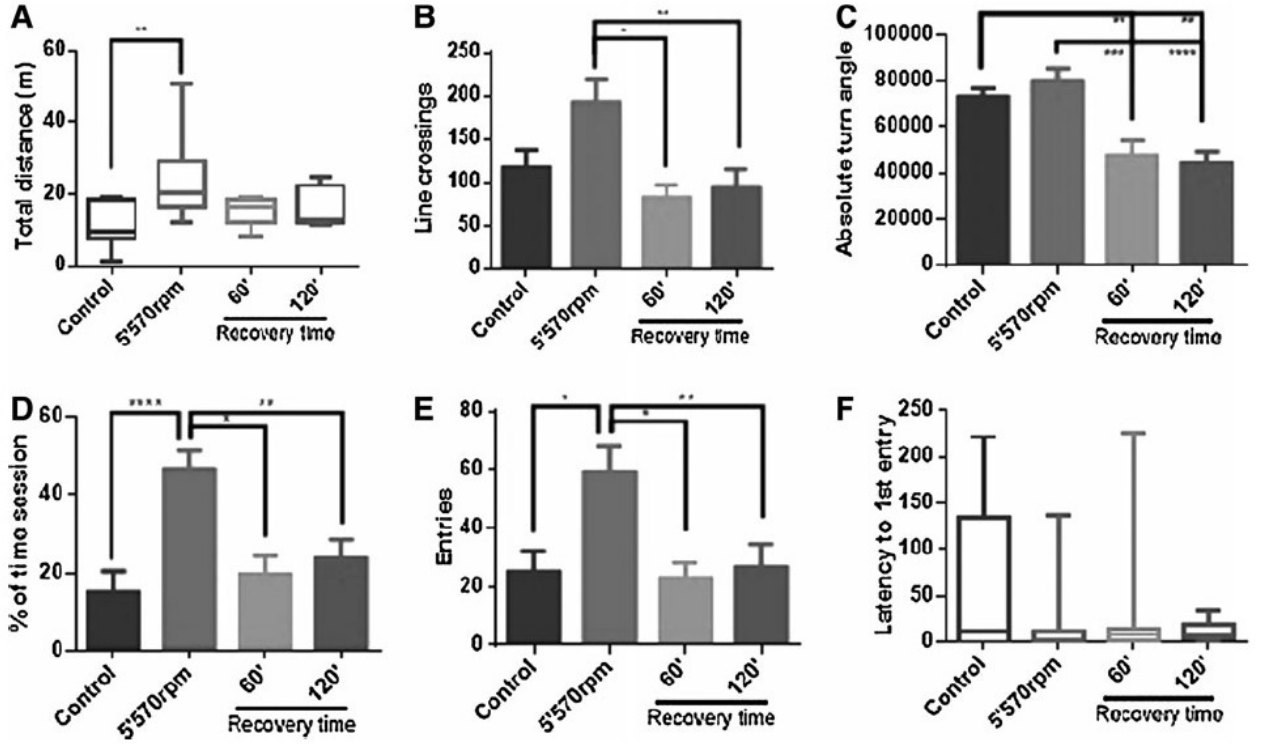

FIG. 7. Locomotor parameters in zebrafish exposed to a spinning test. **(A)** Total distance traveled; **(B)** line crossings; **(C)** absolute turn angle. Relative time at upper zone **(D)**, entries in upper zone **(E),** and latency to first entry in upper zone **(F)**. Data are expressed as mean ± SEM (parametric) and median ± interquartile range (nonparametric) of 12 fish and compared by two-way ANOVA followed by Tukey's multiple range test or by the Kruskal–Wallis test followed by Dunn's multiple comparisons test, depends on data normality. *p* Values in the figure (*$p < 0.05$; **$p < 0.01$; ***$p < 0.01$; and ****$p < 0.0001$).

exercise and muscular activity than other physiological processes.

