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Murilo Sander de Abreu

EFEITOS, PERCEPÇÃO DE FÁRMACOS E COMUNICAÇÃO QUÍMICA EM PEIXES

Santa Maria, RS

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

Orientador: Prof. Dr. Leonardo José Gil Barcellos

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Aprovado em 08 de junho de 2017:

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Leonardo José Gil Barcellos, Dr. (UFSM)

Presidente/Orientador

_____ _____

Anna Maria Siebel, Dr. (UNOCHAPECO) **Bernardo Baldisserotto, Dr. (UFSM)**

_____ _____

Carla Denise Bonan, Dr. (PUCRS) **Denis Broock Rosemberg, Dr. (UFSM)**

Santa Maria, RS.

2017

RESUMO

EFEITOS, PERCEPÇÃO DE FÁRMACOS E COMUNICAÇÃO QUÍMICA EM PEIXES

Autor: Murilo Sander de Abreu

Orientador: Leonardo José Gil Barcellos

Coorientador: Angelo Luis Stapassoli Piato

A presença de fármacos em ambientes aquáticos tem sido estudada há décadas e suas ações têm como consequências alterações de diversos mecanismos fisiológicos. Sabe-se que existe uma complexa comunicação química entre as espécies que habitam esse ambiente, mas a percepção de substâncias das comunicações intra e interespecíficas é desconhecida. Os estudos desenvolvidos objetivam avaliar se os peixes são capazes de identificar diferentes situações estressoras e fármacos, analisando os efeitos neuroendócrinos, comportamentais e osmorregulatórios. Foram elaborados sete estudos: 1) teste de estresse agudo e avaliação osmorregulatória com fluoxetina; 2) teste da ação da fluoxetina em diferentes respostas de estressores (físicos ou químicos); 3) teste de percepção individual a diferentes situações de coespecíficos; 4) teste de preferência a diferentes concentrações de fármacos e detecção pela via olfatória; 5) teste de anosmia experimental em comportamento tipo-ansiedade; 6) teste de anosmia experimental, por $ZnSO_4$, em respostas comportamentais e fisiológicas; 7) teste dos efeitos do estresse agudo sobre comportamento social e "ansiedade" em jundiás (*Rhamdia quelen*). Com base nos resultados, verificou se que a exposição aguda de fluoxetina é capaz de inibir as alterações osmorregulatórias causadas pelo estresse. A fluoxetina atenua a resposta de cortisol a estímulo estressor físico, mas não a estímulo estressor químico. O *zebrafish* pode perceber e desencadear comportamentos aversivos quando em contato com águas condicionadas de estresse físico, químico e alimentar (jejum agudo). O *zebrafish* apresenta atração por psicofármacos como diazepam, fluoxetina, risperidona e buspirona, os quais provavelmente são detectados pela via olfatória. A anosmia experimental temporária (por lidocaína e $ZnSO_4$) modula comportamentos de ansiedade em *zebrafish* adulto. O jundiá pode ser utilizado para estudos comportamentais de "ansiedade" e interação social. De modo geral, esses resultados contribuem para um melhor entendimento dos efeitos e percepções da ação farmacológica e de comunicação química em peixes.

Palavras chave: estresse, ansiedade, fluoxetina, anosmia, *zebrafish*.

ABSTRACT

EFFECTS, PERCEPTION OF DRUGS AND CHEMICAL COMMUNICATION IN FISH

Author: Murilo Sander de Abreu

Advisor: Leonardo José Gil Barcellos

Co-supervisor: Angelo Luis Stapassoli Piato

The presence of drugs in aquatic environments has been studied for decades and their actions have as consequence changes of several physiological mechanisms. It is known that there is a complex chemical communication between the species that inhabit this environment, but the perception of intra and interspecific communication substances is unknown. The studies developed aim to evaluate if the fish are able to identify different stress situations and drugs, analyzing the neuroendocrine, behavioral and osmoregulatory effects. Seven studies were elaborated: 1) acute stress test and osmoregulatory evaluation with fluoxetine; 2) test of the action of fluoxetine in different responses of stressors (physical or chemical); 3) individual perception test to different conspecific situations; 4) testing preference at different concentrations of drugs and detection by olfaction; 5) experimental anosmia test in type-anxiety behavior; 6) experimental anosmia test, by $ZnSO_4$, in behavioral and physiological responses; 7) test of the effects of acute stress on social behavior and "anxiety" in jundias (*Rhamdia quelen*). Based on the results it was verified that the acute exposure to fluoxetine is able to inhibit osmoregulatory changes caused by stress. Fluoxetine attenuates the cortisol response to physical stressor stimulus, but not to chemical stressor stimulus. Zebrafish can perceive and trigger aversive behaviors when in contact with conditioned waters of physical, chemical, and food stress (acute fasting). Zebrafish is attracted to psychotropic drugs such as diazepam, fluoxetine, risperidone and buspirone, which are probably detected by olfaction. Temporary experimental anosmia (by lidocaine and $ZnSO_4$) modulates anxiety behaviors in adult zebrafish. Jundia can be used for behavioral studies of "anxiety" and social interaction. In general, these results contribute to a better understanding of the effects and perceptions of pharmacological action and chemical communication in fish.

Key words: stress, anxiety, fluoxetine, anosmia, zebrafish.

SUMÁRIO

1. APRESENTAÇÃO

A presença de fármacos no ambiente aquático é conhecida há décadas e, consequentemente, o contato desses compostos com as espécies que habitam esses ecossistemas é inevitável. Essa exposição pode provocar alterações importantes como a resposta ao estresse, a atividade osmorregulatória e o comportamento. Além disso, os sentidos (visão, audição, olfato, tato e paladar) são de extrema importância para homeostase, pois a partir deles percebem e detectam alimentos e predadores; assim, a ausência de um sentido, como a perda do olfato, conhecida como anosmia, pode acarretar em alterações comportamentais, pelo aumento da dificuldade em percepção. Com isso, a comunicação intraespecífica e interespecífica é imprescindível para o equilíbrio dos seres vivos que compõem os ecossistemas, pois possibilita que os coespecíficos sejam, por exemplo, avisados sobre um possível predador, zona de risco; garantindo a integridade e continuidade da espécie.

Os resultados aqui apresentados são oriundos de estudos vinculados à linha de farmacologia e toxicologia em peixes do Laboratório de Fisiologia de Peixes da Universidade de Passo Fundo. O trabalho está embasado na apresentação dos resultados finais sob a forma de sete artigos publicados, para fins de defesa de tese de Doutorado, dispondo das seguintes seções: referencial teórico, proposição, materiais e métodos, discussão, conclusão e perspectivas.

A seção de Referencial Teórico abordará sobre a presença de psicofármacos no ambiente aquático; psicofármacos como contaminantes ambientais (benzodiazepínicos, inibidores seletivos da recaptação de serotonina, buspirona, risperidona e etanol); os modelos animais de transtornos mentais (ansiedade e comportamento social); mecanismos fisiológicos de anosmia, resposta ao estresse e osmorregulação; as relações intra e interespecífica; comunicação química em peixes; e finalizando com os modelos animais, *zebrafish* e jundiá.

A seção de Materiais e Métodos descreve de forma geral a metodologia utilizada nos estudos desenvolvidos e, remete aos artigos, os detalhamentos metodológicos de cada estudo. Nas seções Artigo 1 a 7 serão apresentados os materiais e métodos, análise estatística, resultados, discussão e referências bibliográficas específicos de cada estudo.

A seção Discussão apresentará uma análise crítica de todos os resultados obtidos, bem como a correlação desses com a literatura e por fim as seções Conclusão e Perspectivas que abordarão as conclusões obtidas nos estudos com suas perspectivas.

1.1. REFERENCIAL TEÓRICO

1.1.1. Psicofármacos no ambiente aquático

O consumo de antidepressivos, ansiolíticos e estabilizadores de humor pela população tem aumentado exponencialmente nos últimos anos em todo o mundo e, consequentemente, a presença desses compostos e de seus metabólitos na água de efluentes tem crescido (HEBERER, 2002; KOLPIN et al., 2002; CALAMARI, et al., 2003; BROOKS et al., 2005; CALISTO; ESTEVES, 2009; ALONSO et al., 2010; HUERTA-FONTELA; GALCERAN; VENTURA, 2010; CALISTO; DOMINGUES; ESTEVES, 2011). Considerando a diversidade de fármacos encontrados na água com mecanismos de ação variados, esse tipo de contaminação pode causar impactos tanto na população humana usuária desses recursos, quanto nos organismos aquáticos que compõem esses ecossistemas e torna-se prioridade para as agências reguladoras envolvidas na avaliação de risco humano e ecológico (DAUGHTON; TERNES, 1999; HEBERER, 2002; CALAMARI, et al., 2003; BROOKS et al., 2005; JONES, et al., 2005; CALISTO; ESTEVES, 2009; ALONSO et al., 2010; HUERTA-FONTELA; GALCERAN; VENTURA, 2010; CALISTO; DOMINGUES; ESTEVES, 2011; BRODIN et al., 2013).

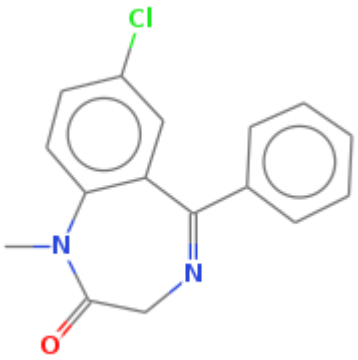
Embora a concentração desses fármacos na água possa ser inferior às concentrações letais, alguns fármacos identificados nos efluentes municipais têm sido encontrados em altas concentrações no encéfalo (BROOKS et al., 2005), músculos (BROOKS et al., 2005; SACKERMAN et al., 2010) e fígado (BROOKS et al., 2005) de diversas espécies de peixes. Há indícios que tais contaminantes possam ocasionar um conjunto de alterações fisiológicas, como neuroendócrinas (como inibição a resposta ao estresse, reprodução) (PARK et al., 2012; PRIETO et al., 2012), bem como comportamentais nos peixes expostos (SACKERMAN et al., 2010; PRIETO et al., 2012, GIACOMINI et al., 2016). Baseando-se nas evidências que apontam sobre a presença e persistência desses fármacos na água na forma intacta ou de metabólitos ativos (HUERTA-FONTELA et al., 2010; CALISTO; DOMINGUES; ESTEVES, 2011), bem como a ação sobre o sistema nervoso central (SNC), torna-se imprescindível a avaliação da ação sobre os ecossistemas aquáticos. Não há relatos sobre a percepção dos peixes em relação à presença desses compostos na água.

1.1.2. Psicofármacos como contaminantes ambientais

1.1.2.1. Benzodiazepínicos

Os benzodiazepínicos são usados como sedativos, amnésicos, ansiolíticos, relaxantes musculares e anticonvulsivantes (GARRET et al., 1994; RAMOS, 2004; GAILLARD et al., 2006; RANG; DALE, 2016; ROCHE, 2010). Em 1958, Leo Sternbach apresentou uma patente para o clordiazepóxido (Librium®), o primeiro do grupo das benzodiazepinas, as quais tornaram-se o maior sucesso da história da indústria farmacêutica; pertencente a esse grupo o diazepam (Valium®) (figura 1) lançado em 1963 (ROCHE, 2010). No Brasil, as capitais brasileiras passaram de um consumo de 2,63 doses diárias para mil habitantes por dia, em 2010, para 3,66 em 2011, chegando a 4,53 em 2012 de ansiolíticos benzodiazepínicos (AZEVEDO et al., 2016).

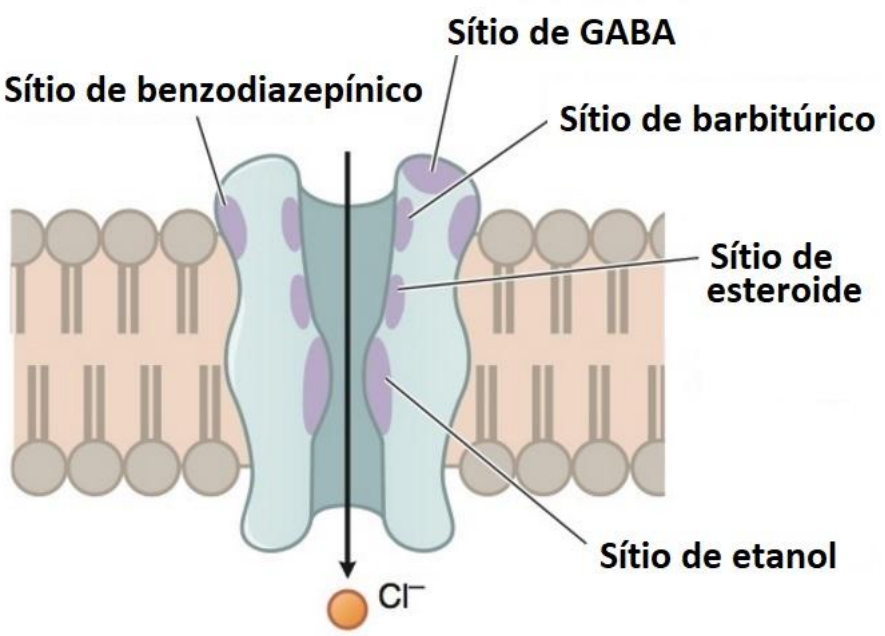
Figura 1. Fórmula estrutural do diazepam. Fonte: http://webbook.nist.gov/cgi/cbook.cgi?Name=Diazepam&Units=SI



Os benzodiazepínicos atuam seletivamente como moduladores positivos em receptores do neurotransmissor GABA (ácido gama-aminobutírico), que medeia a transmissão sináptica inibitória em todo o sistema nervoso central (SNC) (GAILLARD et al., 2006; SIGEL; STEINMANN, 2012). Os benzodiazepínicos intensificam a ação do GABA ao facilitar a abertura dos canais de cloreto, promovendo a hiperpolarização das células onde atuam (figura 2) (GILMAN; GOODMAN, 2001; KATZUNG et al., 2009). O diazepam é metabolizado pelo citocromo P450, através das enzimas 3A4, 2C8 e 2C9, que promovem N-demetilação, produzindo, assim, um metabólito ativo, o nordiazepam. Esse, por sua vez, é hidroxilado,

formando oxazepam, que sofre imediata glicuronidação através de um processo de conjugação (MORGAN et al., 2002).

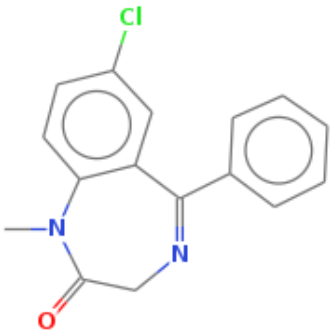
Figura 2. Representação esquemática dos sítios de ligação farmacológica no receptor $GABA_A$, de benzodiazepínicos (diazepam), barbitúricos, esteroides e etanol (figura adaptada). Fonte: GOODMAN; GILMAN, 2012.



1.1.2.2. Inibidores Seletivos da Recaptação de Serotonina (ISRS)

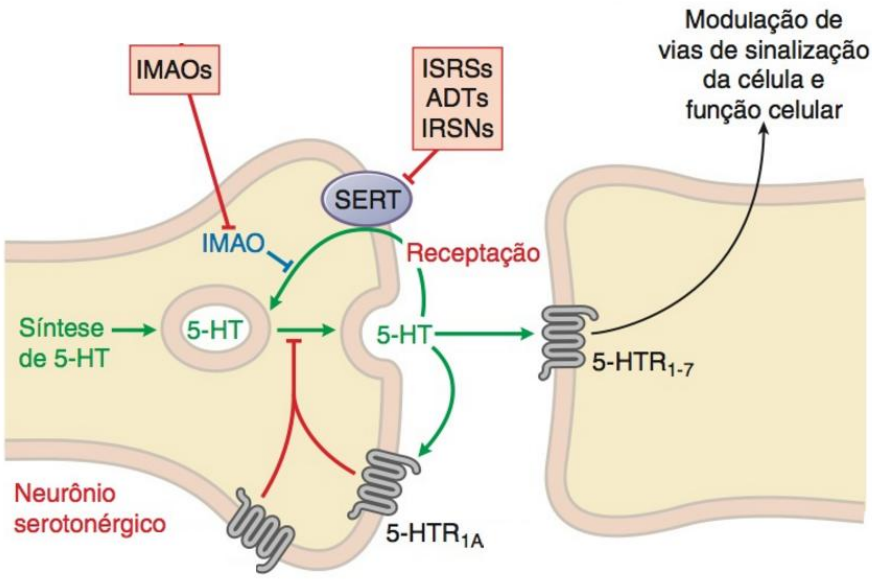
A classe dos inibidores seletivos da recaptação de serotonina (ISRS) é composta por vários fármacos, usados no tratamento de transtornos depressivos e de ansiedade. A fluoxetina (figura 3) é o principal representante dessa classe que foi descrita pela primeira vez como *Lilly 110140*. De acordo com dados da ANVISA (Agência Nacional de Vigilância Sanitária), de 2009 a 2011, no Distrito Federal, o consumo de fluoxetina teve um aumento de 83% (FUCHS; WANNMACHER, 2010).

Figura 3. Fórmula estrutural da fluoxetina. Fonte: http://webbook.nist.gov/cgi/cbook.cgi?Name=Fluoxetine&Units=SI



Os ISRSs atuam inibindo o transportador de serotonina (SERT) responsável pela recaptação de serotonina pelo neurônio pré-sináptico, aumentando a quantidade de serotonina disponível na sinapse. Consequentemente, há aumento global da neurotransmissão serotonérgica no SNC (figura 4) (RANG; DALE, 2016).

Figura 4. Representação esquemática do mecanismo de ação da fluoxetina (figura adaptada). ISRSs, IRSNs e ADTs aumentam a neurotransmissão serotonérgica, bloqueando o transportador de serotonina nos terminais pré-sinápticos (SERT). IMAOs (Inibidores da monoamina oxidase), SERT (transportador de serotonina), ISRSs (Inibidores Seletivos da Recaptação de Serotonina), ADTs (Antidepressivos tricíclicos), IRSNs (Inibidores da recaptação da serotonina e noradrenalina), 5-HT (5-hidroxitriptamina (serotonina)), 5-HTR (receptores 5-HT). Fonte: GOODMAN; GILMAN, 2012.

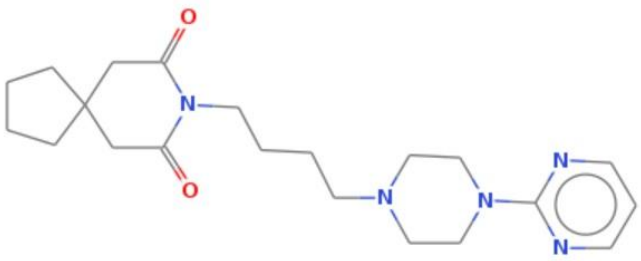


A fluoxetina é extensivamente metabolizada no fígado, principalmente pelas isoenzimas do citocromo P450: CYP2D6 e CYP2C9 à norfluoxetina (HIEMKE; HARTTER, 2000; BRUNTON; LAZO; PARKER, 2006).

1.1.2.3. Buspirona

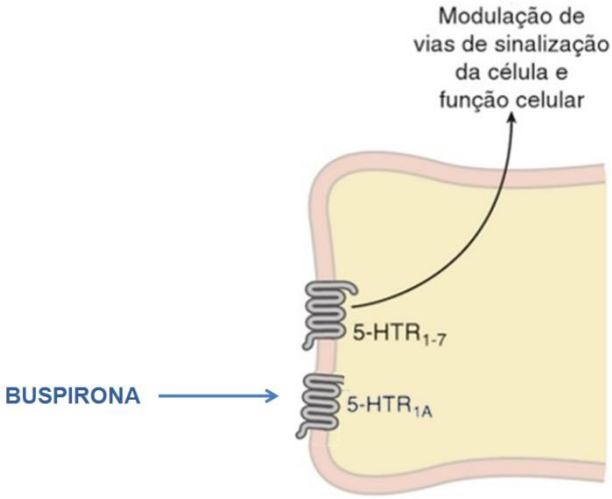
A buspirona (figura 5) é um composto não-benzodiazepínico, pertencente à classe azaspirodecanediona, com propriedades ansiolíticas e sem atividade anticonvulsivante, miorrelaxante e hipnótica (SANTOS et al., 2006; GOA; WARD, 1986). O primeiro fármaco da classe das azapironas foi sintetizado na década de 70 (ANDREATINI et al., 2001; CAIXETA, 1995).

Figura 5. Fórmula estrutural da buspirona. Fonte: http://webbook.nist.gov/cgi/cbook.cgi?Name=Buspirone&Units=SI



A buspirona tem alta afinidade por receptores serotonérgicos do subtipo 5-HT_{1A} (figura 6) e não interage com receptores GABAérgicos (ERHORN, 2008). Além disso, a buspirona produz um aumento na densidade dos receptores D_2 cerebrais na região do corpo estriado, com forte possibilidade de interação entre os sistemas serotonérgico e dopaminérgico (LIMA et al., 2002).

Figura 6. Representação esquemática do mecanismo de ação da buspirona (figura adaptada). 5-HTR (receptores 5-HT). Fonte: GOODMAN; GILMAN, 2012.

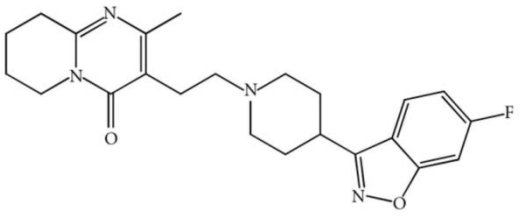


A metabolização da buspirona se da pela enzima CYP3A4 do citocromo P450 (ANDERSON et al., 1996), a qual gera um metabólito ativo, o 1-pirimidinil-piperazina, e um inativo, a 5-hidroxi-buspirona.

1.1.2.4. Risperidona

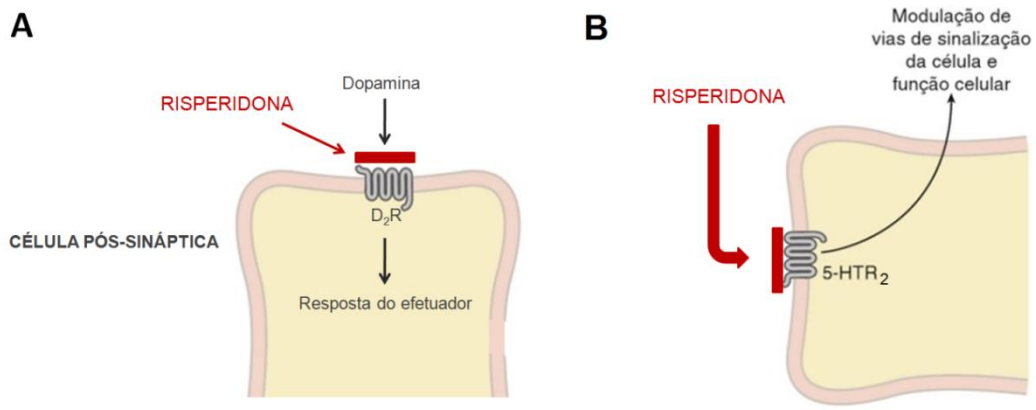
A risperidona é um derivado benzisoxazólico (figura 7), utilizado frequentemente no tratamento da esquizofrenia. A risperidona foi lançada comercialmente em 1993, com o nome de Risperdal®, pela empresa Janssen-Cilag Farmacêutica.

Figura 7. Fórmula estrutural da risperidona. Fonte: https://www.researchgate.net/figure/258398492_fig2_Structure-of-risperidone



A risperidona apresenta efeito bloqueador de receptores de dopamina (D2) (Figura 8A) e serotonina (5-HT_2) (Figura 8B), ligando-se a receptores α_1, α_2, e H1. A risperidona é metabolizada pela CYP2D6 em 9-hidróxi-risperidona. Outra via metabólica da risperidona é a N-desalquilação.

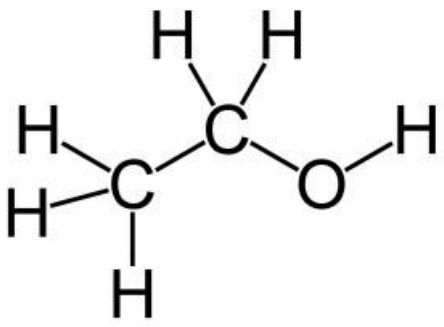
Figura 8. Representação esquemática do mecanismo de ação da risperidona. (A) Bloqueio de receptores de dopamina (D_2R). (B) Bloqueio de receptor de serotonina (5-HT_2) (figura adaptada). 5-HTR (receptores 5-HT). Fonte: O autor.



1.2.2.5. Etanol

Globalmente, estima-se que indivíduos com idade superior a 15 anos consumiram em torno de 6,2 litros de álcool puro (Figura 9) em 2010. No Brasil, o consumo total estimado é equivalente a 8,7 litros por pessoa, quantidade superior à média mundial. Estima-se que no Brasil, os homens consumam 13,6 litros por ano, e as mulheres, 4,2 litros de álcool por ano (OMS, 2014).

Figura 9. Fórmula estrutural do etanol. Fonte: http://www.infoescola.com/quimica/funcoes-organicas



Os efeitos agudos do etanol sobre o SNC são inibitórios, causando relaxamento, redução da ansiedade e sedação. Tais efeitos são mediados por receptores $GABA_A$ (RADCLIFFE et al., 1999; KUMAR et al., 2009), no qual o etanol atua facilitando a inibição GABAérgica (figura 2) e pela redução de glutamato, o principal neurotransmissor excitatório do SNC. A metabolização do etanol inicia-se com a sua oxidação sequencial em acetaldeído e acetato, sofre oxidação e envolve a atividade de uma enzima do citocromo P450, a CYP2E1.

1.1.3. Modelos Animais de Transtornos Mentais

1.1.3.1. Modelos de Ansiedade

O estudo de ansiedade em organismos modelo vem sendo realizado desde a década de 30. No início das pesquisas em psicofarmacologia e ansiedade foram utilizados modelos com ênfase no aprendizado e reflexos condicionados; na década de 50 tiveram-se início os estudos com o enfoque no condicionamento operante (resposta espontânea de animais) (GARCIA, 2007). Assim, várias pesquisas foram realizadas ao longo dos anos, utilizando-se diferentes protocolos para avaliar ansiedade que são classificados de acordo com as respostas condicionadas ou não-condicionadas. Nas condicionadas há o envolvimento da resposta a um estresse e no modelo não-condicionado as reações são espontâneas, ou seja, ocorrem naturalmente. Os modelos utilizados são o teste de campo aberto, que possibilita avaliar a ansiedade do animal através da atividade exploratória em um ambiente não familiar, como uma caixa vazia; o teste de claro/escuro, que avalia o comportamento do animal em uma caixa experimental contendo dois compartimentos, um claro e um escuro, e as transições feitas entre os compartimentos são quantificadas (BOURIN; HASCOËT, 2003), dentre outros. Os modelos de ansiedade mais conhecidos e utilizados em *zebrafish* como, por exemplo, o teste de tanque novo (GIACOMINI et al., 2016; MOCELIN et al., 2015) e o de claro/escuro (MOCELIN et al., 2015) são análogos aos protocolos utilizados em roedores.

1.1.3.2. Modelos de Comportamento Social

O comportamento social é direcionado à interação entre coespecíficos, nas quais são observadas formas de comunicação. Entre os comportamentos sociais, destaca-se o teste de interação social desenvolvido por File e Hyde (FILE, HYDE, 1979), que se baseia em diferentes comportamentos de pares de ratos machos em uma arena iluminada. A frequência e o tempo gasto pelo par de machos em diferentes tipos de interação social podem ser classificados em duas categorias: comportamentos agressivos (agarrar, chutar, boxear ou morder) e comportamentos não agressivos (cheirar e comportamentos de limpeza da face). Nesse teste, ambos os animais são colocados em uma arena com o assoalho marcado em quadrados de forma que se possa quantificar a atividade motora do animal. O aumento na interação social sem o aumento concomitante na atividade motora é um indicador de efeito ansiolítico. Em peixes, como o *zebrafish*, os modelos de interação social mais conhecidos são os testes de preferência de grupo, nos quais os peixes podem ser avaliados de forma individual (GIACOMINI et al., 2016) ou em cardume (GERLAI, 2003), analisando o tempo de aproximação do peixe ao aquário que contém cardume de coespecíficos.

1.1.4. Mecanismos Fisiológicos

1.1.4.1. Anosmia

O sentido olfativo desempenha um papel importante na modulação de comportamentos e em funções cerebrais (WANG et al., 2011). Nos seres humanos, aromas percebidos durante experiências traumáticas podem desencadear ansiedade patológica (HINTON et al., 2004), enquanto que olfato prejudicado por anosmia aguda ou definitiva provoca transtornos afetivos, como ansiedade e depressão (CLEPCE et al., 2012). Déficits olfativos são também associados a muitas outras desordens psiquiátricas tais como a demência, a doença de Parkinson e esquizofrenia (ALBERS et al., 2006; ATANASOVA et al., 2008).

