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EFEITOS NEUROENDÓCRINOS E COMPORTAMENTAIS DO ARIPIPRAZOL EM ZEBRAFISH

Santa Maria, 2019

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM) como requisito parcial para a obtenção de título de Doutora em Farmacologia.

Orientador: Prof. Dr. Bernardo Baldisserotto
Co-orientador: Leonardo José Gil Barcellos

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RESUMO

EFEITOS NEUROENDÓCRINOS E COMPORTAMENTAIS DO ARIPIPRAZOL EM ZEBRAFISH

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O aripiprazol (APPZ) é um antipsicótico atípico de segunda geração, que vem sendo cada vez mais utilizado no tratamento de psicoses, tais como esquizofrenia, autismo, transtorno afetivo bipolar, entre outros. A crescente utilização deste fármaco se dá porque ele provoca menos efeitos extrapiramidais em relação aos antipsicóticos típicos. Associado ao aumento nas prescrições e, consequentemente, no consumo deste medicamento, existe o risco, também crescente, de contaminação ambiental por seus resíduos, seja em forma ativa ou em forma de metabólitos. Estes resíduos no ambiente podem afetar organismos não alvo como os peixes, interferindo de forma negativa na sua homeostase e em seus mecanismos fisiológicos de defesa. Vários estudos têm demostrado a problemática da contaminação de ambientes aquáticos e o impacto sobre a fauna desses ecossistemas por diversos psicofármacos. Nesse contexto, o objetivo geral do presente trabalho foi o de verificar se o APPZ altera as respostas endócrinas e comportamentais ao estresse em *zebrafish* adultos. Nós mostramos, com base nos resultados descritos nos dois artigos produzidos, que os resíduos de APPZ na água produzem efeitos tanto no eixo hipotálamo-hipófise-interrenal (HHI) quanto no comportamento do *zebrafish* adulto. No primeiro artigo, podemos evidenciar que os peixes expostos previamente ao APPZ, quando submetidos ao estímulo estressor agudo, tiveram um claro embotamento da resposta de cortisol ao estresse. Em relação ao efeito do APPZ sobre o comportamento do *zebrafish*, observamos que algumas concentrações reverteram a resposta comportamental ao estresse tanto no aspecto de preferência social, quanto no comportamento do tipo-ansiedade e na percepção de um peixe estímulo, seja predador ou não predador. Assim, concluímos que a exposição aguda ao APPZ é capaz de causar o embotamento do eixo neuroendócrino de estresse em *zebrafish* adultos, bem como alterar o comportamento exploratório e social e a reação frente a uma ameaça predatória. Num contexto ecológico, o somatório desses efeitos endócrinos e comportamentais causados pelo APPZ pode expor os peixes a um maior risco de predação e, consequentemente pôr em risco o equilíbrio ecológico entre as espécies que compõem o ecossistema aquático.

Palavras-chave: *Danio rerio.* Cortisol. Contaminação ambiental. Comportamento exploratório. Comportamento social. Reação anti-predatória.

ABSTRACT

NEUROENDOCRINE AND BEHAVIORAL EFFECTS OF ARIPIPRAZOLE IN ZEBRAFISH

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ADVISOR: Bernardo Baldisserotto

The aripiprazole (APPZ) is a second-generation atypical antipsychotic, which has been increasingly used in the treatment of psychoses, such as schizophrenia, autism, bipolar affective disorder, among others. The increasing use of this drug occurs because it causes less extrapyramidal effects compared to typical antipsychotics. Associated with the increase in prescriptions and, consequently, in the consumption of this drug, there is, consequently, a growing risk of environmental contamination by its residues, either in active form or in the form of metabolites. These residues in the environment can affect non-target organisms such as fish, interfering negatively in their homeostasis and in their physiological defense mechanisms. Several studies have demonstrated the problematic of the contamination of aquatic environments and the impact on the fauna of these ecosystems by several psychotropic drugs. In this context, the aim of the present study was to verify if aripiprazole changes the endocrine and behavioral responses to stress in adult zebrafish. We have shown, based on the results described in the two articles presented, that APPZ residues in water produce effects on both the hypothalamic-pituitary-interrenal (HHI) axis and in the behavior of adult zebrafish. In the first article, we show that the fish previously exposed to APPZ, when subjected to the acute stressor, presented a blunted cortisol response to stress. Regarding to the effect of APPZ on zebrafish behavior, we observed that some of the tested concentrations reversed the behavioral response to stress in both the social preference, the anxiety-like behavior and the perception of a stimulus fish, whether predator or not predator. Thus, we conclude that acute exposure to APPZ is capable of blunting the neuroendocrine axis of stress in adult zebrafish, as well as altering exploratory and social behavior and reaction to a predatory threat. In an ecological context, the sum of these endocrine and behavioral effects caused by APPZ may expose fish to a greater risk of predation and, consequently, increase the risk of broken of ecological balance between species, and consequently impact the aquatic ecosystem.

Key words: *Danio rerio.* Cortisol. Environmental contamination. Exploratory behavior. Social behavior. Anti-predatory reaction.

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LISTA DE ABREVIATURAS E SIGLAS

ACTH – hormônio adrenocorticotrófico

AMPc – adenosina monofosfato cíclica

ANOVA – análise de variância

ANVISA – Agência Nacional de Vigilância Sanitária

APPZ – aripiprazol

BGR – *brain glucocoticoid receptor*

BDNF - *brain-derived neurotrophic factor*

CEUA – Comissão de Ética no Uso de Animais

CNRH – Conselho Nacional de Recursos Hídricos

CONAMA – Conselho Nacional do Meio Ambiente
CONCEA – Conselho Nacional de Experimentação Animal
CRF – *Corticotrophin Releasing Factor*
CRH – hormônio liberador de corticotrofina
Fig. - figura
HHA – eixo hipotálamo-hipófise-adrenal HHI – eixo hipotálamo-hipófise-interrenal

IBGE Pnad - Instituto Brasileiro de Geografia e Estatística -Pesquisa Nacional por Amostra de Domicílio

GABA – ácido gama aminobutírico

IFNλ - interferon gama

IL-1 – interleucina 1 IL-10 – interleucina 10

IL-4 – interleucina 4

Kd – constante de dissociação

Ki – constante de afinidade

MMA – Ministério do Meio Ambiente

POMC – pró opiomelanocortina

S.E.M. – *Standard Error of Mean* (Erro Padrão da Média)

StAR – *Steroidogenic Acute Regulatory Protein*

TCE – teste do claro-escuro

TNF - *tumor necrosis factor*

TPS – teste de preferência social

TTN – teste do tanque novo

SUMÁRIO

1. INTRODUÇÃO

O aripiprazol (APPZ) é um antipsicótico atípico de segunda geração, que vem sendo cada vez mais utilizado no tratamento da esquizofrenia, do autismo, do transtorno afetivo bipolar, entre outros. A crescente utilização deste fármaco se dá porque ele provoca menos efeitos extrapiramidais em relação aos antipsicóticos típicos. Associado ao aumento nas prescrições e, consequentemente, no consumo deste medicamento, existe o risco, também crescente, de contaminação ambiental por seus resíduos, seja em forma ativa ou em forma de metabólitos. Estes resíduos no ambiente podem afetar organismos não alvo como os peixes, interferindo de forma negativa na sua homeostase e em seus mecanismos fisiológicos de defesa. Nesse contexto, a presença do APPZ nas águas tem sido pesquisada e já tem sido descrita na literatura em afluentes e efluentes, tanto na América do Norte quanto na Ásia.

Vários estudos têm demostrado a problemática da contaminação de ambientes aquáticos e o impacto sobre a fauna desses ecossistemas por diversos psicofármacos. O impacto de resíduos do APPZ sobre aspectos endócrinos e/ou comportamentais de roedores e humanos já está bem descrito, mas os impactos sobre os peixes ainda são pouco conhecidos. Acredita-se que pela semelhança neuroendócrina entre peixes e roedores, os efeitos sejam similares, contudo, muito pouco já foi empiricamente testado. Frente à escassez de informações sobre os impactos da contaminação ambiental pelo APPZ nos organismos aquáticos, é que o presente estudo se justifica. Para testarmos a hipótese de que o APPZ impacta no eixo hipotálamo- hipófise-interrenal, homólogo do eixo adrenal dos mamíferos, e no comportamento dos peixes, escolhemos o zebrafish (*Danio rerio*), como modelo experimental.

Assim, o objetivo geral da presente tese é o de elucidar questões sobre o impacto que diferentes concentrações do APPZ na água ocasionam no eixo neuroendócrino de estresse, assim como no comportamento de peixes expostos, destacando-se os aspectos relacionados a ansiedade e medo. A tese se fundamenta na análise do cortisol em peixes expostos ao APPZ *per se* e após um estímulo estressor e na análise das respostas comportamentais do *zebrafish* a testes consagrados como o teste do tanque novo (TTN), teste de preferência social (TPS) e

teste de exposição ao predador. Trata-se de um estudo básico que poderá ser aplicado às demais espécies aquáticas, inclusive para diferentes espécies de teleósteos usados na produção. Da mesma forma, face ao grande potencial translacional do *zebrafish*, esse estudo também poderá contribuir para o melhor entendimento dos efeitos do uso do APPZ em humanos para o tratamento de afecções neuropsiquiátricas.

2. REVISÃO BIBLIOGRÁFICA

2.1. ARIPIPRAZOL

O aripiprazol (APPZ) é um antipsicótico atípico de segunda geração, aprovado para o tratamento de esquizofrenia, transtorno bipolar (BURRIS et al., 2002; TESSLER; GOLDBERG, 2006; BIOJONE et al., 2011), autismo (ACCORDINO et al., 2016; GOEL et al., 2018) e tem sido utilizado também como monoterapia ou adjuvante no tratamento transtorno compulsivo obsessivo (SAHRAIAN; EHSAEI; MOWLA, 2018) e transtornos de ansiedade ou pânico (PIGNON; TEZENAS; CARTON, 2017). Dentre as características pesquisadas sobre este fármaco, destaca-se o controle de efeitos positivos e negativos na esquizofrenia (MAILMAN; MURTHY, 2010) e o controle de irritabilidade, hiperatividade e estereotipias no autismo (ACCORDINO et al., 2016; ICHIKAWA et al., 2018). Em mamíferos também evidencia-se efeitos ansiolítico, panicolítico e anti-aversivo (KOENER et al., 2012; KLING et al., 2014). Os estudos em roedores evidenciam claramente que os efeitos do APPZ são dose- dependentes (BIOJONE et al., 2011).

2.1.1. Características farmacológicas do aripiprazol

A molécula do APPZ, (7-{4-[4-(2,3-dichlorophenyl)-1-piperazinyl]-butyloxy}-3,4-dihydro-2(1*H*)-quinolinone, Figura 1, foi desenvolvida pelos grupos farmacêuticos Otsuka e

Bristol-Myers Squibb (BURRIS et al., 2002; GRADY; GASPERONI; KIRKPATRICK, 2003;

HIROSE; KIKUCHI, 2005b). Este derivado da quinolona foi considerado apto para os estudos farmacológicos de fase III no Japão (GRADY; GASPERONI; KIRKPATRICK, 2003), sendo sua primeira aprovação para o tratamento da esquizofrenia em 2002 pelo US FDA (NEVO et al., 2017).

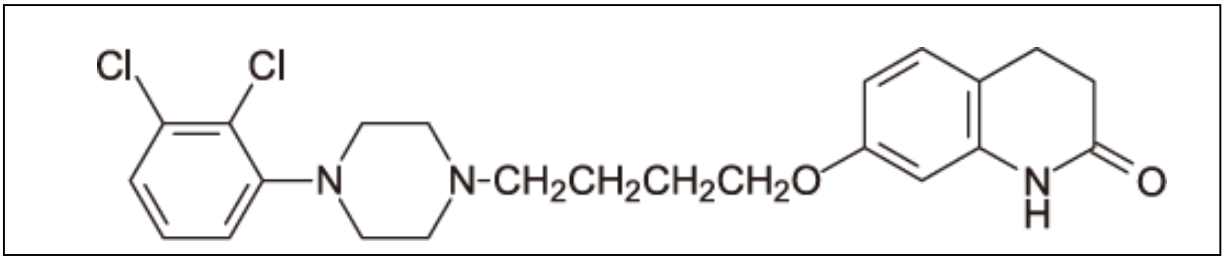

Figura 1 – Estrutura química do APPZ. Fonte: (HIROSE; KIKUCHI, 2005).

2.1.1.1 Farmacodinâmica

A principal característica farmacológica do APPZ é a ação agonista parcial dos receptores dopaminérgicos (TAMMINGA; CARLSSON, 2002; HIROSE; KIKUCHI, 2005; MAMO et al., 2007) e serotoninérgicos (HIROSE; KIKUCHI, 2005). Ou seja, esse fármaco possui a capacidade de se ligar ao receptor bloqueando os efeitos extracelulares da dopamina, ao mesmo tempo que exerce uma reação similar da molécula com uma magnitude de reação menor (HIROSE; KIKUCHI, 2005; HORACEK et al., 2006). Essa capacidade de ligar-se facilmente é devido à afinidade (Ki) do fármaco pelo receptor (KAPUR; SEEMAN, 2000). Os agonistas parciais possuem, além da alta afinidade, uma constante de dissociação (Kd) elevada, o que lhes garante facilidade e rapidez em ligar-se e se dissociar-se dos receptores (KAPUR; SEEMAN, 2000).

Em relação aos receptores dopaminérgicos, o APPZ possui alta afinidade pelos receptores D_2 (K_i = 0,45 nM) (KURAHASHI et al., 2003) com alta taxa ligante à proteína G acoplada, diminuindo o acúmulo de adenosina monofosfato cíclica (AMPc) após a

fosforilação e produzindo um efeito máximo semelhante a dopamina de 85% (BURRIS et al., 2002). A sua taxa de dissociação dos receptores D_2 (K_d = 0,037 nM) (ROTH; SHEFFLER; POTKIN, 2003), também favorece sua eficácia.

Logo, esta afinidade pelos receptores dopaminérgicos faz com que o APPZ seja considerado um estabilizador desse sistema (HIROSE; KIKUCHI, 2005), levando a menores efeitos extrapiramidais em pacientes com esquizofrenia por exemplo (HORACEK et al., 2006). Esses pacientes possuem altas concentrações de dopamina extracelular na região mesolímbica do encéfalo, e baixa concentração no córtex pré-frontal e, nestes casos, o APPZ compete com a dopamina, causando um efeito antagonista na região mesolímbica e um efeito agonista parcial na porção pré-frontal do córtex, ocupando receptores adicionais e ocasionando uma ativação parcial de dopamina (MAILMAN; MURTHY, 2010).

O sistema dopaminérgico é amplamente difuso pelo organismo, entretanto, cada região possui quantidades diferentes de receptores. A hipófise, por exemplo, tem menor quantidade de receptores D_2 que o restante do encéfalo (RANG et al., 2012), motivo pelo qual o APPZ possui menor ação sobre a liberação de prolactina em mamíferos (MAILMAN; MURTHY, 2010). Handley et al. (2016) mostraram também que o APPZ em dose terapêutica não interfere nos eixo hipotálamo-hipófise-adrenal (HHA) de pacientes com esquizofrenia, mantendo a concentração de cortisol semelhante aos de pacientes que receberam placebo. Como o eixo HHA de mamíferos está relacionado com sistema dopaminérgico (BRUNELIN et al., 2008) e o APPZ tem baixa afinidade por receptores nesta área (MAILMAN; MURTHY, 2010), é lógico que o mesmo não promova desregulação endócrina na liberação de cortisol em situações de estresse em mamíferos.

Em relação aos receptores serotoninérgicos, o APPZ possui ação agonista parcial de $5HT_1$ e antagonista dos receptores $5HT_{2A}$ (HIROSE; KIKUCHI, 2005). Entretanto, sua afinidade para tais receptores é menor que pelos receptores dopaminérgicos, a qual pode ser explicada pelo maior valor de sua constante de afinidade (Ki para o 5-$HT1_A$ = 5,6 nM; Ki para 5-HT_2 = 4,6 nM) (ROTH; SHEFFLER; POTKIN, 2003). O APPZ também possui ação agonista parcial em $5HT_7$ e antagonista de $5HT_6$ (TAMMINGA; CARLSSON, 2002; BURDA et al., 2011) mas em menor magnitude. Os receptores $5HT_1$ e $5HT_{2A}$ estão localizados em neurônios

do núcleo da rafe, cuja porção rostral inervam a ponte e o mesencéfalo. A função destes receptores é regular o funcionamento do sono, do humor, assim como os comportamentos emocionais (BEAR, 2012) do tipo ansiedade e agressão (SANDERS-BUSH; HAZELWOOD, 2012). Tais receptores também estão relacionados ao comportamento de ansiedade, pânico e aversão em roedores (BIOJONE et al., 2011). Já os receptores $5HT_6$ localizam-se no hipocampo, no córtex e no sistema límbico, e os receptores $5HT_7$ localizam-se no hipocampo, no córtex, na amigdala e no hipotálamo, sendo esses encontrados também no soma e nos terminais dos neurônios para o ácido gama-aminobutírico (GABA), nos vasos sanguíneos e no sistema gastrintestinal (RANG et al., 2012). Entretanto, a afinidade pelos receptores $5HT_6$ (Ki= 570 ± 95 nM) $_e$ $5HT_7$ (Ki = 10,3 ± 3,7 nM) em roedores é pequena (SHAPIRO et al., 2003).

2.1.1.2 Farmacocinética

O APPZ exerce sua atividade tanto por sua molécula íntegra como pelo seu principal metabólito, o dihidro-aripiprazol (MOLDEN et al., 2006). Apresenta uma taxa de distribuição extra vascular, quando administrado por via intravenosa de 4,9 L/kg (PROMMER, 2017), ligando-se facilmente às proteínas plasmáticas, mais especificamente à albumina (KINGHORN; MCEVOY, 2005). Possui uma biodisponibilidade de 87%, sendo seu metabólito ativo com 40% de atividade semelhante à molécula íntegra (MOLDEN et al., 2006). É metabolizado pelo fígado, mais especificamente via citocromos CYP3A4 e CYP2D6 (KINGHORN; MCEVOY, 2005), atingindo a concentração máxima em meia-hora quando administrado por via intramuscular, e entre 3 a 5 horas por via oral (PROMMER, 2017). Tem uma meia-vida de eliminação de até 75h, e seu metabólito de até 94h (DIAK; METHA, 2008). Apresenta 27% de eliminação renal e 60% fecal, podendo ser eliminado na forma ativa não metabolizada em até 1% na urina e 18% nas fezes (DELEON; PATEL; CRISMON, 2004).

2.2. CONTAMINAÇÃO DO ECOSSISTEMA AQUATICO

A contaminação de ecossistemas aquáticos por diversos tipos de produtos da indústria farmacêutica é uma preocupação mundial crescente desde a década de 90 (DAUGHTON; TERNES, THOMAS, 2009; LIENERT et al., 2011; ARNOLD et al., 2014). Vários contaminantes emergentes de origem farmacológica vêm sendo estudados pois representam uma ameaça ambiental, contaminando o solo e a água e ocasionando efeitos deletérios para o ecossistema e para quem dele usufrui (DAUGHTON; TERNES, THOMAS, 2009; BROOKS et al., 2003; BOXALL, 2004; CALISTO; ESTEVES, 2009; CABEZA et al., 2012; FORD;
HERRERA, 2018). Diferentes métodos de detecção de múltiplos resíduos no ambiente vêm sendo estudados (LÓPEZ-GARCÍA et al., 2018), em face da importância do impacto desses resíduos, mesmo em concentrações ínfimas, no ecossistema (FORD; HERRERA, 2018).

Os resíduos dos medicamentos (e seus metabólitos) podem atingir o ambiente através da excreção nas fezes ou urina dos pacientes usuários (pessoas ou animais), ou através do descarte inadequado (HEBERER, 2002; BOXALL, 2004), por modo direto ou indireto. A contaminação direta ocorre devido a um descarte direto do produto, causada por falhas no tratamento sanitário de medicamentos usados nos hospitais (LIENERT et al., 2011) ou descarte de embalagens vazias ou de restos de medicamentos diretamente na água (HALLING- SORENSEN et al., 1998). Já a contaminação indireta ocorre pelo armazenamento inadequado do produto pela própria indústria, contaminando o solo e atingindo assim lençóis freáticos, açudes, lagos, rios (HALLING-SORENSEN et al., 1998), estuários e oceanos (ARNOLD et al., 2014).

