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RESÍDUOS DE RISPERIDONA NO AMBIENTE: EFEITOS PERSISTENTES E TRANSGERACIONAIS

Santa Maria, RS
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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, RS) como requisito parcial para a obtenção de título de **Doutora em Farmacologia.**

Orientador: Prof. Dr. Leonardo José Gil Barcellos

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Aprovada em 13 de dezembro de 2018

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E aos meus alunos, que hoje são minha fonte de inspiração.

EPÍGRAFE

“'Cause we're not the same and we don't have to try
We're brighter than fireflies, we're gonna light the sky”

Grace VanderWall

RESUMO

RESÍDUOS DE RISPERIDONA NO AMBIENTE: EFEITOS PERSISTENTES E TRANSGERACIONAIS

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ORIENTADOR: PROF. DR. LEONARDO JOSÉ GIL BARCELLOS

Santa Maria, 13 de dezembro de 2018

A contaminação de ambientes aquáticos por componentes farmacêuticos é um motivo crescente de preocupação. Pouco se sabe sobre as consequências da exposição a esses agentes químicos, principalmente durante as fases iniciais do desenvolvimento. A risperidona, um antipsicótico atípico antagonista dopaminérgico e serotoninérgico, foi encontrada em baixas concentrações em diferentes ambientes aquáticos, inclusive em água potável. Utilizando o organismo experimental Zebrafish (*Danio rerio*) tivemos por objetivo simular a exposição de um organismo não alvo a diferentes concentrações de risperidona durante os 5 primeiros dias de vida. Após a exposição, os animais foram colocados em aquários livres de contaminantes até alcançar a fase adulta. Quando adultos, os peixes demonstraram diminuição da responsividade a situações de risco. Após as análises comportamentais, os peixes foram colocados para reprodução e sua prole analisada. A risperidona também alterou os padrões locomotores na prole, comprovando seus efeitos transgeracionais. Concluimos com o estudo que a risperidona pode provocar mudanças consideráveis no comportamento anti-predatório dos peixes resultando em impactos importantes na manutenção e sobrevivência das espécies e ainda que esses efeitos podem ser percebidos através de gerações não diretamente expostas.

Palavras-chave: toxicologia aquática, desenvolvimento embrionário, *Danio rerio*, atividade locomotora, comportamento.

ABSTRACT

RESIDUES OF RISPERIDONE IN THE ENVIRONMENT: PERSISTENT AND TRANSGENERATIONAL EFFECTS

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Contamination of aquatic environments by pharmaceutical components is a growing concern. Little is known about the consequences of exposure to these chemical agents, especially during the early stages of development. Risperidone, an atypical antipsychotic dopaminergic and serotonergic antagonist, was found in low concentrations in different aquatic environments, including drinking water. Using the experimental organism Zebrafish (*Danio rerio*) we aimed to simulate the exposure of a non-target organism to different concentrations of risperidone during the first 5 days of life. After exposure, the animals were placed in aquariums free of contaminants until reaching the adult stage. As adults, fish have shown decreased responsiveness to risk situations. After the behavioral analyzes, the fish were placed for breeding and their offspring analyzed. Risperidone also altered locomotor patterns in the offspring, proving their transgenerational effects. We conclude with the study that risperidone can cause considerable changes in the anti-predatory behavior of the fish resulting in important impacts on the maintenance and survival of the species and that these effects can be perceived through generations not directly exposed.

Key words: aquatic toxicology, embryonic development, *Danio rerio*, locomotor activity, behavior.

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1. INTRODUÇÃO

A utilização de fármacos já faz parte da rotina das pessoas. Fundamentais para o tratamento de doenças e buscando melhorar a qualidade de vida, os componentes farmacêuticos são muitas vezes insubstituíveis, alguns inclusive utilizados diariamente ao longo de toda vida. Por esse motivo, não é difícil compreender o achado de resíduos de fármacos no meio ambiente, visto que muitas vezes são eliminados em sua forma ativa, podendo gerar alguns impactos ao ambiente ou organismos expostos. As concentrações de fármacos são encontradas no ambiente aquático, visto que são eliminados juntamente da urina ou fezes e alcançam facilmente as estações de tratamento de esgoto.

Os níveis contatados de fármacos são descritos em µg/L ou ng/L, e por isso, compreende-se o porquê pouco se fala dos resíduos de fármacos no ambiente aquático. Porém, sabe-se que mesmo encontrados em baixos níveis, esses contaminantes podem alterar padrões comportamentais das espécies expostas, diminuir a eficiência reprodutiva ou ainda aumentar a mortalidade. Concentrações tão baixas podem não somente alterar a fisiologia de espécies não-alvo, como provocar efeitos imprevisíveis, visto que poucos estudos são realizados com esse objetivo.

Estudos que possam detectar as possíveis efeitos deletérios ocasionadas por essas exposições podem esclarecer a importância do correto descarte de fármacos, bem como, incentivar a pesquisa de formas de remoção desses componentes da água.

Frente aos resultados demonstrados por nosso grupo de pesquisa (Figura 1), percebemos que exposições agudas a fármacos e agroquímicos são capazes de bloquear a resposta ao estresse e alterar o comportamento exploratório dos animais, ou ainda a resposta a estímulos externos, como a exposição a predadores, ambiente novo e testes de claro escuro. Experimentos anteriores utilizando embriões de zebrafish demonstraram que baixas concentrações de psicofármacos podem alterar padrões simples de comportamento que podem afetar a sobrevivência dos animais.

Devido à preocupação com a exposição durante fases delicadas do desenvolvimento, selecionamos a risperidona, um antipsicótico atípico já encontrado em ambientes aquáticos, para um estudo toxicológico. O objetivo desse estudo foi

verificar as possíveis alterações provocadas por concentrações da risperidona semelhantes àquelas já encontradas no meio ambiente. Utilizando o zebrafish como modelo animal, verificamos os riscos da exposição à risperidona durante a fase embrionária, analizando o comportamento destes animais após o contato com o fármaco. Após este primeiro momento, permitimos que os animais continuassem seu desenvolvimento até a fase adulta a fim de identificar possíveis alterações comportamentais. Visando avaliar o efeito transgeracional da risperidona, submetemos os animais expostos à reprodução e verificamos o comportamento da prole.

O presente trabalho está fundamentado na apresentação dos resultados finais sob forma de um artigo publicado e um manuscrito submetido, para fins de defesa de tese de doutorado. Nesse documento serão abordadas as seguintes seções: revisão bibliográfica, objetivos, artigo publicado, manuscrito submetido, discussão, conclusão, perspectivas e referências bibliográficas.

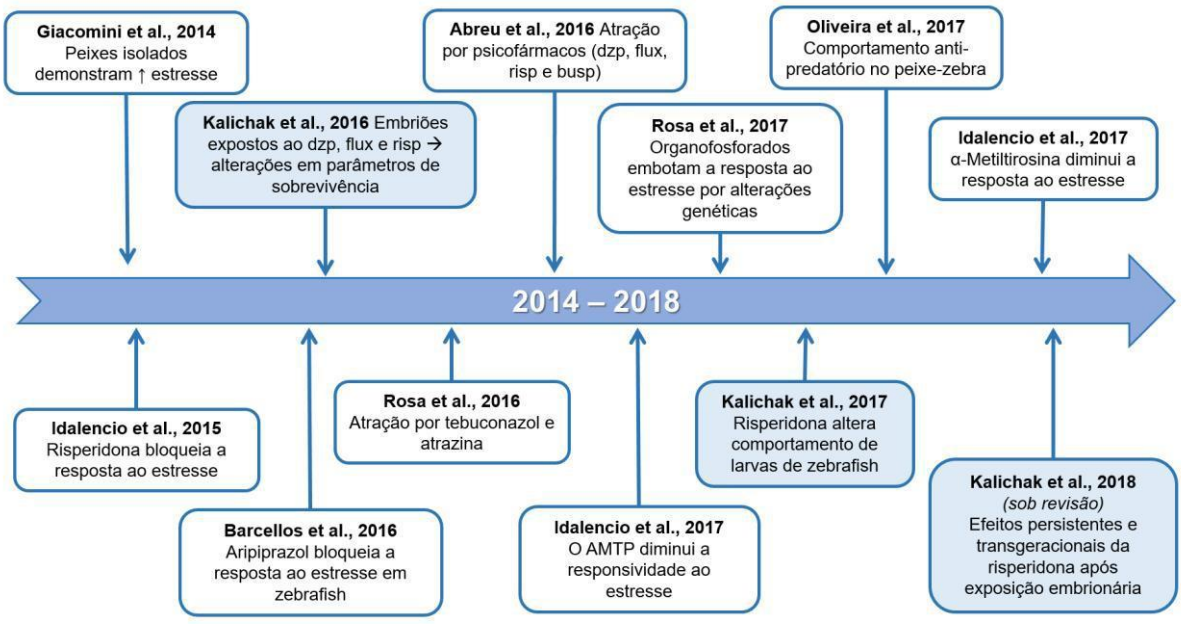


Figura 1 - Principais assuntos de artigos publicados durante o período de mestrado e doutorado, entre 2014 e 2018.
Fonte: Autora, 2018

2. REVISÃO BIBLIOGRÁFICA

2.1. Problemática ambiental

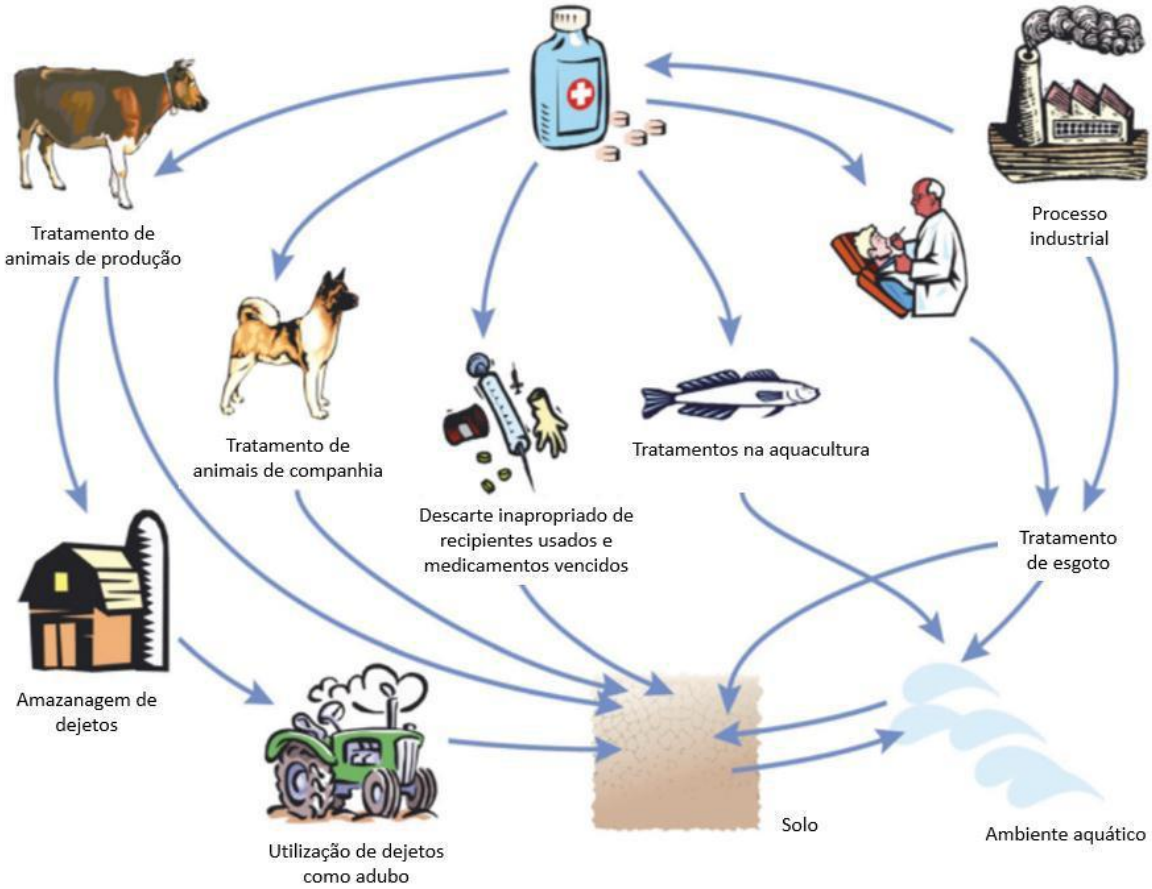
Elaborados para a manutenção da saúde, os fármacos e medicamentos vêm sendo utilizados pela população há centenas de anos. As descobertas farmacológicas desenvolvidas por fisiologistas, médicos e farmacologistas são datadas ainda antes de 1900, com a descoberta da epinefrina, atropina, prazosina e β-bloqueadores como o atenolol e a terbutalina (RUBIN, 2007), alguns deles utilizados até hoje. Com o avanço dos conhecimentos na área, novas moléculas foram registradas, aumentando a possibilidade de uso de fármacos para diferentes objetivos. O uso desses componentes compreende principalmente a melhoria da qualidade de vida, aumento da longevidade e bem-estar e ainda o controle e cura de afecções (FABBRI, 2015).

