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MANIPULAÇÃO AMBIENTAL E FARMACOLÓGICA INDUZ RESPOSTAS COMPORTAMENTAIS E ENDÓCRINAS SIMILARES EM PEIXE-ZEBRA

Santa Maria, RS

2016

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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 **Doutora em Farmacologia.**

Orientador: Profº. Dr Leonardo José Gil Barcellos

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Aprovada em 26 de abril de 2016:

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Santa Maria, RS.

2016

DEDICATÓRIA

À minha família e ao meu anjo da guarda.

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Não é o que acontece com você que importa, mas a maneira com você reage ao que acontece com você.

(Epiteto)

RESUMO

MANIPULAÇÃO AMBIENTAL E FARMACOLÓGICA INDUZ RESPOSTAS COMPORTAMENTAIS E ENDÓCRINAS SIMILARES EM PEIXE-ZEBRA

AUTOR: Ana Cristina Vendrametto Varrone Giacomini

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COORIENTADOR: Angelo Luis Stapassoli Piato

As respostas fisiológicas e comportamentais podem variar de acordo com a forma de alojamento dos peixes e pela presença de fármacos ansiolíticos. O objetivo do estudo foi avaliar a resposta ao estresse agudo em peixes isolados e agrupados e o efeito da fluoxetina, diazepam e enriquecimento ambiental sobre parâmetros comportamentais e endócrino. Foram realizados 6 experimentos: 1. Teste de estresse agudo em peixes-zebra isolados e em grupos; 2. Transferência de peixes-zebra isolados ou em grupo para um ambiente novo; 3. Efeito da introdução de peixes agrupados estressados em um cardume de peixe-zebra residente; 4. Efeito da introdução de um peixe estressado em um cardume de peixe-zebra residente; 5. Efeito da exposição aguda à fluoxetina e diazepam sobre as respostas comportamentais após o protocolo de estresse agudo; 6. Resposta ao estresse agudo em peixes isolados, agrupados e a modulação por fluoxetina, diazepam e enriquecimento ambiental. Com base nos resultados concluímos que grupo é capaz de potencializar a resposta ao estresse, porém, essa resposta é reduzida pela exposição à fluoxetina e diazepam na água e pelo enriquecimento ambiental; a exposição aguda à fluoxetina e diazepam modula comportamentos em peixes-zebra após estresse agudo.

Palavras chave: Cortisol. Comportamento. Psicofármacos. Enriquecimento Ambiental.

ABSTRACT

ENVIRONMENTAL AND DRUG MANIPULATION INDUCES SIMILAR BEHAVIORAL AND ENDOCRINE RESPONSES IN ZEBRAFISH

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The physiological and behavioral responses can vary with the form of housing of the fish and the presence of anxiolytic drugs. The aim of this study was to evaluate the response to acute stress in isolated and grouped fish and the effect of fluoxetine, diazepam and environmental enrichment on behavioral and endocrine parameters. Six experiments were performed: 1. Acute stress test in isolated and groups zebrafish; 2. Transfer of isolated or group zebrafish to a new environment; 3. Effect of introducing grouped stressed fish in a resident zebrafish shoal; 4. Effect of introducing one stressed fish in a resident zebrafish shoal; 5. Effect of acute exposure to fluoxetine and diazepam on behavioral responses after acute stress protocol; 6. Response to acute stress in isolated and grouped fish and the modulation by fluoxetine, diazepam and environmental enrichment. Based on the results we concluded that the group is able to potentiate stress response, however, that response is reduced by exposure to fluoxetine and diazepam in water and environmental enrichment; acute exposure to fluoxetine and diazepam modulates behavior in zebrafish after acute stress.

Keywords: Cortisol. Behavior. Psychoactive drugs. Environmental enrichment.

SUMÁRIO

1 APRESENTAÇÃO

Nos ecossistemas aquáticos a comunicação intraespecífica e interespecífica é imprescindível para o equilíbrio dos seres vivos que compõem as comunidades. Em peixes uma forma de socialização envolve a aglomeração em cardume. Estudos em laboratório não reproduzem o habitat dos peixes nem na forma física nem na socialização. Essa situação pode comprometer a resposta ao estresse e interferir em resultados que buscam elucidar seus mecanismos fisiológicos, neuroendócrinos e comportamentais. Assim, a resposta ao estresse, considerada como um importante mecanismo homeostático pode variar de acordo com a forma de alojamento dos peixes e pode ser modulada pela contaminação ambiental por fármacos ansiolíticos e por enriquecimento ambiental.

Os resultados aqui apresentados representam parte importante oriunda de estudos preliminares e subsequentes vinculados à linha de contaminantes ambientais por fármacos do Laboratório de Peixes do Hospital Veterinário da Universidade de Passo Fundo. O trabalho está embasado na apresentação dos resultados finais sob a forma de dois artigos publicados e um manuscrito submetido, para fins de defesa de tese de Doutorado, dispondo das seguintes seções: referencial teórico, proposição, materiais e métodos, discussão e conclusão.

A seção de Referencial Teórico abordará a comunicação entre coespecíficos, a resposta ao estresse bem como o papel da serotonina e do ácido gama aminobutírico (GABA), a modulação por fluoxetina, diazepam e enriquecimento ambiental, a contaminação ambiental por fármacos e sobre peixes-zebra. Estudos sobre os efeitos comportamentais e endócrinos da fluoxetina e diazepam na água demonstram a importância da avaliação do impacto desse contaminante sobre espécies animais. A seção de Materiais e Métodos descreve de forma sucinta a metodologia utilizada nos estudos desenvolvidos e, remete aos artigos, os detalhamentos metodológicos de cada estudo. Nas seções Artigo 1, 2 e manuscrito submetido 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 a Conclusão do estudo.

1.2 REFERENCIAL TEÓRICO

1.2.1 Relações intraespecíficas

Nos ecossistemas aquáticos muitas espécies de peixes vivem agrupadas em cardume (PITCHER; PARRISH, 1993). Estudos têm demonstrado que formas de agrupamento em peixes podem ocorrer por razões sociais (*shoal*) ou de forma organizada e polarizada (*schools*) (MILLER; GERLAI, 2012). O cardume é importante para o acasalamento e para a redução do risco de predação. Nessa forma de socialização em grupo, diversos tipos de comunicação (química, sonora, mecânica) em indivíduos da mesma espécie (coespecíficos) são necessários principalmente diante de situação de risco eminente (BARCELLOS et al., 2007; 2010; 2011; 2014; BARRETO et al., 2013). Assim a comunicação entre os coespecíficos é interpretada como um mecanismo adaptativo que promove elevação do cortisol em antecipação à ameaça, amplificando a consciência do animal em relação ao ambiente (BARCELLOS et al., 2007; 2010; 2011; 2014).

Em laboratório, inúmeros experimentos são realizados em peixes isolados (EGAN et al., 2009; SACKERMAN et al., 2010; PIATO et al., 2011; PARKER et al., 2012; PAGNUSSAT et al., 2013; ZIV et al., 2013) o que pode interferir nos resultados pela falta de comunicação entre os coespecíficos. As respostas comportamentais e endócrinas envolvidas na resposta ao estresse podem diferir de acordo com a forma de alojamento e assim sofrer influência do agrupamento e/ou do isolamento (PARKER et al., 2012). Assim, o isolamento pode ser uma fonte de estresse e ansiedade (PIATO et al., 2011; PAGNUSSAT et al., 2013; ZIV et al., 2013; ADZIC et al., 2013). Entretanto Parker et al. (2012) verificaram que peixes-zebra isolados apresentaram redução no comportamento tipo ansiedade e nos níveis de cortisol em relação aos peixes agrupados.

Do ponto de vista ambiental, a comunicação entre os coespecíficos pode estar comprometida pela presença de contaminantes ambientais os quais podem causar interrupção endócrina (CERICATO et al., 2008; 2009; LISTER et al., 2009; ROCCO et al., 2011; OLIVEIRA et al., 2013; KOAKOSKI et al., 2014) e assim modificar as respostas comportamentais e endócrinas necessárias para a sobrevivência e equilíbrio dos ecossistemas aquáticos.