It has been reported that metyrapone-treated fish increases muscle glycogen levels, in contrast with the controls suggesting a direct link between plasma cortisol levels and glycogenesis.[23] The activation of this response can be triggered by many stressor stimuli, like increased cellular temperature and metabolic stress,[24] factors which take place in the intense exercise.

Metabolic stress represented by reduction of glucose availability also seems to be involved with exercise-induced stress response.[25] The intense exercise in fish induces a decrease of glycogen levels, with the complete restoration only after ~6 h.[9,26] Regarding the levels of lactate, the group submitted to lower intense exercise presented an increase as exercise becomes more intense.

In this line, Liew *et al.*[27] demonstrated that *Carassius auratus* exposed to burst exercise presented increase of lactate that is concomitant with glycogen and glucose decrease, suggesting the involvement of anaerobic metabolism. At the medium velocity, there was an increase of lactate after 5 min of exercise, which might be related to glycogen and glucose decrease. However, after 10 and 20 min of exercise, the lactate concentrations decrease, and glycogen and glucose stabilize, suggesting a new pathway of glycogenesis since the recovery of white muscle does not depend on Cori's cycle, as glucose mobilization occurs at 10% of glycogen storage.[28]

In the main study, the forced exercise did not induce a significant decrease of glycogen and glucose levels. Importantly, both parameters kept stabilized even with the increase of lactate in forced exercise. However, another metabolic biomarker, the CK activity, increases, indicating that the energy necessary to maintain the exercise might be supported by phosphocreatine, which is the primary source of energy during intense exercise.[29]

We highlight that our forced-exercise protocol alters fish behavior. At the higher velocity, behavioral parameters such as distance, speed, and line crossings are elevated. In the recovery period, the absolute turn angle decreases. These alterations can be interpreted as a state of hyperactivity, suggesting that the forced exercise may lead to an anxiety-like behavior.[30,31] A similar anxiogenic effect of exercise was found in rodents, with probable relation between exercise and neurogenesis.[32]

Besides, neurogenesis can occur in adult teleosts, including after exercise,[33–35] it seems less likely this occurs due to the brief period of exercise in our experiment. In mice, Binder *et al.* (2004)[36] found that in the determined behavioral test, the animals showed anxiety behavior after voluntary exercise for 4 weeks. However, in the same study, an anxiolytic response was observed in another test. This discrepancy of results may occur by nature of test, where additional behaviors, like stress responsiveness and impulsivity, can be evaluated.

In our work, we used behavioral tests that are indicative of anxiety-related behavior. In addition, we assessed these parameters after a protocol of acute forced exercise, a situation that can trigger the release of several neurotransmitters that cause this behavior. Thus, another hypothesis is the involvement of the sympathoadrenal system. The activation of the hypothalamus–pituitary axis provokes the release of adrenocorticotropic hormone (ACTH) into the circulation. Besides promoting the release of corticosteroids, the ACTH is involved in the catecholamine modulation.[37,38] Catecholamines are released after exposure to several stressor stimuli, including high-intensity exercise,[38–40] aiming energy mobilization.

Pacak *et al.* (1995)[41] related a consistent relationship between plasma ACTH and catecholamine in paraventricular nucleus (PVN) microdialysate, in rats, after the activation of hypothalamic–pituitary–adrenal (HPA) axis. The axis activation in consequence to a stress response generates an anxiogenic effect.[42] This activation leads to a defense state that could be interpreted as "anxiety." Studies suggest that the physical effects of anxiety, as hyperactivity and elevated heart rate, are caused by adrenergic system.[43] This hypothesis is supported by experimental evidence, since these effects can be suppressed by administration of beta-adrenergic blockers.[44,45]

Taking into consideration that plasma catecholamines are elevated in acute exercise,[40,41] and given the strict relation between HPI axis and sympathetic nervous system,[37,38] it is plausible to assume that this anxiety-like behavior was caused by the forced exercise protocol.