Após utilizados, os fármacos são metabolizados e excretados por vezes ainda em sua forma ativa e podem ou não sofrer degradação quando em contato com o ambiente. Somado a isso, o crescimento populacional aumenta a demanda de fármacos que de maneira ou outra, passam a contaminar o meio ambiente (BROOKS, 2018). O descarte indevido de medicamentos vencidos diretamente ao lixo ou redes de esgoto sem tratamento específico aumenta a concentração desses contaminantes emergentes nas águas residuais (PUCKOWSKI et al., 2016; FABBRI, 2015). Resíduos de indústrias farmacêuticas, utilização em pesquisas e dejetos de animais tratados também contribuem com essa problemática (SARMAH et al., 2006). As principais formas de contaminação aquática por componentes farmacêuticos são demonstradas na Figura 2.

O crescimento populacional associado ao aumento do consumo de fármacos são alguns dos fatores que levam ao acúmulo desses agentes químicos no meio ambiente (BROOKS, 2018). São poucas as evidências que abordam a conscientização sobre o descarte correto de fármacos e medicamentos, e pouca atenção é dada quando se fala de contaminação ambiental por fármacos (K'OREJE et al., 2016). Alguns países em desenvolvimento não possuem sequer uma legislação específica para o tratamento e retirada de resíduos farmacêuticos antes da liberação da água na natureza (PUCKOWSKI et al., 2016; K'OREJE et al., 2016; FABBRI, 2015).

Alguns estudos vêm sendo realizados a fim de viabilizar a retirada desses contaminantes da água. Vários processos já foram descritos, como a ozonização, oxidação, osmose reversa ou utilização de carvão ativado. Desses processos, o carvão ativado mostra a melhor viabilidade financeira, porém quando em contato com substâncias naturais, pode ocorrer competição pelos sítios de adsorção, diminuindo a sua capacidade de eliminar os contaminantes químicos (TERNES et al., 2002; GOMES et al., 2017). A dificuldade financeira inviabiliza muitos dos métodos descritos, e poucos países realizam a extração desses componentes para o fornecimento de água potável (TERNES et al., 2002; MADIKIZELA et al., 2017).

Figura 2 - Principais formas de contaminação aquática por fármacos.



Fonte: Adaptado de Boxall, 2004.

Os ecossistemas aquáticos são os maiores impactados pelos resíduos de fármacos e esse tipo de contaminação pode trazer consequências para os organismos expostos (FABBRI, 2015; LORENZI et al., 2014; BRUCE et al., 2010). Resíduos de anti-inflamatórios, antibióticos, antidepressivos e estrogênio estão entre

os mais frequentemente detectados no ambiente, devido a grande utilização desses compostos por parte da população (SARMAH et al., 2006). Os produtos farmacêuticos são facilmente transportados pela água, podendo ser levados das áreas urbanas para rurais e vice-versa (MADIKIZELA et al., 2017). Esse fato expõe espécies de organismos não-alvo, gerando consequências na maioria das vezes desconhecidas (K'OREJE et al., 2016; PUCKOWSKI et al., 2016). As concentrações de fármacos encontradas no ambiente são baixas (µg/L ou ng/L), o que permite respostas diferentes daquelas esperadas quando utilizados em doses terapêuticas (CALABRESE, 2019).

As pequenas concentrações de contaminantes podem oferecer risco a organismos não-alvo (CALABRESE, 2019) . Além disso, a presença de fármacos no ambiente aquático pode gerar impacto aos animais expostos, e também à espécie humana, que tem contato crônico a essas substâncias, consumindo diariamente água potável contaminada (WEBB et al., 2003). Esse tipo de exposição pode gerar efeitos pouco impactantes a curto prazo, mas que podem ser percebidos com pequenas alterações fisiológicas ou comportamentais, gerando impactos a longo prazo, relacionados ao comportamento ou alterações fisiológicas (CALABRESE, 2019).

Diversas pesquisas já constataram a interferência desses compostos no crescimento e desenvolvimento embrionário (KALICHAK et al., 2016; GALUS et al., 2013) alterando o comportamento de espécies expostas (LORENZI et al., 2014) e ainda causando alterações endócrinas (ABREU et al., 2015; IDALENCIO et al., 2015; MINGUEZ et al., 2015) ou mesmo genéticas (VELDHOEN et al., 2014, CHAKRAVARTHY et al., 2014).

2.2. Efeitos transgeracionais da exposição aos fármacos

São considerados efeitos transgeracionais aqueles que podem ser passados ao longo de múltiplas gerações. Os mecanismos epigenéticos são atribuidos ao sucesso ou não dessa transmissão de informações genéticas (ALYEA et al., 2013). Incluem-se nos mecanismos epigenéticos a metilação do DNA, modificações pós-traducionais de histonas e ainda RNAs não codificantes (XIN et al., 2015; ALYEA et al., 2013). São esses responsáveis pela herança multi e transgeracional de

fenótipos, e pertubações da vida precoce podem afetar a saúde das gerações subsequentes (XIN et al., 2015).

Estudos relacionados aos efeitos transgeracionais de contaminantes químicos comprovam que a exposição em fases delicadas do desenvolvimento pode alterar a devida eliminação, restabelecimento ou manutenção de marcas epigenéticas aumentando a suscetibilidade a doenças pós-natais. O desenvolvimento inicial requer tempo e ação hormonal para promover crescimento adequado dos órgãos e tecidos, e os contaminantes podem interferir nas atividades endógenas desses hormônios. Se as marcas epignéticas forem interrompidas podem resultar na transmissão de fenótipos adversos através de várias gerações. Além disso, as enzimas envolvidas no metabolismo dos fármacos e os processos de biotransformação e eliminação não estão totalmente desenvolvidas no feto ou no recém nascido, deixando esses organismos ainda mais vulneráveis aos efeitos (XIN et al., 2015).

Determinar os possíveis efeitos subletais e transgeracionais de poluentes se faz necessário para implementação do seu controle biológico. São definidos como efeitos subletais as alterações fisiológicas e ou comportamentais em indivíduos que sobrevivem á exposição a um composto tóxico. Estes efeitos sutis podem prejudicar o comportamento e condições fisiológicas fundamentais como: tempo de vida, taxa de desenvolvimento, reprodução, atividade de alimentação e muitos outros (QU et al., 2014). Os contaminantes inclusive estão relacionados ao aumento de incidência de disturbios endócrinos em humanos, incluindo complicações na gravidez e malformações, além de provocarem alterações epigenéticas nos organismos expostos (XIN et al., 2015).

Os agroquímicos estão dentre os contaminantes mais descritos, e diversos estudos comprovam que a exposição aos mesmos altera o epigenoma de diversas espécies (XIN et al., 2015; ALYEA et al., 2013; CREWS et al., 2007; DARUICH et al., 2001; SCHNUG et al., 2012). Vinclozolina, um fungicida utilizado na agricultura, pode alterar a formação e demonstração de células reprodutivas (ALYEA et al., 2013). Esses resultados inclusive provaram ser sexo-específico, onde se demonstrou que as fêmeas de peixes passaram e preferir machos não expostos (CREWS et al., 2007). Efeitos transgeracionais foram também percebidos em agroquímicos como a imidacloprida e o glifosato, provocando alterações em duas ou mais gerações após a exposição (DARUICH et al., 2001). A mistura de vários

inseticidas provocou ainda efeitos em pelo menos duas gerações subsequentes de minhocas (SCHNUG et al., 2012).

Em relação aos resíduos de fármacos, são poucos os experimentos que analisam os efeitos transgeracionais em concentrações próximas à aquelas encontradas no ambiente. Por isso, pouco se sabe sobre os verdadeiros riscos da exposição a esses contaminantes, a exposição a concentrações subletais pode provocar efeitos desconhecidos.

O etinilestradiol, utilizado com frequência na apresentação de anticoncepcionais, pode provocar em baixas concentrações alterações comportamentais na geração exposta e sua prole, diminuindo a efetividade reprodutiva em peixes (NASH et al., 2004). Além disso, os fármacos são encontrados em baixos níveis, e ainda podem ser detectados em combinações. Combinações de fármacos em níveis já detectados no ambiente podem inibir células humanas de proliferação, causando alterações morfológicas e fisiológicas nos fetos. Esses resultados demonstram um potencial risco para espécies aquáticas que estão diretamente expostas a esses contaminantes (POMATI et al., 2006).

2.3. Efeitos comportamentais da exposição aos fármacos

O comportamento é por definição, a ligação entre os processos fisiológicos internos do organismo e a interação do animal com o ambiente. Por isso, alterações comportamentais têm potencial de impacto direto na boa condição física e perpetuação de uma espécie (ORGER e POLAVIEJA, 2017; CLIFT et al., 2014).

Um dos comportamentos mais conhecidos na natureza é o de presa-predador. Com o objetivo de fugir de predadores e evitar potenciais riscos de vida, o comportamento de fuga é fundamental para a manutenção de uma espécie. Emoções como o medo ou a ansiedade podem ser observadas em todos os vertebrados e evoluem há centenas de anos. Considerando a importância para a manutenção e sobrevivência das espécies, a preservação desses padrões de fuga é observados na maioria dos animais e mudanças ou alterações podem colocar em risco direto os indivíduos envolvidos ou ainda em casos severos a extinção da espécie (STEWART et al., 2013; COLWILL e CRETON, 2011).

Os mecanismos anti-predatórios reduzem a probabilidade de predação e podem ser caracterizados com algumas adaptações comportamentais (BRODIE et

al., 1991). Cores contrastantes da presa podem significar uma fácil localização pelo predador, por isso a intensidade da luz pode interferir na resposta predatória. Ambientes claros para animais contrastantes podem deixar o animal mais exposto (CERRI et al., 1983) e neste caso, a busca por refúgios pode favorecer a presa (BRODIE et al., 1991). Os horários escolhidos para se alimentar também são influenciados pelos hábitos do predador, e períodos noturnos são sempre desfavoráveis para animais contrastantes, visto que a visibilidade deve ser diminuída frente ao predador. Os animais desenvolvem adaptações comportamentais para reduzir os riscos de predação. Podem mudar de habitat a fim de diminuir a probabilidade de encontrar predadores, reduzir comportamentos que podem deixá-los mais expostos, ou passar mais tempo vigilantes (LIMA e DILL, 1990; BRODIE et al., 1991; GOPKO et al., 2017).

A formação de cardume também faz parte dos mecanismos anti-predatórios (CERRI, 1983). Quando agrupados, os peixes conseguem identificar um predador com antecedência ou ainda, inibir o comportamento predatório, confundindo o predador (GODIN e MORGAN, 1985). A identificação do predador em peixes é principalmente visual, ou olfatória, e por isso, a visualização do predador pode interferir no comportamento de um dos animais, que é imediatamente imitado por outros do mesmo cardume (CERRI , 1983; DIXSON et al., 2010).

O comportamento isolado nem sempre se assemelha àquele quando em cardume. Alguns ciprinídeos após perceberem a presença do predador reduzem a velocidade de movimentação e aumentam os períodos de imobilidade, diminuindo a probabilidade de detecção do predador (BRODIE, 1991). Nem todo encontro com predador pode ser fatal e por isso a avaliação de risco é também descrita como anti-predatória. Após perceber que a situação não oferece risco, o animal pode voltar a um comportamento muito próximo do normal (LIMA e DILL, 1990).

As relações presa-predador são dinâmicas e dependem da aprendizagem e adaptação, assim como os mecanismos de prevenção das presas aos predadores (BRODIE, 1991). O resultado dessa interação pode gerar restrições evolutivas, e alterações no comportamento antipredatório podem resultar em impactos ambientais de difícil precisão.

Efeitos comportamentais resultantes da exposição a contaminantes aquáticos já foram descritos em várias espécies de peixes (BARCELLOS et al., 2016; BRODIN et al., 2013; COLWILL e CRETON, 2011; AGUILAR et al., 1997) e ratos

(BARDGETT et al., 2013). A exposição embrionária parece também estar relacionada a alterações comportamentais que podem continuar sendo observadas até a fase adulta, mesmo após a interrupção da exposição (BARDGETT et al., 2013).

2.4. Risperidona

Dentre as categorias de fármacos encontrados no ambiente aquático também são relatados os antipsicóticos (K'OREJE et al., 2016; SNYDER, 2008). O aumento de transtornos psiquiátricos e por consequência a utilização dessa classe de fármacos favorece o acúmulo de resíduos desses componentes no meio ambiente (CALISTO e ESTEVES, 2009).

A risperidona, um antipsicótico atípico utilizado para o tratamento de transtornos psiquiátricos, foi descoberta em 1984 pelo laboratório Janssen Farmacêutica e aprovada para comercialização em 1993. É utilizada principalmente para o tratamento do transtorno do espectro autista e sintomas da esquizofrenia (alucinações, delírios, abstinência emocional, pobreza de discurso) (PRIETO et al., 2014; MANNENS et al., 1994). A preferência pela utilização de antipsicóticos atípicos se deve principalmente pela menor incidência de efeitos colaterais em comparação aos antipsicóticos típicos, como sintomas extrapiramidais, hiperprolactinemia e síndrome neuroléptica maligna (HORACEK et al., 2006).

Pela sua afinidade principalmente por receptores serotoninérgicos do tipo 5-HT_2 e dopaminérgicos D_2, a risperidona é classificada como antipsicótico atípico antagonista serotonina-dopamina (HORACEK et al., 2006). É excretada principalmente pela urina, sendo de 35-45% como risperidona ou como seu principal metabólito ativo 9-hidróxi-risperidona (CORENA-MCLEOD, 2015).