1.2.2 Resposta ao estresse

A resposta ao estresse é desencadeada pelo contato com o agente estressor e envolve a coordenação dos seguintes aspectos: comportamento de esquiva, aumento da vigilância, ativação do sistema nervoso simpático e do eixo hipotálamo-hipófise-adrenal (HHA) (SAPOLSKY, 1998; BARTON et al., 2002). Essa resposta é denominada "reativa" quando ocorre frente a estressores reais, como mudanças no tônus cardiovascular, dor somática ou visceral, inflamação, infecção; ou "antecipatória" quando o estímulo estressor está próximo de ser encontrado como o contato com predador, ambientes não familiares e mudanças sociais (HERMAN et al., 2003). Dessa forma, estímulos estressores fisiológicos, psíquicos ou emocionais ativam os neurônios neurossecretores parvocelulares, situados no núcleo paraventricular (NPV) do hipotálamo os quais liberam o hormônio liberador de corticotrofina (CRH) para a circulação porta hipofisária. Esse peptídeo atua na hipófise estimulando a liberação do hormônio adrenocorticotrófico (ACTH). Esse é liberado no sangue atuando no córtex das glândulas adrenais estimulando a liberação de cortisol. Esse hormônio esteroide atua em receptores de glicocorticoides presentes em tecidos-alvo promovendo a transcrição dos genes relacionados à mobilização de energia, imunossupressão e comportamento. Além disso, o cortisol atravessa a barreira hematoencefálica e atua em várias áreas encefálicas, bem como no hipotálamo, regulando a secreção hipotalâmica de CRH (*feedback negativo*). Os neurônios secretores de CRH também são regulados por estruturas encefálicas como a amígdala, o córtex pré-frontal e o sistema septo-hipocampal (SAPOLSKY; KREY; MCEWEN, 1984, SAPOLSKY et al., 1990; HERMAN et al., 2002; HERMAN et al., 2003; MORRIS, 2007). As informações sensoriais atingem a área basolateral da amígdala onde são processadas e retransmitidas para neurônios do núcleo central. A ativação desse núcleo desencadeia a resposta de estresse via neurônios do núcleo próprio da estria terminal, os quais ativam o eixo HHA e a resposta ao estresse (HERMAN et al., 2003; DABROWSKA et al., 2013). O hipocampo atua de maneira oposta à amigdala sobre o controle do eixo HHA (MORRIS, 2007). A ligação de cortisol a receptores de glicocorticoides (GR) (MORRIS, 2007) no hipocampo causa diminuição da liberação de CRH no hipotálamo (HERMAN et al., 2002; MORRIS, 2007).

Em peixes teleósteos o eixo hipotálamo-hipófise-interrenal (HHI) é análogo ao eixo HHA em humanos, sendo que a resposta ao estresse leva ao aumento do cortisol da

mesma forma (BARCELLOS et al., 2007; 2010; 2011; 2014; CERICATO et al., 2008; 2009; PIATO et al., 2011; KOAKOSKI et al., 2014). Por outro lado, de forma diferente ao eixo HHA de mamíferos, o eixo HHI é utilizado também para osmorregulação (MOMMSEN; VIJAYAN; MOON, 1999). Apesar de estruturalmente diferentes, o Sistema Nervoso Central (SNC) de mamíferos e de peixes-zebra conservam a função de algumas estruturas (LILLESAAR et al., 2009, PIATO et al., 2011; MUELLER, 2012). Peixes-zebra apresentam estruturas análogas ao hipocampo, amígdala e córtex de mamíferos (MUELLER, 2012). A habênula e seus tratos aferentes e eferentes constituem a via diencefálica dorsal transmitindo informações a partir do córtex límbico aos núcleos interpedunculares e aos núcleos dopaminérgicos na área tegumentar e serotonérgicos do núcleo da rafe; recebendo projeções dessas vias, bem como do hipotálamo. Assim a habênula atua como integrador de respostas frente a diferentes situações modulando a resposta ao estresse (OKAMOTO; AGETSUMA; AIZAWA, 2012). A resposta ao estresse promove alterações comportamentais em diversas espécies (LEEA et al. 2014; MASANA et al. 2014; ZHANG et al 2014; SNYDER et al. 2011). Estudos relatam comportamento de ansiedade após exposição a estímulos estressores em peixes-zebra (BENCAN; SLEDGE; LEVIN, 2009; SACKERMAN et al., 2010; GEBAUER et al., 2011; PIATO et al., 2011; PARKER et al., 2012; PAGNUSSAT et al., 2013), roedores (POLTRONIERI; ZANGROSSI; VIANA, 2003; GOBIRA; AGUIAR; MOREIRA, 2013; ZANGROSSI; GRAEFF, 2014) e humanos (ANACKER et al., 2011; PAUL et al., 2014). Vários sistemas de neurotransmissores e neuropeptídios participam dessa resposta devido às aferências ao NPV do hipotálamo (HERMAN et al., 2003). Dentre eles destacam-se a serotonina (5-hidroxitriptamina, 5-HT) e o ácido gama amino butírico (GABA).

1.2.2.1 Papel da Serotonina

A serotonina é um neurotransmissor produzido e liberado por neurônios serotoninérgicos localizados nos núcleos dorsal (NDR) e medial (NmR) da rafe do tronco encefálico que ascendem no feixe prosencefálico medial projetando-se para o hipotálamo, amigdala, hipocampo, córtex pré-frontal (DEAKIN; GRAEFF, 1991; ZANGROSSI; GRAEFF, 2014; PAUL et al., 2014). A síntese de serotonina ocorre a partir do triptofano através das enzimas triptofano hidroxilase e descarboxilase de

aminoácidos L- aromáticos (FAULKNER; DEAKIN, 2014). Após a síntese, a serotonina é armazenada em vesículas e liberada na fenda sináptica para ligação com receptores específicos os quais estão amplamente distribuídos no SNC e classificados em 7 subtipos (5-HT_1 - 5-HT_7). Esses receptores são proteínas transmembrana acopladas à proteína G, exceto os receptores 5-HT_3 os quais são ionotrópicos (ARTIGAS, 2013). Os autorreceptores pré-sinápticos regulam a síntese e liberação de serotonina em todo o encéfalo por *feedback* negativo (ZIGMOND et al., 1999; PAUL; LOWRY, 2013). A ação da serotonina sobre seus receptores é encerrada por inativação enzimática através da enzima monoaminoxidase (MOA) e recaptação para a terminação pré sináptica por transportadores de serotonina (SERT). Clinicamente esses transportadores são alvos de fármacos antidepressivos e ansiolíticos classificados como inibidores seletivos da recaptação de serotonina (ISRS) (ZIGMOND et al., 1999; FUCHS; WANNMACHER, 2010). Além de regular inúmeras funções como percepção sensorial, sono-vigília, dor, humor e apetite, a serotonina está envolvida na modulação de vários comportamentos (ansiedade, agressividade, sexual e dominância no grupo) e na resposta ao estresse (JACOBS; AZMITIA, 1992; LUCKI, 1998; ZIGMOND et al., 1999).