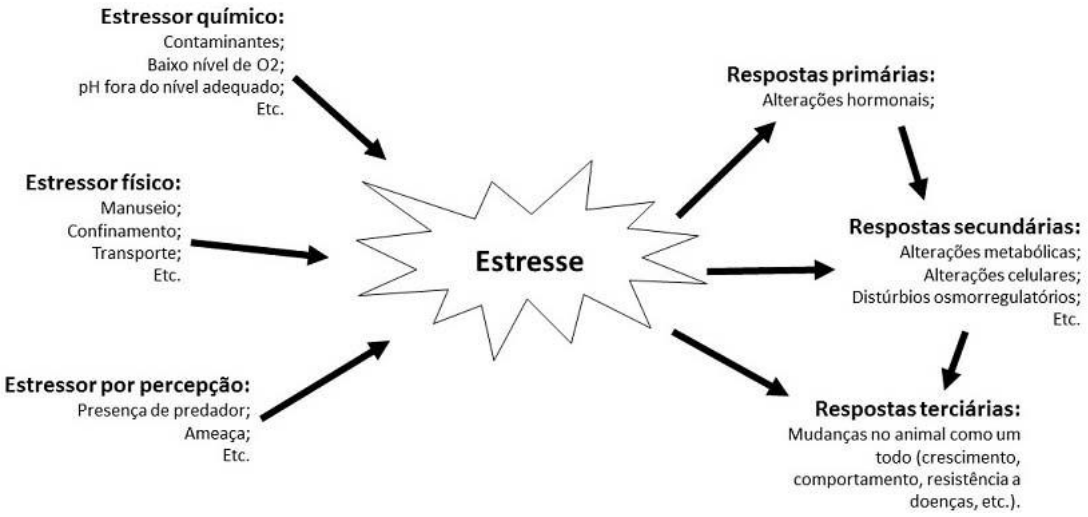
Os roedores são animais macrosmáticos, que dependem do olfato ainda mais fortemente que os seres humanos (RIBEIRO et al., 2014). O olfato desempenha um papel importante na modulação do seu comportamento, incluindo interações sexuais e sociais, defensivas, alimentares e migratórias (DØVING, 1986; KASUMYAN, 2004). A indução de

anosmia como modelo para estudo vem sendo estudada há décadas, com os modelos de anosmia permanente ocasionados por lesão do bulbo olfatório e bulbectomia (HENDRICKS et al., 1994), e anosmia aguda com administração de sulfato de zinco ($ZnSO_4$) (UEBAYASHI et al., 2001) em roedores; em peixes o modelo envolve a anosmia permanente pela bulbectomia (HELLSTRØM; DØVING, 1986).

1.1.4.2. Resposta ao Estresse em Peixes

A resposta ao estresse ocorre pela presença de algum agente estressor (figura 10), que ativa o sistema nervoso simpático e o eixo hipotálamo-hipófise-interrenal (HHI) em peixes. As respostas de estresse são divididas em três categorias: primárias, secundárias e terciárias (figura 10) (BARTON et al., 2002a). As respostas primárias são as hormonais, as secundárias são mudanças nos parâmetros fisiológicos e bioquímicos e as terciárias são as do comprometimento no desempenho, as mudanças no comportamento e o aumento da suscetibilidade a doenças. O estímulo estressor ativa os neurônios hipotalâmicos, os quais liberam o hormônio liberador de corticotrofina (CRH) para a circulação, esse hormônio atua na hipófise, estimulando a liberação do hormônio adrenocorticotrófico (ACTH), que atua no córtex da glândula interrenal, estimulando a liberação de cortisol (RAMSAY et al., 2006; 2009).

Figura 10. Diferentes estressores e respostas de estresse em peixes (figura adaptada). Fonte: BARTON, 2002b.



Além das alterações fisiológicas, a resposta ao estresse promove alterações comportamentais em diversas espécies (SACKERMAN et al., 2010; GEBAUER et al., 2011; SNYDER et al., 2011; PARKER et al., 2012; PAGNUSSAT et al., 2013; LEE et al., 2014; MASANA et al., 2014; ZHANG et al., 2014). A exposição ao estresse promove consequências negativas no comportamento social de camundongos (MASANA et al., 2014); prejudica a função cognitiva, aumenta a ansiedade e agressividade e diminui a interação social em *zebrafish* (PIATO et al., 2011; GIACOMINI et al., 2016). Além disso, tal resposta a agentes estressores pode ser dependente do tipo de linhagem estudada, bem como variar de acordo com o contexto em que é empregado (QUADROS et al., 2016).

1.1.4.3. Osmorregulação

Osmorregulação é a capacidade que alguns animais possuem em manter o equilíbrio iônico, extremamente importante para a homeostase dos peixes. Os peixes de água doce são hiperosmóticos em relação ao ambiente aquático e, consequentemente, apresentam uma entrada excessiva de água por osmose e perda de íons por difusão (BALDISSEROTTO et al., 2008). As alterações nas concentrações iônicas da água podem interferir no comportamento dos peixes, como na reprodução, crescimento e, em situações extremas, sobrevivência (ALMEIDA et al., 2013). Além disso, outros fatores podem vir a comprometer a osmorregulação, como a temperatura, composição da água, níveis de oxigênio dissolvido, e a possibilidade de interferência de fatores externos como estresse (BALDISSEROTTO et al., 2008; BALDISSEROTTO; VAL, 2002; ROSSO et al., 2006).

1.1.5. Relação Intraespecífica e Interespecífica

Nos ecossistemas aquáticos, a comunicação intraespecífica e interespecífica é imprescindível para o equilíbrio dos seres vivos que compõem as comunidades. Os peixes podem comunicar quimicamente ao grupo (coespecíficos) a ocorrência de situação de risco através de substâncias químicas (substância de alarme) produzidas e armazenadas em células epidermais *club* e liberadas na água como resultado da injúria na pele (CHIVERS; SMITH, 1998; KORPI; WISENDEN, 2001; OLIVEIRA et al., 2014), ou pela presença de sangue na água (BARRETO et al., 2013). Entretanto, os estudos sobre a comunicação química frente ao

contato indireto, onde substâncias de distúrbio são liberadas sem a necessidade de lesão, ainda são escassos. Barcellos et al. (2014) verificaram que o contato com o predador promove um aumento no cortisol em indivíduos da mesma espécie por sinais químicos e não visuais (BARCELLOS et al. 2014). Nesse tipo de comunicação, tanto o reconhecimento do risco quanto a capacidade para liberar a substância utilizada em comunicação, são essenciais (JORDÃO; VOLPATO, 2000; BARCELLOS et al. 2011).

1.1.6. Comunicação Química

A comunicação química entre indivíduos da mesma espécie é interpretada como um mecanismo adaptativo que promove a elevação do cortisol em antecipação da ameaça, ampliando a consciência do animal em relação ao meio ambiente (CHIVERS; SMITH, 1998; OLIVEIRA et al. 2014; BARCELLOS et al. 2007; 2010; 2011; 2014; KATS; DILL, 1998). Na relação interespecífica, o contato com o predador pode ser considerado como uma situação de estresse que, além das alterações comportamentais, pode induzir respostas fisiológicas como ativação do eixo neuroendócrino hipotálamo-hipófise-interrenal e, consequentemente, elevação do cortisol no peixe presa. Essa ativação pode se dar a partir do contato direto com o predador por ação das substâncias de alarme (KORPI; WISENDEN, 2001) ou pelo reconhecimento da situação de risco em contatos indiretos com predadores através da ação das substâncias distúrbios (JORDÃO; VOLPATO, 2000), as quais alteram o comportamento e também ativam o eixo HHI em peixes sem nenhum contato direto com o predador (BARCELLOS et al., 2007; 2010; 2011; 2014).

1.1.7. Organismos modelo

1.1.7.1. Zebrafish

O *zebrafish* (*Danio rerio*), também conhecido como peixe-zebra ou paulistinha, é um pequeno teleósteo tropical de água doce, pertencente à família Cyprinidae, de origem asiática (ARUNACHALAM et al., 2013; ENGESZER et al., 2007). Desde o início do século XX, o *zebrafish* vem sendo utilizado como organismo modelo para pesquisas em diversas áreas do conhecimento, entre elas: fisiologia, toxicologia, genética, cardiovascular, embriologia,

metabolismo, oncologia; o que se justifica em razão da homologia genética com seres humanos (HOWE et al., 2013). O *zebrafish* tem sido utilizado também como organismo modelo em neurociências, em estudos sobre doenças neurodegenerativas, comportamento e testes de candidatos a fármacos (BARBAZUK et al., 2000; SHIN; FISHMAN, 2002; MUELLER et al., 2004; GOLDSMITH, 2004; ZON; PETERSON, 2005; EGAN et al., 2009). Com sua elevada homologia, a espécie apresenta todos os neurotransmissores clássicos dos vertebrados (MUELLER et al., 2004) e o sistema neuroendócrino exibe resposta robusta ao estresse (ALSOP; VIJAYAN, 2009), sendo evolutivamente conservado.

1.1.7.2. Jundiá

O jundiá (*Rhamdia quelen*) é um peixe nativo da América do Sul, podendo ser encontrado desde o México até o centro da Argentina, pertencente à família Heptapteridae (SILFVERGRIP, 1996). O jundiá é uma espécie substancial para a aquicultura, uma vez que apresenta capacidade de suportar as baixas temperaturas da região Sul do Brasil durante o inverno, bem como ter seu crescimento potencializado durante o verão (SOSO et al., 2007). É um peixe onívoro de leve tendência carnívora, mas também se alimenta de plâncton e bentos, tendo uma alta preferência por proteína de origem animal (GOMES et al., 2000); habita lagos e fundos de rios, preferindo ambientes com águas mais calmas, com fundo de areia e lama, próximos a vegetação da margem (BALDISSEROTTO; RADÜNZ, 2004). Além disso, o jundiá tem recebido grande atenção por pesquisadores da América do Sul, que têm estudado seus aspectos reprodutivos, como a concentração de esteroides (testosterona) relacionada ao ciclo reprodutivo em machos (BARCELLOS et al., 2002); resposta ao estresse em exposição a um estressor agudo em diferentes estágios de desenvolvimento (KOAKOSKI et al., 2012); assim como metabolismo, no qual avaliam os efeitos do jejum sobre cortisol, glicose, glicogênio hepático e muscular em adultos (BARCELLOS et al., 2010).

1.2. PROPOSIÇÃO

1.2.1. Proposição Geral

Avaliar se os peixes são capazes de identificar diferentes situações estressoras e fármacos, analisando os efeitos neuroendócrinos, comportamentais e osmorregulatórios.

1.2.1.1.Proposições Específicas

- Avaliar se o estresse altera a osmorregulação e se a fluoxetina reverte o efeito do estresse.
- Verificar se a fluoxetina pode modular as diferentes respostas de estressores (físicos ou químicos) em *zebrafish*.
- Identificar a percepção individual a diferentes situações de coespecíficos.
- Verificar se os peixes expostos a diferentes fármacos detectam a presença desses na água, e se presença é detectada pela via olfatória.
- Identificar se a anosmia altera o comportamento tipo-ansiedade em peixes.
- Examinar se a anosmia experimental, por $ZnSO_4$, pode estar associada a respostas comportamentais e fisiológicas.
- Avaliar os efeitos do estresse agudo sobre comportamento social e ansiedade em jundiás.

1.3. JUSTIFICATIVA

Com base na introdução, referencial teórico e proposições expostas, justifica-se a continuidade do foco das pesquisas sobre impactos ambientais causados pela contaminação por fármacos na água em respostas neuroendócrinas, osmorregulatórias e comportamentais; bem como seus mecanismos de percepção e de comunicação química em situações estressantes; além de estudos de novos organismos modelos para análises comportamentais. Esse conhecimento torna-se indispensável, tanto nas alterações causadas em peixes quanto em todo ambiente.

1.4. MATERIAIS E MÉTODOS

1.4.1. Animais e condições de laboratório

Os estudos foram desenvolvidos no Laboratório de Fisiologia de Peixes, da Universidade de Passo Fundo (UPF). Foram utilizados 1522 *zebrafish* (*wild type*), de ambos os sexos com peso entre 0,4 e 0,9 gramas, além de 192 jundiás de ambos os sexos com peso entre 5 ± 1,5 gramas. Os peixes permaneceram na densidade de 0,5 gramas de peso corporal para cada litro de água. *Zebrafish* permaneceram sob fotoperíodo de 14 h claro: 10 h escuro, e aeração constante, foram mantidos a temperatura média de 26 ± 2^{o}C, recebendo ração comercial Alcon® (Alcon® Basic, MEP 200 Complex, Brasil) duas vezes ao dia. Jundiás permaneceram sob fotoperíodo natural e aeração constante, mantidos sob a temperatura de 24 ± 2^{o}C, recebendo ração comercial extrusada (42% de proteína, 3400 kcal kg^{-1} energia digestiva, Brasil) duas vezes ao dia.

1.4.2. Aspectos éticos

Os estudos foram aprovados pela Comissão de Ética no Uso de Animais (CEUA) da Universidade de Passo Fundo, registro nº 012/2012, 029/2013, 010/2014, 029/2014, e 017/2016 (Anexos A - E).

1.4.3. Estudos desenvolvidos

A avaliação do efeito da fluoxetina na água sob a resposta ao estresse e fluxos iônicos em *zebrafish* (artigo 1), foi realizada a partir dos seguintes experimentos: 1) Tempo de curso da resposta ao estresse e osmorregulação; 2) Resposta ao estresse em peixes expostos a fluoxetina; 3) Osmorregulação em peixes expostos a fluoxetina. Ao final dos experimentos, os peixes foram coletados para análise de cortisol de corpo inteiro pelo método descrito por Sink e colaboradores (SINK; LOCHMANN; FECTEAU, 2007), e a água foi coletada para análise de fluxos iônicos (concentrações de Na^{+} e K^{+}) pelo método de descrito por Baldisserotto e colaboradores (BALDISSEROTTO et al., 2008).

A identificação da modulação da fluoxetina sobre as diferentes respostas à estressores (físicos ou químicos) em *zebrafish* (artigo 2) foi feita a partir dos seguintes experimentos: 1) Teste da resposta ao estresse sob estímulos físicos; 2) Teste da resposta ao estresse sob estímulos químicos. A análise de cortisol foi feita pelo método descrito por Sink e colaboradores (SINK; LOCHMANN; FECTEAU, 2007).

A avaliação da percepção individual a diferentes situações de coespecíficos (artigo 3), que foi realizada a partir dos seguintes experimentos: 1) Teste de percepção de estresse físico, químico e contato visual com um predador; 2) Teste de percepção de estresse por privação alimentar. A aferição dos comportamentos foi feita por teste validado para análise de preferência de anestésicos em *zebrafish* (READMAN et al., 2013).

A verificação da atratividade ou aversividade dos peixes por psicofármacos, e da percepção olfatória (artigo 4) foi realizada a partir dos seguintes experimentos: 1) Teste de atração e aversão com aparato quimiotáxico; 2) Teste de atração e aversão com peixes anósmicos. A aferição dos comportamentos foi feita por teste validado para análise de preferência de anestésicos em *zebrafish* (READMAN et al., 2013). A anosmia temporária foi induzida pela aplicação de lidocaína em gel (50 mg/g) nas narinas e superfície olfativa como descrito por Johansen (JOHANSEN, 1985).

A avaliação dos efeitos da anosmia experimental induzidos pela lidocaína sobre o comportamento tipo-ansiedade e os níveis de cortisol em *zebrafish* (artigo 5) foi desenvolvida por análise do comportamento tipo-ansiedade validado em *zebrafish* (KALUEFF et al., 2013), e análise de cortisol pelo método descrito por Sink e colaboradores (SINK; LOCHMANN; FECTEAU, 2007).

A identificação das respostas comportamentais e fisiológicas associadas à anosmia experimental, evocada por $ZnSO_4$ em *zebrafish* adultos (artigo 6) foi desenvolvida a partir dos seguintes experimentos: 1) Teste comportamental no labirinto em Y 1 e 24 h após anosmia; 2) Teste comportamento tipo-ansiedade 1, 24 e 72 h após anosmia; 3) análises histopatológicas e de cortisol 1 h após anosmia. A aferição do comportamento tipo-ansiedade foi feita por teste e análise validados em *zebrafish* (KALUEFF et al., 2013), e análise de cortisol pelo método descrito por Sink e colaboradores (SINK; LOCHMANN; FECTEAU, 2007). As alterações histológicas no epitélio olfatório, inervações nasais e bulbo olfatório foram analisadas a partir do preparo do material com tamponamento (formalina 10%), após descalcificadas, desidratadas (série de etanol graduado), em seguida, incorporadas em parafina; a partir disso coradas com hematoxilina-eosina e examinadas em microscópio óptico AxioCam Erc5 e programa Axio SV40 4.8.2.0 (Carl Zeiss Microscopy GmbH, Jena, Alemanha).

A verificação dos efeitos do estresse agudo sobre comportamento social e ansiedade em jundiás (artigo 7) foi realizada a partir do seguinte experimento: teste de estresse agudo e avaliação comportamental. A aferição dos comportamentos foi feita por teste validado para comportamento tipo-ansiedade e social em *zebrafish* (GIACOMINI et al., 2016).

Os comportamentos de todos os testes (Artigo 3-7) foram filmados por câmera Logitech HD Webcam C525 (Logitech, Romanel-sur-Morges, Suiça), e os vídeos analisados posteriormente com o programa AnyMaze® (Stoelting CO, USA).

1.4.4. Fármacos testados e tempo de exposição

Foram utilizados clonazepam (Rivotril®, ROCHE, Brasil), diazepam (União Química, Brasil), fluoxetina (Daforin, EMS, Brasil), risperidona (Risperidona, EMS, Brasil), buspirona (Ansitec®, LIBBS, Brasil), lidocaína (Lidocaína Gel, EMS, Brasil), etanol, ácido tricloroacético e sulfato de zinco adquiridos a partir de fornecedor comercial.

As concentrações utilizadas nos estudos foram: clonazepam 0,057 e 300 µg /L; diazepam 0,88, 16 e 160 µg /L; etanol 0,25%, 0, 5% e 1%; fluoxetina 1, 25 e 50 µg /L; risperidona 0,00034, 100 e 170 µg/L; buspirona 10, 1000 e 3000 µg/L.

A exposição aos fármacos no teste de atração e aversão (aparato quimiotáxico, artigo 2) foi de 150 segundos, tempo utilizado para a avaliação de preferência de anestésicos (READMAN et al., 2013). Nos experimentos de avaliação de resposta ao estresse e osmorregulação (artigo 1), modulação de diferentes respostas aos estressores (físicos ou químicos) (artigo 2), comportamento tipo-ansiedade (artigo 5), comportamento social e "ansiedade" em jundiás (artigo 7); a exposição à fluoxetina foi de 15 minutos.

1.4.5. Delineamentos, procedimentos e análise estatística

Os detalhes referentes aos delineamentos experimentais de cada estudo desenvolvido, bem como os procedimentos específicos e análise estatística estão descritos nos respectivos artigos.

2. ARTIGOS

2.1. ARTIGO 1 - *Effects of waterborne fluoxetine on stress response and osmoregulation in zebrafish*

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Autores: Murilo S. Abreu, Ana Cristina V. Giacomini, Gessi Koakoski, Thiago A. Oliveira, Darlan Gusso, Bernardo Baldisserotto, Leonardo J.G. Barcellos.

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Short communication

Effects of waterborne fluoxetine on stress response and osmoregulation in zebrafish

Murilo S. Abreu[a], Ana Cristina V. Giacomini[a,b], Gessi Koakoski[a], Thiago A. Oliveira[a], Darlan Gusso[b], Bernardo Baldisserotto[a], Leonardo J.G. Barcellos[a,b,*]

[a] Universidade Federal de Santa Maria (UFSM), Programa de Pós-Graduação em Farmacologia, Campus Universitário, Camobi, Santa Maria, RS, Brazil
[b] Universidade de Passo Fundo (UPF), Programa de Pós-Graduação em Bioexperimentação, Campus I, Bairro São José, Caixa Postal 611, Passo Fundo, RS, Brazil

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ABSTRACT

The presence of fluoxetine in aquatic environments has been reported for decades. Here, we investigate the effects of exposure to fluoxetine on the stress response and osmoregulation in zebrafish. We show that stress response alters osmoregulation and that fluoxetine inhibits these stress-related changes in osmoregulation. The results suggest that the presence of fluoxetine in aquatic ecosystems can cause changes in response to stress and osmoregulation in fish.

1. Introduction

Pharmaceuticals in aquatic environments have been present in detectable amounts since the 1970s (Calisto and Esteves, 2009). The presence of drugs, with varying mechanisms of action, in water may negatively aquatic organisms inhabiting these ecosystems (Daughton and Ternes, 1999). Selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine are found in the water of these ecosystems, with concentrations ranging from 0.012 to 1 μg/L (Kolpin et al., 2002). Fluoxetine exerts anxiolytic effects and can interfere with the neuroendocrine stress axis activity (Abreu et al., 2014).

Stress responses are physiological changes triggered when fish first react, characterized by elevated levels of cortisol (Ramsay et al., 2009), which is an indicator of stress in fish. Cortisol plays a role in various biological processes, such as immune function, metabolism, reproduction, and osmoregulation (Jentoft et al., 2005). Osmoregulation is extremely important for homeostasis in fish. Changes in the ionic concentration of the water can interfere with reproduction, growth, stress, and in extreme situations, survival (Almeida et al., 2013). Zebrafish, *Danio rerio*, has been widely used in several studies because it is genetically homologous to humans (Barbazuk et al., 2000). This study aimed to evaluate the effect of waterborne fluoxetine on acute stress and ionic fluxes in zebrafish.

2. Materials and methods

2.1. Ethic aspect

This study was approved by the Ethics Commission for Animal Use of Universidade de Passo Fundo, Passo Fundo, RS, Brazil (Protocol #20/2013) and meets the guidelines of the National Council for Animal Research Control.

2.2. Subjects

Two hundred adult zebrafish (wild type) of both sexes, weighing 0.8 ± 0.1 g, were bred and maintained in 60 L aquaria with

* Corresponding author at: Universidade de Passo Fundo (UPF), Programa de Pós-Graduação em Bioexperimentação, Campus I, Bairro São José, Caixa Postal 611, Passo Fundo, RS, Brazil.
E-mail addresses: abreu_murilo@hotmail.com (M.S. Abreu), anacvg@upf.br (A.C.V. Giacomini), gessikoakoski@yahoo.com.br (G. Koakoski), thiago_a.oliveira@hotmail.com (T.A. Oliveira), gusso.d@hotmail.com (D. Gusso), bbaldisserotto@hotmail.com (B. Baldisserotto), lbarcellos@upf.br, lbarcellos@upf.br (L.J.G. Barcellos).

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aerated water (DO 7.0 ± 0.5 mg/L, temperature 27 ± 1 °C, pH 7.1 ± 0.2). Stress response and osmoregulation evaluation was based on only peak measurements (Koakoski et al., 2014). Based on established animal welfare guidelines, we selected the one point evaluation methodology to prevent the death of more fish than necessary.

2.3. Experiment 1: time course of stress response and osmoregulation

In this experiment, we determined the peak cortisol levels in zebrafish and osmoregulation after stress. Fish were transferred to individual chambers with 35 mL each (water from a stock tank with DO, temperature and pH identical to fish tank), distributed into six experimental groups: C15, C60, and C240 groups, control without stress maintained for 15, 60, or 240 min in the chamber, respectively; and St15, St60, and St240, acute stress maintained for 15, 60, or 240 min in the chamber, respectively (20 fish per group). The standard acute stressor (air exposure for 60 s) was applied prior to transfer to the individual chambers. Water samples were collected at the beginning of the experiment and at the end of each period.

2.4. Experiment 2: stress response in fluoxetine exposed fish

In this experiment, we aimed to evaluate fluoxetine action at peak cortisol level. Fish were exposed to fluoxetine 1 μg/L (Kolpin et al., 2002) in groups of six fish in a 10-L aquaria for 15 min. (Abreu et al., 2014) (control were not exposed to fluoxetine), transferred to individual chambers, and distributed into four experimental groups: C15 and F15, control and fluoxetine maintained for 15 min in the chamber; and St15 and FSt15, acute stress and fluoxetine maintained for 15 min in the chamber (20 fish per group).

2.5. Experiment **3**: osmoregulation in fluoxetine exposed fish

In this experiment, we aimed to evaluate fluoxetine action at peak change osmoregulation. Fish were exposed to fluoxetine 1 μg/L (Kolpin et al., 2002) for 15 min (control were not exposed to fluoxetine), transferred to individual chambers, and distributed

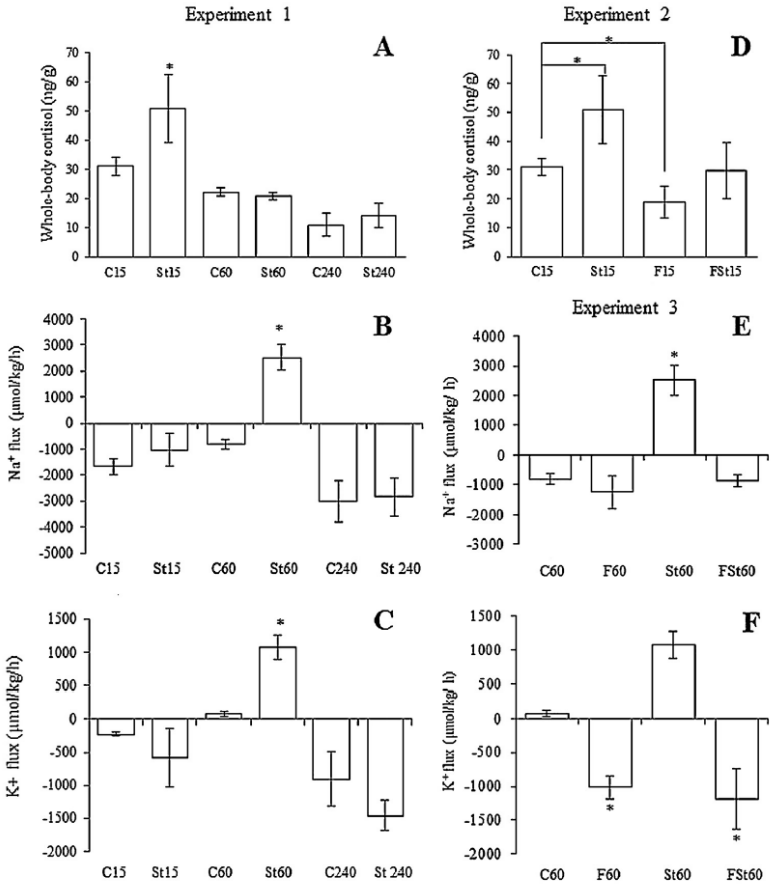

Fig. 1. Whole-body cortisol levels and ion fluxes in stressed (experiment 1) and fluoxetine-exposed zebrafish (experiments 2 and 3). Acute stress increased cortisol levels 15 min after stress (A, $P < 0.001$) and induces influx of Na^+ (B, $P = 0.0061$) and K^+ (C, $P = 0.026$) 60 min after stress. Fluoxetine suppressed the cortisol increase (D, $P < 0.001$). Fluoxetine reversed the influx of Na^+ (E, $P = 0.001$) and K^+ (F, $P = 0.0012$) caused by acute stress. Two-way ANOVA, with acute stress and time after stress as independent variables, followed by a Bonferroni post-test. Bars expressed as means ± SEM.

into four experimental groups: C60 and F60, control and fluoxetine maintained for 60 min in the chamber; and St60 and FSt60, acute stress and fluoxetine maintained for 60 min in the chamber (20 fish per group).

2.6. Whole-body cortisol analysis

After the period in the chambers, the fish were sampled for determining cortisol levels. Whole-body cortisol was extracted using the method described by Sink et al. (2007).

2.7. Net fluxes analysis

Water samples were collected at the start of the experiment and at the end of each period (15, 60, and 240 min) for analysis of net fluxes. Na^+ and K^+ concentrations in water were measured using the method described by Baldisserotto et al. (2008).

2.8. Statistics

Whole-body cortisol concentrations and Na^+ and K^+ fluxes were compared using a two-way ANOVA (with or without acute stress occurrence and "time after stress" as independent variables [Fig. 1(A)–(C)]; with or without acute stress and fluoxetine as independent variables [Fig. 1(D)–(F)]), followed by Bonferroni post-test correction. Differences with $P < 0.05$ were considered statistically significant. Hartley test was carried out to verify the homogeneity of variances, and Kolmogorov–Smirnov test was used to check for normality of the data.

3. Results

3.1. Experiment 1

Zebrafish demonstrated an increase in the whole-body cortisol levels 15 min after acute stress (Fig. 1(A), $P < 0.001$, $F_{2,38} = 12.4$). Acute stress induces influx of Na^+ (Fig. 1(B), $P = 0.0061$, $F_{2,37} = 5.881$) and K^+ (Fig. 1(C), $P = 0.026$, $F_{2,35} = 4.016$) 60 min after stress.

3.2. Experiment 2

Fluoxetine suppressed the stress response and decreased cortisol levels *per se* (Fig. 1(D), $P < 0.001$, $F_{1,26} = 24.66$).

3.3. Experiment 3

Fluoxetine reversed the influx of Na^+ (Fig. 1(E), $P = 0.001$, $F_{1,22} = 14.27$) and K^+ (Fig. 1(F), $P = 0.0012$, $F_{1,20} = 7.58$) caused by acute stress.

4. Discussion

In this study, we show that acute stress alters ionic flux in adult zebrafish and that acute exposure to fluoxetine blocks stress response, consequently, inhibiting the stress-related changes in osmoregulation. The concentration of whole body cortisol measured after 60 and 240 min is similar in both the control values, revealing a peak of cortisol after 15 min stress in the cortisol response during the time of acute stress in zebrafish (Ramsay et al., 2009). The waterborne fluoxetine decreased the peak cortisol values, showing its blocking effect on the stress neuroendocrine axis (Abreu et al., 2014).

It was observed absorption of Na^+ and K^+ in zebrafish 60 min after stress (Fig. 1(C) and (E)), recorded on zebrafish (Cruz et al., 2013), alteration of osmoregulation in zebrafish. Possibly the peak change in osmoregulation occurs 60 min after acute stress. Studies have shown that cortisol stimulates Na^+, Cl^-, and Ca^{2+} uptake (Kumai et al., 2012) and increases the area of chloride cells in the gills.