Diferentes níveis de risperidona já foram relatados em ambientes aquáticos. Registros de até 0,0014 µg/L na água do mar (VIDAL-DORSCH et al., 2012), 0,0029 µg/Lem água de efluentes (BRUCE et al., 2010) ou ainda 0,00034 µg/L na água potável (SNYDER, 2008). Mesmo em concentrações baixas, estes resíduos são indicativos que o tratamento da água não é suficiente para a retirada desses componentes farmacêuticos (BRUCE et al., 2010). Poucos estudos sobre as consequências da presença da risperidona na água e seus efeitos sob espécies de

organismos não-alvo foram realizados até o momento (KALICHAK et al., 2017; IDALENCIO et al., 2015).

2.5. O zebrafish (*Danio rerio*)

O zebrafish, demonstrado na Figura 3, apresenta muitas vantagens como modelo de estudo para ensaios toxicológicos. Caracterizado por ser um organismo de alta prolificidade, rápido crescimento e alcance da faixa adulta (em aproximadamente 3 meses já alcança sua maturidade sexual) e baixo custo de manutenção quando comparado a roedores, o uso da espécie é amplamente aceito pela comunidade científica (AVDESH et al., 2012; RICHENDRFER et al., 2012; EGAN et al., 2009).

O zebrafish pode fazer desovas todos os dias, e uma única fêmea pode depositar até 300 ovos em um único dia. De origem asiática, esses peixes têm preferência pela aclimatação em águas rasas e com terreno arenoso. Possui ovos lisos e transparentes, o que possibilita a avaliação do crescimento desde as primeiras horas após a fertilização (NASIADKA e CLARK, 2012; IRONS, 2011; MACPHAIL et al., 2009; DAHM e GEISLER, 2006).

Figura 3 – Exemplares adultos de zebrafish (*Danio rerio).* (A) Fêmea (B) Macho

Fonte: Kalichak, 2017.

A utilização de embriões e larvas do zebrafish vem ganhando espaço em testes da neurociência comportamental. Em 5 dias após fertilização (dpf), o zebrafish demonstra completa organogênese. Em apenas 7dpf, pode apresentar um tamanho médio de 5mm, demonstrando um amplo leque de comportamentos típicos da espécie, que estão associados ao medo e ansiedade, comportamento social, aprendizado e memória (RICHENDRFER et al., 2012; DAHM e GEISLER, 2006) possibilitando a utilização de ensaios comportamentais que podem ser gravados em vídeo (AHMAD e RICHARDSON, 2013; MACPHAIL et al., 2009).

Com um leque comportamental abrangente, as larvas de zebrafish já são responsivas ao ambiente em 1 dia após fertilização (dpf) e em uma semana de vida já respondem a estímulos como toque, som, movimento da água ou alterações da luz (STEWART et al., 2013; CLIFT et al., 2014). Ao contrário dos adultos, as larvas de zebrafish preferem áreas claras (acredita-se que as áreas escuras simulem a sombra de um predador), evitam áreas de oscilação luminosa e reconhecem quando colocadas em áreas abertas (ORGER e POLAVIEJA, 2017; CLIFT et al., 2014; COLWILL e CRETON, 2011). Estados emocionais como medo e ansiedade são possíveis de serem observados nestes animais. Mesmo durante a primeira semana

de vida as larvas de zebrafish já podem elevar os níveis de cortisol em resposta ao estresse e são sensíveis aos mesmos ansiolíticos usados para ansiedade em humanos (RICHENDRFER et al., 2012; COLWILL e CRETON, 2011).

Pesquisas utilizando esse modelo animal têm um papel de destaque pela sua importância na ciência farmacêutica, toxicológica e genética. O zebrafish possui todos os neurotransmissores clássicos relatados nos vertebrados e apresenta ampla homologia genética com o ser humano, e alterações encontradas neste organismo podem ser de forma ou outra, extrapoladas para outras espécies (SANTOS-FANDILA et al., 2015; CHAKRAVARTHY et al., 2014; NASIADKA e CLARK, 2012; EGAN et al., 2009). Estudos transgeracionais com esse modelo podem avaliar diferentes gerações, necessitando de pouco espaço físico e custo baixo (BAKER et al., 2014). Além disso pesquisas relacionadas à nutrição, crescimento e estresse podem apresentar resultados aplicáveis na aquicultura (DAHM e GEISLER, 2006).

3. OBJETIVOS

3.1. Objetivo Geral

Avaliar se a exposição à risperidona provoca alterações comportamentais persistentes em zebrafish expostos durante as fases de desenvolvimento inicial, e se os mesmos efeitos podem ser percebidos na geração subsequente.

3.2. Objetivos específicos

- Avaliar se a exposição à risperidona afeta a taxa de mortalidade e altera o período de eclosão de embriões.
- Avaliar a frequência cardíaca e número de movimentos espôntaneos realizados pelos embriões durante a exposição à risperidona.
- Analisar as mudanças no comportamento exploratório de larvas de zebrafish utilizando o teste de tanque novo após a exposição à risperidona.
- Avaliar a capacidade de reconhecimento de áreas de risco utilizando o teste de área de estímulo, comparando aos grupos expostos à risperidona.
- Analisar crescimento e desenvolvimento dos animais expostos a risperidona durante a fase embrionária.
- Detectar alterações comportamentais em adultos expostos a risperidona durante a fase embrionária.
- Avaliar a capacidade anti-predatória de animais adultos expostos durante a fase embrionária.
- Avaliar efeitos sob a taxa de mortalidade e eclosão da prole de animais expostos a risperidona.
- Avaliar mudanças no comportamento exploratório de prole dos animais expostos a risperidona.

4. ARTIGO 1 - PSYCHOTROPIC IN THE ENVIRONMENT: RISPERIDONE RESIDUES AFFECTS THE BEHAVIOR OF FISH LARVAE

O artigo intitulado *Psychotropic in the environment: Risperidone residues affects the behavior of fish larvae* foi aceito para publicação na revista Scientific Reports no dia 4 de outubro de 2017, ISSN 2045-2322 (Qualis A1 nas Ciências Biológicas II e fator de impacto de 4,5). O artigo foi anexado no formato publicado pela revista.

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Psychotropic in the environment: risperidone residues affect the behavior of fish larvae

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The ability to avoid and escape from predators are clearly relevant behaviors from the ecological perspective and directly interfere with the survival of organisms. Detected in the aquatic environment, risperidone can alter the behavior of exposed species. Considering the risk of exposure in the early stages of life, we exposed zebrafish embryos to risperidone during the first 5 days of life. Risperidone caused hyperactivity in exposed larvae, which in an environmental context, the animals may be more vulnerable to predation due to greater visibility or less perception of risk areas.

Emotional states such as fear and anxiety are possible to be observed in zebrafish larvae. With a comprehensive behavioral range, these animals are already responsive to the environment at 24 hpf (hours post-fertilization) and within a week of life, they already respond to stimuli such as touch, sound, water movement or changes in light[1,2]. Unlike adults, zebrafish larvae prefer clear areas (dark areas are thought to simulate predator shade), avoid areas of light oscillation and recognize when placed in open areas[2–4]. Even during the first week of life, zebrafish larvae may already are sensitive to the same anxiolytics used for anxiety in humans[3,5].

Behavioral changes have the potential to impact directly on the physical condition and the perpetuation of a species[2,4]. One of the most known behaviors in nature is the prey-predator relationship. To avoid predators and consequently potential life risks, the escape behavior is fundamental to the species maintenance. Emotions such as fear or anxiety can be observed in all vertebrates and are very important to maintainance and survival of the species, and the preservation of these escape patterns is observed in most fish species. Changes or alterations in this response may induce a direct risk to the individuals or even in severe populational consequences[1,3].

In this context, aquatic contaminants are involved in many behavioral changes of exposed species. Several drugs consumed by the population promotes an increase in the amount of drug residues in the aquatic environment[6–9]. Even when detected in low amounts, the ability to cause changes in the physiology of non-target organisms has not yet been fully elucidated, but it is known that even at low concentrations (ng/L or μg/L) these drugs may have effects on exposed species[6,8–10].

Risperidone (RISP), an atypical antipsychotic used mainly for the treatment of schizophrenia and bipolar mood disorder, has already been detected at different levels in aquatic environments[11,12]. RISP levels have already been recorded up to 0.0014 μg/L in seawater[8], 0.0029 μg/L in effluent water[7], and 0.0034 μg/L in drinking water[13]. In Belgium, the highest level of environmental contamination was recorded, presenting 0.364 μg/L in affluents and 0.154 μg/L in Dendre River effluent[14]. A few studies have been carried out to find out the consequences of species exposure and this type of contaminant at low concentrations[15,16]. Despite behavioral changes are common findings in mammals exposed to risperidone during embryonic development[17], no reports about behavioral changes in the RISP-exposed fish were found in the current literature. Thus, in view of the great importance of

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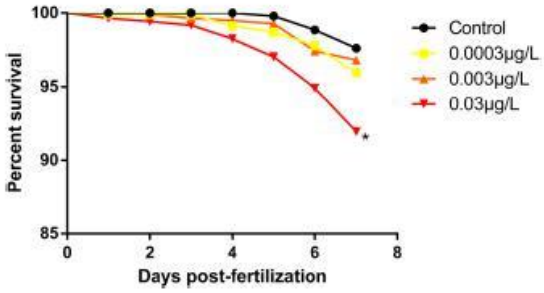

Figure 1. The larvae exposed to the highest concentration of risperidone tested showed increased mortality. Survival curve evaluated by Kaplan-Meier method. *$p < 0.05$. N = 160.

preserving all the behavioral repertoire, we sought to identify the effects of RISP exposition on behavioral parameters in embryos and larvae zebrafish.

Results

Fish exposed to the concentration of 0.03 µg/L showed an increase in mortality when compared to the control group (Fig. 1) ($p < 0.0001$). No differences were found in hatching and heart rate.

During the open field test, RISP at a concentration of 0.003 µg/L increased the total distance traveled ($p = 0.0009$), the number of entries in the central area ($p = 0.0127$) and the mean speed in the final phase of the test (6–12 min) ($p = 0.0009$). The concentration of 0.0003 µg/L increased the immobility time in the settling phase ($p = 0.0542$) (0–6 min), but this result did not last during the final test phase (6–12 min) ($p = 0.6093$) (Fig. 2).

Larvae exposed to the concentration of 0.003 µg/L RISP decreased the response to the aversive stimulus and remained mostly in the stimulus area when compared to the control group ($p = 0.0011$). The other groups did not present alterations (Fig. 3).

At the concentration of 0.003 µg/L larvae decreased the latency for entry into the dark side of the well ($p = 0.0229$), as well as increasing the number of entrances and residence time on this side during the second phase of the test (6–12 min) ($p = 0.1532$). In the initial phase of the test, there was no difference between groups (Fig. 4). There was no difference between the groups tested in relation to spontaneous movement.

Discussion

Here we show that the presence of RISP residues in water can alter the exploratory behavior of zebrafish embryos and larvae. In fact, during the first-time window (0–6 min) in the open field test, aversive stimuli and light/dark tests, larvae exposed to 0.0003 µg/L RISP increased the immobility time. The observed effects of extremely low RISP concentration were surprising since that this concentration was already detected in natural aquatic environments[13], indicating the potential risk for populations exposed to this type of contaminant[10]. Moreover, larvae exposed to higher concentration of RISP displayed an increased mortality (5.68% in relation to the control group) and no significant alteration in hatching. These results are consistent with our previous study, where RISP affects parameters such as survival, hatching, heart rate and total larval length[16].

In the 2nd time window (6–12 min) of the exploration tests, larvae presented increased exploratory activity and decreased response to the environmental stimuli, both indicating hyperactivity. The difference between 1st and 2nd-time window contradicts the actual knowledge that larvae do not present an adaptation period as seen in adults[18]. Further studies should be carried out to validate this hypothesis.

RISP is an atypical antipsychotic antagonist of serotonin and dopamine receptors[19]. Changes in the exploratory activity caused by RISP are common findings in adult rats[17] and adult[20] as well as larvae[21] zebrafish. These changes in exploratory behavior are expected since that activity of dopaminergic and serotonergic neurons is related to motor coordination[22,23].

The effects observed in our larvae appear to be associated with RISP exposure in early stages of development. In fact, the exposure to antipsychotics during the embryonic stage is associated with reduced levels of neurotransmitters leading to functional problems in exposed fish[24] and mammals[17,25,26]. In addition, our larvae were exposed for 5 days and evaluated on the 6th day. This 24h-period without exposure may be related to these effects since the chronic administration of psychoactive drugs also appears to lead to an increase in the activity of dopaminergic and serotonergic receptors shortly after the drug withdrawal[25–31].

In our study, the effects were mainly observed in the intermediary RISP concentration, but not in the higher. In fact, our tested concentrations were lower than plasma levels suitable to cause therapeutic effects[32] or concentrations commonly tested in scientific experiments[17,24,30,31] but exerted effects on zebrafish larvae, like those described with higher concentration. Thus, any comparison between our results and those reported in the literature is difficult, since no reports were found using RISP concentration as low as we used in the present study.