Em relação à resposta ao estresse, de acordo com a hipótese de Deakin e Graeff (1991) estímulos estressores ativam de forma diferente três vias serotonérgicas que se projetam para áreas encefálicas para coordenar as respostas fisiológicas e comportamentais frente a esses estressores. A primeira via é ativada por estímulos aversivos agudos não condicionados como exposição ao predador, dor ou estresse interoceptivo. É representada por neurônios do NDR que se projetam para substância cinzenta periaquedutal (dPAG) para inibir respostas fisiológicas de fuga/pânico. A ação da 5-HT nessa área exerce efeito ansiolítico mediado por estimulação dos receptores 5-HT_{1A} e 5-HT_{2A} no dPAG (DEAKIN; GRAEFF, 1991). A segunda via é ativada pela exposição à estímulo aversivo agudo condicionado e envolve neurônios do NDR que se projetam para a amigdala, hipocampo e córtex pré-frontal para facilitar as respostas de tipo ansiedade de conflito e medo condicionado. A 5-HT nessas áreas aumenta o comportamento tipo ansiedade via receptores 5-$HT_{2A/C}$ e 5-HT_3. A terceira via é ativada por estímulos crônicos condicionados e não condicionados e envolve os neurônios do NmR que se projetam para sistema septo e hipocampal promovendo resiliência ao estresse crônico através da ativação de receptores 5-$HT1_A$ no hipocampo (DEAKIN; GRAEFF, 1991). A disfunção dessas vias serotonérgicas está envolvida na patogênese

e tratamento dos transtornos de estresse em humanos (ANACKER et al., 2011; PAUL et al., 2014).

A relação entre estresse e ansiedade tem sido evidenciada por estudos que mostram o envolvimento o envolvimento do CRH (ZIV et al., 2013) e da serotonina (LEVENTOPOULOS et al., 2009; ANACKER et al., 2011; GRIFFITHS et al., 2012). Neurônios serotonérgicos projetam-se a partir do mesencéfalo e fazem sinapse com neurônios parvocelulares do NPV do hipotálamo onde há receptores 5-HT_{2A} e 5-HT_{1A} (SAWCHENKO et al., 1983; PAN; GILBERT, 1992; VAN DE KAR, 2001). O CRH ativa uma subpopulação de neurônios serotonérgicos no NDR desencadeando comportamento de ansiedade (MELONI et al., 2008; PAUL; LOWRY, 2013) devido às projeções do NDR à formação hipocampal e amigdala (PAUL; LOWRY, 2013).

1.2.2.2 Papel do GABA

O GABA é o principal neurotransmissor inibitório do SNC com neurônios e receptores distribuídos de forma difusa no encéfalo. É sintetizado a partir do glutamato pela enzima ácido glutâmico descarboxilase e armazenado em vesículas na terminação pré-sináptica. Após a liberação na fenda sináptica liga-se à receptores ionotrópicos ($GABA_{A\ e\ C}$) e metabotrópicos ($GABA_B$) (JOHNSTON, 1996; ZIGMOND et al., 1999). Os receptores $GABA_A$ são proteínas de membrana hetero oligoméricas de localização pós-sináptica cuja mudança conformacional promove abertura de canais de Cl^- levando à hiperpolarização da membrana pós-sináptica e, consequentemente, à diminuição da excitabilidade. Os receptores $GABA_B$ localizam-se em membrana pré e pós-sinápticas e estão ligados às vias de sinalização relacionadas à proteína G. A ativação desse receptor resulta na abertura de canais de K^+ reduzindo a excitabilidade dos neurônios pré e pós-sinápticos e diminuição na condutância ao Ca^{++} reduzindo a liberação de neurotransmissores pré e pós-sinápticos (ZIGMOND et al., 1999). Receptores $GABA_C$ estão presentes na retina (JOHNSTON, 1996; CHEBIB; JOHNSTON, 1999; LUKASIEWICZ, 2004) e atuam na modulação da magnitude da transmissão excitatória das células bipolares às células ganglionares (LUKASIEWICZ, 2004). A ação do GABA é encerrada pela dessensibilização dos receptores, pela degradação enzimática (através da GABA transaminase) ou transporte para terminação pré-sináptica ou células da glia circundantes. Os receptores $GABA_A$ apresentam sítios de ligação para o álcool,

neuroesteróides, anestésicos e para os fármacos benzodiazepínicos e barbitúricos os quais potencializam a ação do GABA e, portanto exercem efeito inibitório sobre o SNC (ZIGMOND et al., 1999; FUCHS; WANNMACHER, 2010).

A relação entre GABA e o eixo HHA é evidenciada por estudos imuno-histoquímicos (HERMAN et al., 2002; MIKLÓS; KOVÁCS, 2002; STRATTON; SEARCY; TOBET, 2011), eletrofisiológicos (BOUDABA et al., 1996) e farmacológicos (MAKARA; STARK, 1974; PLOTSKY; OTTO; SUTTON, 1987; MARQUES DE SOUZA; FRANCI, 2008). Traçados neuroanatômicos mostram que as projeções GABAérgicas ao NPV tem origem em locais difusos (CULLINAN; HERMAN; WATSON, 1993) e inervam diretamente os neurônios produtores de CRH os quais expressam subunidades de receptores $GABA_A$ (CULLINAN, 2000) e $GABA_B$ (MARQUES DE SOUZA; FRANCI, 2008) que inibem a secreção de corticosterona (CULLINAN, 2000; MARQUES DE SOUZA; FRANCI, 2008). Estudos eletrofisiológicos a partir de microestimulação por glutamato, registros intracelulares e *patch clamp* confirmam as entradas inibitórias do GABA sobre os neurônios do NPH (BOUDABA et al., 1996). Estudos farmacológicos reforçam o envolvimento na regulação da atividade do eixo. A administração central de GABA reduz níveis de CRH no sangue portal (PLOTSKY; OTTO; SUTTON, 1987) e a infusão no terceiro ventrículo impede a elevação na corticosterona induzida por trauma cirúrgico (MAKARA; STARK, 1974). Dessa forma o GABA é considerado o principal neurotransmissor inibitório que modula a complexa rede de aferências ao NPV do hipotálamo (HERMAN et al., 2002; MIKLÓS; KOVÁCS, 2002; STRATTON; SEARCY; TOBET, 2011) e consequentemente regula os neurônios produtores de CRH (MAKARA; STARK, 1974; PLOTSKY; OTTO; SUTTON, 1987; MARQUES DE SOUZA; FRANCI, 2008).

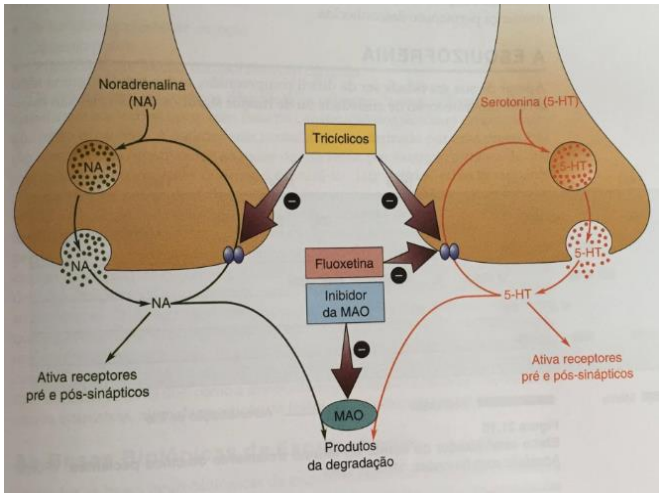
1.2.3 Modulação farmacológica do comportamento

As respostas comportamentais relacionadas ao estresse e ansiedade têm sido estudadas utilizando ansiolíticos, antidepressivos, estabilizadores de humor e estimulantes do SNC (EGAN et al, 2009; SACKERMAN et al., 2010; GEBAUER et al., 2011; PIATO et al., 2011; PRIETO et al., 2012; RICHENDRFER et al., 2012; ZIV et al., 2013).

Em relação aos fármacos antidepressivos e ansiolíticos, estudos demonstram que a fluoxetina altera a expressão de centenas de genes modificando respostas fisiológicas como reprodução, desenvolvimento, comportamento, sistema imunológico, e neuroendócrino (PARK et al., 2012; PRIETO et al., 2012; SACKERMAN et al., 2010; GEBAUER et al., 2011; WONG; OXENDINE; GODWIN, 2013). A fluoxetina é indicada para o tratamento de depressão, transtornos de ansiedade, estresse pós-traumático, Síndrome de Tourette e dor (WONG; BYMASTER; ENGLEMAN, 1995, EAPEN; TRIMBLE; ROBERTSON, 1996, ZIGMOND et al., 1999; WESTENBERG, 2009; FUCHS; WANNMACHER, 2010, ZYCHOWSKA et al., 2015). É metabolizada principalmente por enzimas hepáticas (VON MOLTKE et al., 1997) formando norfluoxetina (o principal metabólito ativo), o qual apresenta atividade farmacológica similar ao fármaco de origem (ALTAMURA; MORO; PERCUDANI, 1994).