In fact, one of the mechanisms by which cortisol interferes with osmoregulation processes is chloride cell differentiation and stimulation of gill Na^+/K^+-ATPase (Dang et al., 2000). Exposure to 500 nM cortisol over two days significantly increased Na^+ absorption in zebrafish (Kumai et al., 2012).

Waterborne fluoxetine reduces the influx of Na^+ and K^+ caused by stress. Fluoxetine blocks stress response, as observed by the lower whole-body cortisol levels, thereby blocking the osmoregulatory effects triggered by stress. In this line, previous studies from Gebauer et al. (2011) and Abreu et al. (2014) have demonstrated that short-term exposures (15 min) to fluoxetine are sufficient to elicit their effects on central nervous system.

5. Conclusion

Our results suggest that the presence of waterborne fluoxetine in aquatic ecosystems may promote ecologically important changes in the stress and osmoregulatory responses in zebrafish.

Conflict of interest

None declared.

Transparency document

The Transparency document associated with this article can be found in the online version.

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2.2. ARTIGO 2 - *Divergent effect of fluoxetine on the response to physical or chemical stressors in zebrafish*

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PeerJ

Divergent effect of fluoxetine on the response to physical or chemical stressors in zebrafish

Murilo S. Abreu[1,*], Ana Cristina V.V. Giacomini[1,2,*], Gessi Koakoski[1,*], Angelo L.S. Piato[3,*] and Leonardo J.G. Barcellos[1,4,*]

[1] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, Santa Maria, Rio Grande do Sul, Brazil
[2] Universidade de Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil
[3] Programa de Pós-Graduação em Farmacologia e Terapêutica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil
[4] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil
* These authors contributed equally to this work.

ABSTRACT

Fluoxetine is a selective serotonin reuptake inhibitor that increases serotonin concentration in the central nervous system and modulates various systems, including the control of sympathetic outflow and the hypothalamus–pituitary–adrenal. However, it is not yet established whether fluoxetine can modulate the responses to stressors stimulants (physical or chemical) that trigger cortisol response in zebrafish. We demonstrate that fluoxetine blunts the response to physical stress, but not to chemical stress.

Subjects Aquaculture, Fisheries and Fish Science, Neuroscience, Pharmacology
Keywords Cortisol, Serotonin, SSRI, Blood, Alarm substance, Stress response, Physical stressors, Chemical stressors

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Corresponding author
Leonardo J.G. Barcellos,
lbarcellos@upf.br

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INTRODUCTION

Fluoxetine (FLU), a selective serotonin reuptake inhibitor (SSRI), increases serotonin concentration in the central nervous system (*Wong, Bymaster & Engleman, 1995*). Serotonin is one of the major neurotransmitters in the central nervous system and modulates various systems, including the control of sympathetic outflow and the hypothalamus–pituitary–adrenal axis (HPA), via serotonergic fibers that innervate structures such as the hippocampus, prefrontal cortex, amygdala, and hypothalamus (*Lowry, 2002*). SSRIs and cognitive–behavioral therapy are both effective treatments for generalized anxiety disorder, and are known to reduce the peak of cortisol in older adults (*Rosnick et al., 2016*). FLU has been shown to blunt the cortisol response (*Abreu et al., 2014*) and, as a consequence, prevent stress-related osmoregulation changes in zebrafish (*Abreu et al., 2015*). In addition, fluoxetine reverses the anxiogenic effects of acute (*Giacomini et al., 2016*) and chronic (*Marcon et al., 2016*) stress in this species.

Stress depends on a stressor stimulus to occur, and in mammals it triggers a stimulatory process in the hippocampus and amygdala (*LeDoux, 2000*, *2007*). In the hypothalamus, stress stimulates the release of corticotropin-releasing factor, which is the key

neurotransmitter regulating the release of adrenocorticotropic hormone from the pituitary, which in turn induces the release of glucocorticoids (cortisol) from the adrenal. In teleost fish like in zebrafish, the hypothalamic–pituitary–interrenal axis is the HPA axis homolog (*Wendelaar Bonga, 1997*).

Stress stimuli can be varied (e.g., social, physical, chemical), such as exposure to neighborhood-level violence, which can influence physiological and cellular markers of stress, even in children (*Theall et al., 2017*). In addition, physical stimuli elicit robust stress responses in fish (*Perry, Reid & Salama, 1996*). Physical stressors such as chasing have been used as standardized stressors (*Abreu et al., 2014*; *Giacomini et al., 2015*, *2016*), and spatial restriction is used as a stress model for behavioral assessment in zebrafish (*Piato et al., 2011*; *Ghisleni et al., 2012*). Stressor stimulus can also be chemical, such as alarm substances, originally described in the minnow (*Phoxinus phoxinus*) (*Frisch, 1941*), which are produced and stored in epidermal "club" cells (*Barbosa et al., 2012*) and are released into the water after skin injuries as those provoked by predator attack (*Chivers & Smith, 1998*; *Korpi & Wisenden, 2001*). Alarm substance is known to induce fear responses in a range of fish species (*Pfeiffer, 1977*). Moreover, blood (*Barreto et al., 2013*) and diamines (putrescine and cadaverine) (*Hussain et al., 2013*) have also been documented as potential chemical stressors. However, it is not yet established whether FLU can modulate the responses to different modalities of stressor stimuli (physical or chemical) that trigger cortisol response in zebrafish.

MATERIALS AND METHODS

Experimental animals

A stock population of 200 mixed-sex (50/50) 180-day-old wild-type zebrafish (*Danio rerio*), weighing 0.45 ± 0.05 g, short-fin (SF) strain, was maintained in two tanks equipped with biological filters, under constant aeration, and with a natural photoperiod (approximately 14 h light:10 h dark). Water temperature was maintained at 26 ± 1 °C; pH at 7.0 ± 0.2; dissolved oxygen at 6.1 ± 0.2 mg/L; total ammonia at <0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22 mg/L $CaCO_3$. This study was approved by the Ethics Commission for Animal Use (CEUA) of Universidade de Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil (Protocol #29/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Experimental protocol

Our aim was to verify whether FLU modulates cortisol changes induced by physical and chemical stressors in zebrafish. After a 15-day period for acclimation to laboratory conditions, fish were randomly distributed into two groups, i.e., untreated fish (control group) and fish exposed to FLU. The latter group was exposed to FLU (Daforin®, EMS, Brazil, São Bernardo do Campo) at a concentration of 50 µg/L for 15 min. before the stressor stimuli (Fig. 1); this concentration and duration of exposure were previously shown to elicit behavioral responses (*Giacomini et al., 2016*) and decrease cortisol response in acute chasing stress (*Abreu et al., 2014*).

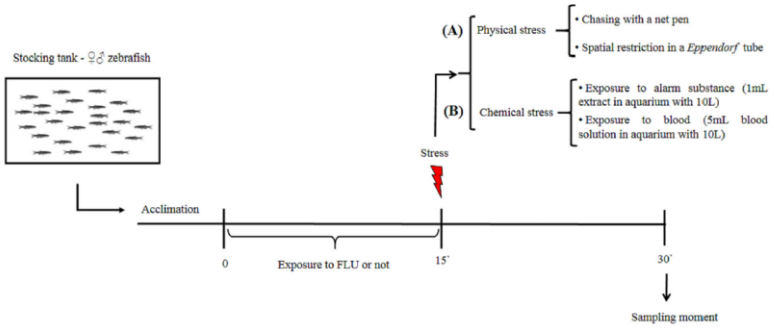

Figure 1 **Schematic representation of the experimental design.**

Physical stimuli on stress response

To evaluate the physical stress response, we then subdivided control and treated fish into groups of 10 animals (duplicate) that were submitted or not to the following types of physical stress: chasing with a net (duration 2 min, and waiting to complete 15 min to sampling); spatial restriction in a microtube (duration 15 min) (Fig. 1A). After the 15 min of exposure to each stressor, fish were captured, euthanized by decapitation with medulla sectioning and immediately frozen in liquid nitrogen for storage at −80 °C until cortisol extraction (Fig. 1). This time interval was based on previous studies showing that cortisol levels peak 15 min following presentation of a stressor stimulus (*Abreu et al., 2014*; *Idalencio et al., 2015*; *Ramsay et al., 2009*).

Chemical stimuli on stress response

To evaluate the chemical stress response, we then subdivided control and treated fish into groups of 10 animals (duplicate) that were submitted or not to the following types of chemical stress: exposure to conspecific blood (duration 15 min); and exposure to alarm substance of conspecifics (duration 15 min). Exposure to blood (5 mL, extracted from zebrafish and jundia (*Rhamdia quelen*)—the use of jundia blood was due to the low yield of zebrafish blood extraction) was in a 10 L aquarium (*Barreto et al., 2013*); and exposure to alarm substance of conspecifics (*Speedie & Gerlai, 2008*) (1 mL, zebrafish) was in a 10 L aquarium (*Barreto et al., 2010*). After 15 min of exposure to each stressor, fish were captured, euthanized, and stored as described above (Fig. 1B). For collection of fish blood (zebrafish and jundia), fish were anesthetized by eugenol (400 mg/L), the anesthesia occurred in less than 1 min and determined by total loss of opercular movement followed by cardiac arrest; then the caudal peduncle was sectioned for the collection of blood. For extraction of alarm substance, fish were quickly killed by medulla sectioning, then shallow cuts were made on each side of fish and the cuts were washed with distilled water; at the end of the process a total of 100 mL of alarm substance in solution were collected (*Speedie & Gerlai, 2008*).

Cortisol analysis

Whole-body cortisol levels were determined using the method described by *Sink, Kumaran & Lochmann (2007)*. Fish were weighed, minced, and homogenized with phosphate buffered saline (pH 7.3). Samples were transferred into tubes with ether, vortexed, centrifuged, and then immediately frozen in liquid nitrogen (three times this last process). The unfrozen portion (ethyl ether containing cortisol) was decanted and transferred to a new tube and completely evaporated, yielding a lipid extract containing the cortisol. The samples were then placed on the plate of enzyme-linked immunosorbent assay kit. 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 = measured value × 1.0604. Whole-body cortisol levels were measured in duplicate for each extraction using the commercially available enzyme-linked immunosorbent assay kit (EIAgen CORTISOL test, BioChem Immunosystems, Rome, Italy). Reading was carried out in microplate reader equipment (ASYS UVM 340, ASYS, Chorley, UK).

Statistical analysis

After testing the homogeneity of variance and normality of data (Hartley and Kolmogorov–Smirnov tests, respectively), we compared the whole-body cortisol levels using two-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Differences were considered statistically significant at $p < 0.05$. The data are expressed as mean + SEM.

RESULTS

Physical stimuli on stress response

Fish exposed to physical stressors (spatial restriction or chasing) displayed an increase in cortisol levels, and FLU blunted the increase in cortisol levels in fish subjected to physical stressors (Fig. 2). Two-way ANOVA revealed significant interaction between the factors ($F_{2,\ 45} = 6.080$, $p = 0.0046$), main effects of drug ($F_{1,\ 45} = 13.89$, $p = 0.0005$) and stress ($F_{2,\ 45} = 12.93$, $p < 0.0001$).

Chemical stimuli on stress response

Fish exposed to chemical stressors (alarm substance or blood) displayed an increase in cortisol levels, but FLU did not blunt the increase in cortisol levels in fish subjected to chemical stressors (Fig. 3). Two-way ANOVA revealed a significant main effect of stress ($F_{2,\ 48} = 5.623$, $p = 0.0064$), but not interaction effect between the factors ($F_{2,\ 48} = 0.7045$, $p = 0.4994$) or a main effect of drug ($F_{1,\ 48} = 0.01718$, $p = 0.8963$).

DISCUSSION

Here we show that fluoxetine blunts the response to physical, but not chemical, stress. Whether physical (*Ramsay et al., 2009*) or chemical (*Teles et al., 2017*) stress increases cortisol levels in zebrafish.

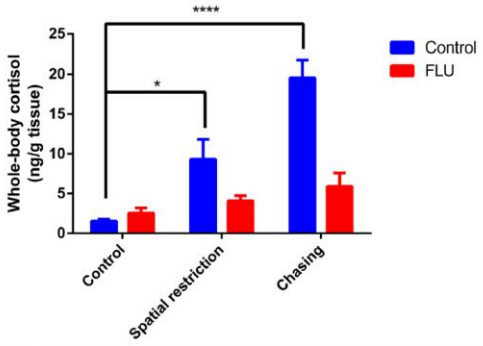

Figure 2 Effects of physical acute stressors (spatial restriction or chasing) on cortisol levels in whole-body zebrafish. Data were expressed as mean + SEM. Two-way ANOVA followed by Dunnett's post hoc test. FLU (fluoxetine). $^{*}p < 0.05$ and $^{****}p < 0.0001$.

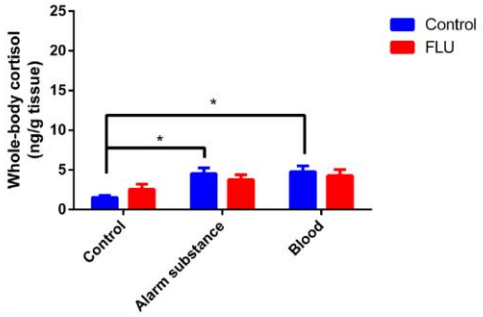

Figure 3 Effects of chemical acute stressors (alarm substance or blood) on cortisol levels in whole-body zebrafish. Data were expressed as mean + SEM. Two-way ANOVA followed by Dunnett's post hoc test. FLU (fluoxetine). $^{*}p < 0.05$.

The greater magnitude of response to a physical stressor could be related to its high impact can cause a clear aversive response in fish (*Abreu et al., 2016*). Besides, confinement stress also resulted in elevated cortisol for being "high-impact stress" (*Silva et al., 2015*), perhaps physical stressors act in dorsolateral and dorsomedial regions of the pallium that have been characterized as functional homologues to the mammalian amygdala and hippocampus (*Goodson & Kingsbury, 2013*; *O'Connell & Hofmann, 2011*; *Vargas, López & Portavella, 2009*), with consequent action under the hypothalamus. On the other hand, chemical stress does not trigger a response of such magnitude (*Silva et al., 2015*). Our hypothesis is that the chemical stressor stimulus depends on more than one sensory pathway (e.g., smell, tactile) for the perception of the stimulus, which would result in a suppression of the stimulation force of the hypothalamic system, with consequent pituitary and later adrenergic stimulation.

We demonstrated that fluoxetine prevents the increase of cortisol in fish in response to physical stressor stimulus. Previously, we showed that fluoxetine blocked cortisol response to acute chasing stress in a dose-dependent manner (*Abreu et al., 2014*) as well as in fish subjected to different forms of housing (*Giacomini et al., 2016*). FLU also blocked the stress response following chronic exposure in zebrafish (*Egan et al., 2009*), besides stress increases serotoninergic activity in the telencephalon in fish (e.g., *Øverli et al., 2004*; *Winberg, Nilsson & Olsen, 1992*). In fact, the levels of serotonin in the brain regions considered homologous to the mammalian hippocampus and amygdala are altered in fish subjected to spatial restriction (*Silva et al., 2015*). This effect reinforces the participation of these regions in response to physical stress, as well as the involvement of serotonin in these pathways.

Still, we have shown that fluoxetine did not block the increase of cortisol in fish in response to chemical stressor stimulus. Alarm substance induced stress responses in *Nile tilapia* (*Oreochromis niloticus*), increasing ventilation rate and cortisol level (*Sanches et al., 2015*) as well as increasing erratic movements in zebrafish (*Speedie & Gerlai, 2008*). The exposure to blood has also been shown to induce antipredator behavior in the fish species *N. tilapia* (*Barreto et al., 2013*). The exposure to alarm substance also increased anxiety-like behavior in the light/dark test in zebrafish and decreased nocifensive behavior, however pretreatment with fluoxetine blocked the anxiogenic effects of alarm substance on the light/dark test and also increased extracellular brain 5-HT (*Maximino et al., 2014*), the same behavioral relationship between alarm substance and serotoninergic system was not observed in the relationship between neuroendocrine and serotoninergic system. Serotonin receptors (5-HT_{1A} and 5-HT_{4}) expressed in steroidogenic cells in the interrenal glands mediate the effects of serotonin on cortisol response (*Herculano & Maximino, 2014*), and this direct mechanism may underlie the effects of fluoxetine observed in physical stress response, namely the inhibition of cortisol release.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Angelo L.S. Piato is an Academic Editor for PeerJ.

Author Contributions

- Murilo S. Abreu conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Ana C.V.V. Giacomini conceived and designed the experiments, performed the experiments, analyzed the data and wrote the paper.
- Gessi Koakoski contributed reagents/materials/analysis tools.
- Angelo L.S. Piato wrote the paper, reviewed drafts of the paper.
- Leonardo J.G. Barcellos conceived and designed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.

Animal Ethics

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

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

Data Availability

The following information was supplied regarding data availability:

The raw data has been supplied as Supplemental Dataset Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.3330#supplemental-information.

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2.3. ARTIGO 3 - *Behavioral responses of zebrafish depend on the type of threatening chemical cues*

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ORIGINAL PAPER

Behavioral responses of zebrafish depend on the type of threatening chemical cues

Murilo S. Abreu[1] · Ana Cristina V. Giacomini[2] · Darlan Gusso[2] · Gessi Koakoski[1] · Thiago A. Oliveira[1] · Alessandra Marqueze[5] · Rodrigo Egydio Barreto[4] · Leonardo J. G. Barcellos[1,2,3]

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Abstract In fish, defensive reactions are induced by different chemical cues that emanate from sense-related stresses [physical, chemical, and visual (visual contact with predator)] or food stresses (acute fasting and chronic food restriction). Using a shuttle box with a two-chamber unmixed laminar flow that allowed fish to remain or flee from a chemical cue, we showed that the avoidance response depended on the type of the chemical cue. We show that zebrafish (*Danio rerio*) retreated from water conditioned with chemical cues released by chemically or physically stressed fish and acutely fasted fish, but not from water with cues from fish experiencing visual contact with predatory fish or fish suffering from chronic food restriction. Our data reinforced the hypothesis that fish use a combination of information and the context of the situation to determine their evasion strategy.

Keywords Defensive behavior · Chemical cues · Attraction · Aversion · Zebrafish

✉ Leonardo J. G. Barcellos
lbarcellos@upf.br

[1] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil

[2] Universidade de Passo Fundo (UPF), Passo Fundo, RS, Brazil

[3] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), Passo Fundo, RS, Brazil

[4] Department of Physiology, Institute of Biosciences of Botucatu, Caunesp, Unesp, Botucau, SP, Brazil

[5] Centro Universitário La Salle-Unilasalle, Programa de Pós-Graduação em Avaliação de Impactos Ambientais, Canoas, RS, Brazil

Introduction

The structural complexity and variations of habitat are known to have significant effects on populations and can shape behavior, morphology, and life history traits (Brown and Braithwaite 2005). An animal's perception of habitat, environment, and its variations is important, and allows it to be equipped to deal with chemical cues of danger that might arise in the environment. Perception of chemical cues can potentially increase survival, allowing individuals to avoid a threatening situation. This is a well-known phenomenon related to prey–predator systems (Chivers and Smith 1998; Korpi and Wisenden 2001).

In fish, several types of chemical cues have been reported as released and perceived by conspecifics leading to defensive responses. These responses include changes in behavior (Chivers and Smith 1998) and induction of stress responses (Pfeiffer and Lamour 1976; Rehnberg et al. 1987; Rehnberg and Schreck 1987; Toa et al. 2004; Barreto et al. 2010; Sanches et al. 2015). One type of threatening chemical cues is called disturbance chemical cues, which are defined as cues released in the water by non-injured fish in stressful contexts (Barcellos et al. 2011), including non-lethal prey–predator encounters (Jordão and Volpato 2000; Barcellos et al. 2014).

Several questions regarding communication via disturbance chemical cues remain unexplored. One such question is: does the stressor that lead to the release of a disturbance chemical cue modulate behavioral responses of the receiver fish? Based on observations of other types of threatening chemical cues, we hypothesized that different forms of chemical communication, such as exposure to water conditioned with chemical cues released by chemically or physically stressed fish or cues from fish experiencing acute fasting or chronic food restriction, can cause stress in receiver

fish and change in behavior, such as avoidance of conditioned water. Fish may respond by immobility (freezing) or dramatically reducing swimming (slowing), or may evade the cue source (Schwarze et al. 2013; Sabet et al. 2015).

A new methodological paradigm allows us to address this question. The use of a two-chamber shuttle box with unmixed laminar flow in the chambers allows fish to remain or flee from a chemical cue (Readman et al. 2013; Abreu et al. 2016). Thus, to answer our question, we investigated zebrafish (*Danio rerio*) behavior in response to exposure to disturbance chemical cues produced by conspecifics experiencing various stressors. We used this species as an animal model because they respond to disturbance chemical cues (Hussain et al. 2013) and have been used as an animal model for research for over three decades in various disciplines, including physiology, toxicology, genetics, embryology, metabolism, oncology, neuroscience, cardiovascular studies, and the study of neurodegenerative diseases (Barbazuk et al. 2000; Mueller et al. 2004; Alsop and Vijayan 2009; Egan et al. 2009; Howe et al. 2013).

Materials and methods

Subjects

The stock population of 200 adult, wild-type zebrafish of the short-fin (SF) strain, weighing 0.65 ± 0.1 g, was housed in a tank. One hundred fish were placed in ten tanks (ten fish per tank) for the preparation of conditional water and the other half was used for the analysis of individual perception in the apparatus (Fig. 1). All fish were supplied constant aeration and biological filtering under a natural photoperiod (approximately, 14 h light:10 h dark). The water was maintained under the following conditions: temperature = 27 ± 1 °C; pH = 7.0 ± 0.2; dissolved oxygen = 6.3 ± 0.5 mg L^{-1}; total ammonia = 0.01 mg L^{-1}; total hardness = 6 mg L^{-1}, and alkalinity = 22 mg L^{-1} CaCO3.

Experimental strategy

We conducted a two-choice experiment, where the tested fish could choose between clean or conditioned water (with a test disturbance substance). We used an experimental apparatus (Fig. 1a) that consisted of an acrylic tank with two separated compartments of unmixed laminar flows of 2 L min^{-1} (Abreu et al. 2016), and fish could move between these compartments. Confirmation (via gentian violet dye) of unmixed laminar flux for all types of conditioned water is shown in Fig. 1b.

Physical stress, chemical stress, and visual contact with a predator

The goal of this study was to determine if fish decide to evade conditioned water (physical stress, chemical stress, visual contact with a predator—detailed in Table 1) and preferentially seek clean water. Fish were individually tested by placing one into an experimental apparatus, distributed into five treatments (ten fish $treatment^{-1}$; see Table 1).

Food deprivation stress

The goal of this study was to determine if fish decided to evade conditioned water (cues from fish experiencing acute fasting, chronic food restriction, or normal nutrition; detailed in Table 2) and preferentially seek clean water. Fish were individually tested by placing one fish into the

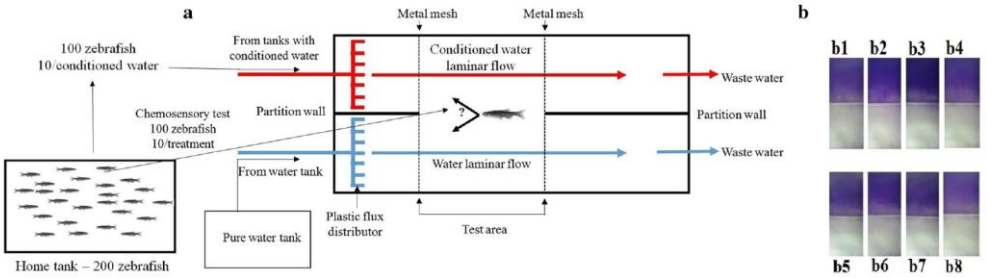

Fig. 1 Experimental setup. **a** Schematic representation of the test chamber. **b** Photographic confirmation of the maintenance of the laminar flow. Images show the stability of laminar flow during dosing. Each compound was stained with a *violet* indicator to visually follow the progression of the compound. *b1* Negative control, *b2* positive control (pH 3), *b3* physical stress, *b4* chemical stress, *b5* contact visual predator, *b6* fish in normal nutritional status, *b7* fish experiencing acute fasting, and *b8* fish experiencing chronic food restriction

Table 1 Different forms of stress (physical stress, chemical stress, visual contact predator)

Group	Experimental protocol	References
Control	Water	Abreu et al. (2016)
pH 3	Water with pH 3	Abreu et al. (2016)
Physical stress	Fish chased with a net for 2 min; after 15 min the water was introduced into the apparatus*	–
Chemical stress	Fish exposed to pH of 5 for 15 min; afterward the water was introduced into the apparatus*	–
Visual of predator display [predator—tiger oscar (*Astronotus ocellatus*)]	Fish viewed the predator for 15 min; afterward the water was introduced into the apparatus*	–

* Aquarium with ten fish 10 L^{-1} for conditioning stimulus-containing water

Table 2 Different forms of food deprivation stress

Group	Experimental protocol	References
Control	Water	Abreu et al. (2016)
pH 3	Water with pH 3	Abreu et al. (2016)
Fish in a normal nutritional status	Water fish fed twice daily*	
Fish experiencing acute fasting	Water from food-deprived fish for 48 h*	–
Fish experiencing chronic food restriction	Water fish fed once a week for 30 days*	–

* Aquarium with ten fish 10 L^{-1} for conditioning stimulus-containing water

experimental apparatus, distributed in five treatments (ten fish $treatment^{-1}$; see Table 2).

Experimental procedures

Individual fish were transferred from the holding tank to the recording apparatus and were acclimated for 150 s. Next, a continuous dose of the test compound was injected into one of the compartments for 150 s. Clean water and conditioned water were alternated between the right and left section of the apparatus among tests to avoid any possible laterality bias caused by a fish preferring to stay on either the left or right side. All fish examined during the test enter in the conditioned side, which guarantee contact with the conditioned water. Thus, thereafter the absence of fish permanency on the conditioned side indicated avoidance of the treatment. After each test, the apparatus was thoroughly washed with water to remove any residual test substance. The location and activity of fish with access to both the treated and untreated sections were recorded with a video camera for the entirety of the experimental period (Abreu et al. 2016). The video camera was positioned directly above the apparatus. The analysis of video recordings of individual fish was performed using AnyMaze® video monitoring system (Stoelting, CO, USA), for both the acclimatization periods (approximately 150 s) and 150 s of exposure. For assessment, the following parameters were collected per test (for clean water and treatments), number of crossings, total distance traveled, mean speed swimming, absolute turn angle, and rotations.

Statistics

The homogeneity of variance and normality were assessed by Hartley and Kolmogorov–Smirnov tests, respectively. Wilcoxon matched-pairs test was conducted to compare subjects' permanency time between compartments. The total number of crossing between compartments, total distance traveled, mean speed swimming, absolute turn angle, and rotations were compared by Kruskal–Wallis test followed by Dunn's post hoc test. Differences were considered statistically significant at $p < 0.05$.

Results

Physical stress, chemical stress, and visual contact with a predator

Figure 2 shows the time spent in the conditioned and clean lanes, and the pre-trial analysis (initial 150 s) before the influx of cues, indicating that attraction or aversion began at the moment of conditioned water influx. In the control (with clean water in both lanes), no preference was detected ($p = 0.6953$), whereas in the positive control, zebrafish showed a clear aversion to pH 3 ($p = 0.001$). Fish perceived water conditioned to a pH of 3 ($p = 0.001$), disturbance cues from chemically stressed fish ($p = 0.0039$), and disturbance cues from physically stressed fish ($p = 0.0313$) as aversive stimuli. Attraction or aversion was not detected for any other treatment.

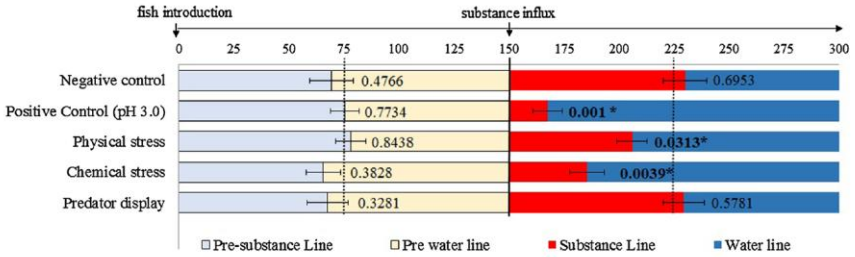

Fig. 2 Time spent (s) in the substance or water lane during the 150-s pre-substance influx and during the 150 s of substance exposure test. The data are expressed as the mean ± SEM for each lane. The means were compared by Wilcoxon matched-pairs signed-ranks test. *p* values are depicted following each *bar*

Table 3 Locomotor activity of zebrafish exposed to different forms of stress (physical stress, chemical stress, visual contact with predator)

Substance	Distance (m)	Mean speed (m s^{-1})	Absolute turn angle (°)	Rotations
Control	13.109 ± 2.103	0.0874 ± 0.014031	35008 ± 2471	28.8 ± 6.06
pH 3	**8.722 ± 0.792**	**0.058 ± 0.0052**	32599 ± 1219	18.9 ± 2.15
Physical stress	13.170 ± 3.091	0.087 ± 0.0206	33067 ± 3328	24.42 ± 6.2
Chemical stress	10.351 ± 0.996	0.069 ± 0.0066	**28107 ± 010**	23.55 ± 2.58
Fish acutely stressed (predator display)	12.928 ± 1147	0.086 ± 0.0075	28990 ± 1910	21.85 ± 2.84

Data expressed as mean ± SEM. Kruskal–Wallis followed by Dunn's post hoc test. Distance travelled ($K = 11.12$; $p = 0.0252$), mean speed ($K = 11.02$; $p = 0.0263$), absolute turn angle ($K = 9.735$; $p = 0.0451$), and rotations ($K = 2.387$; $p = 0.6649$). Significant effects are given in bold

Table 4 Number of crossings of zebrafish exposed to different forms of stress (physical stress, chemical stress, visual contact with predator)

Substance	Number of crossings	
	0–150 s (before substance influx)	151–300 s (during substance influx)
Control	17.6 ± 3.78	30.1 ± 6.21
pH 3	19.125 ± 3.94	**12.09 ± 3.7**
Physical stress	22.375 ± 2.73	30.71 ± 10.73
Chemical stress	20.25 ± 2.51	17.44 ± 3.27
Fish acutely stressed (predator display)	25.85 ± 4.9	27.55 ± 4.11

Data expressed as mean ± SEM. Kruskal–Wallis followed by Dunn's post hoc test. Number of crossing 151–300 s (during substance influx) ($K = 11.98$; $p = 0.0175$). Significant effect is given in bold

Distance traveled, mean speed (pH 3, Table 3), and absolute turn angle exhibited differences (chemical stress, Table 3). The number of crossings were different for the fish in the pH 3 conditioned water (Table 4).