Interpreting our results, we propose three exercise levels as follows: (i) mild exercise (410 rpm/5 min)—stable glucose levels, with the initial metabolism of glycogen, and elevated lactate levels; (ii) moderate exercise (490 rpm/5 min)—the energetic reserves start to decrease, evidenced by the reduction of glucose and glycogen levels. This fact indicates the activation of anaerobic metabolic pathway, verified by consumption of lactate; (iii) intense exercise (570 rpm/5 min)—significant decrease of glucose, glycogen, and lactate. Besides, we observe an increase in CK activity, whole-body cortisol levels, and robust behavioral changes. In the same way, Fuss *et al.* (2009)[32] reported a significant activation of HPA axis in rodents after voluntary exercise, and the increase of circulating corticosteroids in mice after running exercise was also related.[46]

The last comment is about the methodology of measuring metabolic parameters in whole body. Despite the fact that usually these parameters were measured in total blood or specific tissues as white or red muscle, the results obtained in whole fish were within the expected common ranges and respecting the dynamics: consumption of energy storages–elevation of corticosteroids–accumulation of metabolites–recovery.[7,9,10]

Zebrafish present similar neuroendocrine and physiologic structures when compared to other vertebrates, as mammals and humans.[34,47] This similarity makes possible the application of zebrafish in biomedical research, representing a great translational value. Besides, we highlight that the zebrafish is a valuable model organism for ecotoxicological research, acting as a model system to evaluate and monitor environmental pollutants and their biological effects.[48]

Thus, taken together, our results pointed that this adaptation of the spinning test may serve as a valuable tool to evaluate the effects of different drugs on exercised fish. The protocol may be applied both to environmental (to evaluate contaminants that act in fish energy mobilization and recovery after stressors) and translational perspectives (drug or abuse substance effects on exercised or stressed humans).

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CAPÍTULO VI

DISCUSSÃO

Atualmente o uso de pesticidas aumentou muito, acompanhando a modificação das práticas agrícolas e a intensificação da produção (Konstantitinou et al., 2006). As intensas modificações ambientais causadas pela ocupação humana são em grande parte causadas pela contaminação por resíduos das inúmeras substancias utilizadas para diversos fins, como por exemplo os agroquímicos. A utilização cada vez mais intensa de pesticidas na agricultura pode produzir efeitos tóxicos em organismos expostos a essas substâncias, em especial para espécies em ambientes aquáticos, visto que a maioria dos agroquímicos são muito pouco voláteis e altamente solúveis. Segundo Plimmer (1990), apenas de 1 a 3% dos agroquímicos chegam ao seu destino de ação, ocasionando a presença desses compostos e seus resíduos no ambiente. Tendo isso em vista, muitos estudos foram e continuam sendo feitos com o objetivo de elucidar os efeitos dos agroquímicos sobre espécies não-alvo. Estudos *in vitro* de células indiferenciadas precursoras de células neuronais, e em tecido cerebral em estudo *in vivo*, a exposição ao herbicida atrazina demostrou alterar significativamente o nível de catecolaminas sintetizadas por essas células e tecidos (Das et al, 2001). A diminuição de catecolaminas no hipotálamo pode inibir a liberação do hormônio liberador de gonadotrofina, enquanto que a diminuição de dopamina leva a uma diminuição de prolactina (Barraclough, 1992; Ben-Jonathan, 1985), sendo que a prolactina tem o efeito secundário de tornar as células mais sensíveis à ação do o hormônio liberador de corticotrofina (CRF). Muitos pesquisadores ligam ocorrência de neurodegeneração à exposição a ambientes com resíduos de atrazine (Bretaud et al, 2004).