A fluoxetina pertence à classe dos inibidores da recaptação de serotonina cujo mecanismo de ação consiste na inibição da SERT, o que aumenta a quantidade de serotonina disponível na fenda sináptica levando ao aumento global da neurotransmissão serotonérgica no SNC (ZIGMOND et al., 1999; FUCHS; WANNMACHER, 2010) (Figura 1).

Figura 1 - Mecanismo de ação da fluoxetina.



Fonte: BEAR; CONNORS; PARADISO, (2008).

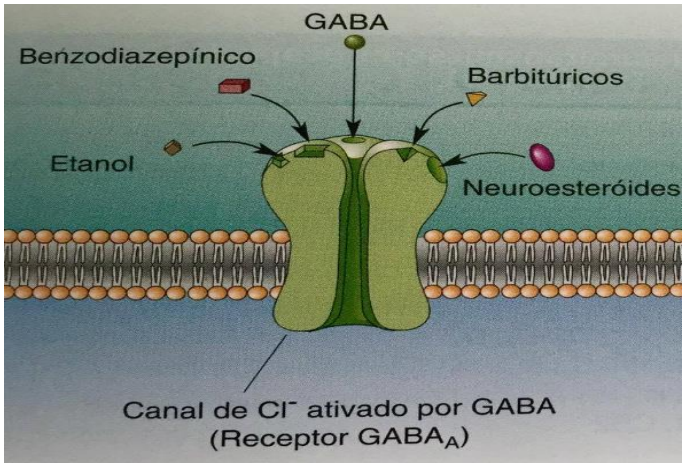
A fluoxetina pode exercer seus efeitos terapêuticos diminuindo a atividade do eixo HHA restaurando a responsividade ao normal. Esse efeito envolve o aumento na

expressão de receptores de glicocorticóides facilitando o *feedback negativo* do cortisol em humanos (ANACKER et al., 2011), em larvas de peixes-zebra (GRIFFITHS et al., 2012) e em roedores (ADZIC et al., 2013). Dessa forma compensa os efeitos da *downregulation* dos receptores de glicocorticóides induzidos pelo estresse e assim, normalizam as respostas comportamentais desencadeadas pelo estresse (LEVENTOPOULOS et al., 2009; ANACKER et al., 2011; GRIFFITHS et al., 2012).

O diazepam pertence à classe dos benzodiazepínicos considerados os fármacos mais frequentemente prescritos devido às suas propriedades ansiolíticas, hipnóticas, sedativas, amnésicas, antiepilépticas e miorrelaxantes (MANDRIOLI; MERCOLINI; RAGGI, 2008). O mecanismo de ação consiste na ligação em receptores $GABA_A$ levando à abertura de canais de Cl^- e assim potencializando o efeito do inibitório do GABA (Figura 2). O diazepam é inicialmente metabolizado por enzimas hepáticas formando os metabólitos ativos nordiazepam e temazepam os quais são respectivamente hidrolisados e desmetilados formando outro metabólito ativo, o oxazepam. A excreção desses metabólitos ocorre após glucoronidação do oxazepam e/ou do temazepam (MANDRIOLI; MERCOLINI; RAGGI, 2008).

Considerando as projeções GABAérgicas ao NPV do hipotálamo, bem como o efeito do diazepam em potencializar a ação do GABA, estudos tem demonstrado a modulação desse fármaco sobre o eixo do estresse em humanos (ROY et al., 1989), em roedores (PIVAC; PERICIC, 1993) e em peixes (ABREU et al., 2014).

Figura 2 - Mecanismo de ação do diazepam.



Fonte: BEAR; CONNORS; PARADISO, (2008).

1.2.4 Modulação por ambiente enriquecido

Ao longo dos anos tem surgido uma forte preocupação quanto ao bem estar animal, tanto daqueles criados em cativeiros quanto aqueles destinados à pesquisa e à produção. De acordo com as agências que regulam os procedimentos de pesquisa e bem-estar animal (Directive 2010/63/EU of the European Parliament and Council of the European Union EC, 2010), animais devem ser mantidos em ambiente com espaço e complexidade que permitam a expressão de seus comportamentos normais. Assim, inúmeros estudos têm sido conduzidos utilizando ambientes enriquecidos em experimentos comportamentais em roedores (KLEIN et al., 1994; VARTY et al., 2000; HAJHEIDARI; MILADI-GORJI; BIGDELI, 2015; GOES; ANTUNES; TEIXEIRA-SILVA, 2015; VARMAN; RAJAN, 2015; KONDO et al., 2015), humanos (WOO et al., 2015; FAVRE et al., 2015) e peixes (NIJMAN; HEUTS, 2000; LEMA et al., 2005; VON KROGH et al., 2010; SCHROEDER et al., 2014; COLLYMORE; TOLWANI; RASMUSSEN, 2015; MANUEL et al., 2015).

Em roedores, estudos mostram que o enriquecimento ambiental diminui os níveis de ansiedade, a resposta ao estresse e à novidade (KLEIN et al., 1994), acelera a habituação em ambientes novos e melhora a aprendizagem espacial (VARTY et al., 2000). Contudo, em espécies aquáticas há poucos relatos sobre as respostas endócrina e comportamental em ambientes enriquecidos. Em peixes, o enriquecimento ambiental aumenta a taxa de proliferação celular no telencéfalo de salmão (LEMA et al., 2005) e de peixes-zebra (VON KROGH et al., 2010), aumenta a recuperação e os efeitos adversos causados por estressores (POUNDER et al., 2016) e a capacidade dos residentes em estabelecer dominância em relação a peixes intrusos (NIJMAN; HEUTS, 2000). Schroeder et al. (2014) mostraram que peixes-zebra preferem ambientes enriquecidos a ambientes sem a complexidade do habitat natural e essa preferência pode variar de acordo com as formas de alojamento dos peixes (COLLYMORE; TOLWANI; RASMUSSEN, 2015)**.**

Estudos sobre os benefícios do enriquecimento ambiental na promoção do bem-estar animal tornam-se importantes para avaliar as respostas aos estressores a que os peixes estão constantemente expostos não somente nos ecossistemas, mas nas manipulações em laboratório e na aquicultura.

1.2.5 Fármacos como contaminantes ambientais

A contaminação dos recursos hídricos, provocada pela presença de resíduos de fármacos nos efluentes, tem sido bastante estudada (HIGNITE; AZARNOFF, 1977; HEBERER, 2002; CALAMARI, et al., 2003; BROOKS et al., 2005; JONES, et al., 2005; CALISTO; ESTEVES, 2009; ALONSO et al., 2010; CALISTO; DOMINGUES; ESTEVES, 2011; ROCCO et al., 2011; BRODIN et al., 2013). Há inúmeros relatos da presença de fármacos e seus metabólitos em águas de superfície e efluentes em diversos países (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). Esse tipo de contaminação provoca impactos negativos tanto na população humana usuária desses recursos, quanto nos organismos aquáticos que compõe esses ecossistemas e tornou-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).