Food deprivation stress

Figure 3 shows the control (with clean water in both lanes), in which no preference was detected ($p = 0.6953$), whereas in the positive control, zebrafish showed a clear aversion to pH 3 water ($p = 0.0010$). Fish perceived only the water conditioned by fish fasted for 48 h ($p = 0.0020$) as an aversive stimulus. Attraction or aversion was not detected for any other treatment.

No significant differences were detected for distance traveled, mean speed (pH 3, Table 5), absolute turn angle, and rotations (Table 5).The number of crossings was significantly different for the pH 3 positive control (Table 6).

Discussion

Herein, we showed that zebrafish avoided water conditioned with chemical cues released by chemically or

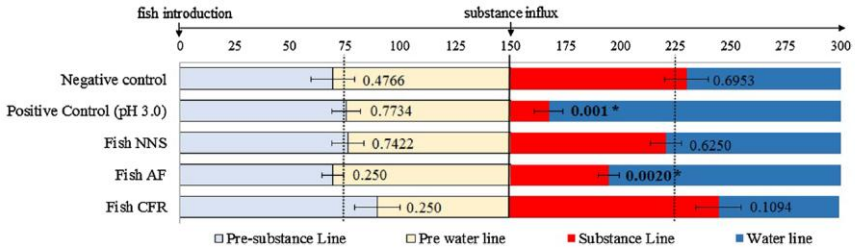

Fig. 3 Time spent (s) in the treated or water lane during the 150-s pre-substance influx and during the 150 s of substance exposure test. The data are expressed as mean ± SEM for each lane. The means were compared by Wilcoxon matched-pairs signed-ranks test. *p* values are depicted following each *bar*. *Fish NNS* fish in normal nutritional status; *Fish AF* fish experiencing an acute fast; *Fish CRF* fish experiencing chronic food restriction

Table 5 Locomotor activity of zebrafish exposed to different forms of food deprivation stress

Substance	Distance (m)	Mean speed (m/s)	Absolute turn angle (°)	Rotations
Control	11.655 ± 0.893	0.087 ± 0.014	35509 ± 2705	30.22 ± .59
pH 3	**7.616 ± 0.545**	**0.0537 ± 0.0028**	32611 ± 1285	18.4 ± 2.31
Fish in a normal nutritional status	12.229 ± 1005	0.0815 ± 0.0067	34738 ± 1658	19.9 ± 1.51
Fish after acute fasting	14.318 ± 1134	0.0954 ± 0.0075	31224 ± 1214	28.8 ± 2.75
Fish after chronic food restriction	13.120 ± 1.761	0.087 ± 0.0117	33379 ± 2022	26 ± 3.13

Data expressed as mean ± SEM. Kruskal–Wallis followed by Dunn's post hoc test. Distance traveled ($K = 21.44$; $p = 0.0003$), mean speed ($K = 16.57$; $p = 0.0023$), absolute turn angle ($K = 4.609$; $p = 0.3298$), and rotations ($K = 7.959$; $p = 0.0931$). Significant effects are given in bold

Table 6 Number of crossings of zebrafish exposed to different forms of food deprivation stress

Substance	Number of crossings	
	0–150 s (before substance influx)	151–300 s (during substance influx)
Control	18.6 ± 4.1	31.11 ± 6.85
pH 3	20. 5 ± 4.23	**10 ± 2.97**
Fish in a normal nutritional status	27.75 ± 4.51	23 ± 3.27
Fish in acute fasting	32.87 ± 3.875	32.7 ± 4.91
Fish in chronic food restriction	28.25 ± 4.15	27.5 ± 4.85

Data expressed as mean ± SEM. Data expressed as mean ± SEM. Kruskal–Wallis followed by Dunn's post hoc test. Number of crossings 151–300 s (during substance influx) ($K = 11.91$; $p = 0.0180$). Significant effect is given in bold

physically stressed fish and by water from acutely fasted fish (disturbance chemical cues). However, the disturbance chemical cues released by a conspecific fish in visual contact with a predator did not elicit any response in terms of evasion or permanency.

The protocol and apparatus for this chemotactic preference test were first validated in an assessment of fish aversion to anesthetics and drugs (Readman et al. 2013; Abreu et al. 2016). We demonstrated that pH 3 decreases the distance and mean speed, effects that had not been previously observed (Abreu et al. 2016), possibly the highest number of experimental groups in relation to this study and to detect a difference there is the need for a stronger effect. The aversive behavioral (avoidance or attraction) paradigm has been used to determine aversive experiences with tests using aversion measures, such as the percentage of time spent in conditioned or clear water. These behaviors are simple and objective measures that are easily quantifiable using fish models for preference or avoidance (Pelkowski et al. 2011). Thus, our methodology was reliable in the detection of fish evasive reactions to chemical stimuli.

Both aversion and permanency responses present intriguing results. We hypothesized that water from

physically and chemically stressed fish and fish experiencing acute fasting would elicit an avoidance behavior in conspecifics because the information is direct. In fact, physical and chemical stress and acute fasting information did not need a specific context or lead to the release of a greater amount of the cue. The communication of the stressful situation has already been described in fish (Toa et al. 2004; Barcellos et al. 2011; Oliveira et al. 2013). Thus, physical and chemical stresses were communicated chemically in the water and were able to stress the fish in the apparatus. It is known that the simple introduction of water from stressed fish (sender) is capable of eliciting a complete stress response in the receiver fish, generalizing the stress response (cortisol increase) to all fish reared in tanks in a recirculating system (Barcellos et al. 2011).

We hypothesized that visual contact with a predator was not detected as an aversive stimulus because numerous predators use prey movement as a visual key to locate the prey and attack (Lima and Dill 1990; Burrows 1994; Burrows and Gibson 1995). Because the predator was not in the same aquarium as the test fish, an effective attack could not occur, and prey did not perceive the situation as life threatening.

Fasting induces change in some aspects of fish metabolism and endocrinology (Barcellos et al. 2010; Rossi et al. 2015), and production and reaction to alarm substances (McCormick and Larson 2008; Barreto et al. 2012). The mobilization of energy reserves from carbohydrates, lipids, or proteins could produce some metabolites that are excreted into the water (Jayaram and Beamish 1992; Lauff and Wood 1996; Wilkie 2002). Fasting promotes the release of the secretogranin II (SGII) precursor, which acts in neuroendocrine cells to stimulate the release of luteinizing hormone and increase locomotor behaviors in fish (Trudeau et al. 2012). Conversely, in chronic fasting, increased utilization of select tissue fatty acids in liver and muscle, increased plasma triglycerides, and decreased liver glucose and glycogen are observed (Pujante et al. 2015). However, chronic fasting trout did not significantly affect protein catabolism in peripheral tissues, indicated by reductions in the level of serum amino acids (Baumgarner and Cooper 2012). In addition, chronic fasting significantly increased pituitary GH expression, contrary to stress, which suppresses this axis (GH) (Malandrakis et al. 2016), suggesting that fish easily adjust their metabolism under situations characterized by chronic fasting. Furthermore, cortisol plays a crucial role in this fasting-induced energy mobilization (Wendelaar Bonga 1997; Mommsen et al. 1999) and is excreted to water via urine, bile (Vermeirssen and Scott 1996), and feces (Turner et al. 2003). Thus, it is plausible that fasted fish release chemical cues that are interpreted by conspecifics as threatening, unsafe, and/or undesirable situations.

Despite the ecological relevance of chemical communication of risk or stressful situations among fish, we found that an isolated fish without any specific context did not react to the majority of the classical chemical cues, especially those related to predator–prey relationships. Our data reinforced the hypothesis that fish use a combination of different information and the context of the situation to determine their evasion strategy.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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2.4. ARTIGO 4 - *Acute exposure to waterborne psychoactive drugs attract zebrafish*

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Acute exposure to waterborne psychoactive drugs attract zebrafish

Murilo S. Abreu [a], Ana Cristina V. Giacomini [a,b], Darlan Gusso [b], João G.S. Rosa [a], Gessi Koakoski [a], Fabiana Kalichak [a], Renan Idalêncio [b,c], Thiago A. Oliveira [a], Heloísa H.A. Barcellos [a,b], Carla D. Bonan [d], Leonardo J.G. Barcellos [a,b,c,*]

[a] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS, 97105-900, Brazil
[b] Universidade de Passo Fundo (UPF), BR 285, Bairro São José, Passo Fundo, RS, 99052-900, Brazil
[c] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), Hospital Veterinário, BR 285, Bairro São José, Passo Fundo, RS, 99052-900, Brazil
[d] Laboratório de Neuroquímica e Psicofarmacologia, Programa de Pós-Graduação em Biologia Celular e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga, 6681, Porto Alegre, RS, Brazil

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ABSTRACT

Psychotropic medications are widely used, and their prescription has increased worldwide, consequently increasing their presence in aquatic environments. Therefore, aquatic organisms can be exposed to psychotropic drugs that may be potentially dangerous, raising the question of whether these drugs are attractive or aversive to fish. To answer this question, adult zebrafish were tested in a chamber that allows the fish to escape or seek a lane of contaminated water. These attraction and aversion paradigms were evaluated by exposing the zebrafish to the presence of acute contamination with these compounds. The zebrafish were attracted by certain concentrations of diazepam, fluoxetine, risperidone and buspirone, which were most likely detected by olfaction, because this behavior was absent in anosmic fish. These findings suggest that despite their deleterious effects, certain psychoactive drugs attract fish.

1. Introduction

Psychotropic medications such as antidepressants, antipsychotics and anxiolytics are widely used (Bocquier et al., 2008) and its prescription has increased worldwide in the last 20 years (Carta et al., 2004; Paulose-Ram et al., 2007; Alonso et al., 2004; la Poza et al., 2013). Consequently, increasing its presence in aquatic environments (Santos et al., 2007) which are monitored especially in urban and hospital wastewater, effluent from water and sewage treatment plants, surface and drinking water (Calisto et al., 2011; Al Aukidy et al., 2012). The main concern is that these contaminants may cause toxicity, affecting the health of non-target humans and animals. Also, many of these drugs are resistant to wastewater treatments and are only partially removed (Palmer et al., 2008; Silva et al., 2011).

The most commonly prescribed, consumed, and consequently detected drugs in aquatic environments are benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), buspirone, risperidone, and ethanol. Benzodiazepines, such as diazepam and clonazepam, potentiate $GABA_A$ receptor function by increasing the channel opening frequency, producing hypnotic effects by acting on the α1 subunit (McKernan et al., 2000) and anxiolytic effects by acting on the α2 subunit (Löw et al., 2000). Fluoxetine is a potent and highly selective inhibitor of the transporter for serotonin reuptake at the presynaptic membrane, causing increases in serotonin concentrations at postsynaptic receptor sites (Wong et al., 1995). Buspirone exerts anxiolytic effects by acting as a partial agonist at serotonin 5-HT1A receptors (Ohlsen and Pilowsky, 2005), and it also interacts to a lesser degree with other receptors, such as the dopamine D2 receptor (Dhavalshankh et al., 2007). The antipsychotic drug risperidone belongs to the benzisoxazole chemical class (Kumar et al., 2008; Courchesne et al., 2007) and has been reported to act therapeutically by blocking serotonin and dopamine receptors (Grant, 2007); thus, it is useful for studying increases in serotonin neurotransmission. Ethanol also has acute anxiolytic effects that are most likely mediated by $GABA_A$ receptors (Radcliffe et al., 1999; Kumar et al., 2009), with depressant effects on the central nervous system at higher doses.

Although the concentrations of these drugs in aquatic environments are lower than the lethal concentrations for most of the species present in these ecosystems, studies have shown that their concentrations in organs such as the brain, liver and muscles are higher than those in the water (Brodin et al., 2013; Brooks et al., 2005; Sackerman et al., 2010). Benzodiazepines and SSRIs may trigger a set of morphological, physiological, neuroendocrine, reproductive, motor and behavioral changes (Brodin et al., 2013; Sackerman et al., 2010; Airhart et al., 2007; Gebauer et al., 2011; Park et al., 2012; Prieto et al., 2012; Abreu et al., 2014; Idalencio et al., submitted for publication).

* Corresponding author.
E-mail address: lbarcellos@upf.br (L.J.G. Barcellos).

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Since these psychoactive drugs are potentially dangerous to fish, we posed the following question: are these drugs attractive or aversive to fish? To answer this question, adult zebrafish were placed into a chamber that allowed them to avoid or to swim into a lane containing contaminated water. This enabled the evaluation of the attraction and aversion paradigm in zebrafish exposed to acute contamination of these compounds.

2. Methods

2.1. Ethics statement

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

2.2. Subjects

A mixed-sex stock population of adult wild-type zebrafish (*Danio rerio*) from the short-fin (SF) strain was used. In the experiment 1, ten fish were subjected to each substance treatment, totalizing 210 fish (21 treatments, each with 10 fish). In the 2nd experiment, ten anosmic fish were subjected to the substances that are attractive or aversive in the 1st experiment and also a saline only control, thus, a total of 200 fish were used in this study.

The fish were fed twice per day at 10:00 and 16:00 h with a commercial flake food until satiation (Alcon® Basic, MEP 200 Complex, Brazil). The mean water temperature in the holding tank was maintained at 24 ± 2 °C, and the dissolved oxygen concentrations varied from 5.6 to 7.2 mg/l (both measured using YSI model 550A oxygen meter; Yellow Springs Instruments, USA). The pH values ranged from 6.2 to 7.4 (measured using a Bernauer pH meter). The total ammonia–nitrogen concentration was less than 0.5 mg/l (measured using a colorimetric test).

2.3. Substances

Clonazepam (Rivotril®), diazepam (União Química, Brazil), fluoxetine (Daforin, EMS), risperidone (Risperidona, EMS), buspirone (Ansitec®, LIBBS) and ethanol were purchased from common commercial suppliers. The details of the substances examined in the experiment are listed in Table 1. The food odor positive controls were prepared using two distinct methods. Positive control 1 was prepared by adding flaked food to the water at a rate of 0.5 g/l, followed by the homogenization and the immediate use of the mixture in specific test trials. Positive control 2 differed from positive control 1 only in that the flaked food remained in the water overnight (12 h) before the mixture was homogenized and used in specific test trials.

2.4. Experimental apparatus

The experimental apparatus consisted of a modified, 30-liter acrylic tank (50 × 25 × 25 cm, length × width × height). Metal mesh was added to prevent the fish from escaping the tank. A short segregation panel and a fine mesh baffle were inserted at the other end of the tank to create two chambers leading to two lanes of water with laminar flow run in parallel without mixing. See the schematic drawing of the apparatus in Fig. 1A and the dye (gentian violet) colored confirmation of laminar flux for all substances in Fig. 1B. The use of the dye aimed to verify if the separate flux was maintained in all drug tests, and drugs were not mixed to the dye during the experiments. A flow rate of 2 l/min was used for each track, and the manifold for each mixing chamber had a single door to allow for the introduction of the test substance.

2.5. Experimental protocol

In experiment 1, individual fish were transferred from the holding tank in a small volume of water. After transfer, the fish were allowed to acclimate for 150 s, and a continuous dose of the test compound was subsequently introduced into one of the mixing chambers for 150 s at a predetermined concentration. During the tests, fish were not fed. The position (left or right) of the clean and contaminated water lanes was switched between each of the trials to prevent a possible bias caused by a fish preference for the left or right lane. The horizontal gradient created by the laminar flow within the tank allowed for the untreated lane to remain uncontaminated, thus creating two lanes between which the fish could move freely (Readman et al., 2013). Following each single fish testing, the system was manually flushed to remove any test substance residue. The location and locomotor activity of the fish with access to both the treated and untreated lanes were recorded with a video camera for the entire experimental period. The video camera was positioned directly above the tank. The analysis of the video recordings was conducted using ANY-maze® video tracking system (Stoelting Co., USA) for both the 150-s acclimation period and the 150-s exposure period to show that the fish responded only after substance introduction, and the results for each test substance were analyzed separately.

The experiment 2 reproduces the 1st one but using zebrafish with temporary anosmia by the application of lidocaine gel (50 mg/g) in the nares and olfactory surface as described by Johansen (Johansen,

Table 1
Effects of substances and concentrations.

Substance	Concentration	Effect	Reference
Water (control)	–	–	–
pH 3 (Trichloroacetic acid)	pH 3	Escape behavior	Readman et al. (2013)
Ethanol	1%	Neuroendocrine changes	Oliveira et al. (2013)
Ethanol	0.5%	Neuroendocrine changes	Oliveira et al. (2013)
Ethanol	0.25%	Neuroendocrine changes	Oliveira et al. (2013)
Clonazepam	0.057 μg/l	Ambient concentration	Almeida et al. (2013)
Clonazepam	300 μg/l	Behavior changes	Gebauer et al. (2011)
Diazepam	160 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Diazepam	16 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Diazepam	0.88 μg/l	Ambient concentration	Calisto and Esteves (2009)
Fluoxetine	50 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Fluoxetine	25 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Fluoxetine	1 μg/l	Neuroendocrine changes	Abreu et al. (2014)
Risperidone	0.00034 μg/l	Ambient concentration	Calisto and Esteves (2009)
Risperidone	100 μg/l	Behavior changes	Magno (2012)
Risperidone	170 μg/l	Neuroendocrine changes	Idalencio et al. (submitted for publication)
Buspirone	10 μg/l	Behavior changes at 1% concentration	-
Buspirone	1000 μg/l	Behavior changes	Gebauer et al. (2011)
Buspirone	3000 μg/l	Behavior changes	Gebauer et al. (2011)

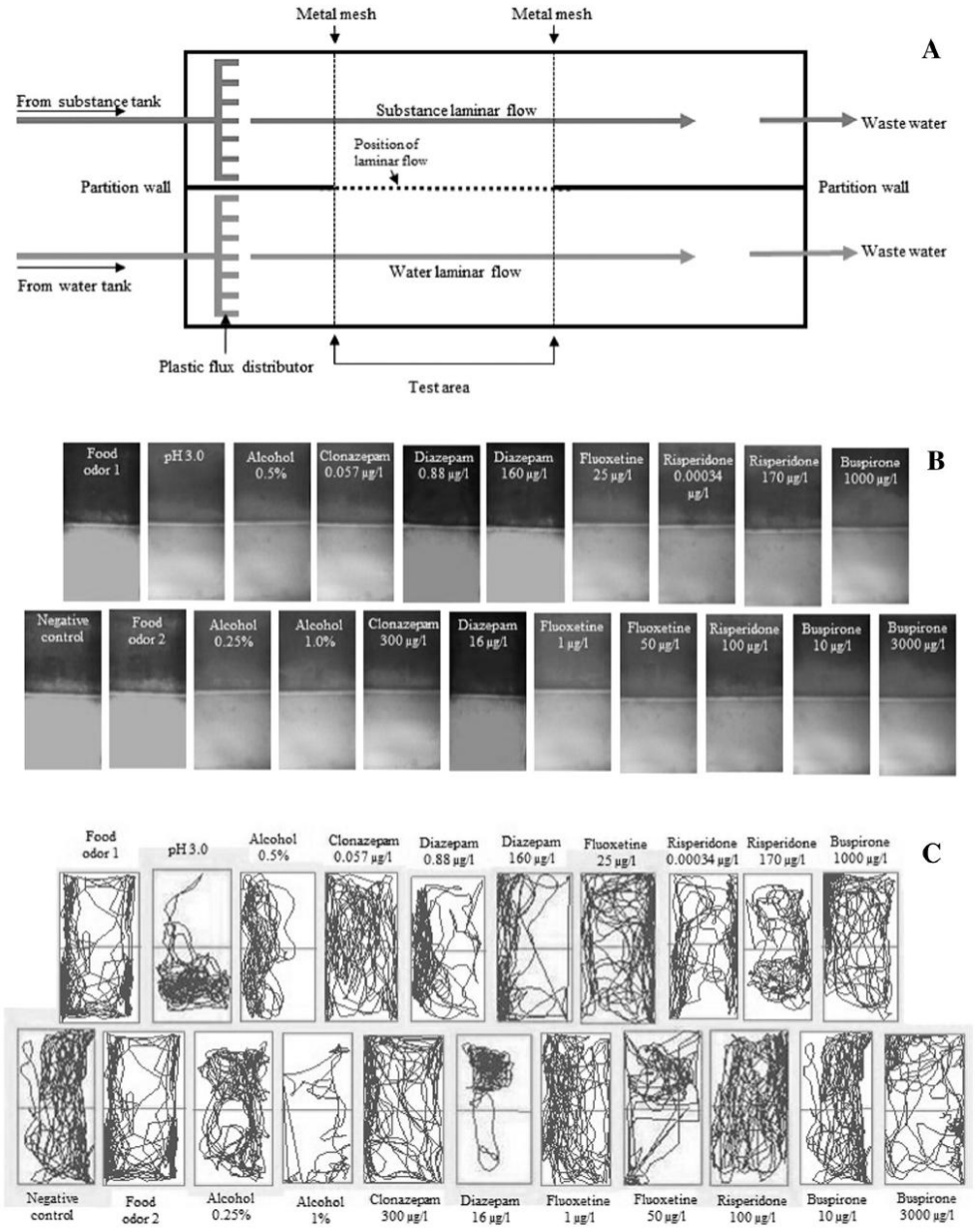

Fig. 1. Schematic representation of the test chamber (A), photographic confirm maintenance of laminar flow during dosage. Images show the stability of laminar flow during dosing. Each compound is dosed with violet as an indicator in order to follow the progression of the compound (B) and representative video tracking the movement of the zebrafish in each treatment (C).

1985). Briefly, each zebrafish was captured and placed on a wet sponge, and the lidocaine gel was gently applied with cotton into the nares. Then, each fish was returned to the aquarium and used immediately in the experiment. To control the influence of the procedure, we repeat the exact temporary anosmic protocol, but with only saline solution in the cotton. The substances tested were those that are attractive to zebrafish in the experiment 1 (diazepam 16 and 160 μg/l, fluoxetine 25 and 50 μg/l, risperidone 100 μg/l and buspirone 1000 μg/l), plus the control situations. As in experiment 1, the time spent in each lane was evaluated. Temporary anosmia is an effective technique to study olfactory participation in odorant detection such as sex pheromones (Souza et al., 1998).

2.6. Statistics

The homogeneity of variance was determined using Hartley's test, and normality was assessed using the Kolmogorov–Smirnov test. For the 150-s analysis intervals (pre- and postdrug influx), the times spent in the two lines were dependent on one another. Thus, the time spent in the treated lane was compared with that spent in the control lane by a paired Student's *t*-test or the Wilcoxon matched-pairs signed-ranks test, depending on data normality. The different drugs and concentrations of the same drug were not compared. The frequency of crossings between the two lanes was compared by the unpaired Student's *t*-test or Mann–Whitney *U*-test, depending on data normality. The locomotor parameters distance traveled, mean speed, absolute turn angle and path efficiency were compared against the control values by one-way ANOVA followed by Dunnet's post hoc test. The differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Experiment 1 — attraction and aversion test

Fig. 2 shows the time spent in the contaminated and clean lanes, and the pre-trial analysis (initial 150 s) before drug influx, showing that attraction or aversion began only at the moment of drug influx. With clean control water in both lanes, there was no preference of the zebrafish for the right or left lane ($p = 0.7214$), whereas in positive control situations, the zebrafish showed a clear aversion to pH 3.0 ($p = 0.0002$) and to two food odor controls ($p = 0.0195$ and 0.0005).

The zebrafish spent more time in the lanes containing diazepam at 16 and 160 μg/l ($p = 0.0413$ and $p = 0.0078$, respectively), suggesting that the fish are attracted by diazepam at these concentrations. Similar attraction was found for fluoxetine (25 and 50 μg/l, $p = 0.0195$ and $p = 0.0222$, respectively), risperidone (100 μg/l, $p = 0.0323$) and buspirone (1000 μg/l, $p = 0.0020$).

No attraction or aversion was detected for ethanol (0.25, 0.50 and 1.0%), clonazepam, or diazepam (0.88 μg/l) or for other concentrations of fluoxetine (1 μg/l), risperidone (0.00034 and 170 μg/l), or buspirone (10 and 3000 μg/l).

Only the fish exposed to pH 3.0, 1% ethanol and 170 μg/l risperidone presented a higher lane crossing frequency than that of the control group exposed to two lanes of clean water (Table 2, $p = 0.0321$, 0.0053 and 0.0311, respectively). Fig. 1C is taken from a representative video and shows the movement of fish tracked during exposure to the substances that elicited significant differences.

No differences were found, except for food odor 1 and 2, in the locomotor parameters (distance traveled, mean speed, absolute turn angle and path efficiency) in all drugs against control values (Table 3).

3.2. Experiment 2 — attraction and aversion test with anosmic zebrafish

The anosmic zebrafish were not attracted by the drugs that were attractive in experiment 1 (diazepam, fluoxetine, risperidone and buspirone). The aversion to food odor was also abolished, whereas the fish maintained the strong aversion to pH 3.0 (Fig. 3A). Fish of control group (identical anosmia protocol but with only saline solution) maintain the attraction verified in the intact ones (Fig. 3B).

Importantly, substances used did not significantly alter pH and DO levels as depicted in Table 4.

4. Discussion

Here, we demonstrated that some psychoactive drugs, such as diazepam, fluoxetine, risperidone and buspirone, were attractive to the fish and that its detection in the water is probably via olfaction. These are very intriguing results if considered from an environmental perspective because the fish did not swim far from the contaminated lanes as expected; in fact, they may have sought these sites. The protocol and apparatus used for this chemotaxic preference test were previously validated in an evaluation of the aversion of fish to anesthetics (Readman et al., 2013), but this is the first study assessing the attraction and aversion paradigm in relation to waterborne psychoactive drugs using a chemosensory chamber test.

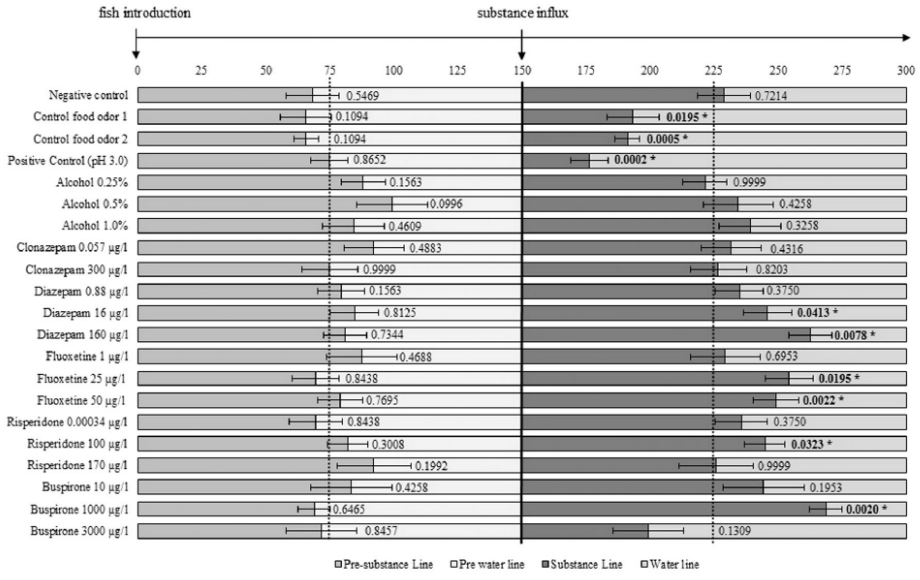

Fig. 2. Time spent (s) in the substance or water lane during the 150-s pre-drug influx and during the 150 s of drug exposure test. The data are expressed as the mean ± SEM for each lane. The means were compared by the paired Student's t test or Wilcoxon matched-pairs signed-ranks test. *P* values depicted following each bar, with 18 degrees of freedom in each comparison.

Table 2
Crossing frequency between contaminated and clean lanes in pre-trial and during drug flux.