Esta contaminação ambiental por poluentes vem sendo detectada em diferentes produtos farmacêuticos, inclusive com os fármacos psicoativos (CALISTO; ESTEVES, 2009; HUERTA-FONTELA; GALCERAN; VENTURA, 2010; CALISTO; DOMINGUES; ESTEVES, 2011; CABEZA et al., 2012; PETRIE; BARDEN; KASPRZYK-HORDERN, 2014), cuja prescrição e consumo aumentaram nas últimas décadas em todo o mundo (FRIEDMAN, 2014; BACHMANN et al., 2016; FORD; HERRERA, 2018). Assim como outros medicamentos, os psicofármacos também são metabolizados e excretados na forma de

metabólitos, ou mesmo na forma íntegra, conjugada ou não (RANG et al., 2012), através das fezes e da urina, e atingem esgotos e afluentes das estações de tratamento (HALLING-SORENSEN et al., 1998). Mesmo após o tratamento sanitário, vários resíduos têm sido encontrados nos efluentes em concentrações variáveis (HALLING-SORENSEN et al., 1998; HEBERER, 2002; HUERTA-FONTELA; GALCERAN; VENTURA, 2010; CABEZA et al.,2012; YUAN et al., 2013; SUBEDI; KANNAN, 2015). Por exemplo, a risperidona e a fluoxetina, foram detectadas na água de beber nos Estados Unidos, nas concentrações de 0,0005 µ/L e 0,00034µ/L, respectivamente (CALISTO; ESTEVES, 2009), enquanto o APPZ foi encontrado nos efluentes de estações de tratamento de água na China, na concentração de 5,56 ng/L (SUBEDI; KANNAN, 2015).

Os fármacos psicoativos foram desenvolvidos para tratamento de controle de ansiedade, distúrbios psiquiátricos e diminuição do estresse em humanos (RANG et al., 2012). Entretanto, resíduos destes medicamentos no ambiente aquático podem causar um impacto negativo tanto na população usuária desses recursos hídricos, quanto nos organismos que compõem os ecossistemas aquáticos (LIENERT et al., 2011).

Alguns estudos já demonstram que, mesmo em baixíssimas concentrações, fármacos psicoativos tais como risperidona (IDALENCIO et al., 2015; KALICHAK et al., 2016; KALICHAK et al., 2017), fluoxetina, diazepam (ABREU et al., 2014; ABREU et al., 2015; (GIACOMINI et al., 2016b), haloperidol (MAGNO et al., 2015; TRAN; FACCIOL; GERLAI, 2016), metilfenidato (ENDRES et al., 2017) e mianserina (BARRETO, 2017) na água interferem de forma negativa nos peixes, prejudicando sua homeostase.

2.2.1 Situação dos contaminantes antipsicóticos emergentes no Brasil

As políticas públicas brasileiras que tratam da qualidade da água no país são regulamentadas pelo CONAMA (Conselho Nacional do Meio Ambiente) resoluções n. 357/2005 e 397/2008, pelo CNRH (Conselho Nacional de Recursos Hídricos) legislação n. 91/2008, pela ANVISA (Agência Nacional de Vigilância Sanitária) legislação RDC n. 306 de 7 de dezembro de 2004, e pelo MMA (Ministério do Meio Ambiente) pela resolução n.

358 de 29 de abril de 2005. Para o manejo de descarte dos resíduos do grupo B (no qual encontram-se os antipsicóticos), a ANVISA orienta que quando não forem submetidos a processo de reutilização, recuperação ou reciclagem, devem ser submetidos a tratamento ou disposição final específicos, para que não promova riscos de contaminação do meio ambiente. Esses órgãos tratam das principais regulamentações para o enquadramento na Lei nº 9.433, que visa garantir a qualidade da água para gerações futuras, com um padrão de qualidade conforme seu uso. Entretanto, segundo informações do IBGE Pnad (2015), cerca de 35 % da população brasileira não possui coleta de esgoto, sendo este descartado diretamente no meio ambiente. Essa destinação inadequada do esgoto dificulta o trabalho destes órgãos que classificam os corpos de água e propõem as diretrizes para as condições e padrões de lançamento de efluentes.

Entretanto, apesar da existência de políticas públicas no país que orientam o destino correto de diferentes tipos de resíduos, vários contaminantes emergentes de origem farmacológica vêm sendo detectados nas águas brasileiras (JANK et al., 2014; FREITAS, 2018). Entende-se por contaminantes emergentes, as substâncias químicas que contaminam os ecossistemas que receberam maior atenção nos últimos anos. Contudo, o termo emergente induz ao pensamento de que tais contaminantes foram introduzidos recentemente no meio ambiente, porém o que está emergindo no país é a maior conscientização da população sobre sua presença no ambiente e sua relação direta com o uso doméstico (FREITAS, 2018). A presença de agrotóxicos (CHIARELLO et al., 2017), antibióticos (JANK et al., 2014; ARSAND et al., 2018), anti-hipertensivos (FREITAS, 2018) e hormônios (SOFIATTI, 2018) tem sido detectada nos ambientes aquáticos brasileiros.

2.3. ALTERAÇÕES DOS FÁRMACOS PSICOATIVOS NO COMPORTAMENTO E NO EIXO NEUROENDÓCRINO DO ESTRESSE

2.3.1. Fisiologia da resposta ao estresse

O estresse é um processo fisiológico de defesa que ocorre em qualquer espécie, em decorrência de algum estimulo estressor, podendo ser benéfico ou deletério (MCEWEN,

2005). O disparo de uma resposta ao estresse agudo é indispensável, por exemplo, para produzir energia e condições fisiológicas adequadas numa situação de fuga da exposição a patógenos e xenobióticos (estímulos abióticos), ou fuga de predadores ou busca por uma presa (estímulos bióticos) (HONTELA, 1998). O estímulo estressor nessas situações desencadeia alterações neuroendócrinas e comportamentais (estado alostático) com o objetivo de promover os ajustes necessários para manutenção da estabilidade do organismo (alostase), visando o equilíbrio fisiológico do sistema (MCEWEN; WINGFIELD, 2003; GRAEFF; ZANGROSSI JR, 2010).

Porém, quando numa determinada situação a resposta ao estresse torna-se prolongada, geralmente promove impactos negativos na população aquática (BARTON; IWAMA, 1991), tais como imunossupressão, pouca procura por alimento, redução no crescimento, supressão da reprodução e aumento da incidência de doenças (ASHLEY, 2007; MCEWEN; WINGFIELD, 2003).

Os efeitos desencadeados pela resposta do organismo ao estresse podem ser divididos em três fases: primários, secundários e terciários. Os efeitos primários referem-se à ativação do eixo hipotálamo-pituitária-adrenal (HPA) em mamíferos, ou hipotálamo-pituitária-interrenal (HPI) em peixes; e a ativação do sistema nervoso simpático para produção de catecolaminas na região medular da adrenal (mamíferos) (MCEWEN, 2005) ou nas células cromafins (peixes) (URBINATI; ZANUZZO; BILLER-TAKAHASHI, 2014). O hipotálamo, em resposta à percepção de um estímulo estressor, libera o hormônio liberador de corticotrofina (CRH), que por sua vez, estimula a glândula pituitária a sintetizar e liberar o hormônio adrenocorticotrófico (ACTH). O ACTH estimula as células do tecido interrenal (localizadas no rim cefálico ou pronéfron) a sintetizar e secretar o cortisol. O CRH estimula também fibras do sistema simpático nas células cromafins a liberarem as catecolaminas (REID; BERNIER; PERRY, 1998; PERRY; CAPALDO, 2011). As células cromafins estão localizadas nas paredes da veia cardinal posterior, próximo às células inter-renais (HONTELA, 1998; URBINATI; ZANUZZO; BILLER-TAKAHASHI, 2014). Esta proximidade entre as células cromafins e o tecido interrenal justifica a relação direta (parácrina) entre as catecolaminas e cortisol: ou seja, concentrações elevadas de cortisol tendem a elevar as concentrações de catecolaminas, bem com a sensibilidade das células cromafins à estimulação colinérgica (REID; BERNIER; PERRY, 1998).

Os efeitos gerados pela estimulação neuroendócrina são os efeitos secundários do estresse, que provocam as alterações nas funções fisiológicas, mais rapidamente observadas pela ação das catecolaminas, adrenalina e noradrenalina. Relacionado ao sistema vascular observa-se aumento na frequência cardíaca e do fluxo sanguíneo para a brânquias, com o objetivo de manter a oxigenação do sangue e dos tecidos (REID; BERNIER; PERRY, 1998). O metabolismo torna-se acelerado, pois reservas hepáticas de glicogênio são consumidas no intuito de elevar a glicose sanguínea, na tentativa de manter a homeostase. O cortisol também estimula a gliconeogênese a partir de aminoácidos e promove a lipólise, como meio de auxiliar na produção de energia na situação estressante (URBINATI; ZANUZZO; BILLER-TAKAHASHI, 2014). Além disso, a ação conjunta dos glicocorticóides e das catecolaminas no cérebro promovem a formação de memória de eventos potencialmente perigosos, os quais o indivíduo deverá evitar no futuro (ROOZENDAAL, 2000).

Entretanto, quando os níveis de cortisol se elevam intensamente ou se mantêm elevados por muito tempo, ocorre um efeito imunossupressor, principal efeito terciário. Além disso, o cortisol elevado desregula o fluxo iônico, ocasionando perturbações osmóticas, com alterações eletrolíticas importantes. Em conjunto com a ativação do eixo hipotálamo-pituitária-interrenal ocorre a liberação de glucagon, hormônio do crescimento e tiroxina (HONTELA; DANIEL; RICARD, 1996). Quando as alterações neuroendócrinas e os efeitos nos órgãos permanecem por tempo prolongado ou são muito intensas, o processo torna-se nocivo (MCEWEN, 2005).

2.3.2 Alterações comportamentais e neuroendócrinas da resposta ao estresse desencadeadas por fármacos psicoativos em zebrafish

Segundo Rang et al. (2012), existem mais de 30 tipos de antipsicóticos, além de vários ansiolíticos e sedativos, para uso clínico na medicina no tratamento de distúrbios mentais. O efeito que promovem nos usuários, sejam os desejáveis ou indesejáveis, podem se manifestar de diferentes formas nas espécies aquáticas, visto que nesse ecossistema chegam resíduos dos medicamentos ou mesmo seus metabólitos (CALISTO; ESTEVES, 2009; BU et al., 2013). Entretanto, poucos antipsicóticos e outros psicofármacos foram avaliados quanto ao efeito na

resposta ao estresse e no comportamento de *zebrafish* adultos. Estes estudos estão sumarizados no Quadro 1 abaixo.

Quadro 1 – Efeitos da presença de psicofármacos na água quanto a resposta endócrina ao estresse e no comportamento em *zebrafish* adultos.

Fármaco	Resposta endócrina ao estresse	Efeito no comportamento
Amilsuprida	-	Aumenta a atividade locomotora *per se* (TRAN et al., 2014)
Haloperiodol	-	Efeito do tipo ansiolítico *per se* (MAGNO et al., 2015)
Risperidona	Diminui a liberação de cortisol nos peixes estressados (IDALENCIO et al., 2015)	Não alterou parâmetros de ansiedade *per se* (TRAN et al., 2014) Efeito do tipo ansiolíticos nos peixes estressados (IDALENCIO et al., 2015),
Metilfenidato	Diminui a liberação de cortisol nos peixes estressados (ENDRES et al., 2017)	Efeito do tipo ansiolíticos nos peixes estressados
Fluoxetina	Diminui a liberação de cortisol e altera a osmorregulação nos peixes estressados (ABREU et al., 2014) Interfere com a osmoregulação do *zebrafish* submetido a estímulo estressor, revertendo o influxo de íons sódio e potássio ocasionado pelo estresse (ABREU et al., 2015)	Diminui a preferência social pelo cardume e produz efeito do tipo ansiolítico ou ansiogênico nos peixes estressados, conforme a concentração (GIACOMINI et al., 2016b)

De forma geral, os psicofármacos embotam a resposta ao estresse, diminuindo a liberação de cortisol e afetam o comportamento dos peixes, interferindo em aspectos locomotores, excercendo um efeito do tipo ansiolítico e diminuindo a preferencia social. Tais efeitos podem ser detectados apenas pela presença do fármaco na água, ou após um estímulo estressor. Em relação ao APPZ, Lee et al. (2011) mostraram que promove alterações morfológicas cardiovasculares em larvas de *zebrafish*, mas em adultos não há nenhuma informação sobre os seus efeitos.

Os resíduos de fármacos psicoativos na água é considerado um dos grupos de contaminantes mais prejudiciais ao ecossistema aquático, mesmo que em pequenas

concentrações (CALISTO; ESTEVES, 2009; FORD; HERRERA, 2018). Tais resíduos atuam como agentes estressores abióticos, e promovem consequências negativas no comportamento de peixes, prejudicando inclusive a função cognitiva (PIATO et al., 2011), aumentando a ansiedade e a agressividade, além dediminuir a interação social em *zebrafish* (GIACOMINI et al., 2016a). Além disso, podem interferir na capacidade de locomoção destes animais, tornando-os mais ativos ou mesmo sedados (MATHUR; GUO, 2017, COLLIER; KALUEFF; ECHEVARRIA, 2016). Os efeitos comportamentais dos fármacos psicoativos podem ocorrer também em larvas de peixes expostas (KALICHAK et al., 2017) e persistir nos adultos que foram expostos na fase larval (KALICHAK et al., [s.d.]).

Entretanto, no ambiente natural, desencadear um comportamento de ansiedade ou medo, e ter sua capacidade locomotora preservada, faz-se necessário. São medidas preventivas anti- predatórias (COLLIER; KALUEFF; ECHEVARRIA, 2016), ou mesmo necessárias para a percepção de agentes tóxicos, tais como agroquímicos ou resíduos de medicamentos na água (ROSA et al., 2016; ABREU et al., 2016).

2.3.3 Testes comportamentais

Para avaliar os efeitos comportamentais exploratórios e relacionados à resposta na presença de um estressor, diferentes testes comportamentais foram desenvolvidos e parametrizados. O teste do tanque novo (TTN) (COLLIER; KALUEFF; ECHEVARRIA, 2016) e o teste de preferência social (TPS) (GERLAI et al., 2000) baseiam-se, respectivamente, na resposta do peixe em explorar ambientes desconhecidos e na preferência pela permanência próximo ao cardume. O equilíbrio dessas ações, exploratórias e de formação de cardume é necessário na natureza para buscar alimentos, parceiros para reprodução e também para a segurança e resolução de interação com competidores (KELLEY; MAGURRAN, 2003).

O teste do tanque novo é empregado de forma bem consolidada na avaliação do comportamento natural de geotaxia, ou seja, um comportamento inato de nado em mergulho e fuga em ambientes novos (EGAN et al., 2009; (CACHAT et al., 2010; (COLLIER; KALUEFF; ECHEVARRIA, 2016; KYSIL et al., 2017), sendo esse teste, análogo ao teste do

campo aberto utilizado em roedores (KYSIL et al., 2017). A exposição ao ambiente novo produz nos peixes uma situação conflitante entre ir para o fundo buscando proteção ou manter-se na superfície explorando o ambiente na busca de alimentos e/ou parceiros novos (COLLIER; KALUEFF; ECHEVARRIA, 2016), por isso, é um dos testes mais indicados para o estudo de modelos de ansiedade em *zebrafish* (MAXIMINO et al., 2010). É um teste consagrado para avaliar o comportamento frente a situações de conflito, e, por consequência, avaliar efeitos da exposição à fármacos de características ansiolíticas ou ansiogênicas (WONG et al., 2010). Para avaliar o comportamento tipo ansiedade, os parâmetros mais indicados são o tempo de permanência no fundo, o número de cruzamentos entre as áreas, o tempo e número de episódios de imobilidade e *freezing* (total imobilidade ou "congelamento"). Já para avaliar o efeito ansiolítico de drogas, a análise do tempo de latência para entrada na superfície e tempo de permanência nessa região são os mais indicados. Também é o teste mais indicado para avaliar características locomotoras, através de parâmetros tais como distância percorrida, velocidade média e ângulo de virada (KYSIL et al., 2017).

O teste de preferência social baseia-se no comportamento social natural do *zebrafish*, quanto à busca pelo cardume (MILLER; GERLAI, 2007; MAXIMINO; DE BRITO; GOUVEIA, 2010) mais especificamente por seus co-específicos (SAVERINO; GERLAI, 2008). Nesse teste, os principais parâmetros comportamentais a serem analisados são o tempo que o peixe permanece no segmento dos co-específicos e a latência para a entrada em cada segmento (SAVERINO; GERLAI, 2008; GERLAI, 2014). Agregar-se ao cardume, para esta espécie, representa uma diminuição do risco de predação em situações ameaçadoras (MIKLÓSI; ANDREW, 2006; MAXIMINO; DE BRITO; GOUVEIA, 2010; GERLAI, 2013),
assim como o aumento de eficácia na busca por alimentos ou mesmo sucesso reprodutivo (MIKLÓSI; ANDREW, 2006; NUNES et al., 2016). Entretanto, algumas situações promovem o isolamento nestes peixes, tais como a exposição a alguns fármacos ansiolíticos. Acredita-se que a exposição aguda à fluoxetina, por exemplo, interfira na percepção do *zebrafish* em relação ao seu cardume (GIACOMINI et al., 2016a) levando a uma situação de isolamento ou indiferença aos co-específicos (GERLAI, 2013).

O teste de exposição ao predador tem por objetivo analisar a capacidade da presa em evitar ou fugir de uma ameaça predatória (BASS; GERLAI, 2008), e baseia-se na capacidade do *zebrafish*, por meio de pistas visuais, de perceber a presença de um peixe estímulo (predador ou não-predador) e afastar-se dele (GERLAI, 2013; KYSIL et al., 2017; ABREU et al., 2018). Nesse teste, outros parâmetros, tais como características do nado (ângulo de virada e nados erráticos), locomoção, distância do predador, formação de cardume e *freezing* (BASS; GERLAI, 2008) fornecem ferramentas para avaliar o comportamento tipo ansiedade e medo (KYSIL et al., 2017), que podem e devem ser desencadeados na presença de uma ameaça predatória (KELLEY; MAGURRAN, 2003).

2.4. ZEBRAFISH

O *zebrafish* (Figura 2) é um teleósteo bem conhecido, de fácil manejo, com menor custo de manutenção em relação aos roedores, com alta prolificidade (SAVERINO; GERLAI, 2008) e que possui alto potencial de estudos translacionais (KALUEFF et al., 2013a). O conhecimento detalhado do seu eixo HHI (RAMSAY et al., 2006; RAMSAY et al., 2009) assim como a compreensão sobre o seu comportamento (GERLAI, 2014; KYSIL et al., 2017) também favorecem a espécie como modelo de pesquisa em diferentes áreas do conhecimento, tais como, fisiologia, genética, toxicologia, embriologia, metabolismo, sistema cardiovascular e oncologia (HOWE et al., 2013).

Figura 2 – Imagem de um cardume de zebrafish (*Danio rerio*) no Laboratório de Fisiologia de Peixes da Universidade de Passo Fundo, RS. Foto: Gelsoli Casagrande, Agecom, UPF (2016).

Por ser a terceira espécie a ter sua caracterização genética completa, tem seu uso facilitado como modelo em pesquisas translacionais (SPITSBERGEN; KENT, 2003). Sua similaridade com outros vertebrados (SEGNER, 2009) e seu comportamento bem conhecido (MIKLÓSI; ANDREW, 2006) tem fixado o *zebrafish* como modelo experimental. Essa espécie tem se mostrado um excelente modelo de estudo sobre alterações e expressões gênicas relacionados a afecções de deficiência do sono, esquizofrenia (PROMMER, 2017), autismo (SICCA et al., 2016; MESHALKINA et al., 2018) e epilepsia (SICCA et al., 2016). Como apresenta um padrão de comportamento bem definido (KALUEFF et al., 2013a; GERLAI, 2010; GERLAI, 2013; KYSIL et al., 2017; MAXIMINO et al., 2010), tem sido utilizado como modelo na compreensão de distúrbios de ansiedade (BLASER; GERLAI, 2006; EGAN et al., 2009; SUBBIAH; KAR, 2013), de aprendizado (LUCHIARI; SALAJAN; GERLAI, 2015); da inter-relação estresse-memória (GAIKWAD et al., 2011), da hiperatividade e do déficit de atenção (NORTON et al., 2016) da depressão (PITTMAN; PIATO, 2016), entre outras afecções e condições neurológicas.