O consumo de medicamentos antidepressivos, ansiolíticos e estabilizadores de humor pela população tem aumentado exponencialmente nos últimos anos em todo o mundo e, consequentemente, a presença dos metabólitos desses fármacos na água de efluentes tem acompanhado esse aumento. Estudos demonstraram que alguns psicofármacos são encontrados na forma ativa, mesmo após terem passado por sistemas de tratamentos da água, como é o caso dos ISRS e benzodiazepínicos (PATERSON; METCALFE, 2008; CALISTO; ESTEVES, 2009; DEBLONDE, COSSU-LEGUILE; HARTEMANN, 2011). Apesar de inúmeros relatos sobre a farmacocinética dos psicofármacos (MANDRIOLI; MERCOLINI; RAGGI, 2008; MANDRIOLI et al., 2012) e sua persistência no ambiente (HALLING-SØRENSEN et al., 1998; ASHTON; HILTON; THOMAS, 2004; TERNES; BONERZ; SCHMIDT, 2001), há uma grande variação na literatura sobre a excreção das formas ativas e inativas e seus metabólitos, bem como a persistência desses fármacos na água (CALISTO; ESTEVES, 2009; KOSJEK et al., 2012). Tais contaminantes ambientais, mesmo em baixas concentrações, têm sido detectados em tecidos de várias espécies de peixes indicando bioacumulação

(BROOKS et al., 2005; PATERSON; METCALFE, 2008; SACKERMAN et al., 2010; BRODIN et al., 2013).

Estudos sobre a captação e depuração da fluoxetina têm sido conduzidos em diferentes espécies de peixes (BROOKS et al., 2005; GAWORECKI; KLAINE, 2008; PATERSON; METCALFE, 2008). Estudos têm mostrado que o diazepam e outros benzodiazepínicos na água são resistentes à fotodegradação, não obstante a presença de matéria orgânica dissolvida e substâncias húmicas (CALISTO et al., 2011) e aos tratamentos fotoquímicos (KOSJEK et al., 2012). A persistência de diazepam pode ser atribuída à presença de halogênios na estrutura química que reduz a biodegradação (JOHNSON et al., 2008) e à deconjugação exercida por bactérias presentes nos efluentes domésticos e nos sistemas de tratamento (TERNES; BONERZ; SCHMIDT, 2001; HALLING-SØRENSEN et al., 1998; ASHTON; HILTON; THOMAS, 2004; CARBALLA et al., 2004).

Considerando o efeito desses fármacos sobre diversos sistemas de neurotransmissão, há indícios que tais contaminantes possam ocasionar um conjunto de alterações fisiológicas, neuroendócrinas (PARK et al., 2012; PRIETO et al., 2012), bem como comportamentais nos peixes expostos (SACKERMAN et al., 2010; GEBAUER et al., 2011; PRIETO et al., 2012; BRODIN et al., 2013). Além disso, em peixes-zebra, os ISRSs, em especial a fluoxetina, alteram a expressão de genes em larvas (PARK et al. 2012) e em adultos (WONG; OXENDINE; GODWIN, 2013), dentre eles os envolvidos na resposta aos estímulos e ao estresse (PARK et al. 2012; WONG; OXENDINE; GODWIN, 2013).

Em peixes-zebra a fluoxetina e o diazepam bloqueiam a resposta ao estresse (ABREU et al., 2014); inibindo as mudanças na osmorregulação relacionadas ao cortisol (ABREU et al., 2015); reduzem a viabilidade das larvas e alteram as fases iniciais no desenvolvimento, efeito evidenciado pelo tamanho das larvas e batimentos cardíacos (KALICHAK et al., 2015) e ainda, esses fármacos são atrativos aos peixes (ABREU et al., 2016). Considerando as 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 comportamento, torna-se imprescindível a avaliação da ação sobre os ecossistemas aquáticos e a população humana usuária da fonte de água contaminada. Essas substâncias podem provocar, inclusive, desequilíbrio nos ecossistemas aquáticos devido aos possíveis efeitos na relação presa-predador.

1.2.6 Peixes-zebra

Os peixes-zebra (*Danio rerio*) conhecidos como *zebrafish* têm sido utilizados em pesquisas em diversas áreas como genética, fisiologia, bioquímica, embriologia, endocrinologia e oncologia devido à homologia genética com seres humanos (HOWE, 2013). Têm sido utilizados também como organismo modelo em neurociências em estudos sobre doenças neurodegenerativas, comportamento e em testes de candidatos a fármacos (ZON; PETERSON, 2005; SHIN; FISHMAN, 2002; BARBAZUK et al., 2000; MUELLER; VERNIER; WULLIMANN, 2004; GOLDSMITH, 2004; EGAN et al., 2009, MACRAE; PETERSON, 2015). Esse organismo apresenta todos os neurotransmissores encontrados nos vertebrados (MUELLER, VERNIER; WULLIMANN, 2004; PANULA et al., 2006) e o sistema neuroendócrino exibe resposta robusta ao estresse (ALSOP; VIJAYAN, 2009; EGAN et al., 2009) e ansiedade (BLASER; GERLAI, 2006; EGAN et al., 2009). As vias serotoninérgicas e GABAérgicas estão bem definidas e formam uma rede complexa dentro das principais áreas do encéfalo sugerindo função similar a dos neurônios de mamíferos (LILLESAAR et al., 2009; MUELLER; WULLIMANN, 2009). Assim os peixes-zebra podem ser considerados um modelo para estudos de ansiedade, estresse e relação presa-predador, pois o eixo HHI está bem caracterizado (ALSOP; VIJAYAN, 2009; FUZZEN et al., 2010; BLASER; GERLAI, 2006; EGAN et al., 2009; GRIFFITHS et al., 2012), assim como os comportamentos anti-predatórios (PFEIFFER, 1977). O estudo do comportamento de peixes-zebra pode ser feito a partir de vários protocolos padronizados e validados na literatura como de ansiedade, memória, comportamento social, preferência, habituação (PRIETO et al., 2012; SACKERMAN et al., 2010; PIATO et al., 2011; WONG et al., 2010; WONG; OXENDINE; GODWIN, 2013; EGAN et al., 2009; COGNATO et al., 2012; GEBAUER et al., 2011; BLASER; ROSEMBERG, 2012; SCHROEDER et al., 2014; BENCAN; SLEDGE; LEVIN, 2009; RICHENDRFER, et al., 2012; MAXIMINO et al., 2011).

O teste de transferência (também chamado de *novel tank test*) para um tanque novo é utilizado para avaliar comportamento tipo ansiedade em peixes (EGAN et al., 2009; SACKERMAN et al., 2010; BLASER; ROSEMBERG, 2012; PARKER et al., 2012; PAGNUSSAT et al., 2013) sendo semelhante ao teste de campo aberto para roedores (EGAN et al., 2009; CACHAT et al., 2010). A transferência para um ambiente

novo promove alterações fisiológicas e comportamentais tais como elevação do cortisol e ansiedade (EGAN et al., 2009; PAGNUSSAT et al., 2013). Quando expostos à novidade os peixes apresentam tendência em permanecer no fundo do tanque até se sentirem seguros para explorar o novo ambiente. Esse protocolo permite avaliar ansiedade utilizando os parâmetros: latência em entrar na metade superior do tanque, entradas no topo do tanque, movimentos erráticos e imobilidade (EGAN et al., 2009). O comportamento tipo ansiedade é atribuído quando há aumento da latência para entrar no topo do tanque, menor tempo gasto no topo, aumento nos movimentos erráticos e imobilidade (EGAN et al., 2009).

Peixes-zebra demonstram capacidade cognitiva e mnemônica cuja identificação dos padrões cognitivos e de seus mecanismos subjacentes é importante para a ciência translacional (COGNATO et al., 2012). Os protocolos disponíveis para estudar a aprendizagem e memória em peixes-zebra são baseados principalmente em longos períodos de treinamento e/ou baseados nos mecanismos de recompensa ou evitação (BLANK et al, 2009; SISON; GERLAI, 2010). O protocolo de labirinto em Y (Y-Maze) apresenta vantagem permitindo o teste específico de memória, pois não envolve aprendizado condicionado minimizando estados motivacionais ou emocionais. Esse teste baseia-se na tendência natural para explorar novidade e esse componente de motivação pode ser avaliado inicialmente em curtos intervalos de treinamento e teste. Uma vez estabelecida a exploração preferencial da novidade, a medida de memória pode ser avaliada utilizando um intervalo mais longo entre o treinamento e o teste (COGNATO et al., 2012).