Substance	Crossing frequency	
	0–150 s (before drug flux)	151–300 s (during drug flux)
Water (control)	12.25 ± 2.91	14.65 ± 4.99
Food odor 1	22.13 ± 2.67	17.90 ± 4.23
Food odor 2	22.13 ± 2.67	15.33 ± 2.89
pH 3	15.90 ± 3.79	7.57 ± 2.86 *
Ethanol 0.25%	11.63 ± 3.19	17.50 ± 8.45
Ethanol 0.5%	7.10 ± 1.33	8.83 ± 3.39
Ethanol 1%	10.88 ± 3.19	6.29 ± 1.71 *
Clonazepam 0.057 μg/l	4.70 ± 2.16	12.75 ± 6.52
Clonazepam 300 μg/l	15.10 ± 3.55	13.72 ± 3.71
Diazepam 0.88 μg/l	17.67 ± 3.23	11.50 ± 2.72
Diazepam 16 μg/l	8.71 ± 1.30	12.20 ± 5.93
Diazepam 160 μg/l	12.11 ± 2.73	10.33 ± 6.19
Fluoxetine 1 μg/l	5.56 ± 1.82	10.10 ± 5.45
Fluoxetine 25 μg/l	15.20 ± 2.16	11.40 ± 3.43
Fluoxetine 50 μg/l	10.89 ± 2.52	10.85 ± 5.47
Risperidone 0.00034 μg/l	12.25 ± 2.49	10.25 ± 3.84
Risperidone 100 μg/l	17.22 ± 2.05	16.50 ± 3.90
Risperidone 170 μg/l	7.40 ± 2.02	7.15 ± 3.11 *
Buspirone 10 μg/l	6.44 ± 3.49	12.56 ± 6.98
Buspirone 1000 μg/l	7.30 ± 2.35	13.50 ± 5.42
Buspirone 3000 μg/l	8.70 ± 2.07	11.45 ± 1.96

If the fish were truly seeking the drug-contaminated sites, the question that is raised is what were they truly seeking? Our main general hypothesis is that the drugs tested at these specific concentrations were attractive to the fish because they evoked a state of well-being. The premise for the formulation of the title of this study was based on the dangerous and/or disruptive effects of these drugs (Brodin et al., 2013; Gebauer et al., 2011; Park et al., 2012; Abreu et al., 2014; Idalencio et al., submitted for publication) and the notion that despite these effects, they are still attractive for fish.

Moreover, each of the tested drugs acts on several neurotransmitter systems at different levels, modulating neurotransmitters such as GABA, serotonin, and dopamine. The reason that these drugs attracted the fish may be related to their activities in the limbic and hypothalamic areas and the brainstem, in which they enhance the reward system (Tan et al., 2011; Abler et al., 2012; Kronenberg et al., 2012; Hsu et al., 2014). Because buspirone does not have sedative effects (Seidel et al., 1985; Bencan et al., 2009), sedation is most likely not the cause of the attractiveness of these drugs. In addition, all of the tested drugs provoked changes in the number of crossings between the clean and contaminated lanes (Table 2). Reinforcing this hypothesis, that discard the sedation as attractiveness cause, all the drugs did not change any locomotor parameter (Table 3). These unchanged locomotor parameters also discard possible neuromuscular effects of the drugs tested.

Regarding buspirone, the intermediary concentration showed a clear attractive effect, whereas the lower and higher concentrations did not attract the fish. Similarly, the intermediary risperidone concentration showed attraction, whereas the lower and higher ones did not. A possible explanation for this pattern is that buspirone and risperidone may provoke a U-shaped dose-response curve similar to that found for diazepam (Abreu et al., 2014) and for the proper risperidone (Idalencio et al., submitted for publication) effects on the stress axis of zebrafish.

The zebrafish displayed a strong avoidance behavior in the positive control situations. This response showed that the zebrafish were able to detect the acidic pH and odors and demonstrated that the test was able to elicit responses to various substances.

The strong aversion for the food odor controls (food odor 1 and 2, Fig. 2), was clearly abolished in anosmic animals (Fig. 3A). First, the food odor was an effective positive odorant control. However, the behavior triggered was the complete opposite of that expected from attraction by food. A possible explanation is that the food used was based on fish flour as the protein source. Perhaps this fish odor was interpreted as the "death odor" that fish consistently avoid as an anti-risk behavior. In fact, dead fish odor triggers a clear stress reaction (Oliveira et al., 2014). Another possible explanation is the absence of feeding motivation, since fish, in stock tank were maintained satiated. In the context of test (exploring the apparatus), fish might be misinterpreting the food odor as death odor as postulated above.

In anosmic zebrafish, the attraction verified in the experiment 1 was abolished, suggesting that the drug detection may have been a result of the stimulation of a chemoreceptor associated with olfaction. Considering the chemosensorial nature of test used the aversion to pH 3.0 is probably associated with touch or taste (Chang et al., 2010). In fact, previous studies show that acidic pH is detected by taste (Chang et al., 2010) and/or olfaction (Hidaka and Tatsukawa, 1989).

Table 3
Locomotor activity of zebrafish exposed do different psychoactive substances.

Substance	Distance (mm)	Mean speed (mm/s)	Absolute turn angle	Path efficiency
Water (control)	6968 ± 573	46.52 ± 3.81	27,087 ± 2159	0.01396 ± 0.00245
Food odor 1	11,341 ± 1149*	75.69 ± 7.66*	33,632 ± 3833	0.00941 ± 0.00186
Food odor 2	12,180 ± 1437*	81.6 ± 9.62*	32,719 ± 2997	0.0067 ± 0.00157
pH 3	7398 ± 795	49.45 ± 5.29	28,206 ± 1714	0.0129 ± 0.00234
Ethanol 0.25%	7771 ± 560	51.77 ± 3.70	33,423 ± 2065	0.0074 ± 0.00154
Ethanol 0.5%	4666 ± 580	31.1 ± 3.91	33,563 ± 2558	0.0202 ± 0.0092
Ethanol 1%	4491 ± 390	29.93 ± 2.59	34,632 ± 1403	0.0174 ± 0.0038
Clonazepam 0.057 μg/l	6072 ± 652	40.5 ± 4.35	33,450 ± 1350	0.01575 ± 0.0016
Clonazepam 300 μg/l	8143 ± 754	54.3 ± 5.03	32,083 ± 2665	0.0083 ± 0.0014
Diazepam 0.88 μg/l	8109 ± 524	54 ± 3.5	31,261 ± 665	0.0086 ± 0.0013
Diazepam 16 μg/l	6205 ± 527	41.64 ± 3.48	29,070 ± 1824	0.01392 ± 0.0021
Diazepam 160 μg/l	6544 ± 753	43.58 ± 5.02	24,468 ± 2115	0.0215 ± 0.007
Fluoxetine 1 μg/l	6995 ± 866	46.6 ± 5.77	30,735 ± 2246	0.0147 ± 0.0034
Fluoxetine 25 μg/l	6988 ± 708	46.7 ± 4.74	30,269 ± 1498	0.0112 ± 0.002
Fluoxetine 50 μg/l	7270 ± 407	48.55 ± 2.74	27,874 ± 1886	0.0144 ± 0.001
Risperidone 0.00034 μg/l	6134 ± 445	40.94 ± 2.98	25,442 ± 1937	0.02 ± 0.004
Risperidone 100 μg/l	8468 ± 584	56.52 ± 3.88	24,744 ± 1664	0.0149 ± 0.001
Risperidone 170 μg/l	5826 ± 557	39 ± 3.69	30,524 ± 2516	0.01327 ± 0.002
Buspirone 10 μg/l	6115 ± 986	40.6 ± 6.55	29,872 ± 2429	0.0158 ± 0.0038
Buspirone 1000 μg/l	7109 ± 808	47.31 ± 5.4	24,031 ± 2155	0.018 ± 0.005
Buspirone 3000 μg/l	5667 ± 685	37.8 ± 4.52	26,487 ± 1351	0.0159 ± 0.003

Data expressed as mean ± SEM. One-way ANOVA followed by Dunnet's post hoc test. (Distance traveled, $F_{20,290} = 5.351$, $p < 0.0001$ and absolute turn angle $F_{20,290} = 2.453$, $p < 0.0001$).

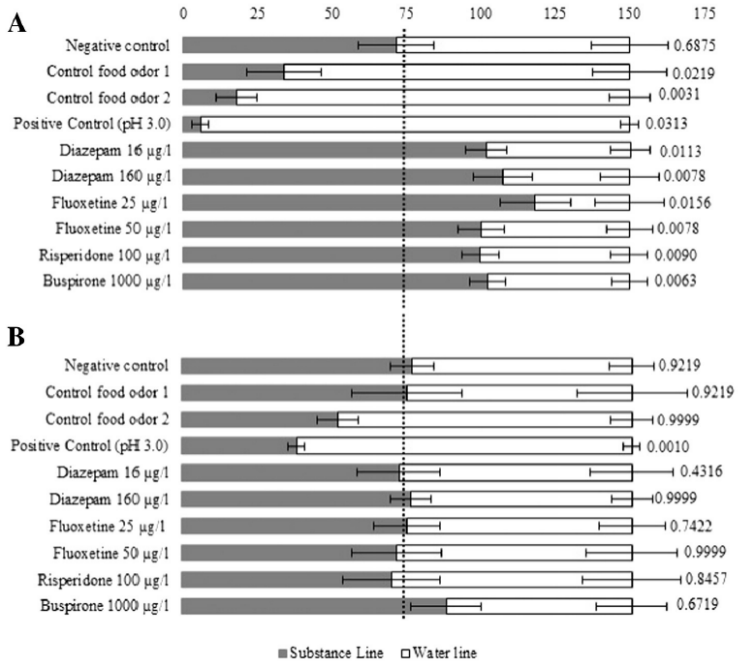

Fig. 3. Time spent (s) in the substance or water lane during the 150-s test in control saline (A) and anosmic zebrafish (B). The data are expressed as the mean ± SEM for each lane. The means were compared by the Wilcoxon matched-pairs signed-ranks test. *P* values in the figure, with 18 degrees of freedom in each comparison.

The absence in the anosmic fish of attraction to the drugs is a very intriguing result. If our hypothesis that attraction was related to a state of fish well-being caused by a drug action on the reward system is true, these drugs need to be absorbed and act in the central nervous system (CNS). However, is the absorption and action related to the olfactory perception of drugs? A possible explanation is a combined sequential effect wherein a previous olfactory perception is necessary to trigger a hedonic effect in the CNS. Another possibility is that olfaction is fundamental to the drug lane choice by fish, and this choice determines that fish spend more time in the presence of the drug and, consequently, absorb more of it. In the absence of olfaction, fish spend less time in the presence of the drug. In fact, a combined action of senses is common, and the most common cases involve a summation of taste with either olfaction or vision (Delwiche, 2012). In addition, the activation of memories and CNS areas related to smell or taste (Shepherd, 2006), including those related to behavioral expression (Chapuis et al., 2007) is also a common phenomenon. Despite these plausible explanations, the mechanism for the involvement of olfaction with attraction to drugs remains to be elucidated.

Considering the reported deleterious and disruptive effects of psychoactive drugs (Brodin et al., 2013; Park et al., 2012; Abreu et al., 2014) in an environmental perspective, we suggest that fish may seek (or at least, not avoid) drug-contaminated places. This can be very dangerous because the fish did not swim far from the contaminated sites as logically expected; in fact, they may have sought these sites. Since the uptake and bioaccumulation of several drugs in fish seems to be time and dose dependent (Lau et al., 2006; Sackerman et al., 2010; Oxendine et al., 2006; Paterson and Metcalfe, 2008; Brodin et al., 2013), a fish that spends more time in the presence of these drugs (attractive or not perceived drugs) tend to absorb higher concentrations than ones that escape from contaminated sites (aversive drugs). Thus, it is difficult to predict the environmental impact of pharmaceutical residues on fish and aquatic environments.

Table 4
pH and DO (mg/l) levels in clean and contaminated water.

	pH		Dissolved oxygen	
Substance	Water	Substance	Water	Substance
Water (control)	6.8 ± 0.1	6.75 ± 0.07	6.2 ± 0.05	6.15 ± 0.1
pH 3 (Trichloroacetic acid)	6.9 ± 0.1	3 ± 0.1	5.7 ± 0.1	5.8 ± 0.05
Ethanol 1%	6.7 ± 0.15	7 ± 0.06	5.9 ± 0.1	5.75 ± 0.15
Ethanol 0.5%	6.2 ± 0.1	6.5 ± 0.08	5.7 ± 0.05	5.65 ± 0.1
Ethanol 0.25%	6.7 ± 0.15	7 ± 0.05	5.6 ± 0.1	5.7 ± 0.07
Clonazepam 0.057 µg/l	7.4 ± 0.03	7.2 ± 0.08	6 ± 0.09	6.2 ± 0.14
Clonazepam 300 µg/l	7 ± 0.04	6.9 ± 0.05	6.2 ± 0.2	6 ± 0.08
Diazepam 160 µg/l	6.7 ± 0.12	6.9 ± 0.2	5.9 ± 0.18	6 ± 0.13
Diazepam 16 µg/l	7.2 ± 0.03	6.9 ± 0.17	6.2 ± 0.17	6.3 ± 0.12
Diazepam 0.88 µg/l	6.9 ± 0.13	6.75 ± 0.14	6.2 ± 0.07	6.1 ± 0.14
Fluoxetine 50 µg/l	6.7 ± 0.14	6.65 ± 0.08	5.9 ± 0.12	6 ± 0.13
Fluoxetine 25 µg/l	6.8 ± 0.09	6.95 ± 0.12	6.2 ± 0.07	6.1 ± 0.14
Fluoxetine 1 µg/l	6.9 ± 0.13	6.75 ± 0.14	7.2 ± 0.03	7 ± 0.17
Risperidone 0.00034 µg/l	7.3 ± 0.06	7.15 ± 0.18	7 ± 0.08	6.8 ± 0.19
Risperidone 100 µg/l	6.7 ± 0.12	6.6 ± 0.18	6.7 ± 0.14	6.5 ± 0.13
Risperidone 170 µg/l	6.4 ± 0.14	6.6 ± 0.08	5.9 ± 0.2	6.2 ± 0.07
Buspirone 10 µg/l	7 ± 0.1	6.8 ± 0.05	5.9 ± 0.14	6.1 ± 0.1
Buspirone 1000 µg/l	6.8 ± 0.2	6.9 ± 0.13	6.7 ± 0.12	6.9 ± 0.04
Buspirone 3000 µg/l	7.2 ± 0.04	7 ± 0.17	6.5 ± 0.16	6.4 ± 0.06

Authors' contributions

L.J.G.B., M.S.A. and A.C.V.G. conceptualized the study and wrote the paper. L.J.G.B., M.S.A., A.C.V.G. and C.D.B. interpreted the data. M.S.A., A.C.V.G., J.G.S.R., G.K., F.K., R.I., T.A.O., D.G. and H.H.A.B. collected and analyzed the data.

Author information

The authors declare no competing financial interests.

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2.5. ARTIGO 5 - *The smell of "anxiety": Behavior modulation by experimental anosmia in zebrafish*

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The smell of "anxiety": Behavioral modulation by experimental anosmia in zebrafish

Murilo S. Abreu [a], Ana C.V.V. Giacomini [a,b], Allan V. Kalueff [d,e,f,g,h], Leonardo J.G. Barcellos [a,b,c,h,*]

[a] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS 97105-900, Brazil
[b] Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS 99052-900, Brazil
[c] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS 99052-900, Brazil
[d] Research Institute for Marine Drugs and Nutrition, College of Food Science and Technology, Guangdong Ocean University (GDOU), Zhanjiang, Guangdong 524025, China
[e] Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg 199034, Russia
[f] Institutes of Chemical Technology and Natural Sciences, Ural Federal University, Ekaterinburg, 620002, Russia
[g] ZENEREI Institute, 309 Palmer Court, Slidell, LA 70458, USA
[h] The International Zebrafish Neuroscience Research Consortium (ZNRC), Slidell 70458, USA

HIGHLIGHTS

- Intact and sham-anosmic fish showed similar behaviors and whole-body cortisol levels.
- Fluoxetine exposed fish displayed a robust anxiolytic-like effect.
- There were no differences in all parameters analyzed in the anosmic fish.
- Anosmia triggers anxiety-like behaviors in zebrafish.
- Experimentally-evoked anosmia modulates anxiety-like behaviors in adult zebrafish.

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ABSTRACT

Olfaction is strongly involved in the regulation of fish behavior, including reproductive, defensive, social and migration behaviors. In fish, anosmia (the lack of olfaction) can be induced experimentally, impairing their ability to respond to various olfactory stimuli. Here, we examine the effects of experimental lidocaine-induced anosmia on anxiety-like behavior and whole-body cortisol levels in adult zebrafish (*Danio rerio*). We show that experimentally-induced anosmia reduces anxiolytic-like behavioral effects of fluoxetine and seems to interact with anxiogenic effect of stress also paralleling cortisol responses in zebrafish. These findings provide first experimental evidence that temporary anosmia modulates anxiety-like behaviors and physiology in adult zebrafish.

1. Introduction

Neuroanatomically confined within the ancient rhinencephalon, olfactory circuits play an important role in modulating behaviors and brain functions [1]. In humans, which are microsmatics, scents perceived during traumatic experiences can trigger pathological stress [2], whereas impaired olfaction (by increasing uncertainty or reducing hedonic behaviors) may cause affective disorders, such as anxiety and depression [3]. Olfactory deficits are also are linked to many other psychiatric disorders, including dementia, Parkinson's disease and schizophrenia [4–5].

Rodents are nocturnal macrosmatic animals that rely on olfaction more heavily than humans [6]. In rodents, olfaction plays a critical role in modulating their behavior, including sexual and social interactions, olfaction-induced fear responses as well as anosmia-induced anxiety/depression-like phenotypes [7–8]. Olfaction is also involved in the regulation of many zebrafish behaviors, including reproduction, defense, foraging, social interaction and migration [9–10]. While olfactory deprivation causes anosmia (the loss of the ability to respond to olfactory stimuli) in fish, their acute anosmia can be induced experimentally (e.g., as an experimental procedure used to study fish chemosensory responses) [11].

* Corresponding author at: Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS, Brazil, 99052-900, Brazil.
E-mail address: lbarcellos@upf.br (L.J.G. Barcellos).

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Studies using zebrafish (*Danio rerio*) as animal models have recently increased exponentially in genetics, developmental biology and behavioral neuroscience [12–14]. Multiple studies have comprehensively evaluated the effects of stressors, drugs (e.g., [15]), chemicals (e.g., [16]) and environmental manipulations (e.g., [17,18]) on zebrafish anxiety-like behavior and endocrine responses. Analyzing zebrafish affective behaviors, the novel tank test represents one of the most commonly used models to access anxiety-like phenotypes in zebrafish [12]. Based on the fish innate protective 'diving' response, this aquatic model provides an efficient test for anxiety-like behaviors in zebrafish, highly sensitive to various experimental manipulations [14, 19]. Given the importance of olfaction in the regulation of animal and human behavior, here we examine the effects of experimentally induced transient anosmia on anxiety-like behavior in adult zebrafish. This study aimed to better understand the role of olfactory modulation in zebrafish affective behaviors, in an attempt to parallel these findings with clinical evidence related to human behavioral regulation by the olfactory system.

2. Materials and methods

2.1. Animals

A stock population of 192 mixed-sex (~50:50 males and females) 1-year old adult wild-type short-fin (SF) zebrafish was housed (1 fish/L) in 100-L tanks equipped with biological filters, under constant aeration and a natural (14 h light:10 h dark) photoperiod (lights on at 20:00 pm). Water temperature was maintained at 27 ± 1 °C, with pH = 7.0 ± 0.1, dissolved oxygen at 6.0 ± 0.2 mg/L, total ammonia at <0.01 mg/L, total hardness at 6 mg/L, and alkalinity at 22 mg/L $CaCO_3$.

2.2. Experimental design

To examine whether anosmia modulates anxiety-like behavior in zebrafish, the three cohorts of fish (intact, anosmic and sham-anosmic zebrafish) were used, as shown in Fig. 1.

In the control group, zebrafish remained in an aquarium for 17 min and then tested in the novel tank task for 6 min. In the odor group, zebrafish were exposed to the food odor for 17 min (see [20] for details) prior to behavioral testing in the novel tank. The FLU group was exposed to fluoxetine (50 μg/L, Daforin® EMS São Paulo, Brazil) for 17 min prior to the novel tank testing. In the stressed group, fish remained in the aquarium for 15 min and then were exposed to an acute stressor (chasing with a net for 2 min) prior to behavioral analyses in the 6-min novel tank test.

2.3. Experimental procedures

2.3.1. Anosmia protocol

The transient experimental anosmia was induced in zebrafish by application of lidocaine gel (Lidocaína Gel, EMS, São Paulo, Brazil) in the nares and olfactory surface, as described in [21]. Briefly, after zebrafish were captured and placed individually on a wet sponge, we applied a cotton ball soaked in lidocaine gel (50 mg/g) into the nares. In the sham fish, we repeated the same procedure but using only saline solution. Intact control fish were experimentally naïve and did not undergo any experimental manipulation or treatment.

2.3.2. Behavioral and cortisol analysis

In all cohorts, fish were transferred individually to a glass transparent test tank (24 × 8 × 20 cm; width × depth × height) and filmed for 6 min using a Logitech HD Webcam C525 camera (Logitech, Romanel-sur-Morges, Switzerland). The test tank was divided into two virtual zones (upper and bottom halves). The videos were then analyzed using ANY-maze® software (Stoelting Co, USA), scoring the following behavioral parameters: the number of upper zone entries, time spent (s) in the upper zone, the number of bottom zone entries, time spent (s) in the bottom zone, absolute turn angle, mean swimming speed (m/s), the number of crossings between the tank zones, and total distance travelled (m), according to the Zebrafish Behavioral Catalog (see [22]). In addition, we assessed the whole-body cortisol concentration in anosmic zebrafish, extracted and determined using the method described previously [23]. This study was approved by the Ethics Commission for Animal Use of the Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #10/2014), and performed in full compliance with the guidelines of Conselho Nacional de Controle de Experimentação Animal (Concea).

2.4. Statistics

The effects of four treatments were compared in intact, anosmic or sham-anosmic zebrafish using the one-way ANOVA followed by post-hoc Tukey's test or Kruskal-Wallis test, depending on the data normality (assessed by the Bartlett's test), for significant effects. In all experiments, P was set at <0.05.

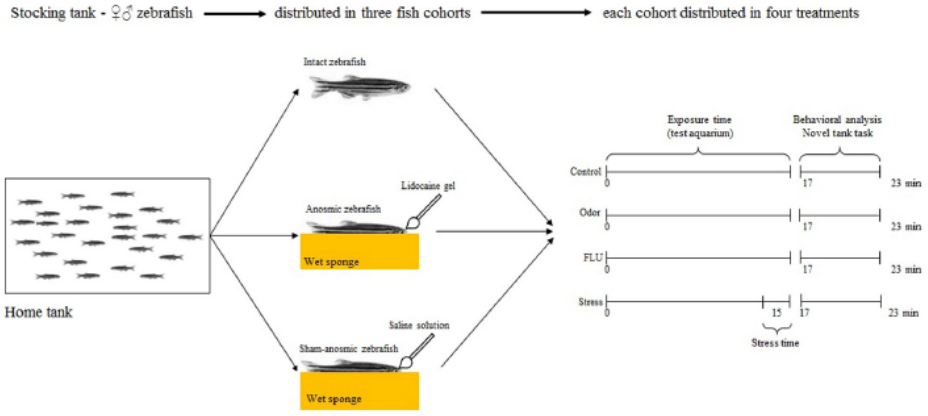

Fig. 1. Schematic representation of experimental design.

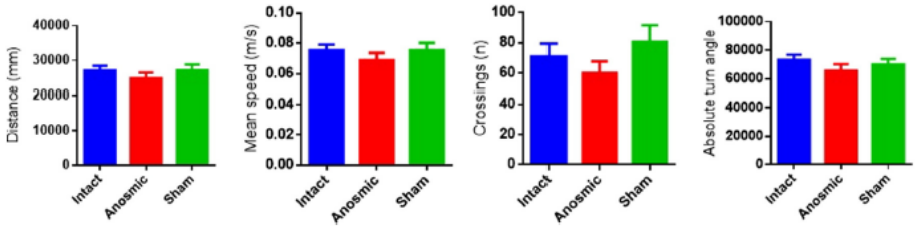

Fig. 2. Locomotor parameters of intact, anosmic and sham-anosmic control fish in the 6-min novel tank test (no significant differences detected by one-way ANOVA, $n = 12$).

3. Results

3.1. Control and intact fish

Overall, no significant behavioral differences were observed in the main locomotor parameters between intact, anosmic and sham-anosmic control fish in the novel tank test (Fig. 2). However, assessing the effects of four experimental treatments, significant differences were found in the intact fish group (Fig. 3), as the control and FLU fish travelled more distance than the stressed group, and showed higher mean speed (also in the food odor group), more crossings, absolute turn angle, as well as entries to the upper and the bottom zones. FLU fish also spent more time in the upper zone (and less, respectively, time in the bottom) vs. controls (Fig. 3).

3.2. Anosmic fish

Although we found no overt behavioral differences between the treatments in the anosmic fish (Fig. 4), all differences previously found between the treatments in intact fish (Fig. 3) were replicated for the sham-anosmic group (Fig. 5). Specifically, the control and FLU fish travelled longer distances than stressed fish, and control fish travelled a longer distance than the odor group, with similar patterns observed for mean speed and absolute turn angle measures. For the number of crossing and entries to the upper and the bottom zones, the control and FLU fish also presented higher values than the stressed fish (Fig. 5).

3.3. Whole-body cortisol in anosmic fish

As shown in Fig. 6, the odor group showed significantly higher cortisol levels than the control and stress groups, but not the FLU group.

4. Discussion

Here, we provide the first evidence that transient experimentally-evoked anosmia modulates anxiety-like behaviors and endocrine responses in adult zebrafish. Importantly, experimental anosmia did not cause sedation here, given the lack of any overt differences in locomotor parameters between control vs. intact, anosmic and sham-anosmic groups (Fig. 2). Intact and sham-anosmic fish showed similar behaviors, with stress predictably reducing their locomotor activity indices, such as distance travelled, crossing, absolute turn angle, entries upper and bottom zones [24]. Consistent with our previous studies [15], the FLU group displayed a robust anxiolytic-like effect, reflected in more time spent in the upper zone (and less time in the bottom) vs. controls. There were no differences between the treatments in all parameters analyzed in the

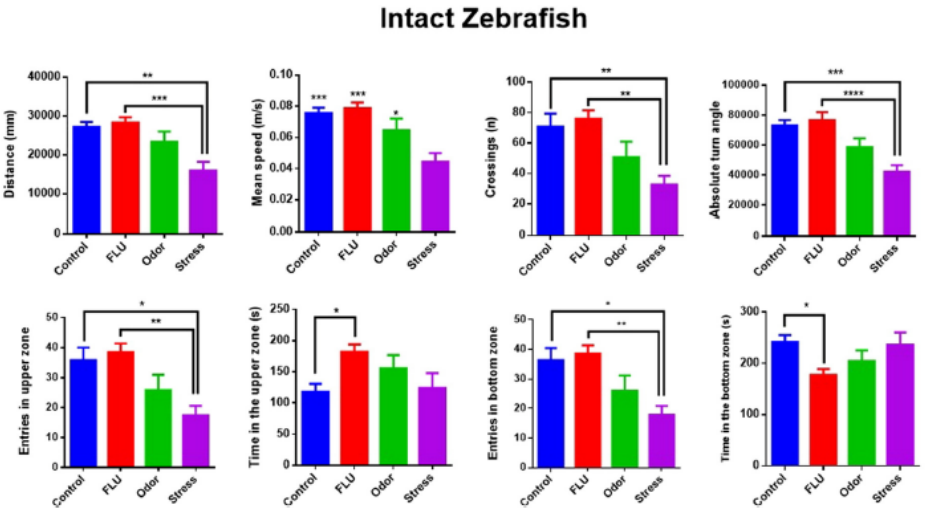

Fig. 3. Locomotor parameters in control, fluoxetine exposed, food odor exposed and stressed fish from the "intact" group in the 6-min novel tank test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ by one-way ANOVA followed by the Tukey's or Kruskal-Wallis test; $n = 12$) for distance travelled ($F_{3,41} = 7.917$; $P = 0.0003$), mean speed ($F_{3,42} = 8.735$; $P = 0.0001$), crossings ($F_{3,42} = 6.561$; $P = 0.0010$), absolute turn angle ($F_{3,42} = 10.47$; $P < 0.0001$), entries to the upper zone ($F_{3,41} = 5.767$; $P = 0.0022$), entries to the bottom zone ($F_{3,41} = 5.747$; $P = 0.0022$), time spent in the upper zone ($F_{3,41} = 3.024$; $P = 0.0403$) and time spent in the bottom zone ($F_{3,41} = 3.041$; $P = 0.0396$).

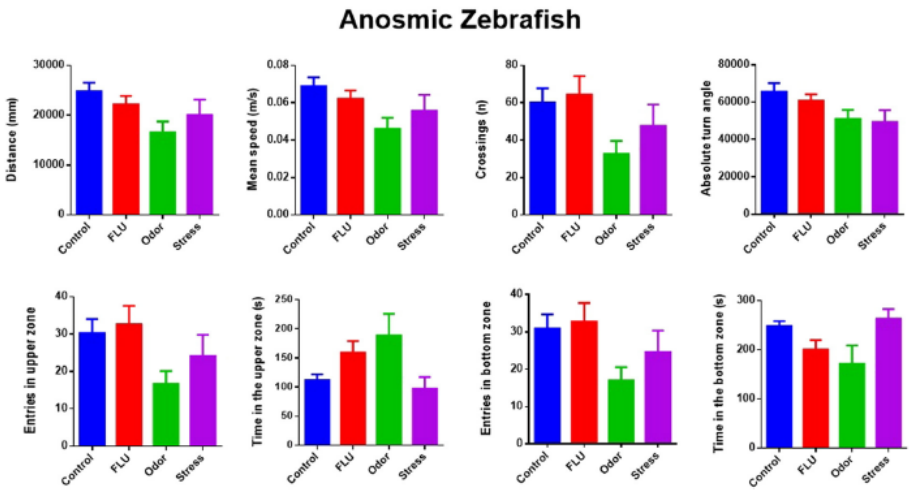

Fig. 4. Locomotor parameters of control, fluoxetine exposed, food odor-exposed and stressed fish from the "anosmic" group in the 6-min novel tank test (no differences found by one-way ANOVA; $n = 12$).

anosmic fish (Fig. 4), suggesting that anosmia may trigger anxiety-like states in fish tested here, because anxiolytic-like effects of fluoxetine were abolished in anosmic zebrafish (Fig. 4). Interestingly, such effects of anosmia on fish anxiety-like behavior may be related to the *habenula* response to the lack of olfactory stimuli, since *habenula* plays a key role in the control of fish emotional behaviors and memory in experience-dependent manners [25]. Additionally, *habenula* also acts as a higher center in fish olfaction that not only modulates the ongoing behaviors, but may also regulate emotionality and state-dependent learning (e.g., [26]).