The last comment is about the possible implications of our results. In fish, dopamine and serotonin are involved in locomotion, attack/defense, learning/memory and eating behavior[33]. The ability to capture prey and escape from predators are clearly relevant behaviors from the ecological perspective, as they directly interfere with the growth and survival of organisms[33,34]. A prey may be more susceptible to predation as a result of non-detection of predators, poor escape performance, reduced resistance, inability to learn and greater visibility

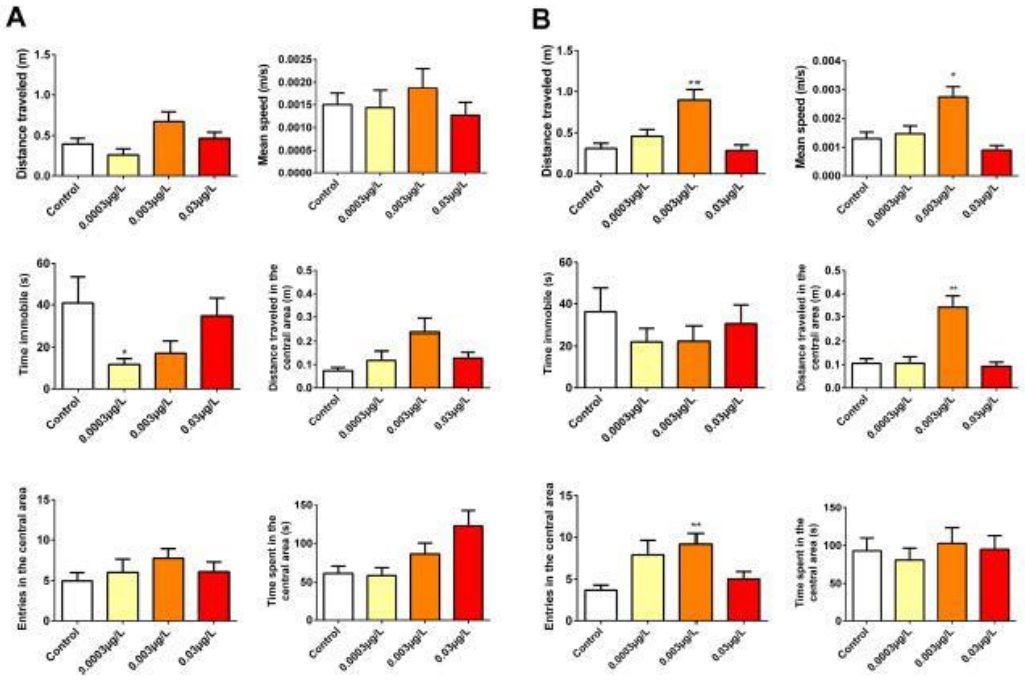

Figure 2. Open field test results. (**A**) The first phase (0–6 min). The concentration of 0.0003 µg/L RISP increased the immobility time of the exposed larvae. (**B**) The second phase (6–12 min). Hyperactivity can be observed by an increase in the distance, average speed and a number of entries in the central area. Means were compared by One-way ANOVA followed by Dunnett's or Kruskal-Wallis test followed by Dunn's were used depending on

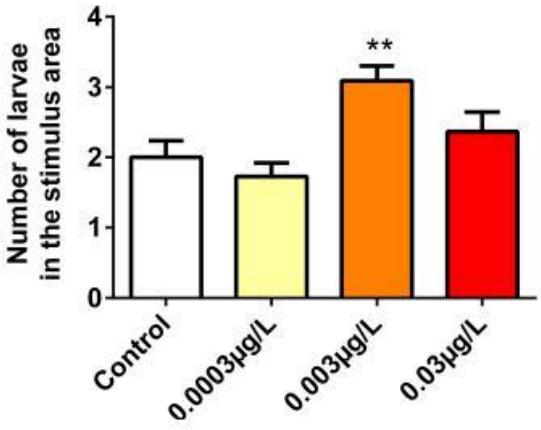

Figure 3. The concentration of 0.003 µg/L risperidone increased the number of animals that remained in the

due to hyperactivity[33,35]. Our results highlight that RISP altered larvae activity patterns, which in an environmental context can directly influence the ability to avoid or evade predatory behavior which may result in significant repercussions on the maintenance of the species as well on the ecosystem.

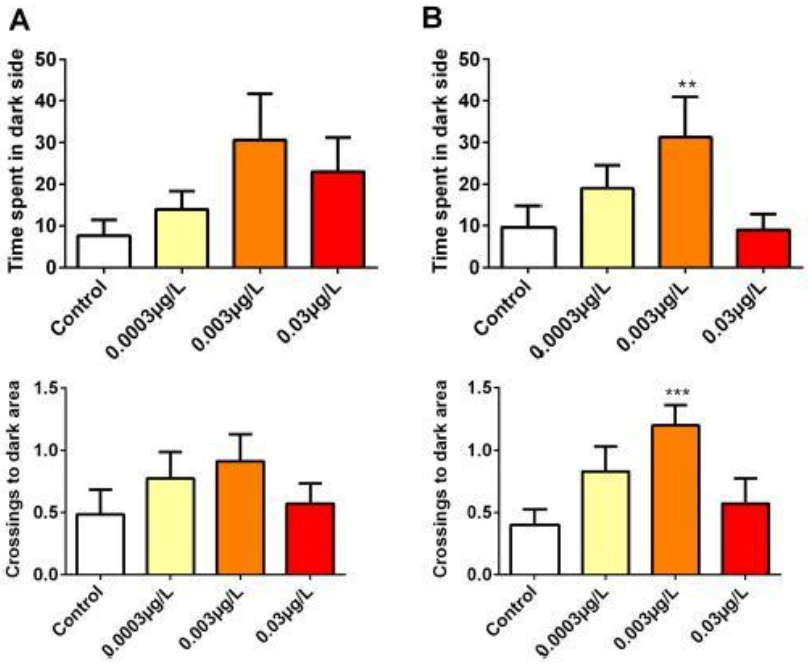

Figure 4. During the LDT, larvae exposed to the intermediate concentration of risperidone increased the time on the dark side and the number of crosses of the center line. Means were compared by One-way ANOVA followed by Dunnett's or Kruskal-Wallis test followed by Dunn's were used depending on data normality. *$p < 0.05$. $N = 35$.

Materials and Methods

Ethical Aspects. This study was approved by the Animal Use Ethics Committee (CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #9/2015) and complied with the guidelines of the National Council for Animal Experimentation Control (CONCEA). Approximately 600 larvae were tested during this experiment.

Study strategy. We exposed embryos and larvae of zebrafish (*Danio rerio*, wild-type) to different concentrations of RISP already detected in the aquatic environment analyzing eventual changes in the larvae behavioral repertoire. This analysis was based on the different behavioral tests as follows: spontaneous movement, open field, light/dark as well as aversive stimulus. We opted for a 5-day chronic exposure to RISP since this period window correspond to the whole period of zebrafish organogenesis (2hpf to 120hpf)[36].

Reproduction and maintenance of embryo. For breeding, healthy zebrafish wild-type, aged between 3 and 18 months were used. The animals were placed in barred bottom aquaria in ratios of 1:1 (males: females). After 12 hours of dark, during the morning the lights were on and after 1 hour the embryos were collected[36,37]. The methods of reproduction and maintenance of embryos are described in the previous work[16].

After collection, the embryos were washed and classified as fertilized and unfertilized with the aid of light microscopy[37,38]. Embryos were maintained in E3 medium (reverse osmosis water + 60 mg/L Marine Ocean Instant Ocean) and distributed in 24 well cell culture plates (3 ml/well), 10 embryos per well and incubated in a water bath at 28 °C[39]. For the tests, embryos of up to 3hpf were accepted. The embryos were exposed to RISP from 3hpf to 120hpf.

Concentrations tested. The RISP concentrations were based on those already registered in the aquatic environment: 0.00034 µg/L[13], 0.003 µg/L, and 0.03 µg/L. These concentrations were previously tested and changed survival, hatching, heart rate and total larval length[16]. The solutions were prepared and stored in amber glass bottles, where they remained heated in a water bath to be replenished in the wells when necessary.

Survival and hatching analysis. For analysis of survival and hatching, we have monitored all animals once a day in the morning for 7 days with the aid of a magnifying glass or optical microscopy. Embryos and larvae that do not show transparency, coagulated or without cell formation, cardiac movement or blood circulation were considered dead. Animals were considered "hatched" when partially or completely outside of the chorion. For this hatching and survival measurements, we analyzed 160 embryos by concentration (control and the three concentrations tested), totalizing 640 embryos

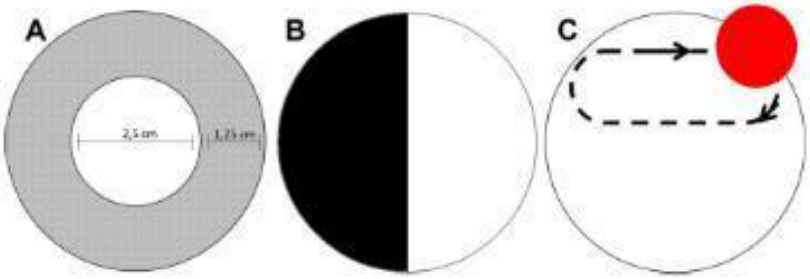

Figure 5. A schematic illustration of the experimental setup used to record zebrafish larvae behavior. (**A**) Open field test - Central and peripheral region established in the software ANY-Maze (**B**) Light/dark test (**C**) Aversive stimulus was produced with a red bouncing-ball.

Spontaneous movement. In 24 hpf the embryos present spontaneous movements of the tail still inside the chorion. They are thus considered because they are induced by the development of the motoneurons without any control by the central nervous system[37,40,41]. These movements were recorded in 1 minute[40] in 64 embryos by group (total of 256 embryos).

Heart rate. Heart rate was assessed at 48hpf in all groups during the morning. The heart rate was manually counted by light microscopy for 1 minute[42] in 48 embryos by group (total of 192 embryos).

Open field test. To perform the open field test, the larvae at 6 dpf were placed in 10 mL wells containing only E3 medium and filmed (Canon EOS Rebel T5 Macro Lens EF 100mm) for 12 minutes. Similar to the behavior seen in mammals, zebrafish larvae also present thigmotaxis and recognize when placed in a new environment[3,4,43].

For thigmotaxic behavior analysis, we filmed 30 larvae by group (total of 120 larvae). In the videos, the well was virtually divided into a central and peripheral area (Fig. 5A)[18] and the period were divided into two phases: adaptation period (0–6 min) and the exploratory period (6–12 min). ANY-maze software was used to analyze the following parameters: total distance travelled, time in the central area, distance travelled in the central area, entries in the central area, immobility time and mean speed.

Light/dark test (LDT). For this test, a 6-well cell culture plate was used with one well (5 ml) divided into a dark (black) area and a clear (white) area. Unlike adults, zebrafish larvae prefer to stay in the white area of the well (Fig. 5B). It is believed that the dark area represents the shadow of a possible predator[42,44].

Thirty-five larvae by group (total of 140 larvae) were placed in the test area and filmed for 6 minutes. The latency for entry into the dark side, the number of entries and dwell time on the dark side were evaluated using the ANY-maze software. As in the open field test, the LDT was divided between the initial phase (0–6 min) and final phase (6–12 min).

Aversive stimulus test (AST). Aversive stimulus aims to test the cognitive ability of the larva to identify areas of danger. Tests with colorations have been used for their ecological relevance, since different species of fish, like zebrafish, use colors to differentiate possible foods, recognize specifics as well as avoid predators[38].

For this test, the larvae were placed in 6-well cell culture plates (5 larvae per well, $n = 55$ by group totalizing 220 larvae) above an LCD monitor. After the adaptation period (2 min), using PowerPoint software (Microsoft Office Professional Plus 2013), we started the exposure to a visual stimulus area with a red sphere of 1.35 cm in diameter with a trajectory that traveled only half the well (Fig. 5C). The animals were stimulated for 5 min and at the end of the test were recorded the number of animals that remained in the stimulus area and those that remained in the non-stimulated area[45].

Statistical analysis. For statistical analysis and graphing we used GraphPad Prism software version 6.01 for Windows. Survival and hatchability data were evaluated by Kaplan-Meier method. For the analysis of the heart rate data, spontaneous movement, open field test, LDT and AST, One-way ANOVA followed by Dunnett's (a group of parametric data) or Kruskal-Wallis test followed by Dunn's were used (a group of non-parametric data). All groups were compared to the control group. Statistical significance was accepted when $p \leq 0.05$.

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Author Contributions

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

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5. ARTIGO 2 - PERSISTENT AND TRANGENERATIONAL EFFECTS OF RISPERIDONE IN ZEBRAFISH

O artigo intitulado *Persistent and trangeneration effects of risperidone in zebrafish* foi submetido para a revista Environmental Science and Pollution Research no dia 9 de novembro de 2018, ISSN 1614-7499 (Qualis B1 nas Ciências Biológicas II e fator de impacto de 2,8). O artigo foi anexado no formato exigido pela revista.

PERSISTENT AND TRANGENERATIONAL EFFECTS OF RISPERIDONE IN ZEBRAFISH

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Abstract

Here we show that zebrafish exposed to risperidone (RISP) in the embryonic and larval stages presented impaired antipredatory behavior during adulthood, characterizing a persistent effect. We also show that some of these behavioral changes are present in the following generation, characterizing a transgenerational effect. This suggests that even short and sub-lethal exposures at essential stages of development can affect the whole life of the zebrafish, including its offspring. Our results highlight the risks and long-term environmental consequences associated with drug residues in water, affecting aquatic life and endangering species that depend on appropriate behavioral responses for survival.