No artigo 1, pôde-se verificar a inibição da expressão do gene *star* no tratamento onde houve exposição ao MP e ao agente estressor. Sendo o cérebro um órgão esteroidogenico, (Sierra, 2004) e levando em conta que neuroesteroides são muito importantes para a manutenção e desenvolvimento de células neuronais (King et al., 2002, 2004; Sierra, 2004), a inibição da proteína StAR nesse tecido é uma evidencia muito importante e preocupante dos efeitos subletais de agroquímicos. Essa diminuição dos níveis de expressão dessa proteína, quando associados a resultados da literatura de interrupção endócrina causada por essa substancia, sugerem que a proteína StAR está envolvida na regulação ou sinalização do eixo HHI. O efeito de compostos químicos causados por interrupção endócrina é pouco estudado e nível de tecido neuronal, sendo que o foco principal é em órgãos esteroidogenicos clássicos como gônadas (Armiliato et al., 2014). No entanto, Lyssimachou e Arukwe (2007)

encontraram valores de StAR diminuídos no cérebro de peixes expostos a etinilestradiol. Nesse mesmo trabalho, onde foi utilizada uma metodologia de PCR semi-quantitativo, foi encontrada diminuição na expressão do gene *hsp70*. A modulação da expressão dessa proteína foi encontrada em outros estudos com exposição a diferentes substâncias (Xing et al, 2013; Olsvik et al, 2014). A diminuição dos níveis de HSP70 no grupo exposto ao MP e ao agente estressor podem indicar que ocorreu estresse celular nas células do tecido cerebral, quando ocorre exposição concomitante do MP e estressor. Esses resultados indicam que a proteína StAR e a HSP70 são parâmetros sensíveis para verificar os efeitos da exposição a substancias químicas poluentes.

O zebrafish é um modelo experimental muito utilizado no campo da ecotoxicologia, devido em grande parte à robustez de suas respostas à manipulação farmacológica e, aliado a isso, as premissas evidenciadas pelo artigo 1 foram exploradas de maneira mais assertiva. Dessa forma, utilizando a metodologia de RT-PCR, os resultados anteriores (Rosa et al., 2013; Rosa et al. 2015) foram confirmados e foi possível demonstrar o mecanismo do efeito toxico do MP sobre o tecido nervoso do zebrafish. Além disso, demonstramos que ao inibir a expressão de determinados genes relacionados ao eixo HHI, ocorre o comprometimento da resposta de cortisol ao estresse. Esses efeitos que caracterizam o MP como desregulador endócrino parecem ser mediados pela hiperestimulação dos receptores muscarínicos, visto que quando é administrado um fármaco antagonista desses receptores, a escopolamina, esse efeito parece ser revertido.

A reposta de cortisol ao estresse é coordenada pelo eixo HHI, portanto qualquer componente desse eixo pode ser alvo de uma substância com potencial de desregulador endócrino, inclusive a proteína StAR (Fuzzen et al. 2010; Pavlids et al. 2015; Lyssimachou e Arukwe, 2007). Além desse, outros genes que codificam componentes intermediários do eixo HHI foram inibidos, como *pomc, bgr* e *hsp70*.

Os hormônios tróficos, como o hormônio liberador de corticotrofina (CRF), agem através de segundo-mensageiros, sendo o mais conhecido o AMPc. Sendo um hormônio trófico, o CRF é o primeiro a ser sintetizado quando ocorre a ativação do eixo HHI, e sinaliza para a síntese de proopiomelanocortina (POMC), através da via do monofosfato de adenosina cíclico (AMPc), que continua a cascata de ativação até o produto final, cortisol. O efeito clássico dos organofosforados, como o MP, é inibir a enzima acetilcolinesterase, evitando a hidrólise da acetilcolina, e levando a um quadro de hiper-ativação colinérgica. Como não ocorre uma ativação especifica, todos os receptores muscarínicos (mAChR) são ativados, inclusive os subtipos M2 e M4, que são inibitórios. Quando ativados esses receptores inibitórios provocam a inibição da síntese de AMPc. Assim como os hormônios tróficos, o gene *star* também é ativado pela mesma via da AMPc (Sierra 2004; Benmessahel et al. 2004;

Stocco 2001; Sandhoff e McLean 1996), e esta estando inibida pelo MP, demonstra ser a causa da inibição tanto de genes chave da ativação do eixo, como *star* e *pomc*, quanto do produto final desse eixo, o hormônio cortisol (Walsh et al. 2000; Benmessahele et al. 2004; Stocco 2001; Sandhoff e McLean 1996). Esse efeito de organosfosforado parece não ser espécie-especifico, visto que em *Rhamdia quelen* exposto ao MP, o mesmo padrão de interrupção endócrina é relatado (Cericato et al. 2009). Com a administração de escopolamina, um antagonista muscarínico competitivo, esses efeitos parecem ser revertidos. Isso acontece pois é encerrada a ativação dos receptores, incluindo M2 e M4, e os níveis normais de AMPc são restaurados a seus níveis normais.