Peixes-zebra exibem comportamento de cardume na natureza e em laboratório. Essa agregação em cardume proporciona múltiplos benefícios incluindo o acesso aos coespecíficos, ao alimento e defesa contra predadores (GRIFFITHS et al., 2004; LEDESMA; MCROBERT, 2008; MORRELL; JAMES, 2008). A resposta de peixes experimentais para um cardume da mesma espécie é associada à preferência social e manifesta-se sob condições neutras ou levemente aversivas (GERLAI, 2003). Embora o peixe-zebra seja uma espécie social apresentando preferência pelo cardume, essa espécie demonstra territorialidade e comportamento agressivo (GERLAI, 2003). A aferição do comportamento de agressividade pode ser feita por um teste validado que quantifica as respostas comportamentais de um peixe experimental ao visualizar sua imagem no espelho (GERLAI, 2003).

O estudo desses protocolos comportamentais já validados em peixes-zebra representa importante ferramenta nas pesquisas que buscam avaliar o impacto de contaminantes ambientais sobre os ecossistemas aquáticos e, do ponto de vista translacional, é importante na elucidação de mecanismos relacionados aos transtornos de estresse, ansiedade e doenças degenerativas.

1.3 PROPOSIÇÃO

1.3.1 Proposição geral

Avaliar as respostas comportamentais e fisiológicas em peixes-zebra alojados individualmente ou em grupo e a potencial modulação por fluoxetina, diazepam e ambiente enriquecido.

1.3.2 Proposições específicas

- Avaliar a resposta ao estresse em peixes alojados individualmente e em grupos;
- Avaliar o comportamento tipo ansiedade, a memória, a preferência por coespecíficos e agressividade de peixes expostos à fluoxetina e diazepam após estresse agudo;
- Verificar a modulação pela fluoxetina, diazepam e ambiente enriquecido na resposta ao estresse em peixes isolados e agrupados.

1.4 MATERIAIS E MÉTODOS

1.4.1 Animais e condições de manutenção

O estudo foi desenvolvido no Laboratório de Fisiologia de Peixes do Hospital Veterinário, da Faculdade de Agronomia e Medicina Veterinária (FAMV) e nos Laboratórios da Área de Ciências Fisiológicas, do Instituto de Ciências Biológicas da Universidade de Passo Fundo (UPF). Foram utilizados 1.648 peixes-zebra (*wild type*),

de ambos os sexos com peso entre 0,5 e 1 grama. Os peixes permaneceram na densidade de um exemplar para cada dois litros de água, em tanques de 100 L de capacidade, sob fotoperíodo natural e aeração constante, abastecidos com água proveniente de poço artesiano. Inicialmente os animais foram mantidos em uma sala de bioensaio com temperatura de 26^{o}C durante 7 dias recebendo alimentação duas vezes ao dia com ração Tetramin® (Tetra, Melle, Germany).

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 009/2014 e 010/2014 (Anexos A e B).

1.4.3 Estudos desenvolvidos

A avaliação da resposta ao estresse em peixes alojados individualmente e em grupos foi realizada a partir dos seguintes experimentos: 1. Teste de estresse agudo em peixes-zebra isolados e em grupos; 2. Transferência de peixes-zebra isolados ou em grupo para um ambiente novo; 3. Efeito da introdução de peixe estressado em um cardume de peixe-zebra residente; 4. Efeito da introdução de peixe estressado individualmente em um cardume de peixe-zebra residente. Ao final dos experimentos os peixes foram coletados para análise de cortisol de corpo inteiro pelo método descrito por SINK; LOCHMANN; FECTEAU (2007).

A avaliação da ansiedade, memória, interação social e agressividade de peixes expostos à fluoxetina e diazepam após estresse agudo foi feita a partir da exposição aos fármacos seguida pelo protocolo de estresse agudo por perseguição com rede. A aferição desses comportamentos foi feita por testes validados para peixes-zebra. O desempenho dos animais durante os testes foi filmado por uma câmera Logitech Quick - cam PRO 9000 e os vídeos foram analisados posteriormente com o programa AnyMaze® (Stoelting CO, USA).

A verificação do efeito da fluoxetina, diazepam e ambiente enriquecido sobre a ~~na~~ resposta ao estresse em peixes isolados e agrupados foi feita utilizando diferentes formas de alojamento dos peixes durante 15 dias: isolados ou agrupados, em aquários sem complexidade ou com enriquecimento ambiental; submetidos ou não à exposição

aos fármacos testados. Durante o período amostras de água foram coletadas para monitoração da presença dos fármacos por cromatografia (LC-MS/MS). Ao final do período experimental os peixes foram coletados para análise de cortisol de corpo inteiro pelo método descrito por SINK; LOCHMANN; FECTEAU (2007).

1.4.4 Fármacos testados e tempo de exposição

Foram utilizados fluoxetina (Daforin®, EMS, Brasil) e diazepam (União Química, Brasil) nas concentrações de 50 μg /L e 16 μg /L, respectivamente (ABREU et al., 2014). A exposição à fluoxetina e diazepam nos experimentos comportamentais foi de 15 minutos, tempo suficiente para desencadear respostas comportamentais (GEBAUER et al., 2011). No experimento de modulação por fármacos e enriquecimento ambiental a exposição à fluoxetina e diazepam foi realizada durante 15 dias após uma única exposição.

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 ARTIGO 1 - MY STRESS, OUR STRESS: BLUNTED CORTISOL RESPONSE TO STRESS IN ISOLATED HOUSED ZEBRAFISH

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My stress, our stress: Blunted cortisol response to stress in isolated housed zebrafish

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HIGHLIGHTS

- Long-term isolated zebrafish presented a reduced cortisol response to stress.
- Stress response of isolated fish depends solely on their own stressor perception.
- The introduction of a stressed shoal in a resident non-stressed shoal induces stress in all fish.
- Stress response of grouped fish was augmented by chemical cues from the other members of the shoal.

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ABSTRACT

Here, we show that individually housed zebrafish presented a reduced cortisol response to an acute stressor (persecution with a pen net for 120 s) compared to zebrafish housed in groups of 10. We hypothesized that the cortisol response to stress was reduced in individually housed zebrafish because they depend solely on their own perceptions of the stressor, whereas among grouped zebrafish, the stress response might be augmented by chemical and/or behavioral cues from the other members of the shoal. This hypothesis was based on previous described chemical communication of stress in fish as well on individual variation in stressor perception and potential individual differences in fish personality.

1. Introduction

In aquatic ecosystems, intraspecific and interspecific communications are essential for the balance of the communities that make up living things. It is well documented that fish can chemically communicate to the group (conspecifics) the occurrence of risk by chemicals (alarm substance) produced and stored in epidermal cells "club" and released into the water as a result of injury to the skin [1–4] and by the presence of blood in the water [5] or by odor [3,6].

However, studies on the chemical communication against indirect contact where disturbance substances are released without injury are still scarce. Barcellos et al. found that contact with the predator promotes a rise in cortisol in conspecifics by chemical and non-visual signals [7]. In this type of communication, both the recognition of risk and the ability to release the substance used in communication are essential [8,9].

This chemical communication to conspecifics is interpreted as an adaptive mechanism that promotes elevation of cortisol in anticipation of the threat, amplifying the consciousness of the animal in relation to the environment [1,4,7,9–12].

Zebrafish (*Danio rerio*) are studied in a wide range of research fields, such as genetics, embryology, metabolism, and oncology. They are also employed to study neurodegenerative diseases, and behavior and drug responses because of their genetic homology to humans [13–18].

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In the natural environment, zebrafish live in shoals and schools. These aggregations represent important forms of socialization that reduce predation risk [19], and isolation can be a source of stress and anxiety.

Laboratory studies do not reproduce the form of socialization in school, since many experimental protocols are performed with fish individually housed [3,18,20–24]. This situation may compromise the response to stress and interfere with results that explain physiological, neuroendocrine and behavioral mechanisms by lack of chemical signaling among conspecifics. Thus, the stress response, an important homeostatic mechanism may vary according to the form of housing fish.