É usado também na avaliação dos efeitos deletérios de drogas de adição, tais como álcool (SCHNEIDER et al., 2017), cocaína e anfetamina (KAWAHARA et al., 2018) e nicotina (STEWART et al., 2015). Estudos sobre a exposição aguda ou crônica ao álcool em *zebrafish*, por exemplo, mostraram sob o aspecto translacional o impacto deletério no aprendizado humano (LUCHIARI; SALAJAN; GERLAI, 2015), assim como na indução de ansiedade por essa droga lícita (MATHUR; GUO, 2017). Já a exposição à cocaína evidenciou os impactos cardiovasculares e comportamentais que esta droga causa em humanos (MERSEREAU et al., 2016).

O *zebrafish* também se assemelha com humanos na resposta ao estresse, pois assim como nos humanos, a estimulação do eixo neuroendócrino desses peixes culmina com a liberação de cortisol como principal glicocorticoide (BARTON; IWAMA, 1991; WENDELAAR- BONGA, 1997; MOMMSEN; VIJAYAN; MOON, 1999; BARCELLOS et al., 2007), o que torna o *zebrafish* um modelo translacional apropriado para o estudo de estresse em humanos.

Este pequeno teleósteo permite também avaliar os efeitos de poluentes emergentes nos ecossistemas aquáticos. Nessa linha, estudos com *zebrafish* mostraram que o cádmio promove estresse oxidativo e imunotoxicidade ocasionando efeitos tóxicos sobre o cérebro, fígado e ovários (ZHENG et al., 2016) e o mercúrio ocasiona desregulação endócrina no eixo hipotálamo-pituitária-gonadal, podendo assim impactar a reprodução da população aquática exposta (ZHANG et al., 2016). A frequente contaminação aquática por hormônios anticoncepcionais, como o 17α-etinilestradiol (DAUGHTON; TERNES, THOMAS, 2009; BOXALL, 2004; PETRIE; BARDEN; KASPRZYK-HORDERN, 2014), também promove impactos nos peixes. O *zebrafish* acumula esse hormônio no tecido e apresenta alterações comportamentais sociais, de agressividade e de ansiedade, além de alterar o níveis de acetilcolina tecidual, comprometendo aspectos reprodutivos e de toxicidade no animal (FENSKE, 2017; SOFIATTI, 2018).

Fármacos psicoativos, tais como a risperidona (IDALENCIO et al., 2015; KALICHAK et al., 2016), fluoxetina, diazepam (ABREU et al., 2014; GIACOMINI et al., 2016c), e metilfenidato (ENDRES et al., 2017) também promovem impactos sobre o eixo hipotálamo- hipófise-interrenal, assim como no comportamento deste teleósteo, podendo

ocasionar impactos severos tanto na locomoção, quanto na busca por alimento ou, quanto na fuga de predadores. Vários desses fármacos são atrativos para os peixes (ABREU et al., 2016), podendo interferir nas suas atividades cotidianas, como na relação presa-predador por exemplo.

O *zebrafish* ainda tem sido muito utilizado em pesquisas sobre fisiologia do estresse e, com enfoque ecológico, na compreensão de aspectos da relação presa-predador, como por exemplo as reações endócrinas à presença direta ou visual de predadores (BARCELLOS et al., 2007) e seu aprendizado em relação a essas situações (BARCELLOS et al., 2010). As reações endócrinas e comportamentais frente à presença de co-específicos mortos (OLIVEIRA et al., 2014) e a participação do cortisol nessa comunicação química do risco de predação também tem sido estudada (BARCELLOS et al., 2014), bem como os impactos de substâncias psicotrópicas, como o álcool, no processo de comunicação química (OLIVEIRA et al., 2013).

3. OBJETIVOS

3.1. OBJETIVO GERAL

Verificar se o APPZ altera as respostas endócrinas e comportamentais ao estresse em *zebrafish* adultos.

3.2. OBJETIVOS ESPECÍFICOS

- Verificar se a exposição aguda ao APPZ altera a resposta endócrina ao estresse do *zebrafish*.

- Verificar se a exposição aguda ao APPZ altera o comportamento do *zebrafish*.

4. ARTIGOS

Neste capítulo serão apresentados dois artigos. O primeiro artigo, intitulado *Waterborne aripiprazole blunts the stress response in zebrafish*, foi publicado na revista Scientific Reports, em novembro de 2016, sob DOI: 10.1038/srep37612, ISSN 2045-2322 (Qualis A1 nas Ciências Biológicas II e fator de impacto de 4.122.

O segundo artigo, intitulado *Waterborne aripiprazole alters zebrafish behavior*, foi submetido para revista Environmental Science and Pollution Research (Qualis B1 nas Ciências Biológicas II e fator de impacto de 2,8).

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4.1. ARTIGO 1:

SCIENTIFIC REPORTS

OPEN

Waterborne aripiprazole blunts the stress response in zebrafish

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Here we provide, at least to our knowledge, the first evidence that aripiprazole (APPZ) in the water blunts the stress response of exposed fish in a concentration ten times lower than the concentration detected in the environment. Although the mechanism of APPZ in the neuroendocrine axis is not yet determined, our results highlight that the presence of APPZ residues in the environment may interfere with the stress responses in fish. Since an adequate stress response is crucial to restore fish homeostasis after stressors, fish with impaired stress response may have trouble to cope with natural and/or imposed stressors with consequences to their welfare and survival.

The consumption of antipsychotic drugs has been growing gradually, especially due to the increasing the number of diagnostics of psychotic disorders in recent decades[1]. These drugs induce serious extrapyramidal effects in patients who are treated in a continuous and prolonged manner[2]. In 2002, the US FDA approved the use of aripiprazole (APPZ), an atypical antipsychotic with considerably lower occurrence and intensity of adverse effects[3,4]. APPZ has been approved for the treatment of schizophrenia, bipolar mania and major depressive disorder[5–9], and is useful for controling positive and negative symptoms in schizophrenia[10], and irritability, hyperactivity and stereotypies in autism[11].

Due to its safety and lower incidence of unwanted side effects, the prescription of APPZ is increasing to replace classical antipsychotics. This increased use justify the concern about the accumulation of APPZ residues in wastewater and, consequently, potential negative effects in non-target organisms such as fish. There are a few studies reporting APPZ concentrations detected in effluents[12,13], and only one study of cardiovascular risk using zebrafish larvae[14]. In addition, there are no reports on the impact of this drug in adult zebrafish (*Danio rerio*).

Endocrine disruptors are compounds that alter the normal functioning of the endocrine system of both humans and wildlife. We have previously shown that other psychotropic drugs such as risperidone[15], fluoxetine, diazepam[16,17] as well as alcohol[18] blunted the response to stress follow acute stress".

Despite the knowledge on the effects of APPZ *in vitro*[18–20], in rodents[21,22] and humans[11,23,24], little is known about its impact as a possible environmental contaminant. Coupled with the increasing use, interest in the study of psychiatry drugs as environmental contaminants[13,25,26] and the impact they have on the aquatic fauna has risk[27–31]. Thus, here we describe, for the first time, the effects of different concentrations of APPZ on the hypothalamic-pituitary-interrenal (HPI) axis in adult zebrafish.

Results

Two-way ANOVA revealed significant main effects for stress ($F_{1,123} = 10.33$, $p < 0.0001$), as well as a significant interaction between the factors ($F_{5,123} = 101.9$, $p < 0.0001$), but not for drug ($F_{5,123} = 1.54$; $p = 0.1822$) (Fig. 1). At concentrations of 0.556 and 556 ng/L, APPZ prevented the increase in cortisol levels in stressed zebrafish. However, this effect was not observed at other concentrations. On the other hand, APPZ *per se* did not interfere with whole-body cortisol levels. We can also observe that the stressor stimulus was effective.

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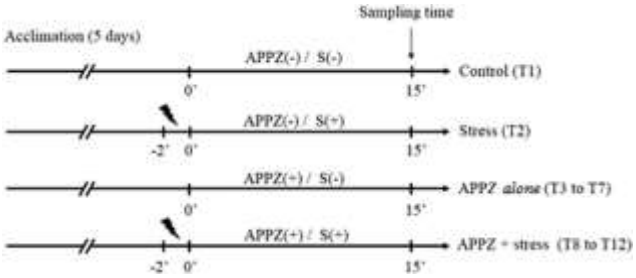

Figure 1. Whole-body cortisol levels in zebrafish exposed to different concentrations of aripiprazole (APPZ, in ng/L) without stressor stimulus (S−) and with stressor stimulus (S+). Each dot represents one independent measurement and the black lines represent the mean. Two-way ANOVA followed by Tukey post hoc test. n = 9–15 **p < 0.01; ***p < 0.001; ****p < 0.0001 vs. control group (S−).

Discussion

Here we provide, at least to our knowledge, the first evidence that APPZ in the water decreases the stress response of exposed fish. The concentration of 0.556 ng/L, ten times lower than the concentration detected in the envi- ronment (5.56 ng/L[13]), blunted the cortisol increase after an acute stressor. This blunting effect of the lower con- centration, in an environmental perspective, points to the necessity of great caution in relation to the delivery of APPZ residues in water bodies.

Interestingly, the HPI-blunting effect was also evident in the higher concentrations of 556 ng/L. This U-shaped concentration-response curve suggests a hormetic effect, similar to other psychotropic drugs such as diazepam[16] and risperidone[17].

In fact, the mechanism of action of APPZ on the HPI axis of zebrafish is not clear. Our hypothesis is that APPZ has a dopaminergic stabilizer effect[20], since it is a partial agonist of dopamine receptors[30], and tends to buffer the effects of endogenous dopamine[31]. Since dopamine receptors regulate hypothalamic-pituitary-adrenal axis activity in rats[32], it is possible that APPZ may also act via this mechanism in fish. Our hypothesis based on the dopaminergic regulation of HPI axis is reinforced since in unstressed fish, the APPZ did not cause any change in cortisol levels, as verified in humans[25]. In addition, zebrafish treated with a dopaminergic antagonist and sub- jected to stress did not increase their cortisol, reinforcing the dopaminergic involvement in the HPI activation[33]. Despite the undetermined mechanism, our results highlight that the presence of APPZ residues in the envi- ronment may interfere in the neuroendocrine axis that coordinates the stress responses in fish. In fish, cortisol plays a key role in the intermediary metabolism, osmoregulation and immune function[34]. Thus, the adequate stress response is crucial to restoring fish homeostasis after stressors; a fish with an impaired response may have trouble to cope with natural and/or imposed stressors with consequences to fish welfare and survival.

A limitation of our study is that we evaluated the effects of acute, but not chronic exposition to AAPZ. Further studies are necessary to better understand the effects of chronic exposition of AAPZ on behavioral and neuroen- docrine parameters in zebrafish.

Furthermore, the zebrafish is a model for translational studies of several human diseases, due to the high homology with the human genome[35]. Although we do not have addressed the mechanism whereby APPZ blocks the stress response, this does not lessen the importance of our initial assessment, since, at least to our knowledge, this is the first report about an *in vivo* APPZ effect in fish HPI axis.

Methods

Ethical note. This study was approved at protocol #20/2016, by the Ethics Commission for Animal Use of Universidade de Passo Fundo (Passo Fundo, RS, Brazil) and all methods were carried out in accordance with the guidelines of National Council of Animal Experimentation Control (CONCEA).

Fish. We used a population of 144 mixed-sex adult wild-type zebrafish (*Danio rerio*), short-fin strain, and 50:50 male and female. Fish were acclimatized for 5 days in 3.9 l glass aquaria (20 × 15 × 14 cm), in groups of three animals, under constant aeration, with a photoperiod of 14 h light: 10 h dark. Water temperature was maintained at 27.4 ± 1.3 °C; pH 6.7 ± 0.3; dissolved oxygen at 5.6 ± 0.5 mg/L; total hardness at 58.9 ± 12.4 mg/L $CaCO_3$, alka- linity at 45 ± 10 mg/L $CaCO_3$ and ionized ammonia was <0.011 mg/L.

Study strategy. Our strategy was to expose zebrafish to five concentrations of APPZ and verify the stress response of these fish to an additional acute stressor. A possible isolated effect of APPZ was accessed by measur- ing cortisol in exposed unstressed fish.

Experimental procedures. We distributed fish in 12 groups. Each group consist of four glass aquaria with three animals each one (n = 12 per treatment). We then exposed fish to five concentrations of APPZ (Aristab , Aché, Brazil). We set two lower and two higher concentrations (at 10-fold basis) in relation to the APPZ concen- tration already detected in the environment (5.56 ng/L[13]). Thus, the five concentrations were 0.0556; 0.556; 5.556; 55.6 and 556 ng/L.

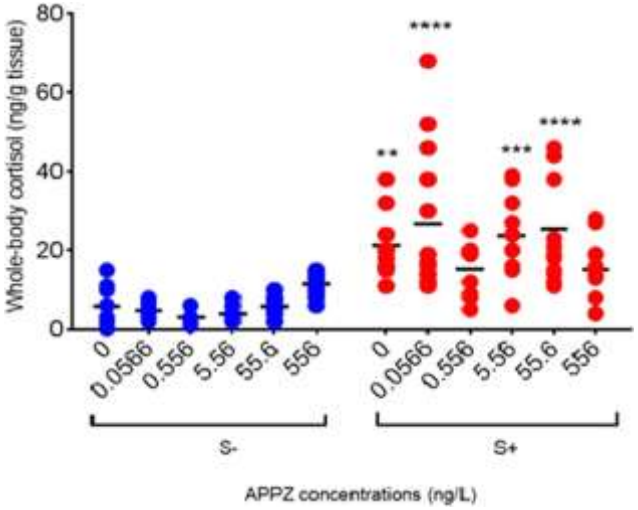

Figure 2. Schematic representation of the experimental design. APPZ (aripiprazole), S(−) without stressor stimulus; S(+) with stressor stimulus.

In the exposed and stressed groups, we administered APPZ directly in the water for a 15 min exposure time and then we applied the acute stress stimulus by chasing fish with a pen net for 2 minutes. After 15 min the ani- mals were euthanized for cortisol measurement (see Fig. 1).

After this period, the fish were gently captured and immediately euthanized with cold water, followed by decapitation and freeze of the whole body at liquid nitrogen for 30 s. Then, we stored samples at −20 °C for cortisol extraction, following the method described in Oliveira *et al.*[18]. The tissue extract was suspended in PBS, and whole-body cortisol was measured using a commercially available enzyme-linked immune sorbent (EIAgen CORTISOL test, Bio Chem ImmunoSystems). This kit is fully validated for zebrafish extracts using the method- ology described by Sink *et al.*[36].

Statistics. The data is expressed as mean ± standard error of mean (S.E.M). The normal distribution of the data was confirmed by Kolmogorov-Smirnov and Levene tests, and results analyzed by two-way ANOVA fol- lowed by Tukey's post hoc test. Two-way ANOVA was used to identify the main effects of stress and treatment, as well as their interactions. Differences were considered significant at $p < 0.05$".

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Author Contributions

H.H.A.B. and L.J.G.B. conceptualize the experiments, wrote the manuscript and prepare the figures. H.H.A.B., F.K., J.G.S.R., T.A.O., R.I., M.S.A., A.C.V.G., M.F., C.V. and M.R. conducted experimental procedures. A.L.P. analyzed the results. G.K. measured cortisol concentrations. All authors have read and approved the manuscript for publication.

Additional Information

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4.2. ARTIGO 2:

Waterborne aripiprazole alters zebrafish behavior

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Abstract

Environmental pollution by antipsychotic residues is a relevant ecologic problem. Even in low environmental concentrations studies revealed that these drugs residues are present in a wide range in different ecosystems and can produce adverse effects in non-target organisms. Thus, here we posed the following question: "What will be the behavioral effects of waterborne aripiprazole (APPZ) in fish?" To answer this question, we exposed adult zebrafish to different APPZ concentrations and evaluated the exploratory, anxiety-like and social behaviors, as well as anti-predatory behavior. Here we show that, despite apparent beneficial reversal of stress- related social impairment and absence of effect on stress-related bottom dwelling, APPZ exposure impairs the anti-predatory reaction of adult zebrafish. Taken together, our results show that APPZ-exposed zebrafish decreases the perception of predator, even at concentrations lower than those already detected in the environment. A failure of the antipredatory response may favor the predator, decreasing the fitness of the prey species and, consequently, affecting the food chain. Our results highlight the risks and consequences associated with APPZ residues in water, which may affect aquatic life and endanger species that depend on appropriate behavioral responses for survival.

Key-words: *Danio rerio*, prey-predator relationship, social behavior, stress, environmental contamination, drug residues.

1. Introduction

Environmental pollution by antipsychotic residues is a relevant ecologic problem (Heberer 2002; Boxall 2004; Küstler and Adler 2014; Martin et al. 2017; Ford and Herrera 2018). Even at low environmental concentrations (Cabeza et al. 2012; Subedi and Kannan 2015), studies revealed that residues of some pharmaceutical drugs are present in a wide range in different ecosystems (Arnold et al. 2014; Martin et al. 2017; Mazzitelli et al. 2018; López-García et al. 2018). These drug residues in the water can produce adverse effects in non-target organisms (KOSTICH; LAZORCHAK, 2007), as endocrine disruption (SABIR; AKHTAR, 2018) and behavioral disturbance (KÜSTLER; ADLER, 2014). Among drugs, the class of psychotropic evokes high environmental concerns (Mazzitelli et al. 2018; López-García et al. 2018), since there are some studies reporting the effects of these drugs, prescribed to human psychiatric disorders, on behavioral and/or neuroendocrinological parameters in fish (Magno et al. 2015; Alvarenga et al. 2017; Abreu et al. 2014; Abreu et al. 2015; Giacomini et al. 2016; Idalencio et al. 2015; Kalichak et al. 2016; Kalichak et al. 2017; Barcellos et al. 2016; Endres et al. 2017).

At behavioral level, risperidone (IDALENCIO et al., 2015), and methylphenidate (ENDRES et al., 2017) cause anxiolytic-like effects in fish. Fluoxetine and diazepam at low concentrations also induce an anxiolytic-like effect (Abreu et al. 2014) and reduce the social preference in stressed fish (GIACOMINI et al., 2016a). In a different way, an acute exposure in a high concentration can induce an anxiogenic effect in fish (MAGNO et al., 2015). This anxiolytic-like effect verified in fish is similar to the anxiolytic effect seem in humans (WONG; BYMASTER; ENGLEMAN, 1995). Despite this is the desired effect in human prescriptions, in an environmental/ecological perspective, an anxiolytic-like reaction may compromise de anti-predatory behavior since prey fish becomes more visible to predator ones (MARTIN et al., 2017).

Anxiety and fear are important emotions associated with risk-assessment behaviors, crucial to the maintenance of species in the environment (MAXIMINO et al., 2010). The stress responses and the risk-assessment behaviors are beneficial to the animals, since they allow that the individual maintain the physiological and behavioral stability, despite fluctuating risk conditions in the environment (McEwen and Wingfield 2003). These risk-assessment and stress behaviors of any animal species, need the brain and the body functions intact, as well an intact behavioral repertoire (Karatsoreos and Mcewen 2011, Sterling 2012).

However, aquatic pollutants, as the antipsychotics drug residues, can change this allostatic state, and adversely affect fish safety, feed or reproduction (GRAEFF; ZANGROSSI JR, 2010). Considering that aripiprazol (APPZ) acts in the Central Nervous System as a partial agonist of dopamine (TAMMINGA; CARLSSON, 2002) and serotonine (HIROSE; KIKUCHI, 2005) receptors, and it is considered as a regulator drug of these systems, it can directly affect the neural basis of behavioral phenotypes (BURDA et al., 2011).

Thus, here we posed the following question: "What will be the behavioral effects of waterborne APPZ in fish? To answer this question, we exposed adult zebrafish to different APPZ concentrations and evaluated the exploratory, anxiety-like and social behaviors, as well anti-predatory behavior, since these behaviors are crucial to prey-predator relationship (Stewart et al. 2013; Colwill and Creton 2011).