Belanger et al (2005), demostraram que a presença de poluentes ambientais exerce efeitos nos processos comportamentais mediados pela olfação, como busca por alimento, comportamento reprodutivo e interação social. No artigo 2, foi verificado um efeito aversivo do herbicida baseado em glifosato, exceto nos 30s iniciais de exposição, sugerindo que a aversão ao local contaminado só inicia após um contato prévio com a substância. Isso pode ser devido a propriedades irritantes da substância. Quanto aos agroquímicos baseados em atrazina e tebuconazole, essas substâncias não apresentaram efeito nem aversivo nem atrativo. Apesar de a exposição ao herbicida a base de glifosato não oferecer maior efeito deletério, por possui efeito aversivo, a exposição aos demais agroquímicos sugere uma maior toxicidade, já que as substâncias não repelem os peixes, que não percebem que estão em água contaminadas, podendo aumentar os efeitos tóxicos. Levando em conta a natureza químico-sensorial do teste, e o fato de ser uma exposição aguda, esse efeito aversivo do herbicida baseado em glifosato pode ser considerado ser originado por um estímulo olfatório. Além disso, sendo o protocolo experimental anteriormente validado em outros estudos (Readman et al 2013; Abreu et al. 2016), e pelo fato de agroquímicos como HBG nao alterarem o pH do meio (Vera et al. 2010), qualquer alteração comportamental verificada pode ser atribuída aos tratamentos utilizados no experimento. O fenômeno da bioacumulação em animais aquáticos parece ser tempo e dose-dependente (Hamelink and Spacie 1977; Geyer et al. 2000; Paterson and Metcalfe 2008), e como pelo menos por algum tempo os agroquímicos não são percebidos, os efeitos que foram evidenciados por exemplo nos artigos 1 e 2, se tornam mais deletérios conforme aumenta o tempo de contato com esses compostos.

Além do potencial aumento dos efeitos de desregulação endócrina, que podem ocorrer mesmo quando há no ambiente baixas concentrações, essas substâncias podem alterar a fisiologia olfatória dos peixes, interferindo em processos ecológicos que dependem desse sistema sensorial, como interação com presa-predador, procura por parceiros ou ainda o comportamento social entre co-específicos. Tierney e colaboradores

(2009) relataram em um estudo que uma mistura de pesticidas contendo HBG provocou atração em zebrafish, e em nosso estudo, a mesma concentração não provocou atração tampouco aversão, no entanto, as metodologias utilizadas são muito distintas e podem explicar essa diferença.

Além disso, na maior concentração do FBT, verificou-se o aumento de parâmetros locomotores como ângulo de giro, que conforme Blazina et al, (2013), são indicativos da função locomotora. O aumento dos níveis de ângulo de giro nos peixes expostos ao FBT pode indicar efeitos neuromusculares, e essa alteração na atividade locomotora pode explicar a resposta não aversiva verificada.

A ausência de detecção, ou a impossibilidade de escapar de locais contaminados são extremamente preocupantes, tanto a nível individual quanto a nível de população, uma vez que os animais ficam sujeitos a uma exposição prolongada. Essa resposta aversiva representa um comportamento defensivo, e uma vez que pode ser medido em ambiente experimental se torna uma ferramenta extremamente interessante para o monitoramento de poluentes aquáticos.

Da mesma forma que apresenta ótimas respostas à manipulação farmacológica, o zebrafish apresenta um repertorio comportamental já estabelecido e decifrado (Rosemberg et al. 2011), e além disso, suas respostas endócrinas a adaptações fisiológicas já estão muito bem caracterizadas (Fuzzen et al. 2010).