Key words: *Danio rerio*, persistent effects, transgenerational toxicology, prey-predator relationship, behavior

1. Introduction

Behavior is, by definition, the connection between the internal physiological processes of an animal and its interaction with the environment (Orger and Polavieja 2017; Clift et al. 2014). Thus, a complete behavioral repertoire is crucial for fish survival and fitness, at both the individual and population level (Stewart et al. 2013).

One of the best-known behaviors in nature is the prey-predator relationship. Escape behavior is fundamental to the maintenance of a species (Kelley and Magurran 2003), helping to avoid predators and potential risks to life. It can be observed in most animals, like rodents (Mondin et al. 2015; Vasilieva et al. 2000), spiders (Bell et al. 2006), and fish (Kelley and Magurran 2003), including the zebrafish (Stewart et al. 2013). Impairments in these behaviors can put individual fish at direct risk or, in severe cases, result in the extinction of a species (Stewart et al. 2013; Colwill and Creton 2011).

Reports of pharmaceutical residues in the environment and their effects on the behavior of exposed species are common. Aquatic ecosystems are affected the most by drug residues, with potential consequences for exposed organisms (Fabbri 2015; Lorenzi, Choe, and Schlenk 2014; Bruce, Pleus, and Snyder 2010). There are several reports on the impact of these compounds on embryonic growth and development (Kalichak et al. 2016; Kalichak et al. 2017; Galus et al. 2013), including altered behavior in exposed species (Lorenzi et al. 2014), as well as endocrine

(Abreu et al. 2015; Idalencio et al. 2015), hormonal (Minguez et al. 2015), or genetic alterations (Veldhoen et al. 2014; Chakravarthy et al. 2014).

Antipsychotics are among the categories of drugs to have been detected in the aquatic environment (K'Oreje et al. 2016; Snyder 2008), including risperidone, an atypical antipsychotic used to treat psychiatric disorders. Levels of up to 0.0014 µg/L risperidone have been reported in sea water (Vidal-Dorsch et al. 2012), 0.0029 µg/L in effluent water (Bruce et al. 2010), and 0.00034 µg/L in drinking water (Snyder 2008).

Risperidone exposure has been shown to be an unsafe for fish, even at low concentrations (Kalichak et al. 2016; Kalichak et al. 2017; Idalencio et al. 2015). In rats, risperidone elicited behavioral changes related to hyperactivity, even when exposed for a short period during embryogenesis (Bardgett et al. 2013). In addition to the direct consequences for exposed animals, drugs and other aquatic contaminants can have persistent effects on future generations (Mccarthy et al. 2018). These effects are not always directly related to the survival of the individual animal, but may also be related to morphology, reproductive inefficiency, and changes in behavior or feeding patterns (Bhandari and Tillitt 2015; Bruner-Tran and Osteen 2011), with consequences that are difficult to measure.

Contamination of aquatic environments with drugs or agrochemicals can affect non-target species like fish. Exposure of fish embryos and larvae may result in chronic metabolic changes that may persist into adulthood, effects that have already been reported for fish endocrinology (Gao et al. 2018; Koakoski et al. 2014) and behavior (Wilson et al. 2016). Although there are some reports related to transgenerational toxicology (Manikkam et al. 2012), little is known of the long-term consequences of exposure to pharmaceutical compounds such as risperidone.

We have previously reported on behavioral changes in zebrafish elicited by exposure to risperidone residues in the larval stage (Kalichak et al. 2017). Given the persistent and transgenerational potential of these changes, here we describe the effects of different concentrations of risperidone on the behavior of adult zebrafish exposed at the embryonic and larval stages, and subsequently in their offspring.

2. Materials and Methods

2.1. Ethical and Legal Note

This study was approved by the Animal Use Ethics Committee (CEUA) of the University of Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol 009.2015 – CEUA) and complied with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA). This research was also registered in the SisGen (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) and complied with their guidelines (registration code: A14E252).

2.2. Study strategy

To evaluate the effects of early exposure to risperidone on adult and larval behavior, we exposed zebrafish (*Danio rerio*) embryos and larvae to different concentrations of risperidone (RISP) already detected in the aquatic environment. We then submitted early-exposed adults (F0 generation) to the following behavioral tests: novel tank test (NTT), social preference test (SPT), prey-predator test (PPT), and dark-light test (DLT). In all behavioral tests, to avoid interference by human activity, the operator exited the experimental room after the fish had been released into the test apparatus.

After breeding the F0 adults, we evaluated F1 embryos and larvae for mortality and hatching rate, spontaneous movement, heart rate, and behavior in the novel tank test (NTT) (see the schematic representation in Figure 1). Approximately 680 F1 embryos and larvae were used.

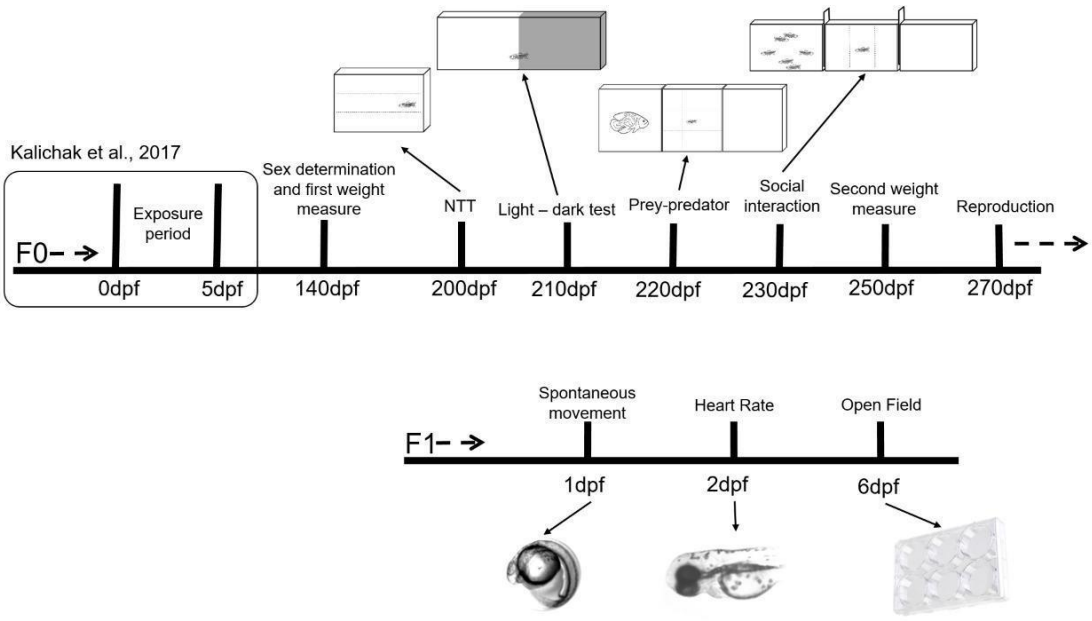


Figure 1. Schematic view of the experimental design. Fish were draw by FK.

2.3. Exposure time and RISP concentrations

We exposed zebrafish embryos to three different RISP concentrations: 0.03, 0.003, and 0.0003 µg/L, during their first 5 d of life, corresponding to the zebrafish embryonic and early larval stages. We then transferred the larvae to clean water (without RISP) for the developing/growing period. A control group had the same handling and care, but were not exposed to the drug.

We used Risperidon™ (1 mg/mL, EMS Laboratory, Hortolândia, SP), the commercial formulation of risperidone, diluted in egg water (reverse osmosis water reconstituted with sea salt; Red Sea Salt, Red Sea™) (WESTERFIELD M. 2000).

2.4. Reproduction and embryo maintenance

We obtained fertilized eggs by natural mating of adult zebrafish. The reproduction and embryo exposure procedures have been described previously (Kalichak et al. 2017).

During the first 20 d, we fed the embryos Sera Micron feed (moisture 5.5%, raw protein 55%, fat extract 7%, and crude fiber 6.4%, Sera™, Heinsberg, Germany). Subsequently, juveniles were fed live brine shrimp nauplii (*Artemia salina*) and Alcon

Basic® and Basic Tabs (moisture 10%, raw protein 45%, fat extract 5%; Alcon™, Camboriú, Brazil) to satiation.

The fish were kept in a recirculation system for the growth period. Temperature, pH, hardness, alkalinity, and oxygen parameters were according to ZFIN Book (WESTERFIELD M. 2000).

At 170 d of age, a batch of fish were weighed and sexed. These same fish were not used in the behavioral tests performed on 250-d-old zebrafish, when a second batch was weighed.

2.5. Behavioral testing

2.5.1. Novel tank test (NTT)

For the NTT, we transferred fish individually to a glass aquarium (24 × 15 × 6 cm; w × d × h) containing recirculation system water to avoid drastic changes in water pH or temperature.

After 1 min of acclimatization, fish were filmed using a web camera (Logitech™ C920) located frontally to the test aquaria. We tested 15 fish per group, with a total of 60 fish. For video analyses, the aquarium was virtually divided into top, middle, and bottom zones. We scored the following behavioral parameters: total distance traveled (m), number of crossings between zones (considering the full body), time spent in the top and bottom zones (s), and transitions to the top zone.

The NTT analysis was divided into three stages: initial (0–2 min), intermediate (2–4 min), and final (4–6 min). These time points were justified due to natural changes that occur in zebrafish behavior over time (Blaser and Gerlai 2006), and allowed for more accurate data analysis. When we placed the zebrafish in a new environment, they initially spent longer at the bottom and, gradually, began to explore the top zone. This behavior is associated with risk analysis, allowing the fish to avoid potentially dangerous areas, such as the top of the tank, as a defensive behavior against predators (Kysil et al. 2017).

2.5.2. Social preference test (SPT)

For the SPT, which consists of observing the fish preference for remaining close to their conspecifics, 48 fish were tested (n = 12 per group). Zebrafish is a

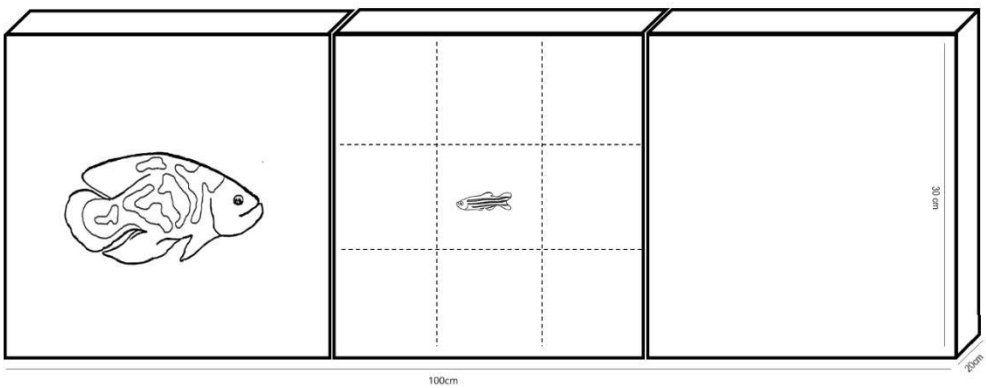
social species, and shoaling is essential for survival because it can reduce the risk of predation, and favor the search for food and mating (Engeszer et al. 2004).

Fish were transferred individually to the test tank (24 × 15 × 6 cm; w × d × h), and acclimatized for 60 s. The opaque partitions were subsequently removed, and the test fish could observe one empty aquarium and one containing 10 conspecifics (as made in Kirsten et al. 2018). Fish were filmed for 60 s, and the time that the zebrafish spent in the conspecific area was scored as an indicator of social preference.

2.5.3. Prey-predator test (PPT)

To analyze the behavioral reaction to a predator, we used a previously conditioned predator fish, the Tiger Oscar (*Astronotus ocellatus).* To avoid behavioral changes and to maintain maximum predatory activity, the predatory fish was trained in the experimental aquarium. The same fish was used for all the experimental trials. Due to the large number of zebrafish (13 per group, totaling 52 fish), and to prevent possible predator fatigue, the experiments were performed over 10 d, with a maximum of 6 prey-predation trials conducted per day. One group of additional 13 zebrafish was used as a control without a predator in the central chamber.

Zebrafish were filmed individually to analyze their antipredatory behavior. They were placed in a central aquarium, with the predator in the left chamber and an empty aquarium on the right (see details in Figure 2). Staying in the zone close to the predator may be indicative of the inability of the zebrafish to recognize the risk zone (Tang et al. 2017). The videos were analyzed in the initial (0–90 s) and final stages (90–180 s). To better observe the immediate aversive response against the predator,



the fish were not acclimatized.

Figure 2: Schematic drawing of the apparatus used for the prey-predator test. Dotted lines indicate the virtual compartments of the aquarium. Fish were draw by FK.

2.5.4. Dark-Light test (DLT)

For the DLT, we used a glass apparatus measuring 45 × 15 × 10 cm (w × d × h), divided into a dark area (where the walls and the back were covered with a black plastic film) and a light area (where the walls and the back were covered by a white opaque plastic film). Fish were initially placed in a central compartment for 1 min for habituation to the apparatus. The central compartment was then carefully removed, allowing the fish to freely explore both compartments. We tested 15 fish per group, filming each fish for 6 min (Mansur et al. 2015).