2. Materials and Methods

2.1. Ethical and Legal Note

This study was approved by the Ethics Commission for Animal Use Committee (CEUA) of University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #020/2016 - CEUA) and complied with the guidelines of National Council for Animal Experimentation Control

(CONCEA). In addition, this research was registered in the SisGen (Sistema Nacional de Patrimônio Genético e do Conhecimento Tradiocional Associado) and complied with their guidelines (registration code A14E252).

2.2. Study strategy

Aiming to verify possible effects of APPZ on zebrafish behavior, our strategy was to expose zebrafish to different APPZ concentrations in water and test the exposed fish in three different behavioral tasks, the Novel Tank Test (NTT), the Social Preference Test (SPT) and the Prey-predator Test (PPT). NTT and SPT were applied in APPZ-exposed fish with and without stress, aiming to verify if this drug affects the well-known stress-induced behavioral phenotype (Gerlai et al. 2000; Kalueff et al. 2013). In addition, PPT was applied in APPZ-exposed fish, aiming to verify if the drug affects the predator perception in zebrafish (GERLAI et al., 2000).

2.3. Animals and housing conditions

A population of 300 mixed-sex 8-month old adult wild-type zebrafish, short-fin strain, weighing 0.3 to 0.5 g, from a heterogeneous breeding stock at the Passo Fundo University, Brazil, was kept at a density of 1 fish/L. The tanks were equipped with biological filters, under constant aeration, and the fish were submitted a photoperiod with 14h light and 10 h dark.

We randomly distribute fish in each experiment using the app RANDOM.ORG. Fish were then acclimatized for 5 days in 4 L glass aquaria (20 × 15 × 14 cm) (n=4 each aquarium). Water temperature was maintained at 27.4 ± 1.1 °C, with pH= 7.2 ± 0.5, dissolved oxygen at 5.7 ± 0.8 mg/L, non-ionized ammonia at ≤ 0.03 mg/L, total hardness at 39±5 mg $CaCO_3$/L, and alkalinity at 35.2 ± 7.3 mg $CaCO_3$/L.

We used a goldfish (*Carassius auratus*) with 12 cm of length as non-predator stimulus

fish due to its peaceful and friendly temperament (KOTTELAT et al., 1996), and an adult tiger oscar (*Astronotus ocelatus)*, a Cichlid fish with strong predatory behavior (SMITH, 1981), with 20 cm of length. Both fish came from a local aquarium market. We acclimatized each one for 5 days in prey-predator aquaria test, in a specific compartment for the stimulus fish. This compartment has 50 L (56 x 30 x 30cm), the water temperature was maintained at 27°C, with pH= 7.2, dissolved oxygen at 6.7 mg/L, non-ionized ammonia at ≤ 0.03 mg/L, total hardness at 39 mg $CaCO_3$/L, and alkalinity at 37 mg $CaCO_3$/L. The other driving conditions were the same of the zebrafish.

We fed zebrafish and goldfish twice a day, *ad libidum*, with a diet containing 48% crude protein (SUPERVIT® - Tropical). The oscar fish was fed with one live zebrafish twice a day, during all period. However, during day of the experiment, the zebrafish were maintained fasted, with all fish receiving food 12 h before the behavioral trials, aiming to avoid interferences of feeding on behavior (DAMETTO et al., 2018).

2.4. Experimental procedures

2.4.1. Drug and concentrations tested

In each behavioral test, we exposed fish to three concentrations of APPZ (Aristab®,Aché, Brazil). We set a concentration previously detected in the environment (5.56 ng/L) (SUBEDI; KANNAN, 2015), and the concentrations that blunt the cortisol response at acute stress stimuli: 0.556 and 556 ng/L (Barcellos et al. 2016). The exposure time to the drug was 15 minutes.

2.4.2. Behavioral testing

For SPT and NTT fish were maintained in 4 L glass aquaria in groups of four. From these aquaria, we captured all fish at the same time and placed them individually in four treatment 1- L beakers. Two fish were no stressed and placed on SPT or NTT, and another two were

stressed and placed in these tests. This strategy was repeated 12 times to reach n = 12 per treatment. The acute stress stimulus was by chasing fish with a pen net for 2 minutes after the 15 minutes of APPZ exposure, and fish were immediately submitted to the behavior test. For prey-predator test (PPT), aiming to avoid isolation effects (Giacomini et al. 2016), we maintained fish in triplets (PAGNUSSAT et al., 2013), but used in the PPT only one fish by aquarium to reach n= 12 per treatment.

To evaluate the behavioral parameters in each test, we videotaped fish using a Logitech Quick cam PRO 9000 camera. To avoid interference by human activity, the operator exited the experimental room after the fish were released into test aquaria in all behavioral tests. The videos were analyzed with the automated tracking software ANY-maze® (Stoelting CO, USA).

2.4.2.1 Social Preference Test (SPT)

To SPT the tank test (30 × 15 × 10 cm, width × depth x height) (SAVIO et al., 2012) was positioned between two equal-sized aquaria, one without fish and the other containing a group of 12 conspecifics, with the same pattern color (ENGESZER; RYAN; PARICHY, 2004). After treatment, fish were individually acclimated to the test tank for 30 s; after this time, their behavior was recorded during 60 s. We virtually divided the tank in three vertically segments to analyze data. The first segment is nearest to conspecifics tank, while the third segment was next to the empty tank. The following parameters were analyzed: time spent in each segment (s), number of entries in conspecifics segment, and distance traveled (m) in this segment.

2.4.2.2 Novel Tank Test (NTT)

In this test, we used rectangular glass aquaria (24 × 8 × 20 cm, width × depth x height) (MOCELIN et al., 2015). We virtually divided the tank test in three horizontals segments to

analyze data. Fish were recorded during 5 minutes and the following parameters were analyzed: time spent in different zones of the tank (top, middle, and bottom) (s), latency to first entry in the top zone(s), number of total crossings, time freezing (s) and total distance traveled (m).

2.4.2.3 Prey-predator test (PPT)

At PPT we used a rectangular glass aquarium (104 × 30 × 30 cm, width × depth x height), divided in three partitions with a glass. The first partion was to predator, with 56 cm of width; the second partion, with 24 cm of width, was to zebrafish test, and the third portion, with 24 cm of witdth was empty, containing only with water. After exposure to APPZ, the zebrafish was introduced in the second partition, and its activity was recorded during three minutes. We virtualy divided the second partition in three segments to analyze data: high risk zone (zone 1, near to predator partition) and low risk zone (zone 3, far from predator, near to empty partition), with an intermediary neutral zone (zone 2). The analyzed parameter was the time spent in high risk zone (seconds). In addition, the analyzes were performed by stages of 60 seconds, called here, first stage (0 to 60 seconds), second stage (61 to 120 seconds) and third stage (121 to 180 seconds), totalizing 3 minutes of recording.

2.5 Statistics

To compare data from SPT, we used a two-way ANOVA followed by a post-hoc Dunnet's. In this same test, we compared non-stressed and stressed fish by Mann-Whitney test. Tocompare data from NTT, we also used a two-way ANOVA followed by Dunnet´s multiple comparison test. Regarding PPT, we first compared all data using two-way ANOVA and, since no interaction or drug effect was evidenced, we compared for each concentration the situations of non-stimulus, goldfish and Oscar presentation by one-way ANOVA followed by Dunnet's post- hoc test. Finally, at this PPT we compared the behavioral pattern of both

stimulus fish using unpaired T test or Mann-Whitney test depending on data normality (assessed by the Kolmogorov-Smirnov test). P was set as <0.05 in all analyses.

3. Results

3.1. Social Preference Test

At first, we observed that the stress reduced the time spent in the conspecific segment (Fig. 1A), as well the number of entries (Fig. 1B) and the distance traveled (Fig. 1C) in this segment.

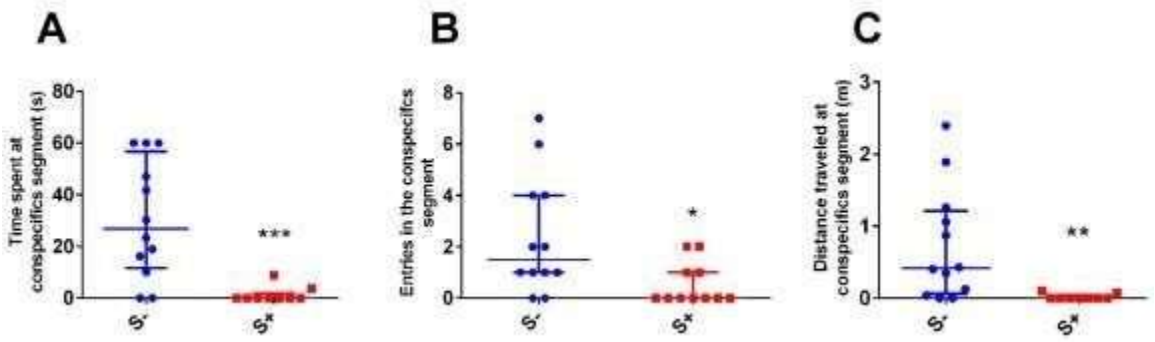

Figure 1. Social preference test of controls (S-) and stressed (S+) zebrafish not exposed to APPZ (A) time spent at conspecifics segment (s); (B) Number of entries at conspecifics segment; and (C) distance traveled at conspecifics segment (m). Data were expressed as median ± interquartile range of 10-12 fish and compared by Mann-Whitney test. (*p<0.05, **p<0.01, ***p<0.001).

The three APPZ concentrations reversed this social impairment induced by the stress, since APPZ-exposed zebrafish spent similar time in the conspecific segment (Fig. 2A), entry similarly, with exception of fish exposed to 556 ng/L of APPZ (Fig. 2B) and swam similar distance in this segment (Fig. 2C) compared to non-stressed control. No differences were found between the APPZ concentrations. In addition, APPZ *per se* at 0.556 ng/L decreased the number of entries in the conspecific segment compared to non-exposed controls (Fig.2B).

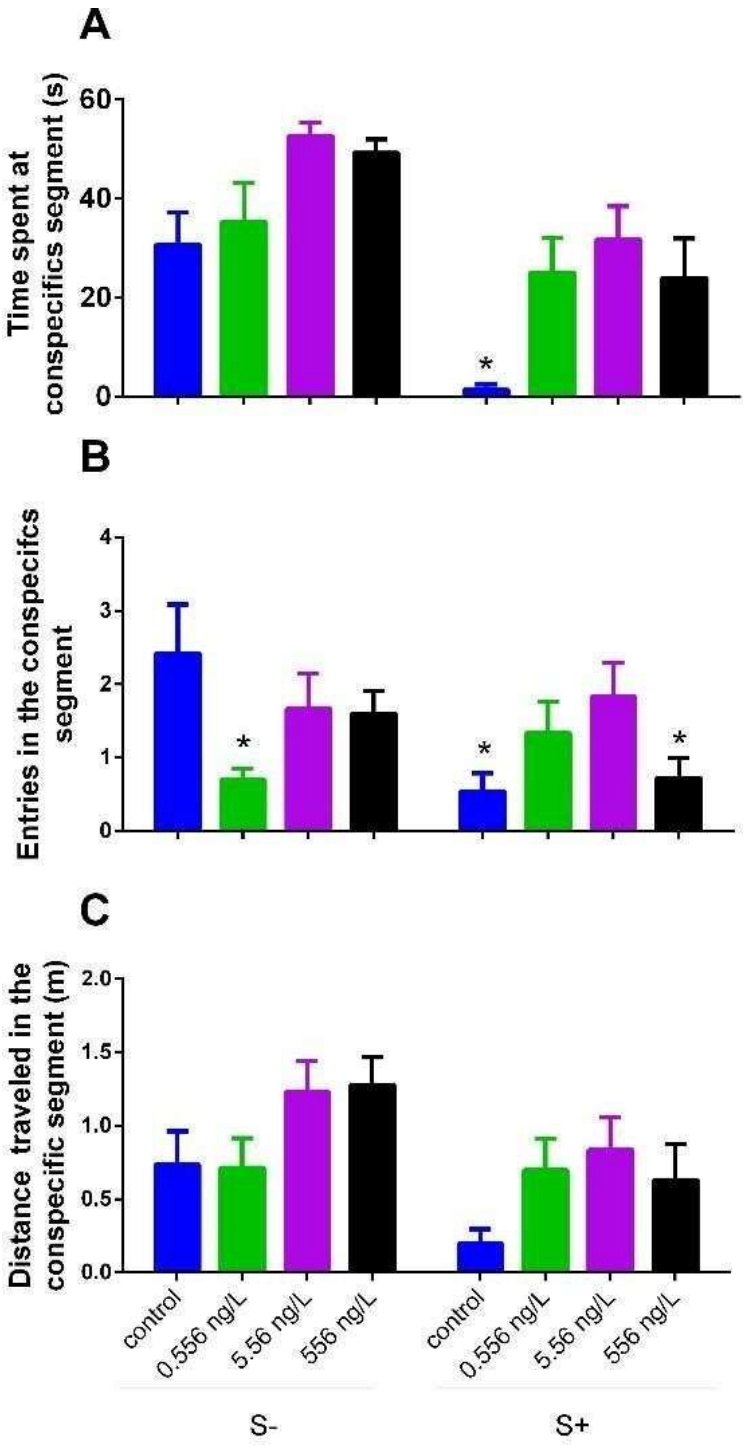

Figure 2. Social preference test of controls (S-) and stressed (S+) zebrafish exposed to different concentrations of APPZ. (A) Time spent at conspecifics segment (s); (B) Number of entries at conspecifics segment; and (C) distance traveled at conspecifics segment (m). Data were expressed as mean ± S.E.M. of 10-12 fish and compared by Two-way ANOVA followed by Tukey's multiple range test. * indicates significant difference against the non-stressed control fish ($p<0.05$).

3.2. Novel Tank Test

Here we observed that at 0.556 ng/L of APPZ the stressed fish increased the time spent at the top zone (Fig. 3A). Despite stress and APPZ exposure not change the crossings between zones (Fig. 3B), latency to entry in the top zone was reduced and bottom dwelling increased in stressed zebrafish. APPZ exposure did not reverse these alterations (Fig. 3C and 3F).The time in freezing (Fig. 3D) and the distance traveled (Fig. 3E) were no different between groups at all APPZ concentrations tested.

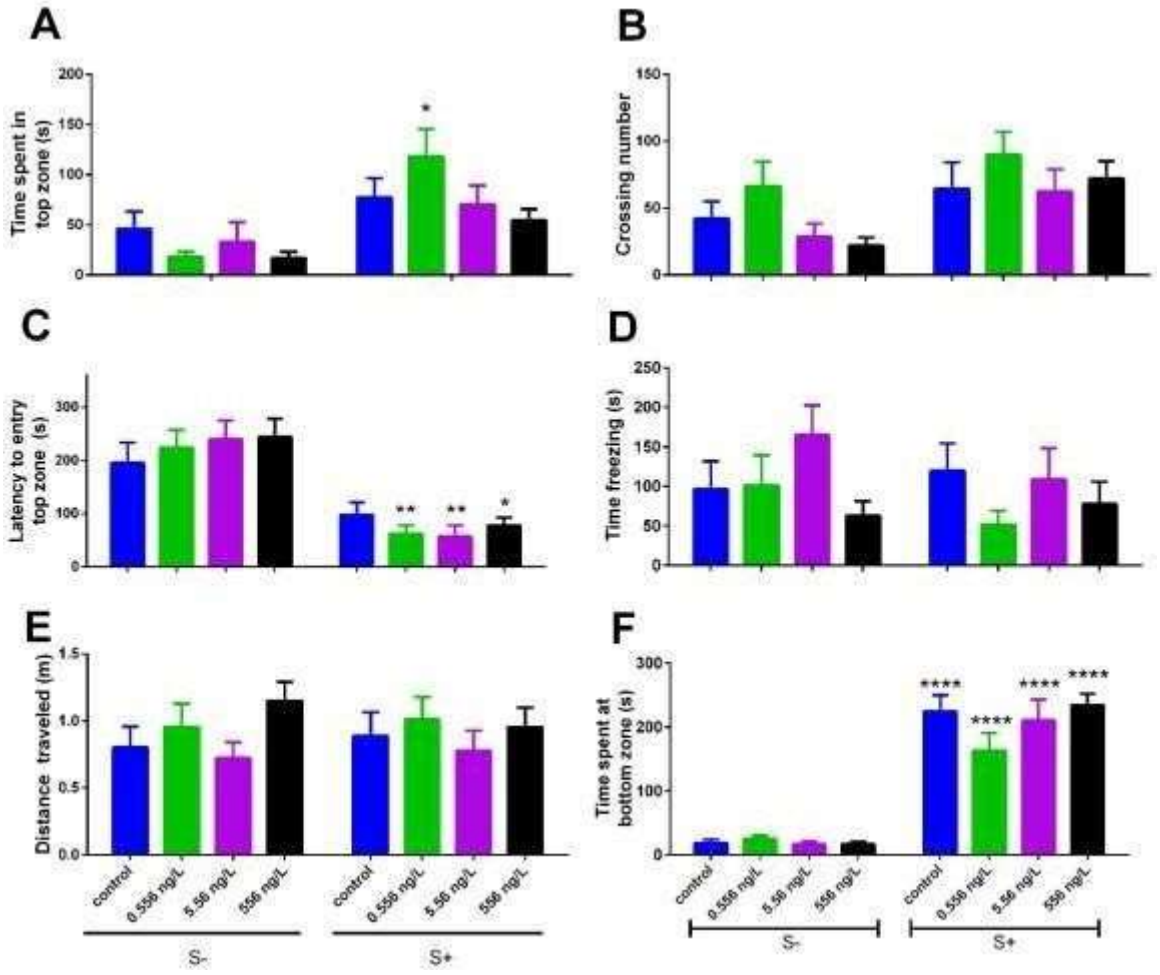

Figure 3. Locomotor parameters at Novel tank test of unstressed (S-) and stressed (S+) zebrafish exposed or not to different APPZ concentrations. A) Time spent at top zone (s); B) Line crossings; C) Latency to top (s); D) Time spent in freezing (s); E) Total distance traveled (m); and F) Time spent at bottom zone (m). Data were expressed as mean ± S.E.M. of 12 fish and compared by Two-way ANOVA followed by Dunnet´s multiple comparison test. The asterisks indicate significant difference against the control non-stressed fish (*p<0.05, **p<0.01, ****p<0.0001).

3.3. Prey-predator test

3.3.1. Behavior of the prey fish

At all APPZ concentrations and in all stages, there was not interaction between stimulus fish and APPZ exposure, thus, we compared the reaction of zebrafish to an empty segment (no stimulus), to a goldfish and to an oscar fish in each APPZ concentration.

At 1st stage (0-60s), control fish spent less time in the high-risk zone when exposed to goldfish, while no differences were found in APPZ-exposed fish (Fig. 4, stage 1). This pattern of control fish was repeated in the second stage, while fish exposed to 0.556 and 5.56 ng/L of APPZ did not change this time in the high-risk zone. However, zebrafish exposed to 556 ng/L of APPZ reduced the time spent in the high-risk zone when exposed to both goldfish and oscar (Fig. 4, stage 2). In the 3rd stage (121-180s) the control zebrafish clearly reacted to the presence of both goldfish and oscar spending less time in the high-risk zone, and this reaction was abolished by the three APPZ concentrations (Fig. 4, stage 3).