Sendo assim, foi realizado um experimento onde foi testado um novo modelo de estresse utilizando como animal experimental o zebrafish. Demonstramos que um “spinning test”, validado previamente por Blazina et al. (2013), onde o peixe é exposto a uma corrente em uma arena circular, pode ser utilizado como modelo estressor. Na velocidade de 570 rpm por um tempo de 5 minutos, ocorre a ativação do eixo HHI com consequente elevação de cortisol que, por sua vez, provoca a mobilização energética característica de uma situação de estresse (Mommsen et al. 1999; Barcellos et al. 2010). Concomitante à elevação de cortisol, ocorre a depleção das reservas de carboidratos, evidenciadas pela redução da glicose e glicogênio. Foi relatado que em peixes tratados com metirapona, um inibidor da síntese de cortisol, ocorre aumento na concentração de glicogênio (Milligan 2003), logo, assume-se uma relação indireta entre a concentração de cortisol e glicogênio tecidual. Outro resultado interessante, que corrobora com os anteriores, é o aumento da concentração de lactato, concomitante à redução de glicose e glicogênio, o que indica o início da atividade anaeróbica, o que ocorre também em outras espécies após exercício intenso Liew et al.(2012). Além disso, ocorre a elevação de outro

biomarcador de atividade anaeróbica, a creatina quinase (CK), indicando a utilização de energia proveniente da fosfocreatina. E como em um protocolo estressor clássico, ocorre alteração de comportamento, onde parâmetros como distância percorrida e velocidade estão elevados e apresentam redução após o período de recuperação pós exercício. A elevação desses parâmetros está relacionada a um estado de hiperatividade, que pode a levar a um comportamento do tipo ansiedade (Maximino et al. 2010; Kalueff et al. 2013). Além disso, o exercício pode provocar a ativação do sistema catecolaminérgico, que assim como a síntese de cortisol, pode ser iniciado pela ação do ACTH (Reid et al. 1996; Reid et al. 1998), e a elevação de catecolaminas pode contribuir para o comportamento do tipo ansiedade verificado. Em roedores, Pacak et al. (1995) relacionou o aumento de ACTH plasmático com a elevação de catecolaminas no nucleo paraventricular, região do sistema nervoso central responsável tanto pela síntese de ACTH, quanto pela promoção de um estado de defesa que pode ser interpretado como ansiedade, mais uma vez corroborando a ativação do eixo HHI pelo protocolo utilizado.

O zebrafish representa um valioso modelo experimental por possuir uma série de características extremamente importantes, além de exibir um grande potencial translacional. Esse novo protocolo estressor demonstrou ser eficaz na ativação do eixo HHI, e representa uma grande possibilidade de utilização em protocolos para investigar os efeitos e mecanismos de toxicidade causados por interruptores endócrinos.

CAPÍTULO VII

CONCLUSÕES

A exposição à agroquímicos organofosforados altera a expressão de genes codificadores de proteínas essenciais à ativação e manutenção do eixo hipotálamo-hipófise-interenal;

O mecanismo pelo qual os organofosforado causam desregulação endócrina é a hiperativação de receptores muscarínicos decorrente da exposição.

Agroquímicos em ecossistemas aquáticos podem alterar a percepção e alterar o repertório comportamental dos peixes.

O modelo de estresse por nado forçado se mostrou eficaz para definir os parâmetros metabólicos, comportamentais e endócrinos basais provocados pela ativação do eixo HHI.

IMPLICAÇÕES DA PESQUISA

Esse novo modelo apresenta alta aplicabilidade na pesquisa de contaminantes ambientais, auxiliando na produção de ferramentas de pesquisa, utilizando aspectos moleculares, endócrinos, metabólicos e comportamentais para produzir um panorama completo dos efeitos de xenobioticos em ambiente aquático, utilizando como referência as respostas fisiológicas dos peixes frente às contaminações ambientais.

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