Short-term isolation is considered a form of stress because cortisol levels have been observed to increase under this condition after transfer to a new tank [23]. Moreover, Parker et al. [22] demonstrated that isolated fish exhibit less anxiety after long-term isolation and that isolation acts as a CNS depressor. Here we evaluate whether the responses to acute stressors differed between grouped and isolated fish over a long-term period (15 to 30 days) and the introduction of stressed fish stress promote a school of fish residents.

2. Materials and methods

2.1. Ethical note

This study was approved by the Ethics Commission for Animal Use (CEUA) of Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #9/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Subjects

A stock population of 876 mixed-sex, adult, wild-type zebrafish (*D. rerio*) of the short-fin (SF) strain was housed in two tanks with constant aeration and biological filtering under a natural photoperiod (approximately 14 h light: 10 h dark). The water was maintained under the following conditions: 26 ± 2 °C; pH 7.0 ± 0.25; dissolved oxygen at 6.5 ± 0.4 mg/L; total ammonia at < 0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22 mg/L $CaCO_3$.

2.3. Experimental protocols

Experiment 1. Acute stress challenge test of isolated and grouped zebrafish

We divided this experiment into two pre-treatment durations: 15 days and 30 days. Individuals were maintained in either isolation or groups of 10 fish prior to applying the acute stress challenge test (persecution with a pen net for 120 s). In each phase, fish from the stock population were distributed into 40 plastic tanks (12 L): 10 containing individually isolated fish without a stress challenge (control), 10 with individually isolated fish subject to an acute stress challenge, 10 with groups of fish without a stress challenge (control) and 10 with groups of fish submitted to an acute stress challenge. Cortisol levels were sampled 15 min after the stress challenge to obtain the peak cortisol concentrations [25]. One fish was sampled at random from each group tank for cortisol level measurement. During the pre-treatment maintenance periods (15 or 30 days), the water quality was monitored and maintained at conditions identical to those in the stock population.

Experiment 2. Transfer of individually isolated or group zebrafish to a novel environment

Fish were distributed into 40 plastic tanks (12 L): 10 tanks with individually isolated fish without transfer to a novel environment (control), 10 tanks with individual fish isolated for 15 days before transfer to a novel environment, 10 tanks with groups of fish without transfer (control) and 10 tanks with individual fish isolated for 15 days before transfer to a novel environment. For the transfer treatments, we killed the fish 15 min after transfer to investigate the effects of the transfer treatment and exploration of the novel environment in individually isolated and grouped fish. One fish was randomly sampled from each group tank for cortisol level measurement.

Experiment 3. Effect of introducing stressed fish into a resident zebrafish shoal

Fish were initially separated into two groups according to fish size: small (0.5 ± 0.05 g) and large (1.0 ± 0.05 g). A total of 72 small fish were categorized as "resident zebrafish (RZf)," and 144 large zebrafish were categorized as "introduced zebrafish (IZf)." The IZf were then distributed into two subgroups of 72 individuals, with each subgroup housed in an 80-L glass aquarium.

The experiment was performed using 24 plastic tanks (12 L). Half of the tanks were stocked with a shoal of six RZf. The remaining 12 tanks received no fish. We sought to investigate the effect of introducing stressed IZf on the cortisol profiles of the RZf. For this purpose, four experimental groups were examined. In the first group, we introduced un-stressed IZf into empty tanks. In the second group, we introduced un-stressed IZf into tanks containing shoals of six RZf. The third group involved introducing stressed IZf into empty tanks, and in the fourth group, we introduced stressed IZf into tanks containing shoals of six RZf. The acute stress was identical to that of the first experiment (persecution with a pen net), and the IZf were transferred immediately after applying the stressor. The IZf and RZf were sampled for cortisol determination 30 min after IZf introduction (i.e., at the peak cortisol moment, [12]; see scheme in Fig. 3).

Experiment 4. Effect of the introduction of individual fish stressed in a shoal of resident zebrafish

As in Experiment 3, fish were initially separated into two groups according to size: small (0.5 ± 0.05 g) and large (1.0 ± 0.05 g). A total of 42 small fish were categorized as "resident zebrafish" (RZf), and 12 large zebrafish were categorized as "introduced zebrafish" (IZf). The experiment was performed using 19 plastic tanks (12 L), 12 tanks with a shoal of six RZf and 7 with IZf, which 6 tanks with one fish (SIso IZf) and one tank with a shoal of six IZf (SG IZf). We sought to investigate the effect of introducing stressed individual IZf on the cortisol profiles of the RZf. For this purpose, two experimental groups were examined. In the first group, we introduced SIso IZf into empty tanks and in the second group, we introduced SG IZf into tanks containing shoals of six RZf. The acute stress was identical to that of the first experiment (persecution with a pen net), and the IZf were transferred immediately after applying the stressor. The IZf and RZf were sampled for cortisol determination 30 min after IZf introduction (i.e., at the peak cortisol moment, [12]; see scheme in Fig. 4).

2.4. General procedures

2.4.1. Cortisol extraction and analysis

Fish were captured and immediately frozen in liquid nitrogen for 10–30 s, followed by storage at −20 °C until cortisol extraction. Whole-body cortisol was extracted following Oliveira et al. [26]. Measurement accuracy was evaluated by calculating the levels recovered from samples spiked with known amounts of cortisol (50, 25 and 12.5 ng/mL). The mean detection of spiked samples was 94.3%. All cortisol values were adjusted for recovery using the following equation: cortisol value = measured value × 1.0604.

Tissue samples were resuspended in 1 mL PBS, and whole-body cortisol levels were measured in duplicate samples of each extract using a commercially available enzyme-linked immunosorbent assay kit (EIAgen™ CORTISOL test, BioChem ImmunoSystems). This kit was

fully validated for zebrafish tissue samples following Sink et al. [27]. The accuracy was tested by repeating the assay 12 times using seven randomly chosen samples on the same plate and calculating the intra-assay coefficient of variation (CV). The reproducibility was tested by assaying the same samples on different plates and calculating the inter-assay CV. To test for linearity and parallelism, the tissue samples were submitted to serial dilutions in the buffer provided with the kit. A strong positive correlation between the curves was observed (R^2 = 0.8918), and the samples yielded low inter- and intra-assay CV values (7–10% and 5–9%, respectively).

2.5. Statistics

Differences among treatment groups in whole-body cortisol concentrations were evaluated using two-way ANOVA, with the type and time of maintenance as factors; Bonferroni post-hoc tests were subsequently applied to discriminate means. Homogeneity of variance was determined using Hartley's test, and normality was tested using the Kolmogorov–Smirnov test. In Experiment 4 values of the cortisol were compared by Student *T* test (A) and Mann-Whitney (B). Differences were considered statistically significant at P values < 0.05. Statistical analysis was performed using the GraphPad Prism statistical package (GraphPad Software, San Diego, CA, USA).

3. Results

Experiment 1. We measured the levels of cortisol in response to stress caused by persecution with a net pen for 120 s in both individually isolated and grouped fish after 15 and 30 days (Fig. 1). For both isolated individuals and those housed in groups, exposure to stress produced a significant increase in cortisol levels relative to that in the control group. However, the elevation in cortisol in response to stress was significantly higher in fish housed in groups relative to those isolated individually. This finding was independent of the duration of the pre-stress housing, as no differences in response were observed between the 15- and 30-day periods.

Experiment 2. The transfer to the novel tank at 15 days (Fig. 2) in both individually isolated and grouped fish significantly increased cortisol levels relative to those of the control (not transferred) groups. The cortisol response was significantly higher in grouped than in individually isolated fish.

Experiment 3. Exposure to stress significantly increased cortisol levels in IZf. The introduction of these stressed IZf into tanks containing RZf elicited an obvious cortisol peak in the RZf (Fig. 3), whereas the introduction of non-stressed IZf had no effect on the RZf cortisol levels.