Interestingly, the whole-body cortisol concentrations were increased in anosmic group exposed to the food odor (Fig. 6), raising the possibility that anosmic fish became more stressed since they needed to use other senses (than olfaction) to detect food. Another possibility is that the exposure to concentrated food extract altered water quality parameters (e.g., pH or osmolarity), thereby indirectly stressing this fish group further.

Finally, olfaction has long been strongly linked to emotional behavior in humans, especially since various olfactory deficits, such as anosmia, hypertrophic turbinates, nasal mucosal atrophy or chronic rhinosinusitis, all present with elevated fear and anxiety [2–3, 27]. The rodent literature also suggests that experimental anosmia evokes animal anxiety-like responses (e.g., [7–8]). Likewise, the critical role of

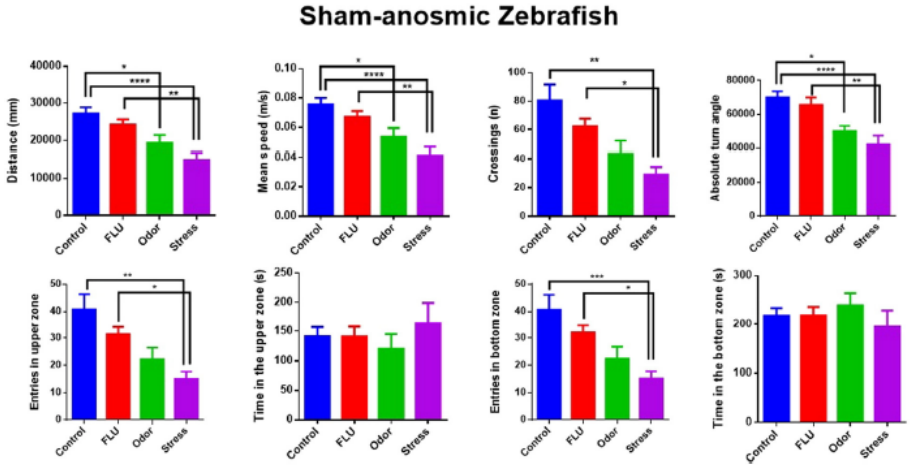

Fig. 5. Locomotor parameters of control, fluoxetine exposed, food odor-exposed and stressed fish from the "sham-anosmic" group in the 6-min novel tank test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ by one-way ANOVA followed by Tukey's or Kruskal-Wallis tests; $n = 12$ per group) for distance travelled ($F_{3,43} = 9.216$; $P < 0.0001$), mean speed ($F_{3,43} = 9.180$; $P < 0.0001$), crossings ($F_{3,47} = 17.23$; $P = 0.0006$), absolute turn angle ($F_{3,43} = 9.625$; $P < 0.0001$), entries to the upper zone ($F_{3,47} = 12.21$; $P = 0.0006$), entries to the bottom zone ($F_{3,47} = 17.44$; $P = 0.0006$), time spent in the upper zone ($F_{3,47} = 1.773$; $P = 0.6208$) and time spent in the bottom zone ($F_{3,47} = 1.773$; $P = 0.6208$).

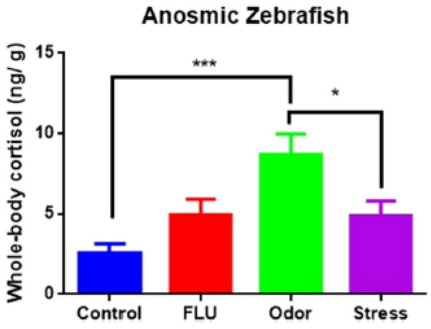

Fig. 6. The whole-body cortisol of control, fluoxetine exposed, food odor-exposed and stressed fish from the anosmic cohort (*$P < 0.05$ and ***$P < 0.001$) by one-way ANOVA ($F_{3,28} = 6.812$; $P = 0.0014$) followed by the Tukey's test; $n = 8$.

olfactory sense in fish prey-predator relationship [28], reproduction [29], preference [21] and responses to chemical cues [30] and tastes [31], is generally consistent with the role of olfaction in modulating fear/anxiety-like behavior in zebrafish. Our present findings (Figs. 2–5) further emphasize the link between experimentally-induced anosmia and anxiety-like behavior in zebrafish.

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2.6. ARTIGO 6 - *Effects of $ZnSO_4$-induced peripheral anosmia on zebrafish behavior and physiology*

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Research report

Effects of $ZnSO_4$-induced peripheral anosmia on zebrafish behavior and physiology

Murilo S. Abreu[a], Ana C.V.V. Giacomini[b], Rubens Rodriguez[b], Allan V. Kalueff[c,d], Leonardo J.G. Barcellos[a,b,e,*]

[a] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS, 97105-900, Brazil
[b] Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS, 99052-900, Brazil
[c] Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, 199034, Russia
[d] Ural Federal University, Yekaterinburg, 62002, Russia
[e] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS, 99052-900, Brazil

HIGHLIGHTS

- Experimental $ZnSO_4$-induced anosmia causes acute, but not prolonged, anxiogenic-like effect on zebrafish behavior.
- $ZnSO_4$ anosmia protocol is stressful, raising whole-body cortisol levels.
- $ZnSO_4$ anosmia protocol causes olfactory epithelium damage with overt basal cell vacuolization and intercellular edema.

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ABSTRACT

Olfaction plays a key role in modulating behavioral and physiological responses of various animal species, including fishes. Olfactory deficits can be induced in fish experimentally, and utilized to examine the role of olfaction in their normal and pathological behaviors. Here, we examine whether experimental anosmia, evoked by $ZnSO_4$ in adult zebrafish can be associated with behavioral and/or physiological responses. We show that experimental $ZnSO_4$-induced anosmia caused acute, but not prolonged, anxiogenic-like effects on zebrafish behavior tested in the novel tank test. The procedure also elevated whole-body cortisol levels in zebrafish. Moreover, ZnSO4 treatment, but not sham, produced damage to olfactory epithelium, inducing overt basal cell vacuolization and intercellular edema. The loss of olfaction, assessed by the fish food preference behavior in the aquatic Y-maze, was present 1 h, but not 24 h, after the treatment. Collectively, this suggests that transient experimental anosmia by $ZnSO_4$ modulates zebrafish behavior and olfaction, which can be used to evoke and assess their stress-related anxiety-like states.

1. Introduction

The olfactory system plays a key role in neurobehavioral adaptations of various fish species [1–3], including zebrafish (*Danio rerio*) [4,5]. Olfactory deprivation, such as experimentally evoked anosmia, has long been investigated in fishes, causing their reversible or permanent, as well as acute or chronic olfactory deficits [6]. In humans, various brain disorders, such as dementia, Parkinson's, schizophrenia and depression, are commonly associated with olfactory deficits, often comorbid with elevated fear and anxiety [7–9]. Various olfactory deficits observed clinically [7–9] or evoked experimentally in animals [4,6], have been consistently linked to increased anxiety-like states, most likely due to increased uncertainty following a markedly reduced input of critical sensory information about the environment. Ablation of olfactory epithelium *via* intranasal administration of zinc sulphate ($ZnSO_4$) has long been utilized to induce experimental anosmia in rodents [10–12]. Similar approaches have also been used in some fish species (*e.g.*, catfish) [13], suggesting it as a useful tool to examine the role of olfaction in complex behaviors across various taxa.

Zebrafish are rapidly becoming a critical novel model organism in translational neuroscience research [14], including studies of

* Corresponding author at: Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS, 99052-900, Brazil .
E-mail address: lbarcellos@upf.br (L.J.G. Barcellos).

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their sensory biology [15,16], stress physiology [17,18] and behavioral syndromes [19,20]. Several models have been developed to evoke olfactory deficits in zebrafish. For example, developmental exposure to cadmium causes long-term deficits in olfactory-dependent predator avoidance, as well alters the expression of brain stress-related proteins [21]. Global down-regulation of genes related to calcium channels and ion transport, G-proteins and olfactory receptors accompanies olfactory deficits following zebrafish exposure to copper [22], whereas reduced epithelial thickness occurs after treating these fish with a detergent, Triton X-100 [23,24]. Although an established method to induce short-term *transient* experimental anosmia in zebrafish involves the application of an anesthetic drug lidocaine into the nares [15], this *per se* increases anxiety-like behavior in zebrafish [4], likely associated with acute reduction in sensory input (*e.g.*, necessary for zebrafish normal locomotion and exploration). Furthermore, evidence shows the effects of acute and/or chronic anosmia in zebrafish induced by other methods, such as using $ZnSO_4$ administration. For example, recently applied to study zebrafish olfaction, this procedure induces acute degeneration of the olfactory epithelium, followed by the restoration of its morphology and function two weeks later [25]. However, the exact impact of $ZnSO_4$-evoked anosmia on zebrafish behavior and related stress responses remains unclear. Here, we examine the effects of $ZnSO_4$–induced experimental anosmia on zebrafish affective behaviors and whole-body cortisol responses.

2. Materials and methods

2.1. Animals

A total of 320 adult 1-year old zebrafish (~50/50 male/female ratio) of the wild-type short-fin (SF) strain, were housed 1 fish/L in 100-L tanks equipped with biological filters, under constant aeration and a natural (14 h light:10 h dark) photoperiod (lights on at 8:00 p.m.). Water temperature was maintained at 27 ± 0.5 °C; pH 7.0 ± 0.15, with dissolved oxygen kept at 6.0 ± 0.1 mg/L, total ammonia at <0.01 mg/L, total hardness at 6 mg/L, and alkalinity at 22 mg/L $CaCO_3$.

2.2. Experimental design

To evaluate the ability of experimental anosmia to modulate anxiety-like behaviors in zebrafish, the study utilized five cohorts of zebrafish (n = 15 each), including intact controls fish, anosmic (lidocaine-treated) fish, sham-anosmic (lidocaine) fish, anosmic ($ZnSO_4$-treated) and sham-anosmic (sham-$ZnSO_4$), as summarized in (Fig. 1). Fish from all five cohorts were individually tested in the novel tank test, to evaluate their anxiety-like behavior 1 h after the anosmic procedure (Fig. 1). The novel tank anxiety test was selected here as one of the most sensitive aquatic paradigms for measuring zebrafish affective behaviors [14]. Cortisol and histopathology analyses were also performed in fish after the novel tank test. Additionally, the intact, anosmic ($ZnSO_4$) and sham-anosmic ($ZnSO_4$) cohorts were evaluated for their anxiety-like behaviors 24 and 72 h after the experimental anosmia procedure, to examine potential *longer-term* consequences of $ZnSO_4$ administration. The functional confirmation of the loss of olfaction was also performed by analyzing the fish food preference behavior 1 h and 24 h after the treatment (note that the anosmia effect on food preference was absent 24 h after treatment, see further).

2.3. Anosmia protocols

Transient experimental anosmia by lidocaine, used here as a 'reference' anosmia protocol, was induced in zebrafish by drug application into the nares, as described previously [4]. Briefly, each zebrafish was placed individually on a wet sponge, followed by inserting a cotton ball soaked in lidocaine gel (50 mg/g) into both nares. In the sham-anosmic fish, the same procedure used only saline solution. Intact control fish were naïve and did not receive any treatment. Experimental anosmia was induced by injecting 5 μL of 5% $ZnSO_4$ [26] in olfactory surface of fish anesthetized as described previously [27] by placing them in 17 °C water, which was slowly adjusted to 12 °C with ice, causing hypolocomotion and immobility in zebrafish. The anesthetized fish were then placed (abdomen down) on the wet sponge, and received injection into nasal cavity, prior to transferring back to their recovery tanks [27].

2.4. Behavioral, cortisol and histopathology analysis

Fish from all cohorts were individually tested in the novel tank apparatus (24 width × 8 depth × 20 height cm), recorded for 6 min using a Logitech HD Webcam C525 camera (Logitech, Romanel-sur-Morges, Switzerland). The videos were then analyzed offline using the ANY-maze® software (Stoelting Co, Wood Dale, USA), scoring the following behavioral parameters: the number of upper zone entries, time spent (s) in the upper zone of the tank, time spent (s) in the bottom zone of the tank, absolute turn angle, mean swimming speed (m/s), and total distance traveled (mm), according to the Zebrafish Behavioral Catalog [19].

The subsequent functional validation of anosmia procedure was performed by assessing potential olfaction deficits in zebrafish based on their food preference in the 5-min aquatic Y-maze test, performed 1 h and 24 h after treatment. Conceptually adapted from rodent olfaction studies, the aquatic Y-maze contained one common 'starting' arm, one arm with a cube of gelatin (control arm) and another arm with a gelatin cube containing fish food, similar to [28]. Five cohorts of zebrafish (n = 10 each), including intact controls fish, anosmic (lidocaine-treated) fish, sham-anosmic (lidocaine) fish, anosmic ($ZnSO_4$-treated) and sham-anosmic (sham-$ZnSO_4$) were evaluated for olfactory behavior 1 h after anosmic procedure; and three cohorts (n = 10 each), including intact control, anosmic ($ZnSO_4$) and sham-anosmic ($ZnSO_4$) were evaluated for olfactory behavior 24 h after anosmic procedure. The food-containing gel (a cube 45 width × 20 depth × 25 height mm) was prepared by mixing 90% gelatin (Dr. Oetker®, São Paulo, Brazil) with 10% (weight/weight) of commercially available tropical fish flake food (Alcon® Basic, MEP 200 Complex, Brazil). The position of the two non-starting arms was switched after each testing, to avoid the arm preference bias. The videos were analyzed offline using ANY-maze® software, assessing the time spent (s) in each arm of the Y-maze.

In addition, we assessed the whole-body cortisol concentration in zebrafish 1 h after evoked anosmia, as described previously [16]. Finally, to detect histological changes in the olfactory epithelium, nasal innervations and olfactory bulb of zebrafish were stored in 10% buffered formalin overnight. Heads of the fish were decalcified, dehydrated through a graded ethanol series, cleared in xylene and then embedded in paraffin. Sections (5-μm thick) were prepared from paraffin blocks using microtome, stained with hematoxylin–eosin, and examined under an optical microscope with camera-attached brand AxioCam Erc5 s and Axio SV40 4.8.2.0 software (Carl Zeiss Microscopy GmbH, Jena, Germany). The study was approved by the Ethics Committee for Animal Use of the University of Passo Fundo, Brazil (Protocol #17/2016), and fully complied with the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA) of Brazil.

2.5. Statistics

The five groups were compared using one-way ANOVA (factor: treatment) followed by a post-hoc Tukey's test or Kruskal-Wallis test followed by a post-hoc Dunnís test, depending on data nor-

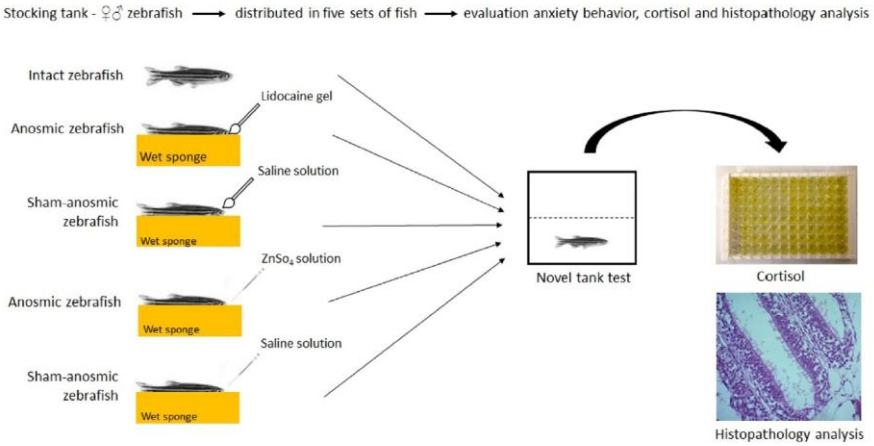

Fig. 1. Schematic summary the experimental design of the study.

mality (assessed by the Kolmogorov-Smirnov test) for significant data. The unpaired Mann-Whitney *U* test was used to analyze time spent in the two arms of the Y-maze for each fish cohort. The Gehan-Breslow-Wilcoxon and Chi-square test were used to analyze zebrafish mortality data. *P* was set as <0.05 in all analyses.

3. Results

3.1. Behavior in Y-maze task 1 and 24 h after anosmia

One hour after either lidocaine- or $ZnSO_4$ administration, the fish spent significantly less time in the control arm, with the lidocaine anosmic group spending more time in the food arm. All three groups differed in time spent in control vs. food arms, also markedly differing from fish subjected to anosmia and to anosmia sham protocol. Both lidocaine- and $ZnSO_4$-anosmic fish spent more time in the food arm, whereas the sham anosmic fish spent more time in the food-free control arm (Fig. 2A). However, 24 h after the anosmia protocols, the responses of all groups were statistically indistinguishable (Fig. 2B).

3.2. Anxiety-like behavior 1, 24 and 72 h after anosmia

Both $ZnSO_4$ groups (the anosmic and the respective sham zebrafish) showed significantly less distance traveled, absolute turn angle, mean speed and entries to the upper zone, compared to the untreated control fish (Fig. 3). In contrast, although intact-, $ZnSO_4$ anosmic- and sham anosmic- fish displayed similar novel tank behaviors, the $ZnSO_4$-anosmic fish displayed 28.57% mortality rate, compared to 0% mortality in control zebrafish ($P = 0.0007$, Chi-square test, Fig. 4A). Similarly to 24-h anosmia, the intact control-, $ZnSO_4$ anosmic- and sham anosmic fish showed no overt behavioral differences in the novel tank test, but displayed 12.5% mortality in $ZnSO_4$-treated fish, compared to intact controls ($P = 0.0036$, Chi-square test, Fig. 4B).

3.3. Whole-body cortisol and histopathological analyses 1 h after experimental anosmia

As shown in Fig. 5 (inset), the $ZnSO_4$ sham group demonstrated significantly higher whole-body cortisol levels then did the control fish. The basal cells of the olfactory epithelium in the $ZnSO_4$ group also showed overt vacuolization (intracellular edema) and separation of the cells by intercellular edema (Fig. 5). In contrast, the nasal innervations and the olfactory bulbs demonstrated no difference in cellular and stromal architecture between the groups (data not shown).

4. Discussion

Overall, the present study shows that $ZnSO_4$-induced peripheral anosmia causes acute deficits in olfactory (Figs. 2 and 3) as well as anxiety-like behaviors in adult zebrafish 1 h, but not 24 or 72 h, after administration (Figs. 3 and 4). This phenotype somewhat differs from that in rodents, where anxiety-like behavioral profile is typically evoked by chronic, but not acute, peripheral anosmia [10]. Notably, experimental anosmia by $ZnSO_4$ did significantly reduce locomotor activity endpoints, such as distance traveled, mean speed and absolute turn angle, in zebrafish, besides entries to the upper zone tested here (Fig. 3). These responses also deviated from our previous observations of unaltered zebrafish locomotor activity for transient lidocaine-induced anosmia [4]. One possible explanation for this discrepancy can be a higher degree of invasiveness of the $ZnSO_4$ injection to, as compared to lidocaine intranasal irrigation of, the olfactory epithelium. In line with this notion, we observed that $ZnSO_4$ administration procedure evoked higher whole-body cortisol levels in zebrafish (Fig. 5), thereby likely to contribute to the stress-evoked reduction of zebrafish locomotion, as suggested recently [29]. Importantly, injection of 5% ZnSO4 into the nares of amphibian species, *Rhinella arenarum*, caused olfactory damage, including vacuolization of the basal cells of olfactory epithelium and intercellular edema (*e.g.* [30]), which also can impact the functional connections the olfactory epithelium to the olfactory bulb and, consequently, trigger a temporary loss of olfaction [10]. As already mentioned, degeneration of the olfactory epithelium due to exposure to $ZnSO_4$ occurs acutely in zebrafish [25] (also see our findings 1 h after anosmia here; Fig. 5), followed by the rescue of the morphology and the function of olfactory epithelium [25].

The mortality observed in the $ZnSO_4$-treated group occurred only 24 h after the treatment (Fig. 4), further supporting the possibility of acute, likely toxic, effects of 5 μL 5-% $ZnSO_4$ in zebrafish

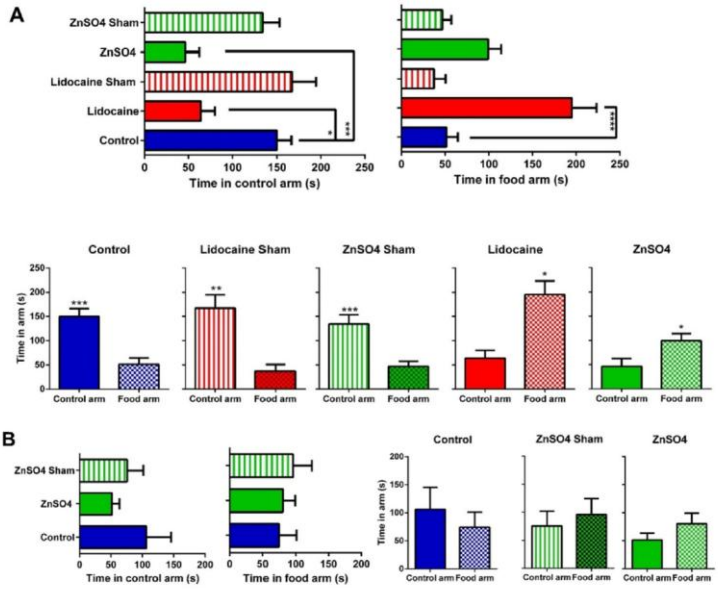

Fig. 2. Olfactory behavior in control, anosmic (lidocaine or $ZnSO_4$) and sham-anosmic (lidocaine or $ZnSO_4$) fish tested in the 5-min Y-maze test 1 h (A) and 24 h (B) after anosmia (n = 10 per group). One way ANOVA statistics for the group effect 1 h later: $F_{4,35} = 8.064$; $P = 0.0001$) for time in the control arm and ($F_{4,35} = 14.31$; $P < 0.0001$) for time in food arm. Time spent in control vs. food arms in each group was significantly different for control ($P = 0.0008$), lidocaine sham ($P = 0.0012$), $ZnSO_4$ sham ($P = 0.0003$), lidocaine ($P = 0.0111$), and $ZnSO_4$ ($P = 0.0292$) groups. One-way ANOVA for the group effect 24 h later: K = 0.4483; $P = 0.7992$), time in food arm ($F_{2,25} = 0.1946$; $P = 0.8244$). Time spent in control vs. food was similar in control ($P = 0.7768$), $ZnSO_4$ sham ($P = 0.9236$) and $ZnSO_4$ ($P = 0.2683$) groups.

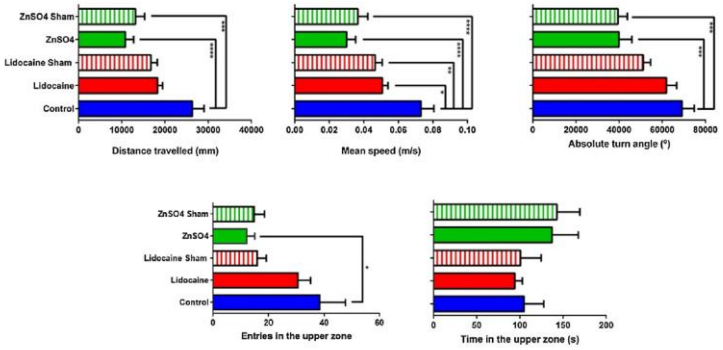

Fig. 3. Locomotor parameters of intact (control), anosmic (lidocaine or $ZnSO_4$) and sham-anosmic (lidocaine or $ZnSO_4$) fish in the 6-min novel tank test 1 h after anosmia n = 15; one-way ANOVA; statistics for distance traveled (K = 21.82; $P = 0.0002$), mean speed ($F_{4,69} = 9.316$; $P < 0.0001$), absolute turn angle ($F_{4,69} = 7.071$; $P < 0.0001$), entries to the upper zone (K = 15.54; $P = 0.0037$) and time in the upper zone (K = 1.518; $P = 0.8235$) groups.

in-vivo. This mortality may be due to administration of $ZnSO_4$ in the olfactory region rich in direct projections (*via* the olfactory nerve) to the olfactory bulbs of the brain, similar to increased mortality in neonatal mice after intranasal $ZnSO_4$ [26]. However, there are some species differences between the fish responses observed here and data obtained in rodent anosmia models. For example, mouse studies using intranasal injection of 5% $ZnSO_4$, similar to the method employed here in zebrafish, showed that in the food-finding task, not all mice are anosmic 1 week after experimentally evoked anosmia [31]. Thus, morphological confirmation of $ZnSO_4$ action on olfactory epithelium in zebrafish, such as performed here (Fig. 5), was essential for correct data interpretation.

Overall, protocols of experimental anosmia induced by lidocaine or $ZnSO_4$ were both effective in evoking olfactory deficits because fish 1 h after procedure show a different pattern of behavior (than controls), clearly *avoiding* food-containing gel cubes. Interestingly, the behavior observed here in zebrafish differed from those typically seen in rodents [32]: while rodent search for the food arm, control fish avoided the arm food, as well as did the sham groups (lidocaine and $ZnSO_4$ shams). The anosmic groups (lidocaine and

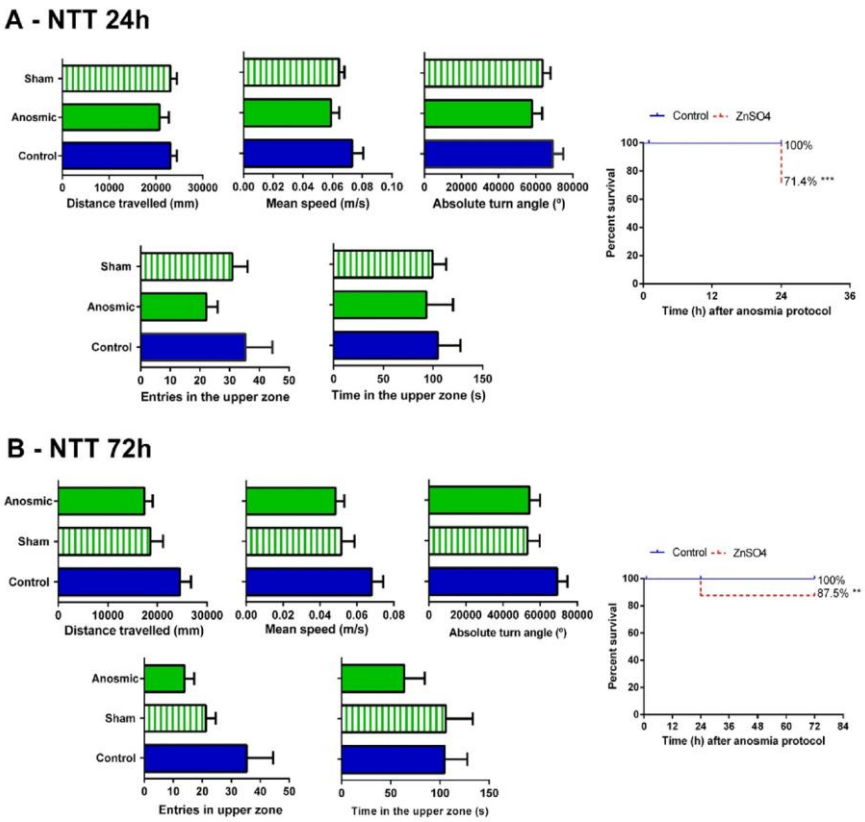

Fig. 4. Locomotor parameters of anosmic ($ZnSO_4$) and sham fish in the 6-min novel tank test (one-way ANOVA; n = 15) 24 h (A) and 72 h (B) after anosmia. Statistics for panel A for distance traveled ($F_{2,36} = 0.7238$; $P = 0.4918$), mean speed ($F_{2,35} = 1{,}503$; $P = 0.2365$), absolute turn angle ($F_{2,35} = 1.115$; $P = 0.3394$), entries to the upper zone ($K = 1.099$; $P = 0.5772$) and time in the upper zone ($F_{2,35} = 0.06939$; $P = 0.9331$), respectively. Kaplan–Meier survival curves for control and $ZnSO_4$ anosmic zebrafish 24 h after the procedure differed significantly at $P = 0.0007$. Statistics for panel B for distance traveled ($F_{2,32} = 3.166$; $P = 0.0556$), mean speed ($F_{2,32} = 3.121$; $P = 0.0578$), absolute turn angle ($F_{2,32} = 2.341$; $P = 0.1125$), entries to the upper zone ($K = 4.336$; $P = 0.1144$) and time in the upper zone ($F_{2,32} = 0.9865$; $P = 0.3839$), respectively. Kaplan–Meier survival curves from control and $ZnSO_4$ anosmic zebrafish 24 h and 72 h after the treatment differed significantly at $P = 0.0036$.