Adult zebrafish prefer dark environments, likely due to the camouflage afforded by these areas (Kysil et al. 2017; Serra, Medalha, and Mattioli 1999). Therefore, fish that remain longer in the light zone may not have identified a risk situation (Kysil et al. 2017). The time spent in the light and dark zones, the number of crossings between the two zones, and the number of fast entries into the light zone were analyzed. A fast entry was considered as a stay in the light zone for less than 1s.

2.6. Reproduction and F1 larval parameters

A batch of fish from the original population of each group (not exposed, and exposed to 0.03, 0.003, and 0.0003 µg/L RISP) were selected for breeding. They were placed in aquaria with perforated bottoms (1:1 males to females, 8 fish per breeding aquarium) for egg collection, since zebrafish eat their eggs after ovulation (Avdesh et al. 2012; Westerfield 2000). The eggs were collected approximately 1 h and 30 min after the light came on in the morning, followed by egg counting and classification. All the born offspring were counted and used in this experiment. Dead and unfertilized eggs were removed (Kimmel et al. 1995) and the water cleaned, following which the embryos were distributed in 24-well plates, with 10 embryos per well. The plates were placed in a water bath with the temperature set to 28°C.

2.6.1. Mortality and hatching

As described in our previous work (Kalichak et al. 2017), embryos were monitored for 6 d to identify possible changes in mortality rates or delays during the hatching period. Embryos and larvae were evaluated daily and debris and dead animals were removed. Embryos without a heartbeat or blood circulation were excluded from the experiments.

2.6.2. Heart rate

We measured embryo heart rate at 48 hours post-fertilization (hpf) under a microscope. Thirty animals were monitored per group. The embryos were returned to their respective wells for growth continuity shortly after heart rate determination.

2.6.3. Spontaneous movements

Within 24 hpf, zebrafish embryos present involuntary movements while still inside the chorion. These movements are essential for the egg breaking and represent a good viability parameter for embryos (Fraysse et al. 2006). This test can be performed using a simple magnifying glass and, therefore, the embryos were not removed from the wells during the analysis. For the test, we observed the embryos for 1 min and registered any tail or body contraction, excluding the eyes. Twenty embryos were observed in each group.

2.7. F1 generation behavioral testing

2.7.1. Open Field Test (OFT)

Larvae (6 dpf) were placed individually in 10-mL wells and filmed for 6 min. Zebrafish larvae naturally present thigmotaxic behavior, associated mainly with a defense against predators when placed in an unfamiliar location (Colwill and Creton 2011). Larvae will eventually explore the entire well, including its central area.

Multiple 6-well plates were used for this test. The video recordings started 30 s after familiarization to the environment. For the video analyses, we considered the total distance traveled, immobility time, distance traveled in the central area (virtually demarcated), entries into the central area, and average speed. To identify possible

time-related changes, the analyses were divided from 0 to 3 and from 3 to 6 min. We tested 15 larvae per group.

2.8. Statistical analyses

Data were compared using one-way analysis of variance (ANOVA) followed either by a post-hoc Dunnett's multiple range test, or a Kruskal-Wallis test followed by a post-hoc Dunn's test, depending on the normality of the data (assessed by the Kolmogorov-Smirnov test). The alpha level was set at 0.05.

3. Results

Here, we first present the results for the adult fish exposed as embryos and larvae, followed by the results for the F1 generation.

3.1. Adult fish exposed as embryos or larvae

3.1.1. Prey-predator test

In the first phase (0-90s), control fish spent less time in the segment near to the predator than control fish without a predator in the central chamber. This pattern occurs also in zebrafish that were exposed to 0.003 μg/L of RISP in the embryonic and larval stages, while zebrafish that were exposed to 0.0003 and 0.03 μg/L of RISP in the embryonic and larval stages, lost this risk perception and remain similar time in the segment near to the predator than control fish not exposed to the predator (Fig. 3a). This pattern was not verified in the 2nd phase (90-180 s), when the zebrafish that were exposed to 0.03 μg/L of RISP in the embryonic and larval stages, spent less time in the segment near to the predator chamber (Fig. 3d). Exposure to a predator increased the distance travelled by control fish compared to control fish without predator, while RISP exposed fish swim similarly to the control not exposed to a predator, during the 1st phase (0-90 s, Fig. 3b). There were no changes at the 2nd stage (Fig. 3e). No changes occur regarding line crossings, both in the initial and final phases.

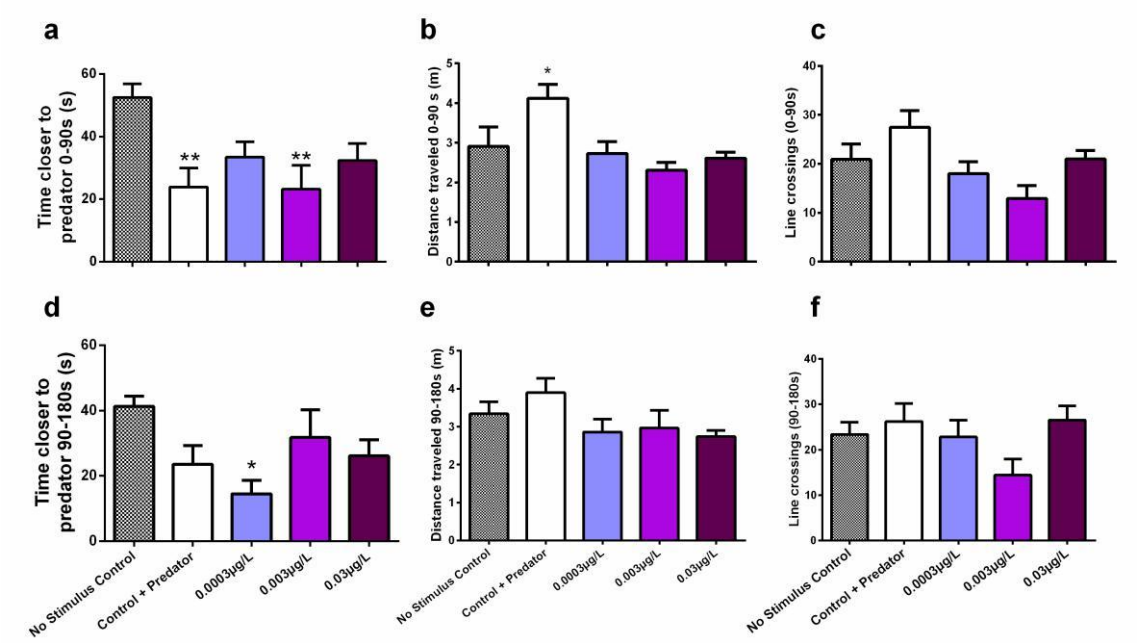


Figure 3. Locomotor and time spent parameters of zebrafish exposed to RISP during the initial development, exposed to prey-predator test. Time spent closer to predator (initial phase, a; final phase, d), distance traveled (initial phase, b; final phase, e) and line crossings (initial phase, c; final phase, f). Data are expressed as mean ± S.E.M. and compared by the Kruskal-Wallis test followed by Dunn's test (**p*<0.05, ** *p*<0.01, ****p*<0.001; n = 13).

3.1.2. Novel tank test (NTT)

No differences were found between RISP-exposed and control groups for all the parameters scored. All groups presented the same pattern of increase in distance traveled in the three NTT time periods: first (0 to 2 min), second (2 to 4 min), and final (4 to 6 min) (Fig. 4).

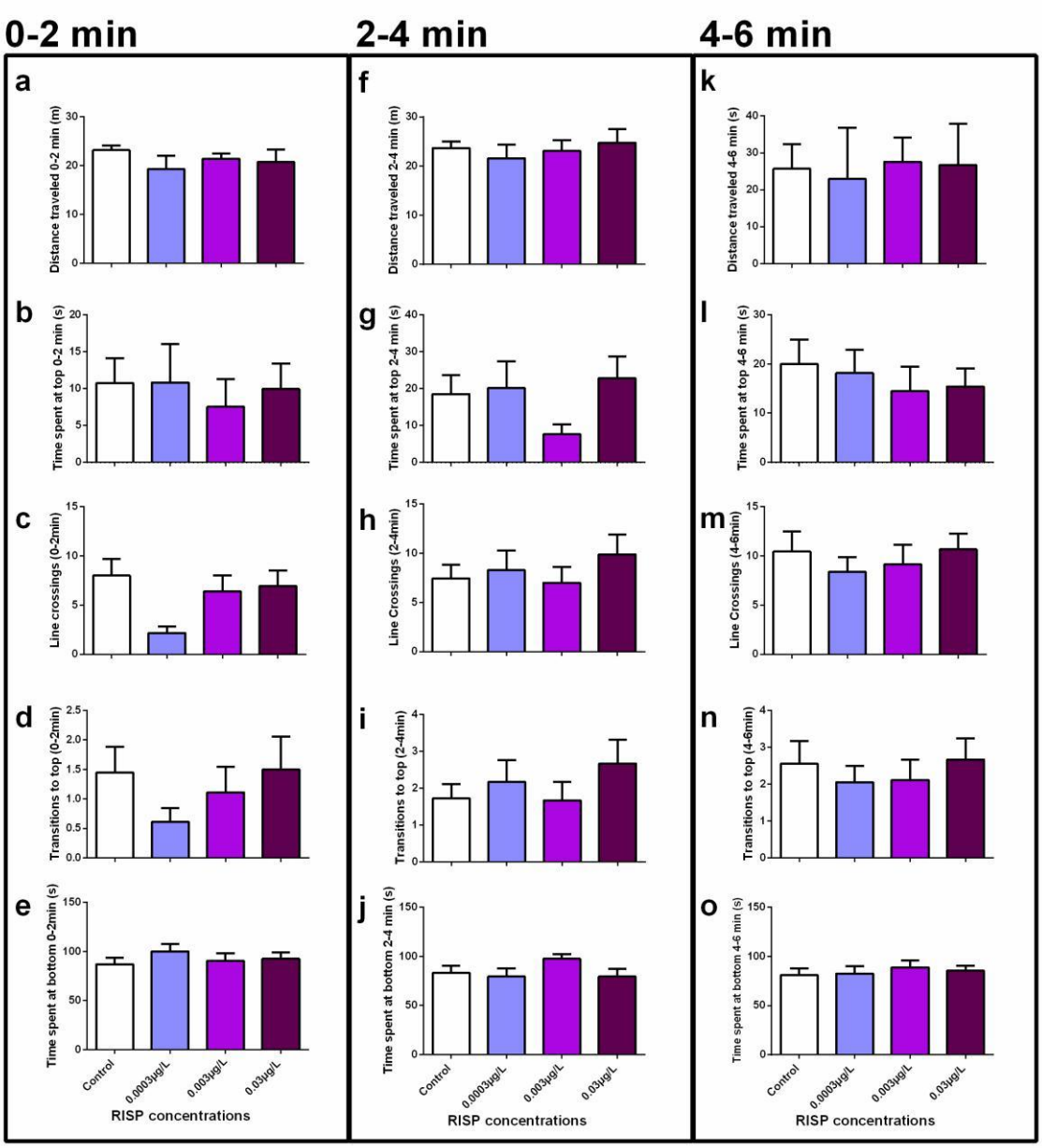


Figure 4. Locomotor and exploratory behavior in the NTT of zebrafish exposed to RISP in the embryonic and larval stages. No differences were found compared to the unexposed control group. Data are expressed as mean ± S.E.M. and compared either by one-way ANOVA followed by Dunnett's multiple range test, or the Kruskal-Wallis test followed by Dunn's test, depending on data normality (n = 15).

3.1.3. Social preference test (SPT)

RISP-exposed fish did not present any changes related to social interaction when compared to unexposed controls (Table 1).

Table 1. Social preference test for fish exposed or not to RISP in the embryonic and larval stages.

	RISP concentrations (μg/l)			
	0 (control)	0.0003	0.003	0.03
Time in shoal zone (s)	26.20 ± 6.86	23.22 ± 9.66	25.25 ± 9.51	20.44 ± 11.47
Line crossings	2.29 ± 3.45	3.20 ± 3.64	2.25 ± 4.18	4.66 ± 4.47
Latency to 1st entry shoal zone (s)	0.52 ± 1.81	0.74 ± 1.22	0.17 ± 0.58	1.08 ± 2.57

Data are represented as mean ± S.D. of 24 fish. Data were compared by one-way ANOVA followed by Dunnett's multiple comparisons test. No differences were found.

3.1.4. Light-Dark test (LDT)

Exposure to RISP did not affect the preference between the dark and light zone. However, at the concentrations of 0.003 μg/L and 0.03 μg/L, the number of crossings decreased (Table 2).

Table 2. Light-Dark test in fish exposed or not to RISP in the embryonic and larval stages.

	RISP concentrations (μg/l)			
	0 (control)	0.0003	0.003	0.03
Time spent in dark zone (s)	145.17 ± 51.08	178.67 ± 55.56	171.67 ± 67.64	175.58 ± 53.75
Center crossings	27.79 ± 10.12	29.29 ± 9.91	19.18 ± 7.32 *	19.33 ± 6.62 *
Fast entries in light zone	2.75 ± 2.70	3.25 ± 2.18	2.17 ± 2.04	1.67 ± 1.72

Data are presented as mean ± S.D. of 24 fish. Data were compared by one-way ANOVA followed by Dunnett's multiple comparisons test (*$p < 0.05$).