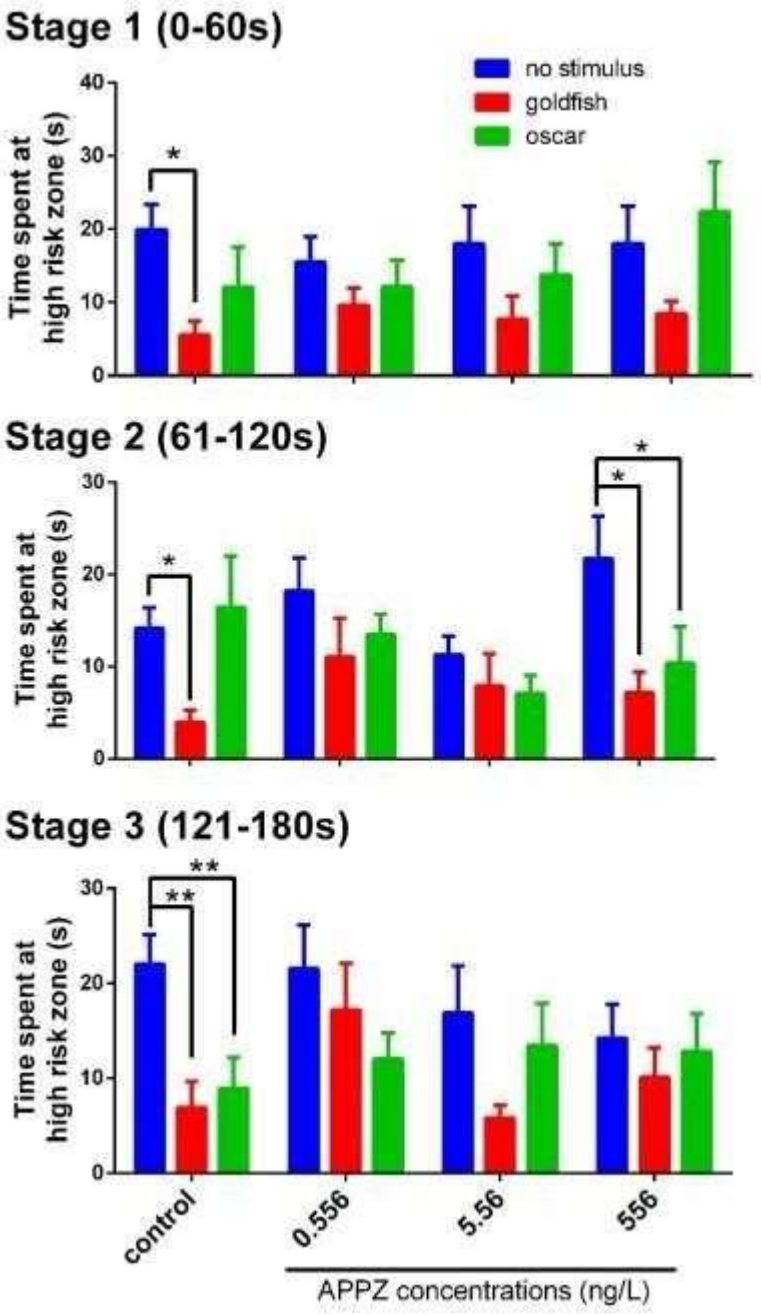

Figure 4. Prey-predator test of zebrafish exposed or not to APPZ. Time spent at high-risk zone of fish exposed to 0.556, 5.56 and to 556 ng/L of APPZ. (1st stage; 2nd stage and 3rd stage). Data were expressed as mean ± S.E.M. of 12 fish and compared by one-way ANOVA followed by Dunnet test in each APPZ concentration (*p<0.05; **p<0.01; ***p<0.001).

3.3.2. Behavior of the stimulus fish

Analyzing the locomotor pattern of stimulus fish, we observed that the goldfish spent less time at top (Fig. 5A), while the oscar spent more time at top zone (Fig. 5B). The goldfish have

greater distance traveled (Fig. 5C), higher turn angle (Fig. 5D) and longer time mobile (Fig. 5E) than the oscar. There were no differences about freezing time between them (Fig. 5F).

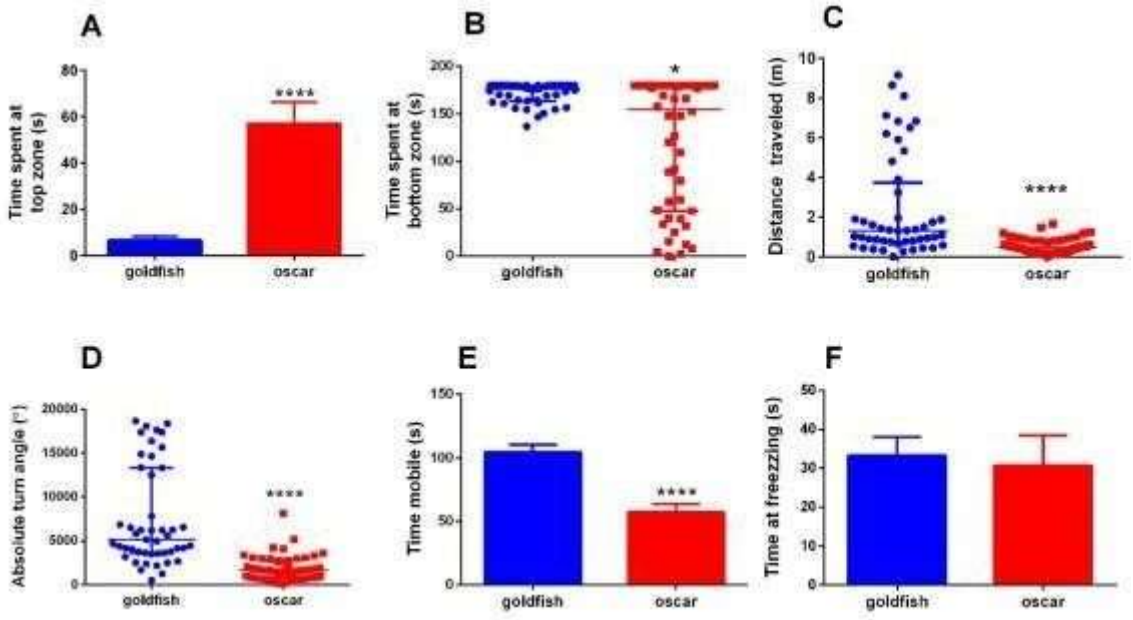

Figure 5. Locomotor behavioral pattern of non-predator and predator stimuli fish. A) time spent at top zone (s); B) time spent at bottom zone (s); C) distance traveled (m); D) absolute turn angle (°); E) time mobile (s) and F) Time at freezing (s). Data were expressed as mean ± S.E.M. and compared by unpaired T test (B and E), or as median ± interquartile range and compared by Mann-Whitney test (A, C, D and F). (* $p<0.05$ and ****$p<0.0001$, n = 48 observations of the same fish).

4. Discussion

Here we show that, despite apparent beneficial reversal of stress-related social impairment and absence of effect on stress-related bottom dwelling, APPZ exposure impairs the anti-predatory reaction of adult zebrafish. In fact, analyzing the prey-predator test, we observed that APPZ decreases the perception of predator by the prey, even at concentrations lower than that detected in the environment (Fig 4, stage 1 to stage 3).

Mechanistically, this effect may be related to the APPZ panicolitic effect (Tamminga and Carlsson 2002; Biojone et al. 2011). The APPZ panicolitic effect is produced because APPZ

is a partial agonist of 5-HT1A receptors and antagonist of 5-HT2A serotoninergic receptors, producing an anti-aversive effect at fear or panic conditions (TAMMINGA; CARLSSON, 2002). This panicolitic-like mechanism may be the same detected in rats (BIOJONE et al., 2011). Interesting, the APPZ-unexposed zebrafish could perceive the goldfish first than oscar, and getaway more distance from the risk area. This first detection of the non-predatory stimulus fish may be due because this fish was more active and spent more time at the bottom, became more visible to zebrafish (KELLEY; MAGURRAN, 2003). When exposed to APPZ the zebrafish lost this capacity, except during the second minute of test, at 556 ng/L APPZ exposure. Perhaps, the zebrafish learn about the inexistence of a potential risk only by visual cues (Kelley and Magurran 2003; Barcellos et al. 2010), but we cannot discard another kind of behavior if exist a combination of visual and chemical cues (Coleman and Rosenthal 2006; Egan et al. 2009; Oliveira et al. 2017).

Another possible mechanism underlining the effects of APPZ on zebrafish behavior is the impairment of cortisol response to stress previously described in adult zebrafish (Barcellos et al. 2016). A link between psychotropic exposure with blunted cortisol response and altered behavior was previously found for risperidone (IDALENCIO et al., 2015), methylphenidate (ENDRES et al., 2017), and bromazepam, fluoxetine and nortriptyline (MARCON et al., 2016), all verified in adult zebrafish.

In the novel tank and social preference tests, we showed that APPZ reversed the stress- induced changes in zebrafish behavior and is concentration dependent, similar to rodents (BIOJONE et al., 2011). The main effect is the reversal of the stress-induced impairment in the social behavior, promoting social preference instead of isolation. In fact, this is a desired therapeutic effect in the treatment of pervasive developmental disorders in humans, improving the symptoms of isolation (Stigler et al. 2004; Erickson et al. 2010; Wink et al. 2010). Apparently, this effect on social behavior may be a positive factor in the

environment,because the group preference (GERLAI et al., 2000) or shoaling (Miller and Gerlai 2011; Soares et al. 2018) reduce predation risk and facilitates foraging (Miller and Gerlai 2012; Kalueff et al. 2013). However, we did not test if APPZ influences the polarization or the dispersive ability of shoal, necessary to detect or avoid a predator (KALUEFF et al., 2013b), so, we cannot affirm that is really a positive reaction to fish.

At the lower APPZ concentration (0.556 ng/L), an anxiolytic-like effect occurred in stressed zebrafish, similar to observed in rats (BURDA et al., 2011) and humans (ERICKSON et al., 2010) treated with APPZ. We verified that APPZ increased time spent and reduced the latency of entry in the top zone in stressed fish at first stage in novel tank test (Fig. 3C). Maybe, this effect is caused because APPZ exerts a partial agonist effect at dopaminergic receptors, with high affinity for D2, reducing the anxiety-like behavior (Burris et al. 2002; Hirose and Kikuchi 2005; Burda et al. 2011). However, this drug is not effective to modify the stress- induced dwelling (Fig. 3F), leading us to think that APPZ exerts an anxiolytic effect only in the initial stage in novel tank test. It is also evident that APPZ did not affect the locomotion activity of fish (Fig 3B and 3E) like seen in rats (KUS et al., 2017).

Taken together, our results show that APPZ-exposed zebrafish decrease the perception of predator, even at concentrations lower than detected in the environment. A failure of the antipredatory response may favor the predator, decreasing the fitness of the prey species and, consequently, affecting the food chain (Kelley and Magurran 2003; Stewart et al. 2013). Our results highlight the risks and consequences associated with APPZ residues in water, which may affect aquatic life and endanger species that depend on appropriate behavioral responses for survival.

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5. DISCUSSÃO

Com base nos resultados descritos nos dois artigos produzidos, podemos mostrar que os resíduos de APPZ na água produzem efeitos tanto no eixo hipotálamo-hipófise-interrenal (HHI) quanto no comportamento do *zebrafish* adulto (Quadro 2). No primeiro artigo (BARCELLOS et al., 2016), podemos evidenciar que os peixes expostos previamente ao APPZ, quando submetidos ao estímulo estressor agudo, tiveram um claro embotamento da resposta de cortisol ao estresse. Em relação ao efeito do APPZ sobre o comportamento do *zebrafish* (Artigo 2 - BARCELLOS et al, 2018 *submitted*) observamos que algumas dessas concentrações reverteram a resposta comportamental ao estresse tanto no aspecto de preferência social, quanto no comportamento do tipo-ansiedade e na percepção de um peixe estímulo (um estressor biótico), seja predador ou não predador.

Em relação aos aspectos neuroendócrinos do estresse, os peixes submetidos ao estímulo estressor agudo apresentaram aumento significativo nos níveis do cortisol corporal total, enquanto nos expostos ao APPZ, os níveis de cortisol permaneceram semelhantes aos dos animais do grupo controle não estressados. Entretanto, não foram em todas as concentrações do grupo estressado que este efeito pode ser constatado. Uma curva em forma de sino ou "U" invertido pode ser caracterizada como um efeito de hormese no grupo estressado (CALABRESE; BALDWIN, 2001), semelhante ao observado com as exposições ao diazepam e fluoxetina (ABREU et al., 2014) e a risperidona (IDALENCIO et al., 2015). Na exposição ao APPZ, contatou-se que as concentrações mais baixas e a concentração mais elevada tiveram o mesmo efeito de embotamento da resposta ao estresse, diferentemente das concentrações intermediárias. O efeito hormético é importante para descrever um tipo de zona biológica de otimização da resposta a um agente, refletindo em padrões de comportamentos e respostas na natureza relacionados as concentrações, que não seguem um padrão de proporção direta (CALABRESE; BALDWIN, 2001); ou seja, pequenas concentrações geram respostas diferentes das concentrações mais elevadas (CALABRESE; BALDWIN, 2003).

Semelhante ao que ocorre em humanos (HANDLEY et al., 2016), o APPZ *per se* não alterou o eixo HHI nos peixes. Provavelmente isso ocorre porque a hipófise tenha menor quantidade de receptores dopaminérgicos (MAILMAN; MURTHY, 2010) e as concentrações de exposição ao APPZ na água foram insuficientes para desencadear algum efeito sobre a

liberação do cortisol pelas células inter-renais. Entretanto, numa situação de estresse, acreditamos que o APPZ tenha exercido sua função de agonista parcial sobre as fibras do sistema dopaminérgico, diminuindo a liberação de cortisol pelas células inter-renais.

Com base nos resultados do primeiro artigo, escolhemos além da concentração já detectada no ambiente e utilizada como referência para estes estudos (5,56 ng/L – (SUBEDI; KANNAN, 2015) mais duas concentrações que embotaram a resposta ao estresse, uma abaixo (0,556 ng/L) e outra acima (556 ng/L) da concentração de referência para realizarmos os testes comportamentais que deram origem ao segundo artigo (BARCELLOS et al., 2018 *submitted*), pois acreditamos que o embotamento da resposta ao estresse no eixo HHI refletiria em alterações comportamentais.

Constatamos inicialmente que o APPZ apresentou um efeito diferente conforme a condição do animal, não estressado e estressado. Nos animais não estressados, observamos um efeito per se menos robusto do que nos animais estressados (Quadro 2).

Quando avaliamos os efeitos do APPZ *per se*, constatamos que não houve efeito no sistema endócrino e na consequente liberação do cortisol. Entretanto, o APPZ *per se* promoveu alterações no comportamento. Na menor concentração testada, houve uma nítida redução do número de buscas pelo cardume (artigo 2 – Figura 2B), entretanto não afetou o tempo de permanência no segmento dos co-específicos (Artigo 2 – Figura 2A). Por outro lado, para todas as concentrações testadas, durante a exploração inicial do ambiente novo (no teste do tanque novo), os peixes mostraram um efeito tipo ansiolítico, caracterizado pela exploração vertical, como menor latência para explorar a superfície (Artigo 2 - Figura 3C). Entretanto, com o passar do tempo, esse efeito perde força e não é suficiente para alterar o tempo de permanência no fundo, um efeito tipo-ansiogênico clássico de um animal frente a um ambiente novo, buscando no mergulho para o fundo a sua proteção (KYSIL et al., 2017).

Porém, sob condições de estresse agudo, o impacto da exposição ao APPZ foi bem mais evidente. O estresse, como esperado, produziu um efeito ansiogênico, aumentando o tempo de fundo no tanque novo (artigo 2- Figura 3F) e promovendo um afastamento do cardume, constatado pelo diminuição do tempo de permanência, assim como o número de entradas no segmento dos co-específicos no teste de preferência social (artigo 2 – Figura 1A e 1B). O APPZ nas concentrações de 0.556 e 5.56 ng/L, reverteu o isolamento provocado pelo estresse (Artigo 2 Figura 2A) e em todas as concentrações promoveu uma latência menor para explorar a superfície (Artigo 2 Figura 2C). Esses efeitos tipo-ansiolítico e pró-social que foram

verificados em nosso estudo, são semelhantes aos efeitos desejáveis do aripiprazol nos organismos alvo,ou seja, em seres humanos com desordens psíquicas caracterizadas por comportamentos antissociais (NICKEL et al., 2006) e de ansiedade (KATZMAN, 2011).

Em relação ao estresse biótico, provocado pela presença de um peixe-estímulo, pode- se perceber uma reação condizente com as fases dos comportamento anti-predatório nos *zebrafish* não expostos ao APPZ: a detecção do risco (fase 1), passando pelo reconhecimento e inspeção da ameaça (fase 2), até a evitação do ataque (fase 3) (KELLEY; MAGURRAN, 2003). Os peixes não expostos ao APPZ detectaram visualmente a possível ameaça através do tamanho e do movimento do peixe estímulo (BASS; GERLAI, 2008). Hipotetizamos que, o fato do *goldfish* ser bem mais ativo e permanecer mais no fundo do aquário, aparentemente facilitou a sua visualização (fase 1 anti-predatória) ainda no primeiro estágio do teste (entre 0 e 60 segundo), levando o *zebrafish* à fuga para a área mais distante da área de risco (fase 3 anti- predatória) desde o início do teste (Artigo 2 – figura 4). Como o oscar permaneceu menos móvel e no topo do aquário, sua percepção visual e reconhecimento da ameaça (fases 1 e 2 anti- predatória) ocorreu mais no final do teste (estágio 3, entre 121 e 180 segundo). Salientamos que a escolha do peixe-estímulo foi baseada em outros estudos (OLIVEIRA et al., 2013; ABREU et al., 2018) que utilizaram o oscar e com a descrição de seu comportamento predatório (SMITH, 1981) e o *goldfish* descrito como gentil e não predador (KOTTELAT et al., 1993). Ambas as espécies foram utilizadas no nosso estudo como estímulo estressor biótico, sendo o impacto sobre o comportamento do *zebrafish* diferente de acordo com o tipo de atividade e posição do peixe estímulo no aquário (Artigo 2, figura 5).

Em relação a esse estresse biótico, o APPZ nitidamente interferiu no comportamento dos peixes expostos. Na presença do APPZ, o *zebrafish* parece perder as capacidades anti-predatórias (Artigo 2 – figura 4). Uma hipótese que explicaria isto é a capacidade de localização espacial do *zebrafish* (DE PERERA, 2004) associada à detecção da falta de eficácia dos ataques dos peixes estímulos, diminuindo a resposta de fuga da presa (BARCELLOS et al., 2010). Ou seja, conseguiram detectar que a presença daquele peixe-estímulo não era uma ameaça no local onde se encontravam. Essa hipótese explicaria também porque na concentração mais elevada, durante o segundo estágio do teste, os peixes permanecem mais afastados da área de risco, mas retornaram a exploração no estágio 3. Ou seja, os *zebrafish* visualizaram a movimentação dos peixes estímulo entre 61 e 120 segundos e detectaram que não era uma ameaça no local após 121 segundos de teste. Esse

“aprendizado” sobre a relevância das situações de risco já foi descrito para o *zebrafish* (BARCELLOS et al., 2010).

Outra hipótese, menos plausível que a primeira, está relacionada ao efeito hormético do APPZ, ou seja, mesmo em concentrações inferiores às terapêuticas para humanos (DIAK; METHA, 2008), o APPZ pode ter ocasionado um déficit visual pela diminuição do diâmetro da pupila, como constatado em humanos sem distúrbios psiquiátricos (KOLLER et al., 2018). Nessa hipótese, consideramos que diâmetro diminuído da pupila tenha prejudicado a visualização da possível ameaça. Em não havendo a visualização (fase 1 anti-predatória) para o reconhecimento de um risco (fase 2 anti-predatória), os *zebrafish* não manifestaram o comportamento de evitação (fase 3 anti-predatória).

Todos esses efeitos observados no comportamento do *zebrafish* provavelmente estão relacionados às áreas de atuação do APPZ no sistema dopaminérgico e serotoninérgico do encéfalo. O sistema dopaminérgico na região mesocorticolimbica é responsável pelo controle dos comportamentos adaptativos, tais como aprendizado, fuga e emoções do tipo ansiedade (SANDERS-BUSH; HAZELWOOD, 2012). Sendo o APPZ um agonista parcial de receptores D2, atuaria como um estabilizador funcional da liberação de dopamina, regulando esses comportamentos (KILTS, 2002). Em relação ao sistema serotoninérigico, os receptores $5HT1_A$ também são os responsáveis pelo controle da ansiedade; e os $5HT2_A$ por comportamentos hiperativos (SANDERS-BUSH; HAZELWOOD, 2012). Provavelmente, o efeito ansiolítico e anti-aversivo no peixes, tal como constatado em roedores (BIOJONE et al., 2011; BURDA et al., 2011), foi por ação tanto no sistema dopaminérgico quanto serotoninérgico.