$ZnSO_4$) also did not show the expected random (50:50%) preference between the arms, but presented avoidance to the food arm, thereby differing from controls and the sham lidocaine and $ZnSO_4$ groups. On the one hand, the random distribution would have been the most logical outcome of a complete experimental anosmia. Thus, a possible explanation for these differences can be that $ZnSO_4$ and lidocaine did not completely block olfaction, but nevertheless markedly reduce it (as was also confirmed for $ZnSO_4$ by the histopathological lesions here; Fig. 5). Noting that the food has been characterized as aversive for untrained zebrafish [15], it is likely that the food used was based on fish odor that triggers a stress reaction [33] perceived as the "death odor" (that fish consistently avoided as an anti-risk behavior). Thus, combined with the anxiogenic effect of evoked sensory (olfactory) deficits, the anxiety-like state induced in fish by exposing them to the novelty of the testing apparatus might differentially modulate fish behavior, *i.e.*, making the food arm less aversive to the $ZnSO_4$ group (thereby also differing from the sham $ZnSO_4$ group, which showed no such aversion, in that aspect predictably resembling the control group on Fig. 2a). Another well-established behavioral test for zebrafish is the olfactory assessment test based on the use of alarm pheromone [34,35] or its major chemical component, hypoxantine 3-N-oxide [36,37]. Although the use of such treatments may indeed be interesting, the present study chose the food odor instead, aiming to minimize potential synergistic (and therefore behaviorally confounding) stress-evoking action of alarm cue and anxiogenic impact of anosmia procedure.

Discussing potential factors that may contribute to apparent species differences in $ZnSO_4$ anosmia effects, one may consider divergence between zebrafish and rodent neurons in their ability to regenerate. For example, zebrafish have active neurogenesis throughout the adult brain, compared to neurogenesis in rodents occurring only in several brain regions in adults [38,39]. Furthermore, given a more superior overall brain neuroregenerative potential in teleost fishes vs. mammals and humans [40,41], it is possible that 'adaptive' neuroregeneration in olfactory epithelium of zebrafish tested here could have already counterbalanced the damage caused by $ZnSO_4$ 24 h, and especially 72 h, after administration, thereby minimizing long-term behavioral and physiological effects of anosmia in this study.

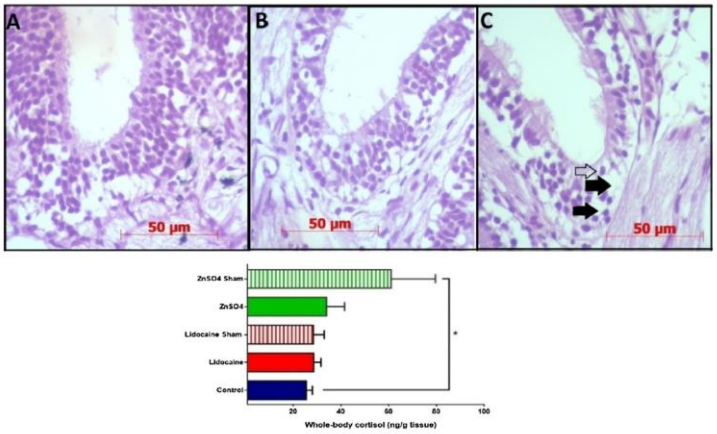

Fig. 5. Photomicrographs show nasal epithelium sections, stained by hematoxylin-eosin (×400), from representative control (A), $ZnSO_4$ sham (B) and $ZnSO_4$-treated (C) zebrafish 1 h after anosmia. Note normal olfactory epithelium in control and $ZnSO_4$ sham fish groups, and overt basal cells vacuolization (white arrows) and intercellular edema (black arrows) in all $ZnSO_4$-treated fish tested (the photographs represent selected representative images of the typical observed lesions). Inset: whole-body cortisol levels in control, lidocaine, lidocaine sham, $ZnSO_4$ and $ZnSO_4$ sham fish 1 h after experimental anosmia procedure by Kruskal-Wallis test ($K = 10.01$; $P = 0.0402$) followed by Dunnís post-hoc test ($n = 8$ in each group, $^* P < 0.05$).

Finally, there are several additional potential implications of this study. For example, based on our results, despite overt behavioral (anxiety-like) effects caused by lidocaine gel application to the olfactory epithelium, this method can be recommended to induce transient anosmia, as compared to the less efficient (but more stressful) $ZnSO_4$ anosmia protocol, which is also associated with higher zebrafish mortality. Our data are also in line with several recently published studies on zebrafish olfaction [23–25], suggesting developing zebrafish-based models of smell as a promising direction of research, albeit requiring further tuning of experimental protocols. Taken together, our findings support the growing value and utility of zebrafish as a research model for neuropsychiatric and neurological diseases [42], including both olfactory deficits and related anxiety-like states [7].

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2.7. ARTIGO 7 - *Evaluating "anxiety" and social behavior in jundiá (Rhamdia quelen)*

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Evaluating "anxiety" and social behavior in jundiá (*Rhamdia quelen*)

Murilo S. Abreu [a], Ana C.V.V. Giacomini [a,b], Gessi Koakoski [a], Angelo L. Piato [c], Leonardo J.G. Barcellos [a,b,c,d,*]

[a] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Av. Roraima, 1000, Cidade Universitária, Camobi, Santa Maria, RS 97105-900, Brazil
[b] Universidade de Passo Fundo (UPF), BR 285, São José, Passo Fundo, RS 99052-900, Brazil
[c] Programa de Pós-Graduação em Farmacologia e Terapêutica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90050-170, Brazil
[d] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo(UPF), BR 285, São José, Passo Fundo, RS 99052-900, Brazil

HIGHLIGHTS

- The *Rhamdia quelen* has been researched regarding several aspects of its physiology;
- However, experiments using validated tests to evaluate their behavior are scarce;
- Acute stress increased cortisol, induced anxiety and decreased social behavior;
- The antidepressant fluoxetine prevented the effects of stress on social behavior;
- Here we provide a behavioral evaluation using computerized analysis.

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ABSTRACT

Jundiá (*Rhamdia quelen*) is a suitable species for aquaculture in regions of temperate or subtropical climate. This species has received great attention regarding several aspects of physiology as well as an organism to study the impact of environmental contaminations. However, experiments using validated and objective tests to evaluate the jundiá behavior are scarce. The effects of acute stress have been studied in other fish species, such as zebrafish (*Danio rerio*), however, the effects in jundiá are lacking. Thus, we evaluated the effects of acute stress (net chasing) on anxiety-like and social behavior in jundiá. For these purpose, all behavioral analyses were carried out using automated tracking software. We showed that the acute stress protocol increased cortisol levels and induced anxiogenic-like behavior in the novel tank test, and decreased social behavior in jundiá. The antidepressant fluoxetine was able to prevent the effects of acute stress on social behavior. Here we show a behavioral evaluation of *Rhamdia quelen* using consolidated tests and computerized analysis, which allows more measurable, reliable and comparable results.

1. Introduction

The jundiá (*Rhamdia quelen, Pimelodidae, Siluridae*, Teleostei) is a fish species with a neotropical distribution, from southeast Mexico to southern South America [1]. Since it is a suitable species for aquaculture in regions with a temperate or subtropical climate [2], the jundiá has received great attention by South American researchers, who investigated aspects of its reproductive physiology [2–3], stress response [4–10], metabolism [11] and nutrition [12–14].

The impacts of environmental contaminants, such agrichemicals, on stress response [15–18], oxidative stress [19] and immune system [20–22] have also been studied. The impact of water quality [23–27] and exposure to plant essential oils [28–30] on several endpoints were also evaluated. The jundiá growth performance was studied in cage culture [31] and in ponds in mono- [32] and polyculture [33–35].

However, experiments aiming to evaluate the jundiá behavior are scarce, especially using validated and objective protocols. In fact, some studies report changes in jundiá behavior through descriptive observations, without any measurable parameters that can be compared statically. These subjective methods present low reliability due to variations in the individual perceptions of the observers evaluating the tests.

Behavioral tests on fish are well characterized for some species, such as zebrafish (*Danio rerio*), but for the jundiá there are not established

* Corresponding author at: Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo(UPF), BR 285, São José, Passo Fundo, RS 99052-900, Brazil.
E-mail address: lbarcellos@upf.br (L.J.G. Barcellos).

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0031-9384/

protocols to evaluate anxiety-like and social behaviors. Thus, our aim was to evaluate the effects of acute stress on anxiety-like and social behavior in jundiá using an automated tracking software.

2. Materials and methods

2.1. Animals

A stock population of 192 mixed-sex, 90–180-day-old, jundiá (*Rhamdia quelen*), weighing 5 ± 1.46 g, full length of 6.69 ± 0.65 cm, were held in tanks equipped with biological filters, under constant aeration, and with a natural photoperiod. Water temperature was maintained at 24 ± 2 °C; pH 6.8 ± 0.3; dissolved oxygen at 6 ± 0.5 mg/L; total ammonia at <0.01 mg/L.

2.2. Experimental design

Since stress modulates fish behavior [36–37], we submitted undisturbed and stressed animals to consolidated behavioral tests in order to better characterize jundiá behavior. We also exposed fish to the antidepressant fluoxetine, since this drug presents anxiolytic-like effects in fish [36,38], reverting the stress-related behavioral changes [36].

For this purpose, we randomly distributed fish into four groups: control undisturbed (Control group), fluoxetine exposed (FLU group), acute stress (Stress group) and acute stress exposed to fluoxetine (Stress + FLU group). All groups were tested immediately and 15 min after the acute stress. Fluoxetine (Daforin® EMS São Paulo, Brazil) was used at a concentration of 50 μg/L. We found that the behavior changes occurs immediately after stress, so do not realize the 15 min after stress protocol for social behavior. We also based our decision on animal welfare guidelines established to prevent more fish death than necessary.

2.2.1. Acute stress protocol and behavioral evaluation

For the behavioral evaluation immediately after stress, the control group was kept in the aquaria for 17 min and then transferred to test aquarium. The FLU group was exposed to FLU for 17 min. After 15 min, the stress groups (Stress and Stress + FLU) were submitted to the acute stress protocol (chasing with a net for 2 min, [39]). Upon transfer to the test aquarium, all four groups (with and without stress) were submitted to behavioral analysis (Fig. 1A). A similar experimental schedule was applied to another animal set, but behavioral testing was carried out 15 min after the end of the acute stress protocol (Fig. 1B).

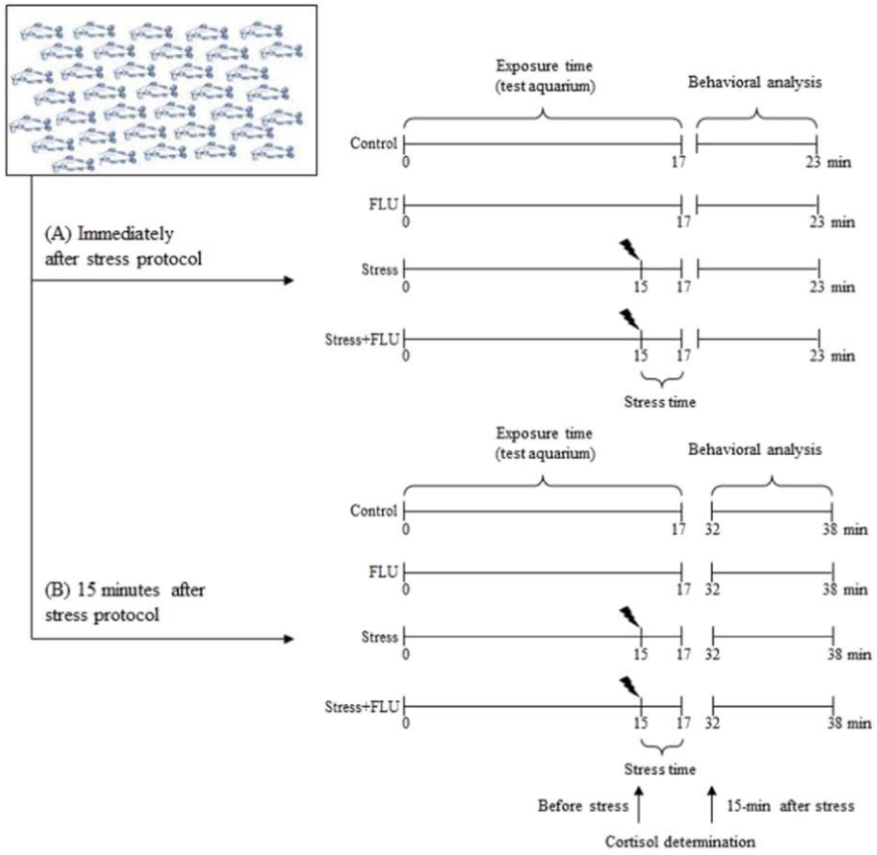

Fig. 1. Schematic representation of experimental design. (A) Immediately after stress protocol; (B) 15 min after stress protocol.

Table 1
Novel tank test in jundiá 15 min after acute stress, exposed or not to fluoxetine. Significant differences in bold font.

Parameters	Treatments				Comparison	P	F	DF
	Control	FLU	Stress	Stress + FLU				
Distance	8.07 ± 1.34	10.52 ± 1.48	7.99 ± 1.56	8.33 ± 1.39	Interaction	0.4712	0.5220	1,47
					Stress effect	0.4459	0.5656	1,47
					Drug effect	0.3460	0.9060	1,47
Mean speed	0.02 ± 0.001	0.03 ± 0.001	0.02 ± 0.001	0.02 ± 0.001	Interaction	0.3530	0.8798	1,47
					Stress effect	0.3530	0.8798	1,47
					Drug effect	0.2661	1.2670	1,47
Line crossings	58.93 ± 11.18	76.69 ± 13.77	63.43 ± 15.45	70.10 ± 14.36	Interaction	0.6926	0.1582	1,47
					Stress effect	0.9405	0.0056	1,47
					Drug effect	0.3854	0.7676	1,47
Absolute turn angle	30,844 ± 5659	39,514 ± 6013	30,986 ± 6632	39,800 ± 7416	Interaction	0.9910	0.0001	1,47
					Stress effect	0.9737	0.0010	1,47
					Drug effect	0.1815	1.8390	1,47
Max speed	0.15 ± 0.01	0.22 ± 0.05	0.16 ± 0.01	0.20 ± 0.07	Interaction	0.7418	0.1099	1,47
					Stress effect	0.7971	0,0660	1,47
					Drug effect	0.1484	2.1590	1,47
Path efficiency	0.02 ± 0.01	0.02 ± 0.001	0.02 ± 0.01	0.01 ± 0.001	Interaction	0.9267	0.0086	1,47
					Stress effect	0.8543	0.0340	1,47
					Drug effect	0.4050	0.7060	1,47
Upper zone								
Time	52.48 ± 16.81	66.12 ± 21.62	65.60 ± 23.09	55.31 ± 20.76	Interaction	0.5723	0.3234	1,47
					Stress effect	0.9563	0.0030	1,47
					Drug effect	0.9369	0.0063	1,47
Entries	13.86 ± 3.05	18.54 ± 3.38	23.30 ± 4.65	17.64 ± 5.55	Interaction	0.2169	1.5560	1,47
					Stress effect	0.3066	1.0690	1,47
					Drug effect	0,9061	0.0141	1,47
Distance	3.42 ± 0.74	4.63 ± 0.84	3.46 ± 0.55	4.43 ± 0.96	Interaction	0.8767	0.0243	1,47
					Stress effect	0.9167	0.0108	1,47
					Drug effect	0.1630	2.0090	1,47
Latency 1st entry	69.98 ± 21.94	104.48 ± 35.13	35.29 ± 10.82	60.70 ± 22.51	Interaction	0.8619	0.0305	1,47
					Stress effect	0.1379	2.2780	1,47
					Drug effect	0.2550	1.3280	1,47
Latency 1st exit	80.56 ± 23.79	105.86 ± 35.08	37.49 ± 11.06	63.44 ± 22.55	Interaction	0.9896	0.0001	1,47
					Stress effect	0.0920	2.9590	1,47
					Drug effect	0.3077	1.0630	1,47
Middle zone								
Time	39.79 ± 7.31	45.61 ± 8.44	40.44 ± 9.58	63.06 ± 13.54	Interaction	0.3856	0.7671	1,47
					Stress effect	0.3498	0.8919	1,47
					Drug effect	0.1447	2.1990	1,47
Entries	29.36 ± 5.63	39.08 ± 6.65	39.30 ± 6.27	34.79 ± 7.41	Interaction	0.2783	1.2030	1,47
					Stress effect	0.6652	0.1919	1,47
					Drug effect	0.6898	0.1612	1,47
Distance	2.91 ± 0.38	2.88 ± 0.42	3.53 ± 0.38	2.92 ± 2.8	Interaction	0.8030	0.0629	1,47
					Stress effect	0.7765	0.0814	1,47
					Drug effect	0.7831	0.0766	1,47
Latency 1st entry	31.61 ± 16.09	34.42 ± 23.48	14.92 ± 6.03	40.33 ± 19.76	Interaction	0.5176	0.4251	1,47
					Stress effect	0.7536	0.0996	1,47
					Drug effect	0.4192	0.6641	1,47
Latency 1st exit	32.98 ± 16.12	35.57 ± 23.41	18.88 ± 5.62	41.91 ± 20.01	Interaction	0.5581	0.3490	1,47
					Stress effect	0.8253	0.0492	1,47
					Drug effect	0.4629	0.5479	1,47
Bottom zone								
Time	266.69 ± 21.13	235.90 ± 29.13	253.97 ± 28.76	241.62 ± 30.64	Interaction	0.7400	0.1114	1,47
					Stress effect	0.8997	0.0160	1,47
					Drug effect	0.4838	0.6098	1,47
Entries	16.71 ± 2.91	23.77 ± 3.70	20.10 ± 2.98	18.14 ± 3.40	Interaction	0.1759	1.8800	1,47
					Stress effect	0.7344	0.1164	1,47
					Drug effect	0.4411	0.6036	1,47
Distance	3.94 ± 0.52	4.62 ± 0.92	4.74 ± 1.08	3.30 ± 0.68	Interaction	0.2240	1.5180	1,47
					Stress effect	0.7638	0.0913	1,47
					Drug effect	0.6607	0.1951	1,47
Latency 1st entry	0.61 ± 0.47	0.47 ± 0.23	5.39 ± 2.82	3.04 ± 1.64	Interaction	0.5230	0.4131	1,47
					Stress effect	**0.0378**	**4.5690**	1,47
					Drug effect	0.4726	0.5244	1,47
Latency 1st exit	31.99 ± 16.04	37.19 ± 23.24	23.62 ± 7.83	44.00 ± 19.31	Interaction	0.6627	0.1923	1,47
					Stress effect	0.9642	0.0020	1,47
					Drug effect	0.4632	0.5471	1,47

2.3. Cortisol measurements

The whole-body cortisol was evaluated in a different set of animals 15 min after acute stress protocol for all groups (see Fig. 1B). Whole-body cortisol was extracted using the method described by Oliveira et al. [40]. 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

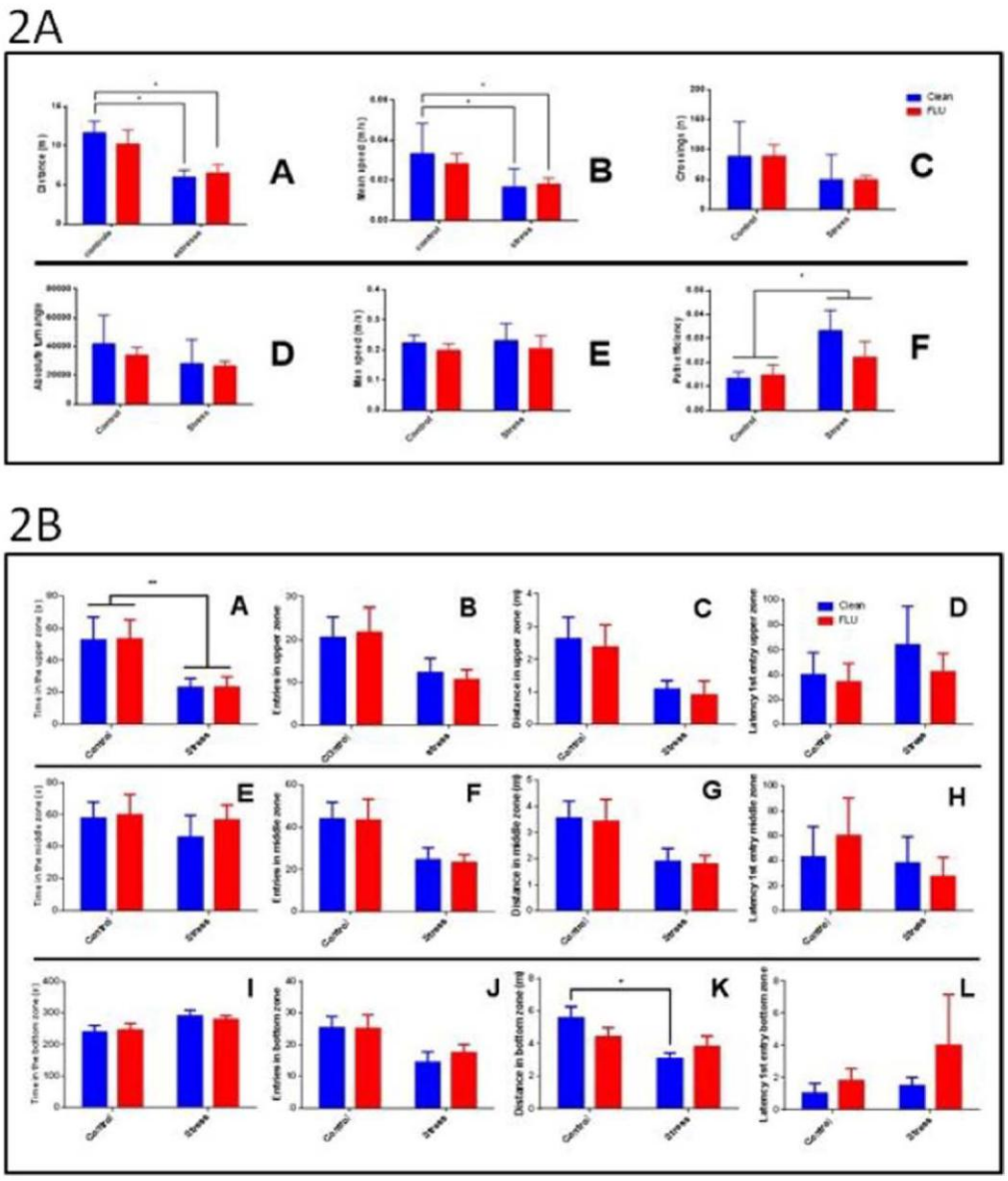

Fig. 2. Novel tank test in jundiá immediately after acute stress, exposed or not to fluoxetine. Panel 2A (locomotor activity) (A) Total distance traveled; (B) mean speed; (C) number of crossings; (D) absolute turn angle; (E) maximum speed and (F) path efficiency. Panel 2B depicts the fish movement in the three tank zones. Time, entries, distance travelled in the zone and latency to the 1st entry in upper (A, B, C and D, respectively), middle (E, F, G and H, respectively) and bottom zone (I, J, K and L, respectively). The * indicates statistical difference verified by two-way ANOVA followed by the Tukey test. Data are expressed as mean ± SEM of 12 animals per group.

samples was 94.4%. All of the cortisol values were adjusted for recovery with the following equation: cortisol value = measured value × 1.0604. Whole-body cortisol levels were measured in duplicate for each extraction using the commercially available enzyme-linked immunosorbent assay kit (EIAgen CORTISOL test, BioChem Immunosystems). This kit was fully validated for jundiá tissue extracts using the methodology described by Sink et al. [41].

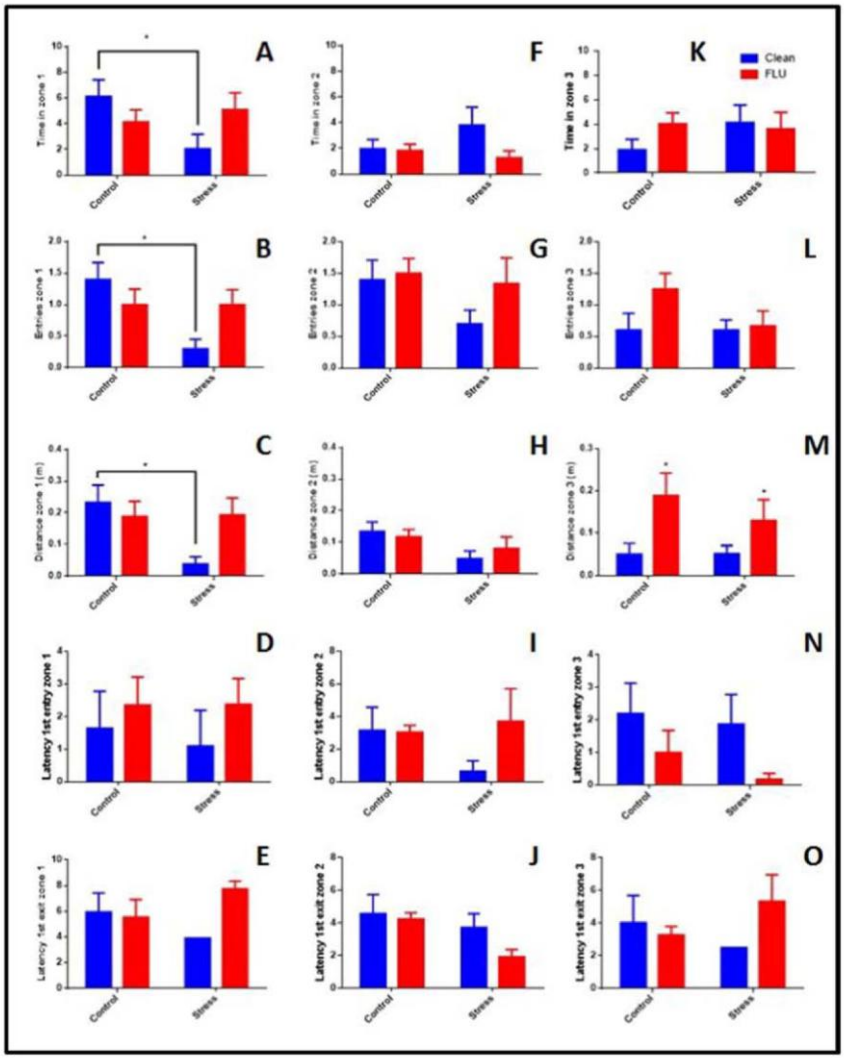

Fig. 3. Jundiá response to the social interaction test immediately after acute stress, exposed or not to fluoxetine. Time, entries, distance travelled, latency to the 1st entry and latency to the 1st exit of zone 1 (A, B, C, D and E, respectively), zone 2 (F, G, H, I and J, respectively) and zone 3 (K, L, M, N and O, respectively). Differences were depicted by asterisks (two-way ANOVA followed by the Tukey test). Data are expressed as mean ± SEM of 9–12 animals per group.

2.4. Behavioral tests

2.4.1. Novel tank test

For the novel tank task, fish were transferred individually to a test aquarium (24 × 8 × 20 cm; width × depth × height) and recorded for 6 min [36]. The following parameters were analyzed: distance traveled, mean swimming speed, maximum swimming speed, number of crossings, absolute turn angle, path efficiency, time in the zones (upper, middle and bottom), entries in the zones (upper, middle and bottom), latency to enter the zones (upper, middle and bottom).

2.4.2. Social interaction test

In this task, fish were transferred individually to the test aquarium (30 × 15 × 10 cm; width × depth × height). The test tank was positioned between two equal-size tanks, one without fish and the other containing a group of 15 conspecifics. After transfer, fish were acclimated to the test aquarium for 30 s, and then behavior was recorded for 10 s [36]. For video analysis the test tank was virtually divided into three vertical segments. The first segment (zone 1) is the nearest to conspecifics, while the third segment was next to the empty tank. The following parameters were analyzed: time

in the zones, entries in the zones, distance moved in the zones, latency to zone entries and exits (Table 1).

In both tasks, behavior was recorded using a Logitech HD Webcam C525 camera (Logitech, Romanel-sur-Morges, Switzerland), and the videos were analyzed using ANY-Maze® tracking software (Stoelting CO, USA).

2.5. Statistical analysis

Data were analyzed by two-way ANOVA followed by Tukey post hoc test. The homogeneity of variance was determined using Hartley's test, and normality was assessed using the Kolmogorov–Smirnov test. Statistical significance was set at $p < 0.05$.

2.6. Ethical note

This study was approved by the Ethics Commission for Animal Use of the Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #12/2012) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal.

3. Results

In the novel tank task (Fig. 2), the acute stress protocol decreased total distance traveled and average swimming speed (Fig. 2A) when testing was carried out 15 min after stress. Moreover, this protocol decreased the time spent in the upper zone and distance traveled in the bottom of the tank (Fig. 2B). Fluoxetine *per se* and in stressed animals was devoid of effects in these parameters (Fig. 2).

In the social interaction task (Fig. 3), the acute stress protocol decreased the time, number of entries and distance in the zone 1 (the nearest to social stimuli) (Fig. 3A, B and C, respectively). Fluoxetine reversed the effects of acute stress protocol on time, entries and distance in the 1st zone (Fig. 3A, B and C, respectively).

Regarding cortisol levels, both unexposed and fluoxetine-exposed jundiá displayed increased cortisol levels 15 min after stress (Fig. 4).

4. Discussion

Here we show, for the first time, the feasibility of use of well-known zebrafish behavioral tests to evaluate anxiety-like and social interaction in the jundiá. Using these tests, we observed that acute stress induced anxiety-like and decreased social behavior in jundiá and that fluoxetine was able to prevent the stress effect on social behavior. Studies from our group using zebrafish showed that acute stress protocol induces anxiety-like behavior and alters social behavior, while fluoxetine reduces social interaction and decreases anxiety-like behavior [36].