3.1.5. Weight of parental fish at 170 and 250 dpf

Embryonic and larval exposure to RISP resulted in reduced weight gain, impairing the growth of zebrafish exposed to all the tested concentrations (Table 3). These changes were not gender-dependent (data not shown).

Table 3. Weight at 170 and 250 d of fish exposed or not to RISP in the embryonic and larval stages.

	Weigth at	
	170 days	250 days
Control	0.139 ± 0.073	0.310 ± 0.161
0.0003 μg/l	0.078 ± 0.083 ****	0.165 ± 0.128 **
0.003 μg/l	0.080 ± 0.046 ****	0.216 ± 0.097 *
0.03 μg/l	0.089 ± 0.037 ****	0.195 ± 0.094 *

Data are expressed as mean ± S.D. of 20 to 214 fish, and were compared by the Kruskal-Wallis test followed by Dunn's multiple comparisons test (170 d), and by one-way ANOVA followed by Dunnett's multiple comparisons test (250 d) (*p<0.05, **p<0.01, **** p<0.0001, compared to the unexposed controls).

3.2. Offspring evaluation

3.2.1. F1 embryonic parameters

Parental RISP exposure in the embryonic and larval stages resulted in a reduced survival rate in the F1 embryos. The concentration of 0.0003 µg/L directly decreased the number of live embryos (Fig. 5a), while the concentration of 0.03 µg/L decreased the rate of hatching (Fig. 5b), heart rate (Fig. 5c), and number of spontaneous movements within the chorion (Fig. 5d).

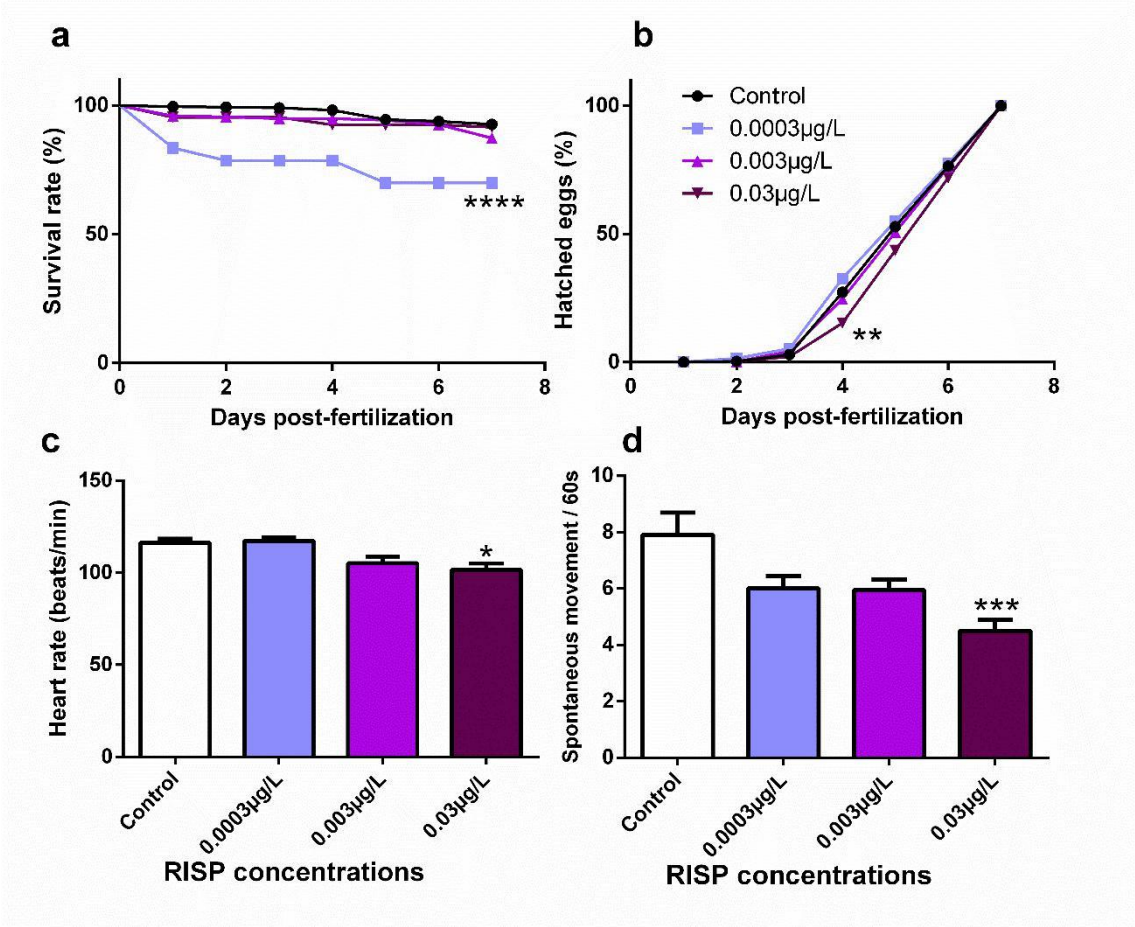
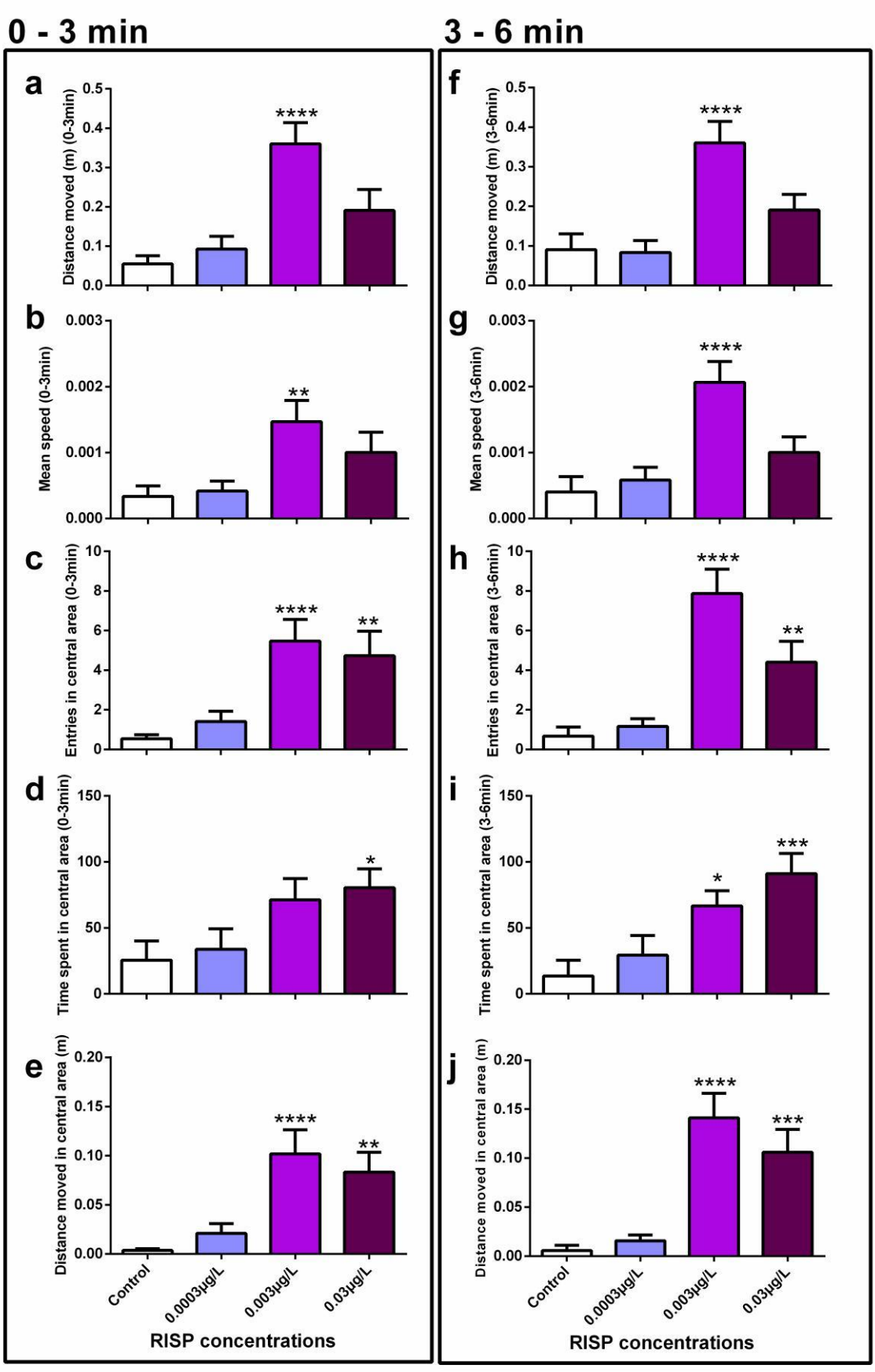


Figure 5. The F1 embryos of parents exposed to the highest concentration of RISP in the embryonic and larval stages showed decreased heart rate (n = 30), spontaneous movement (n = 20), and hatched embryos (n = 113 – 183). The survival rate (n = 113 – 183) of offspring of fish exposed to 0.0003 µg/L RISP was lower than the other groups. Data are expressed as mean ± S.E.M. and compared by the Kruskal-Wallis test followed by Dunn's test (*p<0.05, ** p<0.01, and ***p<0.001).

3.2.2. Open-Field test (OFT)

In the OFT, F1 larvae of fish exposed to RISP concentrations of 0.003 and 0.03 µg/L presented higher exploratory activity, independently of the stage (Fig. 6A initial stage, and 6B final stage). This was evidenced by the recorded increase in distance traveled (Fig. 6a and f) and mean speed (Fig. 6b and g), as well as the increase in the number of entries (Fig. 6 c and h), time spent (Fig. 6 d and i), and distance traveled in the central area (Fig.6 e and j).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Time spent in central area (0-3min)
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Entries in central area (3-6min)
Time spent in central area (3-6min)
Distance moved in central area (m)
Control
0.0003µg/L
0.003µg/L
0.03µg/L
RISP concentrations

Figure 6. Larval open field test at initial stage and final stage. Data are expressed as mean ± S.E.M. and compared either by one-way ANOVA followed by Dunnett's multiple range test, or the Kruskal-Wallis test followed by Dunn's test, depending on data normality (**p*<0.05, ***p*<0.01, ****p*<0.001, and *****p*<0.0001; n = 15 larvae per group).

4. Discussion

As reported in our previous study, RISP exposure affected the survival and behavior of fish exposed during the embryonic stage (Kalichak et al. 2017). Here, we show that early-exposed fish presented an impaired antipredatory reaction during adulthood, characterizing a persistent effect. We also show that some of these behavioral changes are present in the following generation, characterizing a transgenerational effect.

Behavioral changes in fish caused by psychoactive drug residues are not uncommon. Even at below the therapeutic index, animals may exhibit atypical behavior when exposed to compounds like typical and atypical antipsychotics (Barcellos et al. 2016; Idalencio et al. 2015; Giacomini et al. 2016), serotonin reuptake inhibitors (Kalichak et al. 2016; Abreu et al. 2015; Weinberger II and Klaper 2014; Painter et al. 2009), or even antidepressants like Mianserin, a serotonin agonist (Barreto 2016).

In an environmental context, changes in antipredatory behavior resulting from risperidone exposure can have direct effects. During evolution, animals have developed different antipredatory behaviors (Kelley and Magurran 2003); in fish, these include camouflage and a preference for dark areas or environments. To avoid predators, animals may exhibit erratic movements, making it difficult for a predator to predict their movements (Serra et al. 1999), or they may shoal, which can intimidate a predator (Herbert-Read et al. 2018). 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, Cardenas, and Mchenry 2013).

In fact, in the prey-predator test, zebrafish that were exposed to 0.003 μg/L of RISP in the embryonic and larval stages, spent less time in the segment near to the

predator, while zebrafish that were exposed to 0.0003 and 0.03 μg/L of RISP, lost this risk perception and remain similar time in the segment near to the predator than control fish not exposed to the predator. This type of U-shaped curve-response was commonly found in pharmacological studies and are commonly associated with receptors activation (Calabrese and Baldwin 2001). Similar U-shaped curve responses were found for RISP (Kalichak et al. 2016; Kalichak et al. 2017; Idalencio et al. 2015), and for other psychoactive drugs as the atypical antipsychotic aripiprazole (Barcellos et al. 2016).

In addition to the impact on antipredatory behaviors, RISP-exposed fish grew less than unexposed fish. This size difference seemed to be compensated for over time, since the difference verified at 170 dpf was not observed at 250 dpf. In its clinical use throughout human embryogenesis, RISP did not show evidence of altered growth or delay in sexual maturation in children (Dunbar et al. 2004; Coppola et al. 2007), and it is approved for use by pregnant women.

We showed that the effects of RISP were persistent until the adult stage and could be observed in the following generation, characterizing a possible transgenerational pattern. The offspring of early-exposed adults exhibited reduced survival, hatching, and heart rates, as well as increased exploratory behavior, as had their parents when evaluated as embryos and larvae (Kalichak et al. 2017), and as adults (this study). Our results point to the persistence of RISP effects into adulthood and to transgenerational effects in offspring not directly exposed to the drug.