Comparando as informações dos dois artigos (Quadro 2), podemos constatar que o efeito do APPZ no eixo HHI pode ser um dos mecanismos por trás das alterações comportamentais observadas nos peixes estressados expostos ao medicamento. As concentrações que promoveram o embotamento da resposta ao estresse (Artigo 1), também promoveram diferentes alterações comportamentais relacionadas aos aspectos de preferência social, do tipo ansiedade e do tipo medo. O APPZ embota a resposta neuroendócrina ao estresse, promove a aproximação do *zebrafish* estressado ao cardume, manifesta um comportamento ansiolítico inicial, mas exerce um efeito anti-aversivo na presença de uma ameaça predatória. Se o *zebrafish* conseguir manter-se na formação de cardume (fato que ainda não está elucidado) e menos ansioso, com uma iniciativa exploratória, mesmo que apenas na fase inicial, aumentará as chances de reprodução e busca por alimento (KELLEY;

MAGURRAN, 2003), o que aparentemente pode ser benéfico. Entretanto, o efeito anti-aversivo na presença do predador poderá acarretar uma diminuição da segurança do cardume, aumentando o risco de predação (KELLEY; MAGURRAN, 2003).

Em termos de mecanismo, o estresse, a ansiedade e o medo são controlados por áreas diferentes do encéfalo, apesar de serem modulados pelo sistema dopaminérgico e serotoninérgico. No estresse, o controle do disparo da fase primária da resposta ao estresse ocorre através do hipotálamo e hipófise (URBINATI; ZANUZZO; BILLER-TAKAHASHI, 2014), enquanto a ansiedade é controlada principalmente pelo sistema límbico (KUHAR; COUCEYRO; LAMBERT, 2015) e o medo pela amigdala (SANDERS-BUSH; HAZELWOOD, 2012). Em todos esses locais há receptores dopaminérgicos D2, e o APPZ apresenta afinidade atuando como estabilizador funcional da dopamina (LAWLER et al., 1999; KILTS, 2002) e assim promovendo os efeitos no comportamento do peixe.

Quadro 2 – Esquema de comparação dos resultados do cortisol dos *zebrafish* submetidos ao estimulo estressor agudo (artigo 1) e do comportamento da exposição ao APPZ (artigo 2).

	APPZ S-						APPZ S+					
Parâmetro	Controle	0,0556	0,556	5,56	55,6	556	Controle	0,0556	0,556	5,56	55,6	556
Cortisol												
Tanque Novo												
Tempo de topo												
Número de cruzamentos												
Latência para o topo												
Tempo de *freezing*												
Distância percorrida												
Tempo de fundo												
Preferência social												
Tempo no segmento dos co-específicos												
Número de entradas no segmento dos co-específicos												
Distância percorrida no segmento dos co-específicos												
Presa-predador 1º estágio												
Tempo na zona de alto risco sem peixe estímulo												
Tempo na zona de alto risco na presença do goldfish												
Tempo na zona de alto risco na presença do oscar												
Presa-predador 2º estágio												
Tempo na zona de alto risco sem peixe estímulo												
Tempo na zona de alto risco na presença do goldfish												
Tempo na zona de alto risco na presença do oscar												
Presa-predador 3º estágio												
Tempo na zona de alto risco sem peixe estímulo												
Tempo na zona de alto risco na presença do goldfish												
Tempo na zona de alto risco na presença do oscar												

Legenda : aumenta diminui não altera não utilizado

6. CONCLUSÃO

A exposição aguda ao APPZ é capaz de causar o embotamento do eixo neuroendócrino de estresse em *zebrafish* adultos, bem como alterar o comportamento exploratório e social e a reação frente a uma ameaça predatória. Num contexto ecológico, o somatório desses efeitos endócrinos e comportamentais causados pelo APPZ pode expor os peixes a um maior risco de predação e, consequentemente pôr em risco o equilíbrio ecológico entre as espécies que compõem o ecossistema aquático.

7. PERSPECTIVAS

Considerando que as informações disponíveis sobre os efeitos da contaminação ambiental com APPZ em peixes são escassas, buscaremos aprofundar esses conhecimentos, utilizando o *zebrafish* como modelo experimental. Como foi constatado que o APPZ aumenta a preferência social numa situação de estresse, o próximo estudo terá como objetivo avaliar se isso interfere na formação do cardume. Para tal a avaliação do comportamento de agrupamento em cardume (*shoalling*) torna-se uma boa alternativa, tanto expondo apenas um peixe do cardume ao APPZ, quanto o cardume todo e avaliar indicies importantes tais com a coesão do cardume e a polarização.

Além disso, temos como objetivo futuro, a avaliação do impacto sobre a reprodução, analisando os efeitos sobre taxa de eclosão, sobrevivência das larvas, e eventuais efeitos na morfologia dessas larvas. Na sequência, pensamos em analisar se a exposição aguda ao APPZ causa impactos persistentes e nas gerações futuras, como verificado recentemente em nosso grupo para o antipsicótico atípico risperidona (KALICHAK, 2018). Para tal, pensamos em manter essas larvas expostas e reproduzi-las, avaliando os efeitos na primeira geração.

Apesar do nosso estudo não evidenciar efeitos locomotores em decorrência da exposição ao APPZ, essa análise foi feita em um tanque com água parada. Mas e se o peixe é exposto a um fluxo de água corrente, teria o APPZ influenciado na sua locomoção? Para testar tal hipótese, pensamos em expor esses peixes ao nado forçado como o utilizado por Rosa et al. (2017).

Nosso estudo evidenciou que as alterações comportamentais e endócrinas são muito semelhantes as observadas em roedores e em humanos. Mas devido a algumas diferenças entre o sistema dopaminérgico do *zebrafish* com estas espécies, faz-se necessário um estudo mais aprofundado do mecanismo pelo qual o APPZ atua no *zebrafish*.

Futuramente, a exposição crônica ao APPZ também deverá ser explorada, visto que com a crescente prescrição e consumo deste fármaco o risco de contaminação ambiental por resíduos aumenta. Com o passar do tempo, a concentração ambiental a ser detectada no ambiente provavelmente será maior, e tais impactos deverão ser avaliados também.

Não esquecendo também que podemos ter interações medicamentosas com outros fármacos, tais como risperidona, fluoxetina, entre outros, uma vez que todos estão sendo

detectados no ambiente aquático. Avaliar os efeitos destas interações faz-se importante, pois tais resíduos estão presentes em conjunto nos ambientes aquáticos.

Estas perspectivas e tantas outras ideias podem consubstanciar minha linha de pesquisa na carreira de pesquisadora. Acredito que o conhecimento aprofundado do impacto de cada fármaco no ambiente faz-se necessário como auxílio para medidas de controle e prevenção de prejuízos ao ecossistema aquático.

8. ESTUDO COMPLEMENTAR

Além dos dois artigos incluídos na presente tese, realizei outro trabalho,vinculado a disciplina APG 998 - Publicação em revista científica, que resultou na publicação "*The effects of auditory enrichment on zebrafish behavior and physiology*", na revista *PEERJ*, v. 6, p. e5162, 2018. O trabalho se contextualiza com a presente tese pela questão da manutenção dos peixes experimentais, uma vez que versa sobre os efeitos do enriquecimento ambiental sobre aspectos de bem-estar animal. Peixes mantidos em boas condições ambientais que propiciem alto grau de bem-estar podem fornecer resultados mais acurados nas pesquisas as quais são modelo.

Como metodologia, expusemos os *zebrafish* à duas horas de uma seleção de músicas do compositor italiano Antonio Vivaldi, duas vezes ao dia, durante 15 dias. Após esse período, avaliamos os níveis de cortisol corporal, as respostas comportamentais aos testes de tanque novo e claro-escuro e a expressão gênica de alguns genes chave do eixo HHI e do sistema imunológico dos peixes, tais como IL-1, IL-4, IL-10, IFNλ, TNF, BNDF, cFOS, CRF,POMC, BGR e StAR.

Constatamos que no TTN, os peixes submetidos ao enriquecimento ambiental prévio ficaram mais calmos, permanecendo mais tempo na superfície e menos tempo no fundo do tanque. Além disso, apresentaram-se mais exploratórios na superfície (com maior distância percorrida, maior tempo de mobilidade, maior ângulo de virada), mergulhando menos vezes no fundo do tanque. Tais comportamentos evidenciam um efeito tipo ansiolítico da música clássica nos peixes. No TCE, confirmou-se tal efeito, pois não houve diferença entre o tempo que permaneceram no claro ou no escuro, e ao analisarmos o comportamento no compartimento claro, constatamos que nadaram menos e tiveram menor número de rotações neste local.

Contudo, o enriquecimento ambiental não influenciou apenas o comportamento destes peixes, mas promoveu um impacto benéfico sobre outros genes, relacionados à imunidade, cognição, atividade cerebral e ao estresse. Em relação à imunidade, houve diminuição dos genes pró-inflamatórios (IL-1B e INF-λ) sem alterar a expressão dos genes anti-inflamatórios (IL-4, IL-10 e TNF-α). Ocorreu também um aumento da expressão do BNDF, gene que está relacionado à ativação do hipocampo e aumento da cognição, contribuindo para o efeito tipo ansiolítico. E por fim, constatamos que não houve aumento

da expressão do cFOS, nem da StAR, genes relacionados com a reatividade cerebral ao estresse, e à cascata do cortisol, respectivamente, evidenciando a não interferência desse tipo de música no eixo HHI, culminando com a não alteração dos níveis de cortisol corporal dos peixes expostos à música.

Concluímos que o enriquecimento ambiental com música clássica, assim como verificado em outras espécies, reduz o estresse e aumenta o bem-estar dos organismos aquáticos, propiciando inclusive benefícios sobre os aspectos imunológicos.

A relação desse trabalho com a presente tese está justamente em propiciar uma ambiente de bem-estar animal, principalmente relacionado às condições prévias da experimentação. O Laboratório de Fisiologia de Peixes da UPF, assim como outros locais, faz a reprodução para produção dos animais experimentais. A adoção de uma medida como essa, associada a outras de enriquecimento ambiental, favorecem a manutenção da saúde dos animais em condições de melhor bem-estar. Vários locais ao redor do mundo preconizam a utilização de música ambiental em biotérios e ambientes de produção animal, para diferentes espécies (primatas, aves, suínos, bovinos, equinos, roedores, entre outras), sendo, inclusive, obrigatória em diversos países europeus. Vários estudos também mostram os efeitos benéficos para as pessoas, porém para peixes, o efeito da música em ambiente externo ao aquário ainda não havia sido estudado. Nosso trabalho teve um objetivo bem específico, que foi de mostrar que os peixes conseguem escutar a música mesmo esta estando em ambiente externo ao aquário, e que essa medida traz benefícios para os peixes, e com isso melhorar a qualidade da experimentação com o *zebrafish*.

The effects of auditory enrichment on zebrafish behavior and physiology

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ABSTRACT

Environmental enrichment is widely used to improve welfare and behavioral performance of animal species. It ensures housing of laboratory animals in environments with space and complexity that enable the expression of their normal behavioral repertoire. Auditory enrichment by exposure to classical music decreases abnormal behaviors and endocrine stress responses in humans, non-humans primates, and rodents. However, little is known about the role of auditory enrichment in laboratory zebrafish. Given the growing importance of zebrafish for neuroscience research, such studies become critical. To examine whether auditory enrichment by classical music can affect fish behavior and physiology, we exposed adult zebrafish to 2 h of Vivaldi's music (65–75 dB) twice daily, for 15 days. Overall, zebrafish exposed to such auditory stimuli were less anxious in the novel tank test and less active, calmer in the light-dark test, also affecting zebrafish physiological (immune) biomarkers, decreasing peripheral levels of pro-inflammatory cytokines and increasing the activity of some CNS genes, without overt effects on whole-body cortisol levels. In summary, we report that twice-daily exposure to continuous musical sounds may provide benefits over the ongoing 50–55 dB background noise of equipment in the laboratory setting. Overall, our results support utilizing auditory enrichment in laboratory zebrafish to reduce stress and improve welfare in this experimental aquatic organism.

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INTRODUCTION

Numerous studies consistently show benefits of music, especially classical music, to humans (*Binns-Turner et al., 2011*; *Smolen, Topp & Singer, 2002*; *Villarreal et al., 2012*; *Cervellin & Lippi, 2011*). For example, classical music increases human wellbeing, reduces stress, and anxiety, as well as normalizes blood pressure, immune function, and cognitive performance (*Rickard, Toukhsati & Field, 2005*). Musical "auditory" environmental enrichment can be also used to improve welfare of laboratory animals, with clear positive behavioral effects and overall stress relief reported in multiple species, including dogs, primates, pigs, horses, and rodents (*Alworth & Buerkle, 2013*). In contrast, uncontrollable chronic noise exposure in the laboratories may impair welfare of the experimental animals (*Patterson-Kane & Farnworth, 2006*), and therefore represents a detrimental factor in neurobehavioral studies (also see *Kettelkamp-Ladd, 1993*).

Like mammals, fish have a well-developed auditory system (*Fay & Popper, 2000*). Fish perceive various sounds within aquatic environment, demonstrating selectivity for music tempo (*Catli, Yildirim & Turker, 2015*) and discriminating sound intensity, frequency, and the source location (*Fay & Popper, 2000*). Fish hearing involves otolith organs (saccule, lagena, and utricle), and their "auditory filters" operate in the range <40 Hz to >1 KHz, depending of the species (*Fay & Popper, 2000*).

Despite the negative effects of noise on many fish species (*Vazzana et al., 2017*; *Celi et al., 2016*; *Buscaino et al., 2010*; *Filiciotto et al., 2014*), classical music exposure accelerates reproduction in several fish species (*Papoutsoglou et al., 2007*; *Papoutsoglou et al., 2010*; *Imanpoor, Enayat Gholampour & Zolfaghari, 2011*; *Catli, Yildirim & Turker, 2015*) by positively modulating their physiological and metabolic states (*Papoutsoglou et al., 2010*). The reaction of fish to music has also been examined in some earlier studies. For example, exposed to classical music in culture ponds, carps (*Papoutsoglou et al., 2007*), and turbots (*Catli, Yildirim & Turker, 2015*) grew larger and fed more efficiently. In addition, fish are capable of hearing sounds from the aquatic ambient (*Popper & Fay, 2011*). However, there are scarce in-depth systematic studies of potential effects of environmental music exposure on behavioral and physiological biomarkers in fishes and of the impact of aquatic research and housing laboratory environments on such fish phenotypes.

The zebrafish *(Danio rerio)* is a widely used animal model organism in neuroscience research (*Papoutsoglou et al., 2007*; *Sicca et al., 2016*; *Levitas-Djerbi & Appelbaum, 2017*; *Uchiyama et al., 2012*; *Kalueff et al., 2013*). They are genetically and physiologically similar to others vertebrates, such as rodents and humans (*Howe et al., 2013*), and possess a well-described behavioral repertoire (*Kalueff et al., 2013*) and stress neuroendocrine axis (*Stewart et al., 2014*; *Kalueff, Stewart & Gerlai, 2014*; *Alsop & Vijayan, 2009*). In zebrafish, environmental enrichment research is only beginning to emerge.

For example, enrichment using other sensory modalities is known to blunt zebrafish stress responses and improve welfare (*Schroeder et al., 2014*; *Collymore, Tolwani & Rasmussen, 2015*; *Manuel et al., 2015*; *Giacomini et al., 2016*). However, little is known about the impact of sound exposure, and its potential as an auditory enrichment, on zebrafish behavior and physiology. In addition to raising a scientific interest, this question also becomes important practically since zebrafish research facilities routinely utilize aquatic systems with circulating water and/or stationary tanks with aerators and water filters, each generating significant background noise. Although critical from an animal welfare and data reproducibility standpoints, these aspects have not been systematically assessed in zebrafish laboratories. Likewise, despite the well-known positive effects of musical environmental enrichment in rodents and other species, there are no studies assessing the effects of music on zebrafish behavior and physiology. To address this knowledge gap, here we examine the effects of auditory environmental enrichment via chronic classical music exposure on zebrafish behavioral and physiological responses. Specifically, we wanted to assess how repeated exposure to such auditory enrichment can modulate zebrafish stress/anxiety-related behavior in two different behavioral models, fish endocrine (cortisol) and physiological (immune) responses as well as the expression of selected CNS genes, compared to the control group of fish unexposed to auditory enrichment.

MATERIALS AND METHODS

Animals

A total of 36 mixed-sex (1:1 female:male ratio) adult one-year old wild-type short-fin outbred zebrafish were used in this study. Fish were bought from a local commercial supplier (Recanto dos Peixes, Marau, Brazil) and were acclimated to the University of Passo Fundo animal facility for six months prior to testing. The animals were housed for 20 days in the UPF aquatic laboratory facility (including a five-day acclimation and a 15-day testing). The fish were kept, in groups of three, in 12 3-L glass tanks (20 height × 15 depth × 14 width cm), under constant aeration and a 14 h L: 10 h D cycle. Water temperature was maintained at 27.5 ± 1.3 °C, with pH 7.7 ± 0.08, dissolved oxygen at 5.6 ± 0.5 mg/L and ionized ammonia <0.022 ppm. Water was partially (30%) changed every two days throughout the entire experimentation period. Relevant to the goals of this study, the baseline noise levels in the laboratory were 50–55 dB (with frequency varying from 240 to 420 Hz), and mostly consisted of sounds produced by fish husbandry equipment, such as aerators and water pumps. Control fish were kept away from the room used for music exposure of the experimental ("enriched") cohort. No other sounds were presented to the control group, and their only difference from the experimental group was the lack of music exposure during the experiments.

Ethical note

All experimental procedures were performed in accordance with the guidelines of the National Council of Animal Experimentations Control (CONCEA) of Brazil. This study

Table 1 Summary of Vivaldi's music classical collection utilized in the present study.

Concert	Music
In C major	Allegro molto 5.18
	Larghetto 3.10
	Allegro 1.35
N.1 "Spring"	Allegro 3.29
	Largo 2.54
	Danza pastorale: Allegro 4.26
For mandolin, strings, and basso continuo no.1	Allegro 2.56
	Largo 3.0
	Allegro 3.03
For two violin, strings, and harpsichord	Allegro 3.09
	Andante 2.46
	Allegro 2.43 For
two oboes, bassoon, two horns, violin, strings, and organ	Allegro 4.26
	Largo 1.32
	Allegro 4.05
N.10	Allegro 4.13
	Largo, Larghetto 3.20
	Allegro 3.29

was approved by the Ethics Committee for Animal Use of the University of Passo Fundo, Brazil (UPF protocol 040/2017).

Experimental procedures

Our study aimed to assess zebrafish behavioral and endocrine (cortisol) responses and the expression of selected immune and hypothalamus-pituitary-interrenal axis-related genes in the brain. Behavioral testing utilized the novel tank (NTT) and the light-dark test (LDT) tasks following a 15-day repeated exposure to music. For this, fish were divided into two groups kept in six glass tanks (three fish per tank, n = 18 per group). One group was subjected for 15 days to two sessions of 2-h selection of Vivaldi's music (Table 1), chosen here as the representative "Popular collection." The intensity level of the music was arbitrarily set at 65–75 dB (with frequency varying from 330 to 506 Hz), based on considerations of safety and overall pleasantness of sounds for human ears (*Brookhouser, 1994*). Music and background noise intensities and frequencies in this study were assessed outside the water using the Sound Level Meter Application (available online from Google Play at https://play.google.com/store/apps/details?id=com. bolshakovdenis.soundanalyzer) on a Samsung Galaxy S6 smartphone (Samsung Brazil, Brası´lia, Brazil, 2017). The morning daily session started at 8:30 am, followed by the second (afternoon) daily exposures at 17:00 pm. All fish were fed twice a day, 30 min prior to each the music exposure sessions, to mitigate the effect of hunger on their behavior. On the final day, fish were fed at 8:00 am and submitted to behavioral assays (NTT or LDB test, n = 10–12 per group each) at 10:30 am. After testing in either assay for 6 min,

Table 2 The qPCR primers used in the present study.