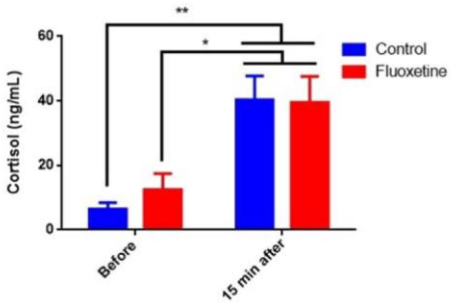

Fig. 4. Whole-body cortisol concentrations in jundiá 15 min after stress, exposed or not to fluoxetine. Differences were depicted by asterisks (*$p < 0.01$, **$p < 0.001$, two-way ANOVA, followed by Tukey's test).

Zebrafish is a diurnal, social species that prefers to swim near to the water surface [42], whereas jundiá is a nocturnal fish, without a marked shoal behavior and prefers to live near to the bottom under woods and rocks [43]. Thus, a question arose: why jundiá and zebrafish presented similar responses in the novel tank task? The response seems to be related to the fact the jundiá fingerlings present a group behavior similar to zebrafish and tend to swim around to explore the novel environment (personal observation).

We also show that behavioral changes occurred immediately after the stress while the increased cortisol concentrations occur 15 min after. This time is considered the peak cortisol concentration in jundiá at this age [7,9].

A surprising result was that fluoxetine did not influenced the cortisol concentration after acute stress protocol. However, fluoxetine impairs the cortisol response to an acute stress in zebrafish [44]. This different response may be related to drug exposure time that seems to be insufficient to impair jundiá HPI axis. Reinforcing this hypothesis, Ziv et al. [45] found an impaired stress response only after four days of exposure. In addition, in jundiá, 96 h of exposure to agrochemicals was necessary to affect HPI responsiveness to stress [15–18]. In fact, the impacts of fluoxetine exposure on fish were species dependent [46].

Interestingly, despite fluoxetine did not alter the cortisol concentrations after 15 min, the behavior was changed by the exposure to this drug. In fact, changes in behavioral parameters are not necessarily linked to hormonal concentrations [47]. Cortisol is a hormone synthetized as a response of adrenal stimulation by ACTH [37] that triggers protein synthesis [48], thus a hormonal response tends to be slower in relation to a behavioral response.

A last comment is that we used, for the first time, tests that evaluate anxiety-like behaviors and social interaction in the jundiá. Until now, behavioral tests were based only in observational assessment. Examples are the studies by Andrade et al. [49] and Golombieski et al. [25] that evaluated the effect of antibiotics and sodium chloride on the behavior and survival of jundiá infested by *Ichthyophthirius multifiliis* and *Aeromonas hydrophila*, and that determined the preferred pH range of juvenile jundiá. Here we presented a behavioral evaluation using consolidated tests and computerized analysis, which allows more measurable, reliable and comparable results.

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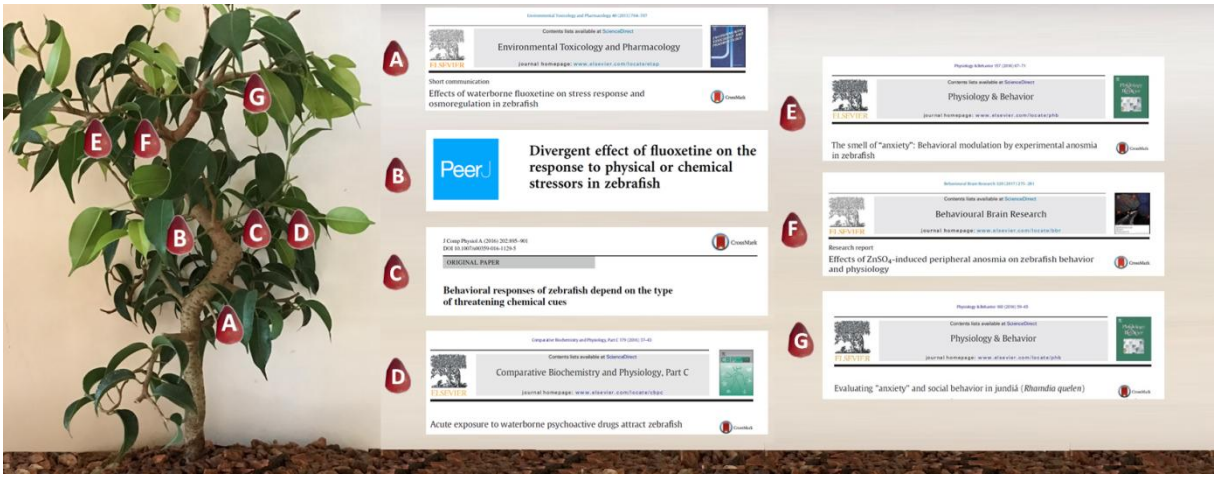
3. DISCUSSÃO

O estresse agudo altera os fluxos iônicos em *zebrafish* adulto e a exposição aguda à fluoxetina bloqueia a resposta ao estresse, consequentemente, acarreta inibição das alterações osmorregulatórias relacionadas ao estresse (Fig. 12A), com isso consequências diretas na osmorregulação, no eixo neuroendócrino e no comportamento desses peixes. Ademais, as respostas de estímulos estressores (físicos ou químicos) desencadeiam uma resposta de cortisol em *zebrafish*, a qual pode ser modulada, atenuada, pela fluoxetina em resposta a um estímulo estressor físico, mas não ao estressor químico (Fig. 12B).

O *zebrafish* pode perceber e desencadear reações defensivas induzidas por diferentes estímulos químicos desencadeados por estímulos estressores (estresse físico, químico ou visual (contato visual com predador)); ou estresse alimentar (jejum agudo ou restrição alimentar crônica); esses estímulos desencadeiam comportamentos aversivos (como estresse físico, químico e o estresse alimentar (jejum agudo)), reforçando a hipótese de que os peixes usam uma combinação de informações e o contexto da situação para determinar sua estratégia de evasão (Fig. 12C). Ademais, a presença de fármacos psicoativos nos ecossistemas aquáticos (CALISTO; ESTEVES, 2009), como diazepam, fluoxetina, risperidona e buspirona, podem ser atrativos aos peixes e sua detecção na água é, provavelmente, através de olfato (Fig. 12D). Assim, a evidência de que a anosmia experimental por lidocaína em gel (Fig. 12E) e pelo $ZnSO_4$ (Fig. 12F) modula comportamentos de ansiedade em *zebrafish* adulto, demonstra o ciclo de alterações desencadeadas pelos fármacos no ambiente aquático. Além disso, novos organismos modelos podem vir a ser estudados como o jundiá (*Rhamdia quelen*), em estudos comportamentais de "ansiedade" e interação social, a partir de testes consolidados e análise computadorizada, que permitem resultados mais confiáveis e comparáveis (Fig. 12G).

Figura 12. A árvore representa de forma figurativa o esquema estrutural da discussão dos estudos desenvolvidos, no qual os galhos representam a linha de estudo e os frutos os artigos publicados. (A) Efeitos da fluoxetina na água na resposta ao estresse e osmorregulação em *zebrafish*: apresentando uma temática quanto aos fármacos em ambiente aquático, alterações neuroendócrinas, osmorregulatórias e comportamentais; (B) Efeito divergente da fluoxetina sobre a resposta a estressor físico ou químico; (C) Respostas comportamentais do *zebrafish* dependem do tipo de sinais químicos ameaçadores: na temática de estímulos químicos desencadeados por estímulos estressores; (D) Exposição aguda a fármacos psicoativos na água atraem *zebrafish*: apresentando uma temática quanto à bioacumulação e atração de fármacos, bem como anosmia; (E) O cheiro da "ansiedade": modulação

comportamental por anosmia experimental em *zebrafish;* (F) Efeito da anosmia periférica induzida por $ZnSO_4$ sobre o comportamento e fisiologia do *zebrafish*; (G) Avaliando comportamento de "ansiedade" e social em jundiá (*Rhamdia quelen*): como a temática de organismos modelos para estudos de doenças neuropsicológicas e neurológicas, incluindo déficits olfatórios.



O cortisol de corpo inteiro apresentou elevação dos níveis 15 minutos após o estresse, momento do pico de cortisol em *zebrafish* (RAMSAY et al., 2009). A fluoxetina diminuiu os níveis de cortisol, demonstrando efeito de bloqueio sobre o eixo neuroendócrino do estresse (ABREU et al., 2014) . O estresse agudo causou uma absorção de Na^+ e K^+ 60 minutos após estresse, assim hipotetizou-se que a alteração osmorregulatória por estresse agudo em *zebrafish* ocorra 60 minutos após a exposição ao estresse. Ademais, o cortisol pode estimular o influxo de Na^+, Cl^- e a captação de Ca^{2+} (KUMAI et al., 2012). Com isso, a fluoxetina ao bloquear a resposta ao estresse, altera a osmorregulação pela redução no influxo de Na^+ e K^+.

Nesse contexto, verificou se que a fluoxetina modula (atenua) a resposta ao estresse físico, mas não químico; mesmo o estresse físico (RAMSAY et al., 2009) ou químico (TELES et al., 2017) aumentando os níveis de cortisol no *zebrafish*. A maior magnitude da resposta do estressor físico deve estar relacionada pelo seu confinamento (SILVA et al., 2015), possivelmente estressores físicos atuam nas regiões dorsolateral e dorsomedial do palium, regiões de homologia funcional à amígdala e hipocampo em mamíferos (GOODSON; KINGSBURY, 2013), com ação consequente sobre o hipotálamo. Por outro lado, o estresse químico não desencadeia uma resposta de tal magnitude (SILVA et al., 2015), com a hipótese de que dependa de mais de uma via sensorial (por exemplo, o odor, tato) para a percepção do estímulo, o que resultaria em uma supressão da força de estimulação do sistema hipotalâmico, com consequente estimulação hipofisária e posterior adrenérgica.

A fluoxetina atenuou o aumento do cortisol em *zebrafish* em resposta ao estímulo estressor físico; e a atenuação a resposta ao cortisol é concentração dependente sobre um estresse agudo (perseguição) (ABREU et al., 2014). A fluoxetina também bloqueou a resposta ao estresse após exposição crônica em *zebrafish* (EGAN et al., 2009), além do estresse aumentar a atividade serotoninérgica no telencéfalo em peixes (ØVERLI et al., 2004). De fato, os níveis de serotonina nas regiões cerebrais homólogas ao hipocampo e à amígdala dos mamíferos estão alterados em peixes submetidos a restrições espaciais (SILVA et al., 2015). Esse efeito reforça a participação destas regiões em resposta ao estresse físico, bem como o envolvimento da serotonina nessas vias. Ainda assim, a fluoxetina não bloqueou o aumento do cortisol em peixes em resposta ao estímulo estressor químico. As respostas de estresse induzidas pela substância de alarme na tilápia do Nilo (*Oreochromis niloticus*) aumentaram a taxa de ventilação e os níveis de cortisol (SANCHES et al., 2015), bem como o aumento de movimentos erráticos em *zebrafish* (SPEEDIE; GERLAI, 2008). A exposição ao sangue também demonstrou induzir o comportamento anti-predatório em tilápia do Nilo (BARRETO et al., 2013). Os receptores serotoninérgicos (5-HT_{1A} e 5-HT_4) expressos em células esteroidogênicas nas glândulas inter-renais medeiam os efeitos da serotonina na resposta ao cortisol e esse mecanismo direto pode estar subjacente aos efeitos da fluoxetina observados na resposta ao estresse físico, nomeadamente a inibição da liberação de cortisol.

O *zebrafish* pode perceber e evitar a água condicionada por estímulos químicos liberados por peixes estressados (quimicamente ou fisicamente) e pela água de peixes submetidos ao jejum agudo (sinais químicos de perturbação). O protocolo e o aparato experimental, teste de preferência quimiotáxica, foram validados em estudo de preferência a anestésicos em *zebrafish* (READMAN et al., 2013). Hipotetizou-se que a água do estresse físico e químico, e os peixes no jejum agudo provocaram um comportamento de evitação (aversão), uma vez que a informação é a partir de um estímulo direto, por serem estímulos que não precisam de um contexto específico para a liberação. A comunicação da situação de estresse já foi descrita em peixes (BARCELLOS et al., 2011, OLIVEIRA et al., 2013), assim o estresse físico e químico foram capazes de estressar os peixes comunicados quimicamente pela água ao peixe no aparato, pois já se sabe que a simples introdução da água de peixe estressado (emissor) é capaz de provocar uma resposta de estresse em peixes receptores, generalizando a resposta ao estresse (aumento de cortisol) a todos os peixes criados em todos os tanques do sistema de recirculação (BARCELLOS et al., 2011). O jejum induz algumas alterações no metabolismo dos peixes, na endocrinologia (BARCELLOS et al., 2010, ROSSI

et al., 2015) e na produção e reação a substâncias de alarme (MCCORMICK; LARSON, 2008; BARRETO et al., 2012). A mobilização de reservas de energia a partir de carboidratos, lipídios ou proteínas pode produzir alguns metabólitos que são excretados para a água (JAYARAM; BEAMISH, 1992; LAUFF; WOOD, 1996; WILKIE, 2002). Em jejum aumenta o precursor de secretogranina II (SGII), que atua em células neuroendócrinas estimulando a liberação de hormônio luteinizante e aumentando os comportamentos locomotores em peixes (TRUDEAU et al., 2012). Além disso, o cortisol desempenha um papel crucial na mobilização de energia induzida pelo jejum (WENDELAAR BONGA, 1997; MOMMSEN, 1999), e é excretado para a água através da urina, bile (VERMEIRSSEN; SCOTT, 1996) e fezes (TURNER et al., 2003). Assim, é muito plausível a hipótese de que os peixes em jejum liberam algumas substâncias químicas que são interpretadas por coespecíficos como uma situação ameaçadora, insegura e/ ou indesejável. Reforçando a hipótese de que os peixes usam uma combinação de diferentes informações e contextos para determinar sua estratégia de evasão.

Nesse contexto de percepção de substancias na água, a presença de fármacos na água é preocupante, além de intrigante, uma vez que fármacos psicoativos apresentam ser atraentes, pois os peixes não nadam evitando a pista contaminada como esperado; na verdade, buscam esses fármacos. O protocolo e aparatos usados para esse teste de preferência quimiotáxico já foram previamente validados (READMAN et al., 2013). A hipótese geral para que os peixes busquem os fármacos é a de que as concentrações eram atrativas para os peixes, por evocar um estado de bem-estar. Além disso, cada fármaco testado atua em diferentes sistemas, modulando neurotransmissores como GABA, serotonina e dopamina. A razão pela qual esses fármacos atraem os peixes pode estar relacionada com áreas que estimulam a recompensa como límbica, hipotálamo e tronco cerebral (TAN et al., 2011.; ABLER et al., 2012.; KRONENBERG et al., 2012.; HSU et al., 2014). Pelo conhecido fato da buspirona não ter efeitos sedativos (SEIDEL et al.,1985; BENCAN et al., 2009), é mais provável que a sedação não seja a causa da atração desses fármacos. Além disso, todos os fármacos testados provocaram alterações no número de cruzamentos entre o lado com fármaco e o lado com somente água; reforçando a hipótese que a sedação não seja o fato da atração, todos os fármacos não alteraram nenhum parâmetro locomotor, descartando possíveis efeitos neuromusculares. No que diz respeito à buspirona e risperidona, somente a concentração intermediária apresentou efeito atrativo. Uma possível explicação para esse padrão é que a buspirona e risperidona podem provocar uma curva de concentração-resposta em forma de U,

similar à encontrada para o diazepam (ABREU et al., 2014) e para a risperidona (IDALENCIO et al., 2015) sobre o bloqueio da resposta ao estresse *zebrafish*. O *zebrafish* apresentou comportamento de aversão frente ao controle positivo, demonstrando que são capazes de detectar o pH ácido e odores. A aversão quando em contato com o odor alimentar foi abolida em peixes anósmicos.

Ademais, em *zebrafish* anósmico, a atração verificada pelos fármacos (diazepam, fluoxetina, risperidona e buspirona) foi abolida, sugerindo que a percepção de fármacos possa ter sido como resultado da estimulação de um quimiorreceptor associado ao olfato. Considerando-se a natureza quimio-sensorial do teste utilizado, a aversão ao pH 3,0 é provavelmente associada ao contato (tátil) ou sabor (paladar) (CHANG et al., 2010). Estudos mostram que o pH ácido é detectado pelo gosto (CHANG et al., 2010) e/ou olfato (HIDAKA;TATSUKAWA, 1989). Em peixes anósmicos a ausência de atração aos fármacos é um resultado intrigante, se nossa hipótese de que a atração foi relacionada a um estado de bem-estar causado por uma ação do fármaco sobre o sistema de recompensa, certamente, atribui-se à absorção e atuação no sistema nervoso central (SNC). Outra possibilidade, é que olfato é fundamental para a escolha da pista com fármacos, e essa escolha determina que peixes passem mais tempo na presença do fármaco e, consequentemente, absorvam mais do mesmo. Na ausência do olfato, os peixes permaneceram menos tempo na presença dos fármacos. Na verdade, uma ação combinada dos sentidos é comum, envolvendo um somatório entre sabor com olfato ou visão (DELWICHE, 2012). Além disso, a ativação de memórias e áreas do SNC relacionadas com cheiro ou sabor (SHEPHERD, 2006), incluindo as relacionadas com a expressão comportamental (CHAPUIS et al., 2007) é também um fenômeno comum. Apesar destas explicações plausíveis, o mecanismo para o envolvimento do olfato com atração pelos fármacos deve ser elucidado.

Nesse sentido, também se verificou a evidência de que a anosmia experimental modula comportamentos de ansiedade em *zebrafish* adulto, demonstrada por seu efeito ansiogênico, o qual não foi abolido pela fluoxetina. Esse tipo de efeito da anosmia sobre o comportamento de ansiedade em peixes pode estar relacionado com a resposta da habênula à falta de estímulos olfativos, uma vez que a habênula desempenha um papel fundamental no controle de comportamentos emocionais e aprendizagem dependente do estado em peixes (OKAMOTO et al., 2012). A anosmia experimental não causou sedação, devido à ausência de diferenças nos parâmetros locomotores com controle. Peixes intactos e manipulados apresentaram comportamentos semelhantes, com estresse reduzindo a atividade locomotora

(MOCELIN et al., 2015). As concentrações de cortisol de corpo inteiro foram aumentadas no grupo anósmico expostos ao odor alimentar, levantando a possibilidade de que o peixe ao se tornar anósmico, necessitou utilizar outros sentidos (não o olfato) para detectar o alimento. Outra possibilidade é que a exposição ao odor alimentar altera os parâmetros de qualidade da água (por exemplo, pH).

Além disso, a anosmia periférica induzida por $ZnSO_4$ também provoca déficits agudos nos comportamentos olfativos, bem como comportamentos semelhantes à ansiedade em adultos zebrafish no período de 1 h, mas não nos períodos de 24 ou 72 h, após a administração. Esse fenótipo difere do que ocorre em roedores, onde o perfil comportamental de ansiedade é normalmente evocado por anosmia crônica, mas não aguda (MCBRIDE et al., 2003). Notavelmente, o procedimento de administração de $ZnSO_4$ evocou níveis mais elevados de cortisol no *zebrafish*, contribuindo assim para a redução evocada pelo estresse da locomoção, como sugerido recentemente (MOCELIN et al., 2015). Importante, a injeção de 5% de $ZnSO_4$ nas narinas de espécies anfíbias, *Rhinella arenarum*, causou danos olfatórios, incluindo vacuolização das células basais do epitélio olfatório e edema intercelular (YOVANOVICH et al., 2009), o que também pode afetar as conexões funcionais do epitélio olfatório para o bulbo olfatório e, consequentemente, desencadear uma perda temporária de olfato (MCBRIDE et al., 2003). Como já mencionado, a degeneração do epitélio olfativo devido à exposição ao $ZnSO_4$ ocorre de forma aguda no peixe-zebra (HENTIG; BYRD-JACOBS 2016), seguido pelo resgate da morfologia e função do epitélio olfatório (HENTIG; BYRD-JACOBS 2016).

Em geral, os protocolos de anosmia experimental induzida pela lidocaína ou $ZnSO_4$ foram ambos eficazes na evocação de déficits olfatórios, uma vez que os peixes 1 h após o procedimento mostraram um padrão de comportamento diferente (aos controles), evitando claramente cubos de gelatina contendo alimentos. Curiosamente, o comportamento observado no *zebrafish* diferiu daqueles tipicamente vistos em roedores (ZOU et al., 2015). Os grupos anósmicos (lidocaína e $ZnSO_4$) também não mostraram uma preferência aleatória esperada (50:50%) entre os braços, mas evitaram o braço alimentar, diferindo assim dos controles e dos grupos manipulados (lidocaína e $ZnSO_4$). Assim, uma possível explicação para essas diferenças pode ser que $ZnSO_4$ e lidocaína não bloquearam completamente a olfação, mas, no entanto, reduziram-na acentuadamente (como confirmado para $ZnSO_4$ pelas lesões histopatológicas). O alimento utilizado é baseado em odor de peixe uma vez que, tem como

base farinha de peixe na formulação, o qual desencadeia reação de estresse (OLIVEIRA et al., 2014) percebida como o "odor da morte" (comportamento anti-risco).

Na contextualização de análise comportamental em animais, como comportamento tipo-ansiedade e social, mostrou-se, pela primeira vez, a viabilidade do uso da metodologia já bem estabelecida em *zebrafish* para o estudo comportamental de jundiás. Usando essas metodologias, o estresse agudo induziu ansiedade e diminuiu o comportamento social no jundiá, e a fluoxetina foi capaz de prevenir o efeito do estresse sobre o comportamento social. *Zebrafish* apresenta um comportamento tipo-ansiedade quando em estresse agudo e altera o comportamento social, enquanto a fluoxetina reduz a interação social e diminui o comportamento tipo-ansiedade (GIACOMINI et al., 2016). O *zebrafish* é uma espécie diurna e social que prefere nadar perto da superfície da água (GERLAI et al., 2000), enquanto o jundiá é um peixe noturno, sem um comportamento marcado e prefere viver no fundo entre rochas; assim, surgiu uma questão: por que jundiá e *zebrafish* apresentaram respostas semelhantes quando em um novo aquário? A resposta parece estar relacionada ao fato de que os alevinos de jundiá apresentam um comportamento de grupo semelhante ao *zebrafish* e tendem a explorar o ambiente novo (obs. pessoal). Mostramos também que mudanças comportamentais ocorreram imediatamente após o estresse, enquanto que as neuroendócrinas (aumento dos níveis de cortisol) ocorrem 15 minutos após, momento do pico de cortisol em alevinos de jundiá (BARCELLOS et al., 2012; KOAKOSKI et al., 2012).

4. CONCLUSÃO

A fluoxetina atenua a resposta de cortisol a estímulo estressor físico, mas não a químico, com consequente alteração na reposta osmorregulatória; por perceber estímulos estressores desencadeia comportamentos aversivos quando em contato com águas condicionadas de estresse (físico, químico e estresse alimentar (jejum agudo)). Por outro lado, *zebrafish* apresenta atração por psicofármacos como diazepam, fluoxetina, risperidona e buspirona; os quais provavelmente são detectados pela via olfatória, que na sua ausência (anosmia experimental temporária (por lidocaína e $ZnSO_4$)) modula comportamentos de "ansiedade". Por fim, o jundiá (*Rhamdia quelen*) pode ser utilizado como organismo modelo para estudos comportamentais de "ansiedade" e interação social.

5. PERSPECTIVAS

Os estudos aqui apresentados contribuirão para futuros estudos que poderão vir a elucidar os impactos ambientais causados pela contaminação por fármacos na água; direcionar estudos referentes à comunicação química ou situações estressantes entre os peixes, bem como a participação das vias serotonérgicas na percepção de estímulos estressores; dar sequência a estudos de anosmia, visto que o *zebrafish* se apresenta como um organismo modelo de pesquisa para doenças neuropsicológicas e neurológicas, incluindo déficits olfatórios; e utilizar jundiás em estudos comportamentais de "ansiedade" e interação social.

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ANEXO A – PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS Nº 012/2012, PROTOCOLO Nº 005, 2012

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

PARECER Nº 012/2012

A Comissão de Ética no Uso de Animais da Universidade de Passo Fundo, em reunião no dia 20/04/12, analisou o projeto de pesquisa "**Alterações endócrinas e oxidativas sobre o eixo hipotálmo-hipofise-interrenal em jundiás: mecanismos de ação de defensivos agrícolas e persistência/recuperação destas alterações sobre a resposta ao estresse",** registro na CEUA Nº 005/2012, de responsabilidade do pesquisador **Leonardo José Gil Barcellos.**

Em relação aos aspectos éticos, a Comissão considerou o estudo 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 e dos "*Princípios Éticos para o Uso de Animais de Laboratório*" preconizados pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL).

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, 23 de abril de 2012.

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

ANEXO B – PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS Nº 029/2013, PROTOCOLO Nº 020, 2013

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

PARECER Nº 029/2013

A Comissão de Ética no Uso de Animais da Universidade de Passo Fundo, em reunião no dia 29/11/13, analisou o projeto de pesquisa "**Avaliação do efeito de agente estressor nos fluxos iônicos em peixe zebra (*Danio rerio*)",** registro na CEUA Nº 020/2013, 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, 03 de dezembro de 2013.

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

ANEXO C – PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS Nº 010/2014, PROTOCOLO Nº 010, 2014

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

PARECER Nº 010/2014

A Comissão de Ética no Uso de Animais da Universidade de Passo Fundo, em reunião no dia 16/05/14, analisou o projeto de pesquisa "**Toxicologia e efeitos endócrinos e comportamentais pela exposição à água contaminada por diazepam e fluoxetina**", registro na CEUA Nº 010/2014, de responsabilidade do pesquisador **Leonardo José Gil Barcellos**.

Com o objetivo de aumentar o conhecimento existente sobre as consequências da contaminação ambiental por resíduos de fármacos benzodiazepínicos oriundos da utilização humana e seus impactos nos sistemas endócrinos, comportamentais, citológicos, bioquímicos e genotóxicos em peixes, 3.480 peixes zebra de 6 meses de idade, machos e fêmeas serão submetidos a experimentos para verificar efeitos dos fármacos sobre o estresse, comportamento e toxicidade aguda e crônica

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, 16 de maio de 2014.

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

ANEXO D – PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS Nº 029/2014, PROTOCOLO Nº 023, 2014

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

PARECER Nº 029/2014

A Comissão de Ética no Uso de Animais da Universidade de Passo Fundo, em reunião no dia 21/11/14, analisou o projeto de pesquisa "**Comunicação química e percepção de fármacos em peixes expostos"**, registro na CEUA Nº 023/2014, de responsabilidade do pesquisador **Leonardo José Gil Barcellos.**

O projeto tem como objetivo verificar se os peixes são capazes de identificar a presença de substâncias de alarme ou distúrbio bem como de psicofármacos e se essas substâncias causam atração ou aversividade. Para isso serão utilizados 640 peixes da espécie *Danio rerio*, de ambos os sexos com peso entre 0,5 e 1 grama, provenientes do Laboratório de Piscicultura do Centro de Extensão e Pesquisa Agropecuária (Cepagro-UPF).

Em relação aos aspectos éticos, a Comissão considerou o projeto relevante e de relação custo-benefício adequada. O pesquisador responsável 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, 27 de novembro de 2014.

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

ANEXO E – PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS Nº 017/2016, PROTOCOLO Nº 017, 2016

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

CERTIFICADO

Cerificamos que a proposta intitulada "Efeitos da indução de anosmia por $ZnSO_4$ em peixe-zebra", registrada com o nº 017/2016, sob a responsabilidade de **Leonardo José Gil Barcellos**, e que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos) para fins de pesquisa, encontra-se de acordo com os preceitos da Lei nº 11.794 de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **aprovado** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE DE PASSO FUNDO (CEUA-UPF), em reunião de 24/06/2016.

Finalidade: Pesquisa	Vigência da autorização: 01/07/2016 a 01/07/2017
Espécie/linhagem/raça: *Peixe-zebra (Danio rerio)*	Origem: Laboratório de Piscicultura CEPAGRO - UPF
Sexo: Machos e fêmeas	Idade/peso: 12 meses / 0,5 g
Número de animais: 165	

Resumo:

Para avaliar se os diferentes protocolos de anosmia induzidos experimentalmente modulam comportamento tipo-ansiedade em peixes-zebra, serão utilizados cinco grupos experimentais: intacto (controle), anósmico (lidocaína), sham-anósmico (lidocaína), anósmico (ZnSO4) e sham-anósmico (ZnSO4). A anosmia experimental temporária com lidocaína será induzida em peixes-zebra por aplicação de lidocaína nas narinas e na superfície olfativa. Cada peixe-zebra será capturado e colocados individualmente em uma esponja molhada e, em seguida, aplicará se algodão embebido em lidocaína gel (volume 50 mg/g) nas narinas. A anosmia com ZnSO4 será induzida pela administração de 5 µL na superfície olfativa, usando uma administração injetável de ZnSO4-5%, nos peixes anestesiados Após análises comportamentais os peixes serão abatidos por secção da medula espinhal para coleta e análise de Cortisol.

Passo Fundo, 24 de junho de 2016.

Profa. Dra. Ana Cristina Vendrametto Varrone Giacomini
Coordenadora – CEUA – UPF

ANEXO F – PERMISSÃO PARA INCLUSÃO DO ARTIGO NA TESE

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