RISP is a dopaminergic (D2) and serotoninergic (5-HT_2) receptor antagonist. Although the effects of RISP exposure at low doses (as shown in our study) are poorly understood, antagonism of these receptors can cause functional alterations in the exposed generation that may persist until adulthood (Singh and Singh 2017; Bardgett et al. 2013; Prieto et al. 2012; Zuo et al. 2008).

Although transgenerational effects of drug residues in aquatic environments have been reported (Galus et al. 2013; Baker, Barron, and Kasprzyk-hordern 2014; Pomati et al. 2006), little is known about the mechanisms of persistence in subsequent generations. Epigenetic changes have been proposed as a possible explanation for these transgenerational effects, but environmental factors, disease, or even eating habits can cause inheritable changes in the functional genome (Santangeli et al. 2016; W. Tang and Ho 2007; Ojha et al. 2015). These changes are important in evolution because they can define the phenotype of the organism and

ensure that information is transmitted through generations (Tang and Ho 2007). However, epigenetic changes resulting from exposure to contaminants or pollutants can interfere directly with the functional aspects of an organism (Santangeli et al. 2016; Martinez-Sales, Garcia-Ximenz, and Espinós 2015). Concentrations of bisphenol A already present in the environment were shown to affect the female zebrafish reproductive system and deregulate epigenetic mechanisms (Santangeli et al. 2016). In this line, zebrafish exposed to water samples collected from drinking water reservoirs presented decreased reproductive efficiency, both in the exposed generation and their offspring (Martinez-Sales et al. 2015).

In our study, is that we did not evaluate the mechanisms by which RISP caused the persistent, as well the transgenerational effects on zebrafish. We also not know how far these transgerational changes can last. However, this does not lessen the importance of this initial evaluation, since data about persistent and transgerational effects of drug residues on fish development and behavior are very scarce.

Here, we concluded that RISP exposure at early life stages alters the behavioral responses of the adult zebrafish, and that these effects could be observed in the following generation. This suggests that even short and sub-lethal doses, at essential stages of development, can affect the whole life of the fish, as well as its offspring. Our results highlight the risks and long-term environmental consequences associated with drug residues in water, which may affect aquatic life and endanger species that depend on appropriate behavioral responses for survival.

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6. DISCUSSÃO

Com base nos artigos produzidos demonstramos que a risperidona, mesmo quando utilizada em concentrações já verificadas no ambiente aquático, pode alterar o comportamento de larvas de zebrafish e ainda interferir negativamente no número de animais vivos após a exposição. Além disso, esses efeitos apresentaram persistência até a fase adulta e alteraram o comportamento da prole, sugerindo um efeito transgeracional da risperidona. Esse resultado é importante para análise dos riscos de toxicidade destes fármacos no ambiente visto que as baixas concentrações de risperidona utilizadas neste trabalho já foram detectadas em ambientes aquáticos (VERGEYNST et al., 2015; VIDAL-DORSCH et al., 2012; BRUCE et al., 2010; SNYDER, 2008).

Levando em consideração o primeiro artigo, relatou-se que a exposição a risperidona durante a fase embrionária pode trazer prejuízos relacionados à sobrevivência, além de diminuir a responsividade das larvas de zebrafish a situações de risco. Os animais expostos passaram a explorar áreas centrais e zonas de estímulo, podendo esse ser um indicativo de que estão mais predispostas à predação.

No segundo artigo, baseado no desenvolvimento e reprodução das larvas expostas no primeiro, os efeitos percebidos nos embriões expostos a risperidona foram persistentes até a fase adulta, diminuindo o crescimento dos animais. A exposição a risperidona aumentou o tempo de permanência próximo ao predador, sugerindo a inabilidade da identificação de situações de risco.

Após a reprodução dos animais, ainda no segundo artigo, percebemos diminuição das taxas de sobrevivência, eclosão, movimentação expontânea e frequência cardíaca na prole dos animais expostos. Mesmo sem contato direto com o contaminante, as larvas demonstraram alterações comportamentais, permanecendo por mais tempo em áreas de estímulo.

Os efeitos na atividade exploratória encontrados em nosso trabalho são resultados bastante comuns na exposição a risperidona ou a outros antipsicóticos atípicos. A atividade neuronal dopaminérgica e serotoninérgica tem como principal função a modulação motora, por isso, é justificável que muitos dos achados sobre a risperidona estejam relacionados a esse tipo de comportamento (IGARTÚA et al.,

2015; BARDGETT et al., 2013; MCLEAN e FETCHO, 2004). Em uma breve revisão de literatura, encontramos que as concentraç intermediárias da risperidona demonstraram efeito excitatório, aumentando a movimentação de larvas de zebrafish com 4dpf (IGARTÚA et al., 2015) e em experimentos com ratos, os animais jovens que receberam risperidona apresentaram alterações sobre receptores dopaminérgicos, serotoninérgicos e glutamatérgicos as quais podem persistir até a fase adulta mesmo com a interrupção do tratamento (SINGH e SINGH, 2017; CHOI et al., 2010; CHOI et al., 2009; MORAN-GATES et al., 2007).

A exposição ao fármaco durante as fases iniciais de desenvolvimento também parece explicar os efeitos encontrados. Os antipsicóticos quando utilizados durante a fase embrionária estão associados a uma redução do nível de neurotransmissores em áreas específicas do cérebro, provocando problemas funcionais na geração exposta, os quais permanecem até a fase adulta (SINGH e SINGH, 2017; BARDGETT et al., 2013; PRIETO et al., 2012; ZUO et al., 2008).

Pela sua afinidade principalmente por receptores serotoninérgicos do tipo 5-HT_2 e dopaminérgicos D_2 a risperidona é classificada como antipsicótico atípico antagonista serotonina-dopamina (HORACEK et al., 2006). Neurônios dopaminérgicos já podem ser encontrados em embriões de zebrafish antes das 18hpf. Em 24hpf receptores do tipo D1 já podem ser detectados na medula espinhal e diencéfalo ventral (IRONS, 2011). Porém os neurônios do tipo serotoninérgico só podem ser encontrados a partir do 2dpf (PRIETO et al., 2012). A falta de atividade serotoninérgica ou as baixas concentrações utilizadas do fármaco podem justificar a não atividade da risperidona na movimentação espontânea (realizada em 24hpf), mas alterações encontradas nos testes de tanque novo, estímulo aversivo e claro-escuro (realizados no 5dpf) já são justificados pela atividade dos dois tipos de neurônios.

Mesmo as concentrações testadas nos dois trabalhos estarem muito abaixo das doses terapêuticas, ou ainda comumente testadas em experimentos científicos, a risperidona provocou efeitos sobre as larvas de zebrafish em ambos os artigos desenvolvidos, semelhantes a aqueles descritos na literatura. Conhecidos por efeitos horméticos, doses muito baixas podem exercer efeitos sobre os receptores e provocar alterações na atividade celular. Efeitos horméticos são caracterizados quando uma ação é percebida nas concentrações mais baixas utilizadas, diferente das concentrações mais altas. Resultados semelhantes foram descritos com a

utilização de concentrações subletais de imidacloprida (QU et al., 2014). Esses efeitos podem ser semelhantes àqueles já esperados, ou ainda, totalmente contrários ou desconhecidos, já que poucas pesquisas são realizadas utilizando baixas doses de fármacos e componentes químicos. Conhecidos por curva dose-resposta, os efeitos descritos em U invertido ou mesmo respostas bifásicas são bastante conhecidos na farmacologia e podem auxiliar no esclarecimento de efeitos horméticos (CALABRESE e BALDWIN, 2002; CALABRESE e BALDIWN, 2001; DAVIS e SVENDSGAARD, 1990).

Por isso, as alterações encontradas em nosso trabalho são de extrema importância para a caracterização do risco da exposição a risperidona, visto que as larvas e adultos expostos demonstraram alterações comportamentais. Os trabalhos que avaliam o risco ecológico da exposição a substâncias químicas são importantes para compreensão de fenômenos comportamentais anormais, não só no ponto de vista celular e molecular mas também comportamental, para conscientização da população na proteção de ecossistemas e ainda sobre o risco da exposição toxicológica a esse tipo de componente (WEIS et al., 2001; FUIMAN e MAGURRAN, 1994; MESA et al., 1994).

Nos peixes, a serotonina, dopamina, acetilcolina e GABA estão envolvidos na locomoção, agressão, aprendizado e comportamento alimentar. A capacidade de capturar presas e evadir de predadores são comportamentos claramente relevantes sobre o ponto de vista ecológico, pois interferem diretamente no crescimento e sobrevivência dos organismos (WEIS et al., 2001; FUIMAN e MAGURRAN, 1994). Uma presa pode ser mais susceptível a predação como resultado da não detecção de predadores, mau desempenho de fuga, resistência reduzida, incapacidade de aprendizado e maior visibilidade (MESA et al., 1994; WEIS et al., 2001).

Assim, considerando também os dados obtidos previamente (KALICHAK et al., 2016), a RISP afeta diretamente a geração exposta durante a fase inicial causando claro prejuízo à eclodibilidade e viabilidade dos embriões e larvas. Além disso afeta os sobreviventes para o resto de sua vida, uma vez que esses animais crescem menos e ainda continuam apresentando déficits no seu repertório comportamental, especialmente referente aos comportamentos componentes da relação presa-predador. O segundo artigo ainda mostrou que mesmo conseguindo reproduzir, sua prole apresenta déficits comportamentais nítidos, especialmente no comportamento de percepção e evitação de risco.

Os prejuízos causados pela exposição a risperidona ainda não podem ser estimados. Em um contexto ambiental, os animais que são pouco responsivos ou mudam seu comportamento em virtude de poluentes, podem estar suscetíveis a predação, ou ainda, incapacitados da exploração normal de novos ambientes, demonstração de comportamento reprodutivo ou busca de alimento. Além disso, sabendo-se que a risperidona também já foi detectada em água potável, pode-se interpretar que humanos e animais expostos a água contaminada podem também estar em risco, possibilitando o desenvolvimento de comportamentos alterados, em concentrações subletais.

7. CONCLUSÃO

A exposição de embriões e larvas de zebrafish provoca diminuição na sobrevivência e eclosão e também causa alterações comportamentais, aumentando a velocidade e locomoção dos peixes expostos e diminuindo a resposta a estímulos externos ou normalmente nocivos para a espécie. Durante a fase adulta, os peixes expostos a risperidona apresentaram efeitos persistentes, diminuindo o crescimento e a responsividade ao estímulo predatório. A risperidona teve efeitos também sob a prole, que mesmo sem exposição direta, apresentou aumento de mortalidade e aumento do comportamento exploratório, permanecendo por mais tempo na área considerada de risco. Em um contexto ambiental essa reposta pode influenciar no comportamento antipredatório e pode diminuir as chances de sobrevivência dos animais expostos.

8. PERSPECTIVAS

Os estudos aqui demonstrados deixam claros os riscos oferecidos por resíduos farmacêuticos no ambiente aquático. Mesmo em baixas concentrações, a risperidona provocou alterações comportamentais no *zebrafish*, as quais demonstraram ser persistentes e capazes de serem transmitidos através de gerações. Poucas informações são disponíveis a respeito dos impactos causados pela presença desses contaminantes, por isso, a importância do desenvolvimento de novos estudos relacionados ao tema, a fim de conscientizar os riscos da exposição a esses poluentes, mesmo em baixas concentrações.

Devido os interessantes resultados demonstrados, serão analisadas ainda possíveis alterações hormonais decorrentes da exposição ao psicofármaco. Para isso, utilizaremos como base os níveis de cortisol dos peixes adultos expostos durante a fase embrionária, a fim de detectar possíveis alterações na resposta ao estresse.

Estudos futuros relacionados a resposta cognitiva dos animais expostos a risperidona também devem ser realizados, e relacionados ao aprendizado e memória. Possíveis alterações epigenéticas ou ainda a expressão de receptores no SNC também são questões que devem ser esclarecidas em futuras pesquisas.

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ANEXO – PROTOCOLO APROVAÇÃO CEUA PARECER N° 009/2015

UNIVERSIDADE DE PASSO FUNDO
VICE-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

PARECER Nº 009/2015

A Comissão de Ética no Uso de Animais da Universidade de Passo Fundo, em reunião no dia 19/06/15, analisou o projeto de pesquisa "**Efeitos de resíduos de psicofármacos na água sobre o desenvolvimento inicial de *zebrafish***", registro na CEUA Nº 005/2015, de responsabilidade do pesquisador **Leonardo José Gil Barcellos**.

Em relação aos aspectos éticos, a Comissão considerou o projeto relevante e com relação custo-benefício adequada. O pesquisador e seus colaboradores estão comprometidos com a observância dos procedimentos para o uso científico de animais estabelecidos na Lei 11.794 de 8 de outubro de 2008.

Diante do exposto, a Comissão, de acordo com suas atribuições definidas na Lei 11.794 de 8 de outubro de 2008, manifesta-se pela aprovação do projeto de pesquisa na forma como foi proposto.

O pesquisador deverá apresentar relatório à CEUA ao final do estudo.

Situação: PROTOCOLO APROVADO

Passo Fundo, 26 de junho de 2015.

Prof. Ana Cristina Vendrametto V. Giacomini
Coordenadora – CEUA – UPF