Gene	Primer (5′–3′)	Efficiency (%)	Accession number
StAr	F: CCTGTTTTCTGGCTGGGATG R: GGGTCCATTCTCAGCCCTTAC	101	NM_131663.1
POMC	F: CGCAGACCCATCAAGGTGTA R: CGTTTCGGCGGATTCCT		AY125332.2
CRF	F: ACGCACAGATTCTCCTCGCC R: TCCGCGGCTGGCTGATT		NM001007379.1
cFOS	F: CAGCTCCACCACAGTGAAGA R: GCTCCAGGTCAGTGTTAGCC	97	DQ003339.2
BGR	F: ACAGCTTCTTCCAGCCTCAG R: CCGGTGTTCTCCTGTTTGAT		DQ017615.1
BDNF	F: CGCCGTTACTCTTTCTCTTGG R: CCATTAGTCACGGGGACCTTC	102	NM_001308648.1
b-2-microglobulin	F: GCCTTCACCCCAGAGAAAGG R: CGGTTGGGATTTACATGTTG		NM_131163.2
TNF-*a*	F: GACCACAGCACTTCTACCG R: ACATTTTCCTCACTTTCGTTCAC		NM_212859
IL-1*b*	F: GCTGGAGATGTGGACTTC R: ACTCTGTGGATTGGGGTTTG	100	NM_212844
INF-*g*	F: TGCCTCAAAATGGTGCTACTC R: AATCGGGTTCTCGCTCCTG		AB158361.1
IL-4	F: TCTCTGCCAAGCAGGAATG R: CAGTTTCCAGTCCCGGTATATG		AM403245.2
IL-12	F: CTGTAGGATCCATCCAAACATCT R: CACTGGCACTTCTACCCTATTT		AB183002.1
IL-10	F: CTCTGCTCACGCTTCTTCTT R: GCTCCCTCAGTCTTAAAGGAAA		BC163038.1
b-Actin	F: GCAAAGGGAGGTAGTTGTCTAA R: GAGGAGGGCAAAGTGGTAAA	99	AF057040.1

the fish were individually removed by the net and immediately euthanized with ice-cold water, decapitated and stored at liquid nitrogen for 30 s. The 6-min behavioral testing used here in both assays is a standard, commonly used testing protocol in zebrafish neurobehavioral analyses (*Egan et al., 2009*). Their trunks were then stored at -8 °C for cortisol analyses, and their heads stored at -80 °C for RNA and DNA extraction and analyses of the genes expression using the real-time PCR (Table 2). The control group underwent the same housing, handling, and testing procedures, but was unexposed to music throughout the study. The selection of "no-music" control (rather than exposing controls to other types of music or noise) for our study was based on the specific research question we aimed to address. The main focus of our study was to examine the potential of music exposure as an environmental enrichment. Respectively, for the stated experimental design, the selection of Vivaldi (vs. other composer) was not critical, serving as an example of a mild relaxing music frequently used in auditory enrichment studies in other species (*Rickard, Toukhsati & Field, 2005*; *Papoutsoglou et al., 2007*).

Because we wanted to assess whether repeated exposure to music in general can affect

fish physiology and behavior, only direct comparison of music-exposed vs. unexposed fish groups was appropriate. Albeit interesting and clearly meriting further scrutiny, comparing Vivaldi's music with other music or sounds was beyond the scope of the present study.

The novel tank test

The novel tank test was a rectangular glass tank (24 width × 8 depth × 20 high cm), as described previously (*Mocelin et al., 2015*). Fish were video-recorded for 6 min by a Logitech Quickcam PRO 9000 camera located in front of the tank, and their videos were then analyzed offline by automated ANY-maze® software, assessing time spent in top, middle, and bottom zones (s), number of bottom entries, distance traveled in each zone (m), absolute turn angle in each zone (°), total time spent in mobility (s), according to the Zebrafish Neurobehavioral Catalog (*Kalueff et al., 2013*).

The light-dark test

The LDTwas a rectangular apparatus (45 width × 10 depth × 15 high cm), with a five-cm central area separated by two sliding doors (*Magno et al., 2015*). The apparatus was filled with a five-cm deep water, and fish were individually introduced into the central chamber for 30 s for acclimation. The partition was then raised one cm above the tank floor, to allow zebrafish to swim freely between the sides of the apparatus. Fish were filmed for 6 min and their videos were then analyzed offline using ANY-maze® software, assessing the light zone rotations (complete 360º circling), distance traveled (m),
mean speed (m/s), and time spent in zone (s).

Cortisol extraction and measurement

The procedure was performed according to (*Sink, Lochmann & Fecteau, 2008*) using body trunk samples previously stored at -8 °C. Cortisol levels were determined by enzyme- linked immune sorbent assay kit (EIAgen CORTISOL test; BioChem ImmunoSystems, Rome, Italy) from tissue extracts re-suspended in PBS buffer (*Oliveira et al., 2014*). The accuracy was tested by calculating the recoveries 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 with the following equation.

Cortisol value ¼ Measured value — 1:0604:

RNA extraction, cDNA synthesis, and gene expression analysis

The brains of three fish per sample were pooled (total n = 6 samples per an 18-fish group) and used for RNA extraction. The protocol consisted of tissue lysis using the Tissuelyser LT® (Qiagen, Hilden, Germany), RNA extraction using RNeasy® Mini Kit (Qiagen, Hilden, Germany), and DNAse I amplification grade treatment (Invitrogen, Carlsbad, CA, USA) to eliminate genomic DNA. The RNA quality and concentration was measured by spectrophotometry (Nanophotometer Pearl®; IMPLEN, Munich, Germany). For cDNA synthesis, one mg of total RNA was used for the reverse transcription assay, using QuantiTect® III Reverse Transcription kit (Qiagen, Hilden, Germany). The real time PCR (qPCR) was performed using Rotor-Gene Q equipment (Qiagen, Hilden, Germany) with

initial denaturing at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. At the end, a standard melting curve was included to confirm the specificity of the amplified product. The amplification of the mRNA of the selected genes (Table 2) was compared to *b*-actin, used as a housekeeping gene. For the calibration curve, each gene was cloned and transformed into competent One Shot TOP10 *E. coli* and cultured in LB supplemented with ampicillin. The cloning was confirmed by PCR and the resulting plasmid was extracted. Then, the calibration curve consisted of decimal dilutions (1:10) of each cloned gene. To compare the results from different groups, the same threshold value (0.10) was used. The relative quantification of gene expression was performed using the $2^{-\Delta\Delta ct}$ formula (*Rao et al., 2013*). The following genes were selected here for analyses based on their established roles in neuroinflammation and/or neuroendocrine functions: *c-fos* (a neuronal marker of activation/arousal, often upregulated in stress), genes of pro-inflammatory cytokines interferon INF-*g*, tumor necrosis factor TNF-*a* and interleukins (IL) IL-1*b* (often upregulated by stress), genes of anti-inflammatory cytokines IL-10, IL-4, neurotrophin brain-derived neurotrophic factor (BNDF), selected HPI axis-related genes encoding Steroidogenic acute regulatory protein (StAr), Pro-opiomelanocortin (POMC), brain glucocorticoid receptor (BGR), and stress hormone corticotropin-releasing factor (CRF). The primers used for these genes are presented in Table 2.

Statistical analysis

Data were analyzed using the unpaired *t*-test or Mann–Whitney *U*-test, depending on data normality, as assessed by the Kolmogorov–Smirnov test, and homogeneity of variance, determined using the Hartley's test. p was set at < 0.05 for all tests.

RESULTS

Overall, fish exposed to music clearly preferred the top NTT zone ($p = 0.002$) and spent significantly less time at the tank bottom ($p = 0.0116$). In the top, they also travelled longer distance ($p = 0.0370$), spent more time moving (mobile) ($p = 0.0019$), they showed higher absolute turn angle ($p = 0.0011$), compared to unexposed controls. In the bottom zone of the NTT, the number of entries into this area ($p = 0.0095$) was significantly lower than controls (Fig. 1), collectively suggesting an anxiolytic-like behavioral profile evoked by music exposure in the experimental group.

In the LDT, there were no differences between the groups in time spent in light ($p = 0.1267$), although fish exposed to music appeared calmer as they travelled shorter distance in the light zone ($p = 0.0299$) and showed fewer rotations ($p = 0.0004$, Fig. 2).

The CNS gene expression results are presented in Fig. 3. Overall, affecting the group of immune genes, auditory enrichment decreased the expression of pro-inflammatory IL IL-1*b* ($p = 0.0173$) and INF-*g* ($p = 0.0022$), but did not affect other cytokines IL-4 ($p = 0.1797$, NS), IL-10 ($p = 0.3016$, NS), and TNF-*a* ($p = 0.4740$, NS). Additionally, music exposure elevated the expression of *BNDF* ($p = 0.0260$), but not *c-fos* ($p = 0.2229$, NS) or selected HPI axis-related genes *StAr* ($p = 0.6571$, NS), *POMC* ($p = 0.4961$, NS), *BGR* ($p = 0.8983$, NS), and *CRF* ($p = 0.6063$, NS).

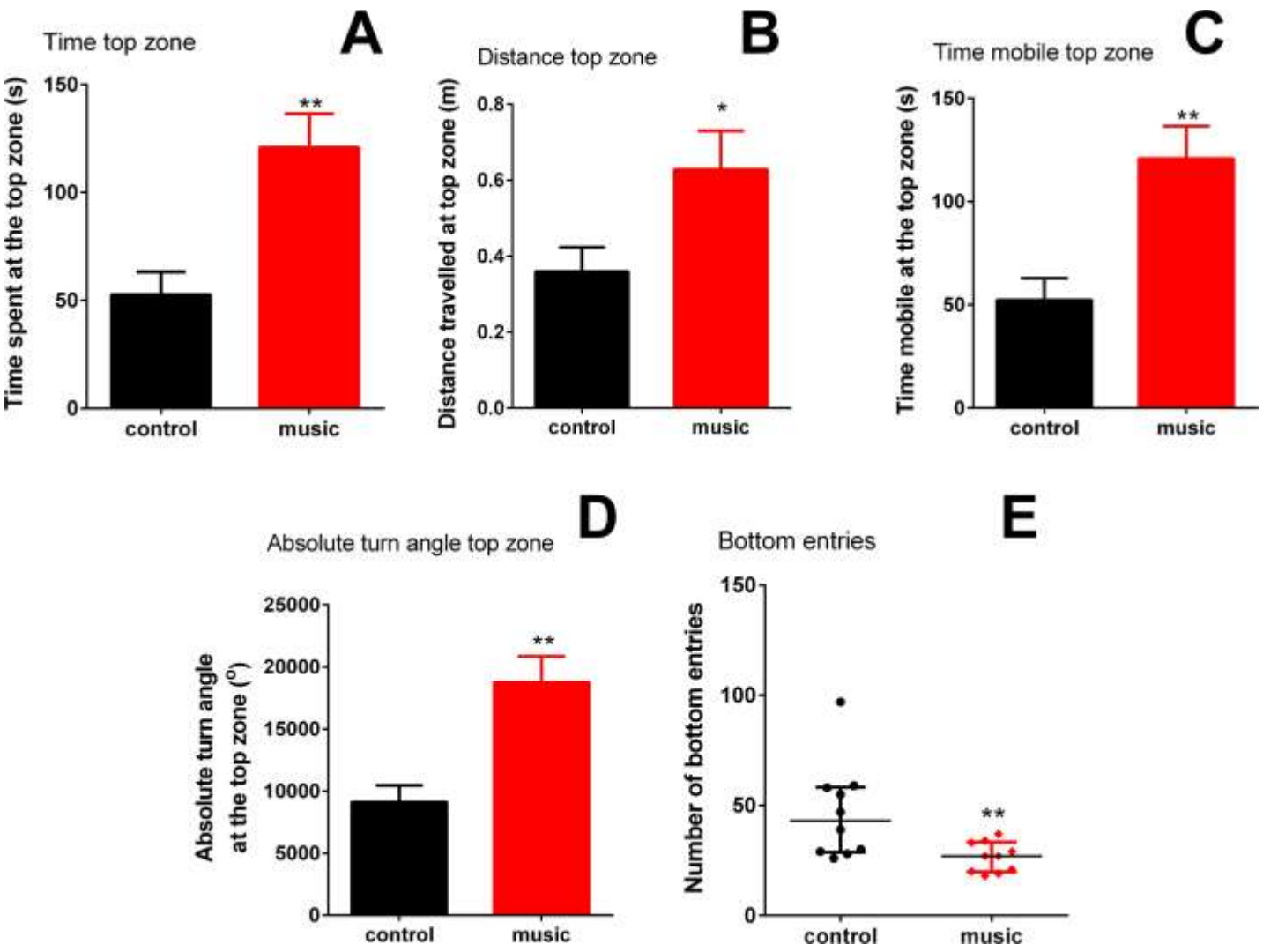

Figure 1 Behavioral performance of zebrafish in the novel tank test (NTT) following daily exposure to auditory enrichment (Vivaldi's music) for 15 days. Data from top zone ((A) time spent at the top zone; (B) distance travelled at the top zone; (C) time mobile at the top zone and (D) absolute turn angle at the top zone) are expressed as mean ± S.E.M. and analyzed by unpaired *t*-test. Data from the NTT bottom zone ((E) number of the bottom entries) are expressed as median ± interquartile range and analyzed by Mann–Whitney *U*-test. $^{m}p < 0.05$; $^{mm}p < 0.01$ vs. unexposed control ($n = 10$).
Full-size DOI: 10.7717/peerj.5162/fig-1

Finally, the trunk cortisol levels did not differ between the groups ($p = 0.5371$, $n = 8$), with fish exposed to music yielding 11.88 ± 1.41 vs. control 10.25 ± 2.1 ng/g tissue.

DISCUSSION

Mounting evidence supports the role of various types of environmental enrichment in zebrafish models (*Schroeder et al., 2014*; *Collymore, Tolwani & Rasmussen, 2015*; *Manuel et al., 2015*). To the best of our knowledge, the present study is the first report examining the role of auditory enrichment, such as 15-day repeated classical (Vivaldi) music exposure, on zebrafish behavior and physiology. In the NTT, fish chronically exposed to this type of auditory enrichment were less anxious and most active, compared

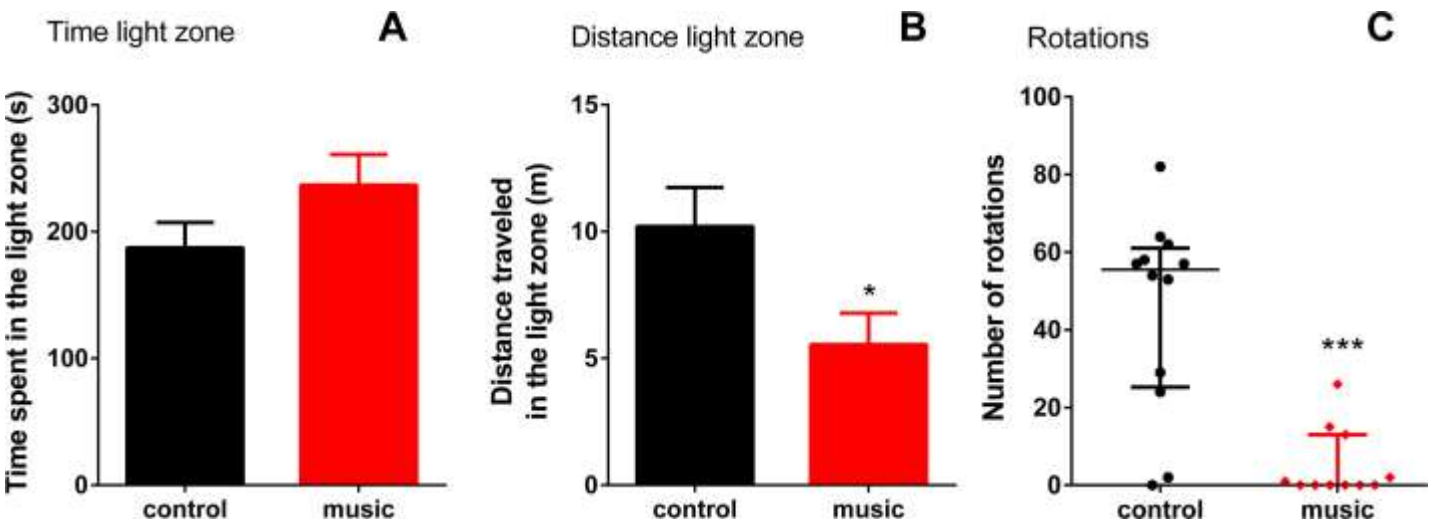

Figure 2 Behavioral performance of zebrafish in the light-dark test (LDT) following daily exposure to auditory enrichment (Vivaldi's music) for 15 days. Data from time spent (A) and distance travelled in light zone (B) were expressed as mean ± S.E.M. and analyzed by unpaired *t*-test. Number of rotations in the light zone (C) were expressed as median ± interquartile range and analyzed by Mann–Whitney test. $^{m}p < 0.05$; $^{mmm}p < 0.001$ vs. unexposed control ($n = 12$). Full-size DOI: 10.7717/peerj.5162/fig-2

to unexposed control group (Fig. 1). In addition, the exposed group showed no overt stress responses (vs. control) in whole-body cortisol assay and unaltered expression of CNS genes related to stress response (Fig. 3B). The baseline behavioral response of control fish tested in the NTT (e.g., spending more time in the bottom, Fig. 1) resembled other studies using this model (*Egan et al., 2009*) and was generally expected, since the test novelty is a stressful factor for zebrafish (*Kysil et al., 2017*). In contrast, fish exposed to specific auditory enrichment (Vivaldi's music) used here were clearly less anxious even facing the NTT novelty, strikingly paralleling "anxiolytic" effects of Mozart's music in humans (*Rickard, Toukhsati & Field, 2005*) and rodents (*Alworth & Buerkle, 2013*). While the two composers clearly differ in their styles, the overall high level of auditory harmony of their music is widely recognized (*Mammarella, Fairfield & Cornoldi, 2013*) and likely contributed to the similar behavioral effects observed here. However, comparing present auditory enrichment with other types of music and/or non-music sound stimulation in zebrafish was beyond the scope of this study.

Interestingly, although the LDT results somewhat differed from the NTT findings (Fig. 2) described above, the fact that music-exposed fish were less active than controls suggests that they were also generally calmer in the light zone. This response may also reflect the fact that LDT has a limited ability to detect anxiolytic responses, compared to zebrafish NTT (*Kysil et al., 2017*), and the LDT inherent limitation as a model since substantial portion of fish behaviors in the dark section of the apparatus remained unaccounted for in this test.

Furthermore, specific type of auditory enrichment used here also affected the immune genes expression in zebrafish vs. unexposed controls (Fig. 3A), similar to music effects reported earlier in rodents (*Lu et al., 2010*; *Uchiyama et al., 2012*). Here, fish exposed

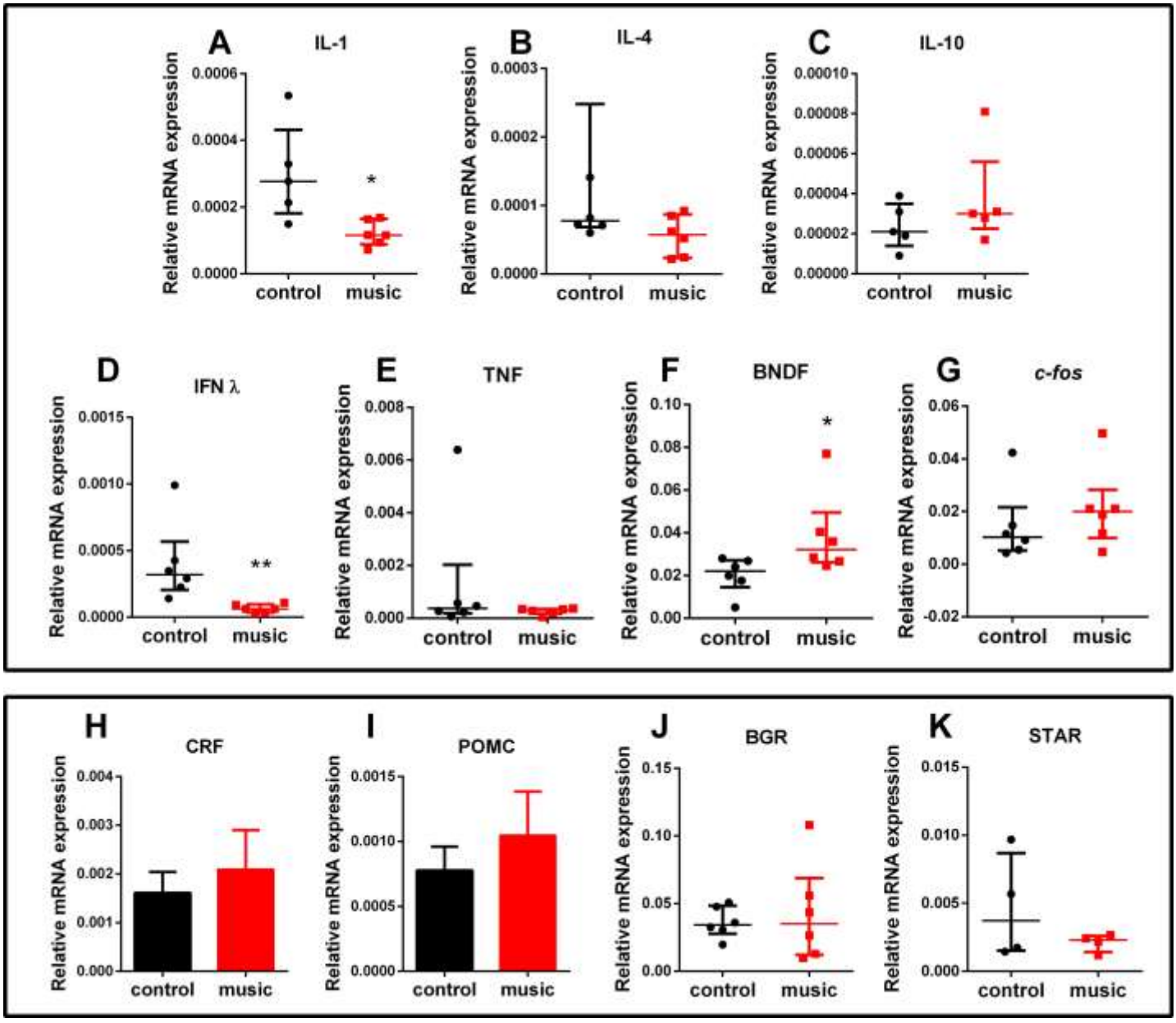

Figure 3 Relative mRNA expression of immune and HPI axis-related brain genes in zebrafish exposed daily to auditory enrichment (Vivaldi's music) for 15 days. (A) IL-1; (B) IL-4; (C) IL-10; (D) IFNZ; (E) TNF; (F) BDNF; (G) *c-fos*; (H) CRF; (I) POMC; (J) BGR and (K) StAR. Parametric data for *POMC* and *CRF* expression are expressed as mean ± S.E.M. and analyzed by unpaired *t*-test. Data for other genes are non-parametric and expressed as median ± interquartile range, analyzed by Mann–Whitney test. $^{m}p < 0.05$; $^{mm}p < 0.01$ vs. unexposed control ($n = 6$). Abbreviations of the genes are as in Table 2.

Full-size DOI: 10.7717/peerj.5162/fig-3

to auditory enrichment showed lower expression of some pro-inflammatory genes (IL-1*b* and IFN-*g*), but without affecting anti-inflammatory genes *IL-10* and *IL-4* (Fig. 3A).

Notably, both Vivaldi's and Mozart's music seem to positively modulate neuronal activation at hippocampal and enhance spatial cognition ability in rodents, based on their up-regulation of *BDNF* (*Xing et al., 2016*), which can also contribute to anxiolytic-like profile observed here in zebrafish (Fig. 2). In contrast, we did not observe the effect of

music on *c-fos* expression in the brain. Although this early proto-oncogene is a well-established marker of stress reactivity in the brain (*Bouwknecht et al., 2007*) and can be upregulated by noise stress in rats (*Babb et al., 2013*), the baseline differences in stress reactivity in music-exposed vs. control fish may not be robust here, especially since zebrafish trunk cortisol levels also remained unaltered. Overall, the observed behavioral phenotypes (Figs. 1 and 2) suggest that auditory stimulation may have an anxiolytic-like effect in zebrafish, compared to unexposed controls. Furthermore, our method of auditory stimulus presentation differs from that of other groups (*Papoutsoglou et al., 2007*; *Imanpoor, Enayat Gholampour & Zolfaghari, 2011*) who introduced hydrophones directly into the aquatic environments. While the latter method requires an expensive experimental equipment, our easier and cheaper method (utilizing a simple MP3 player) can be advantageous from the practical point of view.

One limitation of our study is that it did not measure the intensity level of the sound signal coming into the fish tank water. However, this technical aspect does not negate the overall relevance of our results, for the first time revealing the role of repeated musical auditory environmental enrichment in zebrafish. As already mentioned, the 65–75 dB sound range in the laboratory room was chosen as pleasing to humans (*Brookhouser, 1994*), but it remains unclear how zebrafish perceive it. Testing more loud sounds (e.g., using the same music but at different loudness levels) may also be interesting, and can be performed in subsequent follow-up studies. However, such studies are rather problematic in the research facility, and are unlikely feasible or practical for other laboratories as an auditory enrichment, since it would create a major discomfort to researchers and technicians, and may also distress all species of laboratory animals.

Nevertheless, we note that fishes can discriminate sound intensity and frequency, as well as localize the sound source and analyze auditory signal spectra (*Fay & Popper, 2000*). Several questions remain open for future studies in zebrafish models. For example, would other composers and even music types evoke similar, or different, behavioral profiles, in fish? Will these responses be similar with those of another species, like rats (*Otsuka, Yanagi & Watanabe, 2009*) or birds (*Watanabe & Nemoto, 1998*)? And, if there were a difference, to what extent the behavioral outcome recorded would depend on baseline housing factors, such as background noise present in specific laboratory environments, as well as whether inter-laboratory differences in such auditory backgrounds may affect the observed behavioral outcomes? Indeed, the effect of other husbandry factors, such as lighting, have been reported to affect stress responsivity in rodents (*Bouwknecht et al., 2007*). Thus, the possibility of similar effects of "sound background" in rodent or fish models remains unclear, and merits further scrutiny in zebrafish tests.

Likewise, in addition to *c-fos* and cortisol assays, other hormonal and molecular biomarkers, such as neurochemical alterations and/or stress-related peripheral or central cytokines, may be examined in-depth in the follow-up studies. The patterns of brain gene expression and epigenetic modifications may also be examined in such studies, including recently developed methods such as differential gene expression analyses (*Gutha et al., 2018*). Furthermore, music exposure for a longer period of time (e.g., 5–10 weeks) and/or more frequently (e.g., 3–4 h twice a day) may be utilized in

future studies, to more fully characterize long-term auditory enrichment effects in zebrafish. Clearly, the latter protocols may be more relevant to prolonged sound exposure in laboratory housing environments, providing important novel insights into zebrafish husbandry and their phenomics. Again, using additional control groups, including exposure to white noise as well as other musical and non-musical sounds, can be a useful future line of research in this model. Finally, combining behavioral and physiological analyses in such studies with additional neuromorphological assays relevant to brain plasticity, such as examining synaptic density, neuronal arborization, and/or dendritic spines, may also be warranted in zebrafish and other aquatic species.

CONCLUSION

In summary, zebrafish exposed to specific type of auditory enrichment (twice daily exposure to Vivaldi's music for two weeks) were less anxious and more active, compared to their unexposed control counterparts. The exposed fish also showed upregulated pro-inflammatory genes *IL-1b* and *INFg*, as well as the neurotrophin *BNDF* gene in the brain. Taken together, these findings suggest that the used auditory enrichment in zebrafish may be a potential factor modulating their behavioral and physiological responses. In essence, we report that twice daily exposure to continuous 65–75 dB sounds may provide benefits over the ongoing background noise of equipment in the laboratory setting. From the practical standpoint, these results support using musical environmental enrichment in zebrafish, similar to auditory enrichment currently used in rodents Moreover, it has still not been established that the melodic content of the music is responsible for the effects reported here, although some studies show that animals react differently to music and other sounds,
such as static (*Kettelkamp-Ladd, 1993*). For example, it has been repeatedly demonstrated that non-musical sound alone may have a beneficial effect on animals (*Robbins & Margulis, 2014*; *Robbins & Margulis, 2016*; *Pysanenko et al., 2018*), and therefore our conclusions are limited to auditory enrichment in general, rather than to music more specifically.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Helo´ısa H. A. Barcellos conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Gessi Koakoski conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Fabiele Chaulet performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Karina S. Kirsten performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Luiz C. Kreutz analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Allan V. Kalueff conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Leonardo J. G. Barcellos conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All methods were carried out in accordance with the guidelines of National Council of Animal Experimentations Control (CONCEA). This study was approved by the Ethics Commission for Animal Use of University of Passo Fundo, Brazil (UPF protocol 040/2017).

Data Availability

The following information was supplied regarding data availability:

The raw data and statistics are included in the Supplemental Dataset Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5162#supplemental-information.

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9. DEMAIS PUBLICAÇÕES

1. Fior, D.; Dametto, F.; Fagundes, M.; Santos Da Rosa, J.G.; Sander De Abreu, M.; Koakoski, G.; Idalencio, R.; **Barcellos, H.H.A.**; Piato, A.; Barcellos, L.J.G. Divergent action of fluoxetine in zebrafish according to responsivity to novelty. Scientific Reports, v. 8, p. 13908, 2018.

Trabalho principal da dissertação de mestrado em Bioexperimentação (UPF), de Débora Fior. Nesse trabalho participei de todas as etapas desde os experimentos até a análise dos dados. Mostramos que o teste de reconhecimento do objeto novo pode discriminar o zebrafish em neofóbicos e neofílicos dentro de uma população. E que o zebrafish não mantém o seu comportamento na sequência dos testes e apenas os neofóbicos retornam ao seu comportamento responsivo inicial quando expostos a fluoxetina. Essas diferenças de fenótipos de comportamento podem aumentar a variabilidade da resposta individual, e interferir nas respostas de diversos testes comportamentais. Esses dados reforçam a validade em determinar a personalidade do zebrafish, uma vez que mostramos claramente a diferença na resposta comportamental de zebrafish neofílico e neofóbico submetidos a fluoxetina.

2. Santos da Rosa, J.G.; **Barcellos, H.H.A.**; Fagundes, M.; Variani, C.; Rossini, M.; Kalichak, F.; Koakoski, G.; Oliveira, T.A.; Idalencio, R.; Frandoloso, R.; Piato, A.L.; Barcellos, L.J.G. Muscarinic receptors mediate the endocrine-disrupting effects of an organophosphorus insecticide in zebrafish. Environmental Toxicology, v. 32, p. 1964- 1972, 2017.

Trabalho integrante da tese de doutorado em Farmacologia (UFSM) de João Gabriel Santos da Rosa. Nesse trabalho participei principalmente na elaboração e realização da metodologia experimental, assim como na discussão dos resultados e elaboração das conclusões. Mostramos que o inseticida a base de metil-paration causa desregulação na resposta endócrina ao estresse, alterando a expressão de genes da esteroidogenese, especificamente uma diminuição da expressão da StAR, da hsp70 e do POMC. Mostramos também que isto parece ser mediado via receptores muscarínicos, visto que a escopolamina (um antagonista muscarínico) bloqueou este efeito.

3. Endres, H.C.; Rosa, J.G.S.; Kabaselle, C.; **Barcellos, H.H.A.**; Bertol, C.D.; Barcellos, L.J.G.; Rossato-Grando, L.G. First evidence that waterborne methylphenidate alters endocrine and behavioral stress responses in zebrafish. Neuroscience Letters, v. 650, p. 114-117, 2017.

Trabalho de conclusão de curso de graduação em Farmácia (UPF), de Helena Cristina Endres. Nesse trabalho participei na elaboração do projeto, na realização da metodologia experimental, e análise dos dados. Mostramos que o metilfenidato (MPH) embota a resposta ao estresse em *zebrafish*. Além disso, também evidenciamos o efeito hormético do MPH *per se*, Também o MPH modula o comportamento tipo ansiedade, promovendo um efeito ansiolítico nos peixes estressados. Concluímos que o MPH na água pode alterar as respostasneuroendócrinas e comportamentais, podendo impactar a sobrevivência e o bem-estar dos peixes.

4. Idalencio, R.; **Barcellos, H.H.A.**; Kalichak, F.; Rosa, J.G.S.; Oliveira, T.A.; Abreu, M.S.; Fagundes, M.; Dametto, F.; Marcheto, L.; Oliveira, C.M.; Barcellos, L.J.G. α-methyltyrosine, a tyrosine hydroxylase inhibitor, decreases stress response in zebrafish (*Danio rerio*). General and Comparative Endocrinology, v. 252, p. 236-238, 2017.

Trabalho integrante da tese de doutorado em Farmacologia (UFSM) de Renan Idalencio. Nesse trabalho participei da parte experimental assim como de análise dos resultados, e da discussão sobre as conclusões. Mostramos que a α-metil-L-tirosina (AMPT), um inibidor da tirosina hidroxilase, diminui a resposta do eixo neuro-endócrino do estresse do *zebrafish* na presença de um estressor agudo. A AMPT *per se* não interfere nos níveis de cortisol basais, porém no peixe estressado diminui a resposta ao estresse após 15 minutos da estimulação. Esses resultados sugerem um mecanismo catecolamina-glicocorticoide na resposta neuroendócrina do peixe.

5. Kalichak, F.; Idalencio, R.; Rosa, J.G.S.; **Barcellos, H.H.A.**; Fagundes, M.; Piato, A.; Barcellos, L.J.G. Psychotropic in the environment: risperidone residues affect the behavior of fish larvae. Scientific Reports, v. 7, p. 14121, 2017.

Trabalho integrante da tese de doutorado em Farmacologia (UFSM) de Fabiana Kalichak. Neste trabalho participei desde o processo de reprodução dos peixes para obtenção dos embriões, passando pelos experimentos comportamentais e análise dos resultados e discussão das conclusões. Expusemos os embriões à risperidona durante os primeiros 5 dias de vidas, realizando os testes comportamentais de campo aberto, tigmotaxia e aversividade, no 6º dia de vida. Mostramos que a risperidona causou hiperatividade nas larvas, o que num contexto ambiental, pode torna-la mais vulnerável a predação devido maior visibilidade pelo predador, ou mesmo por sua menor percepção de áreas de risco.

6. Rosa, J.G.S.; Abreu, M.S.; Giacomini, A.C.V.; Koakoski, G.; Kalichak, F.; Oliveira, T.A.; **Barcellos, H.H.A.**; Barreto, R.E.; Barcellos, L.J.G. Fish aversion and atraction to selected agrichemicals. Archives of Environmental Contamination and Toxicology, v. 71, p. 415-422, 2016.

Trabalho integrante da tese de doutorado em Farmacologia (UFSM) de João Gabriel Santos da Rosa. Minha atuação neste trabalho foi relacionada a análise dos resultados e participação nos debates de discussão sobre o assunto, auxiliando na elaboração das conclusões. Mostramos que apenas o herbicida a base de glifosato (GBH) foi aversivo ao peixe, enquanto o herbicida a base de atrazina (ABH) e o fungicida a base de tebuconazole (TBF) não causaram nem atração nem aversividade para o *zebrafish*. Logo, o GBH não impõe um risco de toxicidade extra, visto que não é atrativo para os peixes. Entretanto, o ABH e o TBF são mais deletérios, visto que não induzem uma resposta aversiva ao peixe. E como esses agroquímicos promovem bioacumulação e parecem ser tempo e concentração-dependentes, um peixe que permaneça mais tempo em contato, tende a absorver concentrações maiores em relação aqueles que fogem de locais contaminados.

7. Rosa, J.G.S.; **Barcellos, H.H.A.**; Idalencio, R.; Marqueze, A.; Fagundes, M.; Rossini, M.; Variani, C.; Balbinoti, F.; Tietböhl, T.M.H.; Rosemberg, D.B.; Barcellos, L.J.G. Just keep swimming: neuroendocrine, metabolic, and behavioral changes after a forced swimming test in zebrafish. Zebrafish, v. 14, p. 51-59, 2017.

Trabalho integrante da tese de doutorado em Farmacologia de João Gabriel Santos da Rosa (UFSM). Nesse outro trabalho, participei de todas as etapas, desde a elaboração do projeto para a CEUA, atuando nos experimentos com o vórtex, na extração e análise do cortisol, bem como no comportamento. Também participei da análise dos resultados, da discussão e elaboração das conclusões. Foi um trabalho apresentado nas disciplinas de Seminários em Farmacologia I e II. Mostramos que o vórtex, como uma adaptação do teste de nado forçado pode ser utilizado com modelo de estudo no paradigma exercício-exaustão e recuperação em peixes. Esse teste em diferentes rotações por minuto e tempo de exercício, promove mudanças no funcionamento do eixo HHI, no metabolismo intermediário, assim como no comportamento, tanto durante o exercício quanto nos períodos de recuperação. Essa pode ser uma ferramenta útil para avaliar os efeitos dos poluentes emergentes no exercício do peixe. Pode ser um protocolo tanto para os estudos ambientais (para avaliar os contaminantes que atuam na mobilização da energia dos peixes e na recuperação de estressores) quanto para estudos de perspectivas translacionais (para avaliar efeitos de fármacos em humanos após exercício ou estressados).

8. Abreu, M.S.; Giacomini, A.C.V.; Gusso, D.; Rosa, J.G.S.; Koakoski, G.; Kalichak, F.; Idalencio, R.; Oliveira, T.A.; **Barcellos, H.H.A.**; Bonan, C.D.; Barcellos, L.J.G. Acute exposure to waterborne psychoactive drugs attract zebrafish. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, v. 179, p. 37-43, 2015.

Trabalho integrante da tese de doutorado em Farmacologia de Murilo Sander de Abreu (UFSM). Neste trabalho atuei na manutenção dos animais experimentais e na discussão dos resultados e conclusões. Mostramos que o teste de aversão através de uma câmara que permite o peixe escapar ou ficar em contato com água contaminada. Testamos então a aversividade ou preferência do zebrafish por clonazepam, diazepam, fluoxetina, risperidona e buspirona. Mostramos a atratividade por algumas concentrações de diazepam, fluoxetina, risperidona e buspirona que podem ser detectadas pelo olfato, visto que os peixes foram submetidos a anosmia, e nesta condição não foram atraídos para o compartimento desses fármacos. Esses achados sugerem que apesar dos efeitos deletérios, estas drogas psicoativas atraem os peixes.

9. Idalencio, R.; Kalichak, F.; Rosa, J.G.S.; Oliveira, T.A.; Koakoski, G.; Gusso, D.; Abreu, M.S.; Giacomini, A.C.V.; **Barcellos, H.H.A.**; Piato, A.L.; Barcellos, L.J.G. Waterborne Risperidone Decreases Stress Response in Zebrafish. Plos One, v. 10, p. e0140800, 2015.

Trabalho principal da dissertação de mestrado em Bioexperimentação de Renan Idalencio (UPF). Neste trabalho atuei auxiliando na manutenção dos peixes experimentais, na extração e mensuração do cortisol, e na análise e discussão dos resultados. Neste estudo investigamos os efeitos da exposição aguda a risperidona na resposta ao estresse e comportamental do *zebrafish*. Mostramos que a risperidona também apresenta um efeito hormético, e que a concnetração intermediária embota o eixo HHI e desencadeia um efeito tipo ansiolítico no *zebrafish*. Logo, a presença da risperidona nos ambientes aquáticos pode alterar o perfil neuroendócrino e comportamental do estresse.

10. Kalichak, F.; Idalencio, R.; Rosa, J.G.S.; Oliveira, T.A.; Koakoski, G.; Gusso, D.; Abreu, M.S.; Giacomini, A.C.V.; Barcellos, H.H.A.; Fagundes, M.; Piato, A.L.; Barcellos, L.J.G. Waterborne psychoative drugs impair the initial development of zebrafish. Environmental Toxicology and Pharmacology, v. 41, p. 89-94, 2016.

Trabalho principal da dissertação de mestrado em Farmacologia de Fabiana Kalichak (UFSM). Neste trabalho, auxiliei na avaliação dos parâmetros morfológicos e vitais das larvas, assim como na análise e discussão dos resultados e elaboração da conclusão. Mostramos que a fluoxetina, o diazepam e a risperidona afetam o desenvolvimento inicial do *zebrafish*. Todos esses fármacos aumentam a taxa de mortalidade e frequência cardíaca, e diminuem o tamanho das larvas. A risperidona e a fluoxetina tornam os ovos mais opacos, diminuindo a eclosão. Esses resultados apontam para um efeito negativo nas fases iniciais do desenvolvimento do *zebrafish*, visto que a viabilidade larval diminui, promovendo efeitos a nível populacional. Nossa hipótese é de que os ovos e as larvas absorvem os fármacos, exercendo um efeito no sistema nervoso central, tendo implicações no ambiente aquático.

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