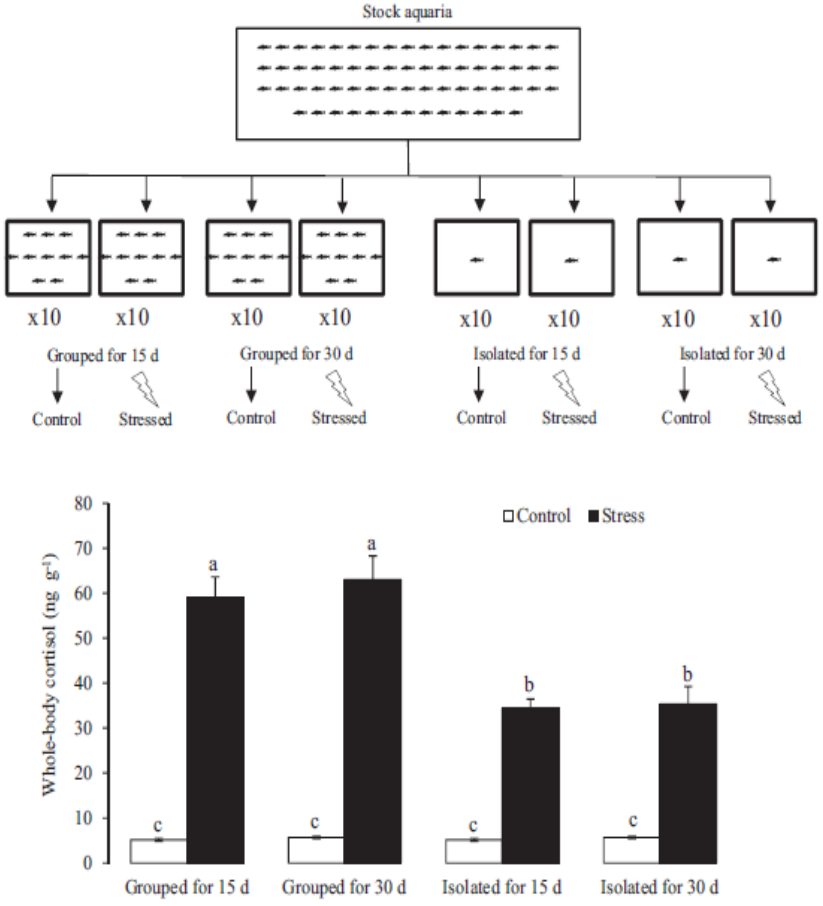

Fig. 1. Experiment 1. Schematic representation of the experimental design and peak cortisol concentrations (mean ± SEM) following an acute stress test by persecution with a pen net for 120 s in individual and grouped zebrafish housed for 15 and 30 days. The cortisol profiles were affected by both acute stress ($P < 0.0001$; $F_{1,18} = 399$) and housing conditions (isolated or grouped) ($P < 0.0001$, $F_{3,36} = 12.71$), and there was a significant stress*housing interaction ($P < 0.0001$, $F_{6,72} = 12.69$).

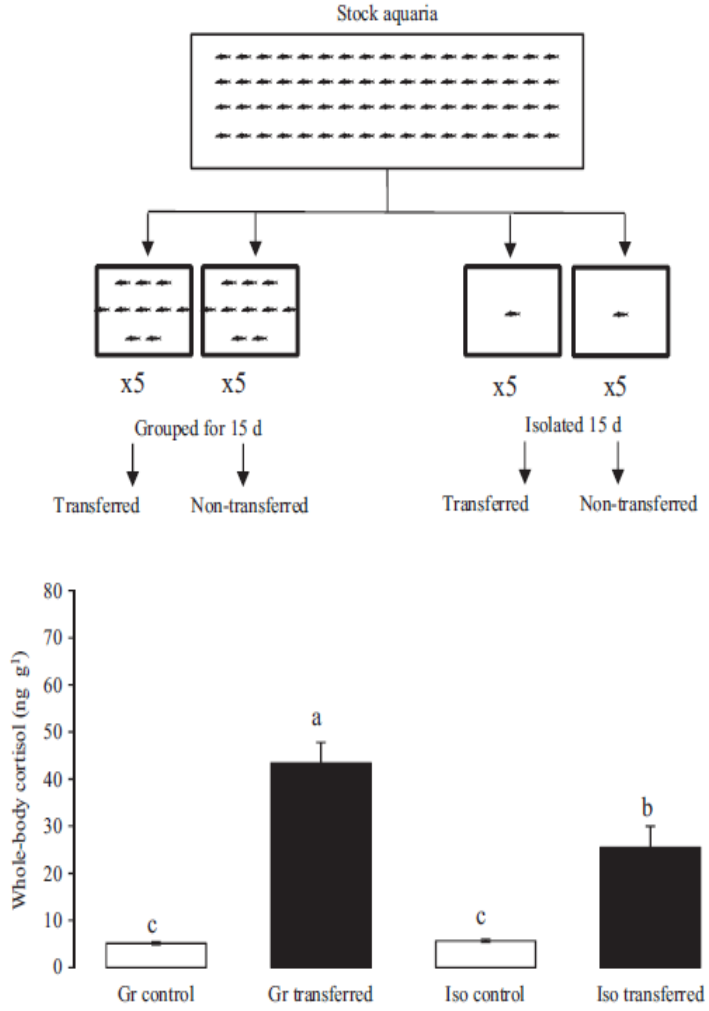

Fig. 2. Experiment 2. Schematic representation of the experimental design and peak cortisol concentrations (mean ± SEM) after transfer to a novel environment in zebrafish either housed individually or in groups for 15 days. The cortisol profiles were significantly affected by both acute stress ($P < 0.0001$; $F_{1,16} = 84.77$) and housing conditions (isolated and grouped) ($P = 0.0114$, $F_{1,16} = 8.15$), and there was a significant stress*housing interaction ($P = 0.013$, $F_{3,16} = 7.82$).

Experiment 4. The introduction of IZf in the tank containing RZF was not able to cause increased levels of cortisol resident group (Fig. 4).

4. Discussion

Here we show that individually housed zebrafish presented a reduced cortisol response to an acute stressor relative to individuals housed in groups of 10. We hypothesize that the cortisol response to stress is reduced in an individually housed zebrafish because it depends only on the individual's own perception of the stressor, whereas in grouped fish, the stress response might be augmented by chemical and/or behavioral cues from the other individuals in the group (indicated by the finding that the introduction of a group of stressed zebrafish stressed the resident zebrafish, Experiment 3). This hypothesis assumes individual variation in stressor perception and possible individual differences in fish personality.

Zebrafish live in association with conspecifics, and shoaling behavior is exhibited from early life [28]. Disrupting this evolved behavior is considered to be stressful in this species [23]. The cortisol levels of fish individually exposed to a novel environment have been observed to be higher and more variable than those observed in trios of fish, a result attributed to the disruption of innate zebrafish shoal strategies [23].

The question therefore arises why, in the present study, did zebrafish exposed to a handling stressor in 10-fish shoals exhibit higher cortisol levels than did fish exposed individually? We suggest that this difference may reflect the type of stressor imposed. The stressful stimulus employed by Pagnussat et al. [23], involving exposure to a novel apparatus, was likely milder than the handling administered in our study. In addition, methodological differences between studies (immediate transfer from a group versus maintenance over 15 and 30 days) may also have contributed to the observed difference between studies.

Exploring a new environment triggers neuroendocrine cascades, yielding changes at both the behavioral and hormonal levels [18,20,23]. In our study, individually housed and grouped fish were acclimated for 15 and 30 days, providing sufficient time to explore and adapt to the new environment, as well as acclimate to shoal deprivation (in the individually stressed fish). Therefore, the cortisol response can be attributed only to the imposed stressor and not to environmental exploration and adaptation or shoal deprivation.

Similarly, resting cortisol levels were found to be higher in grouped fish than in individually housed fish in an experiment that included a 2-week adaptation period to single, paired and grouped housing conditions [22]. However, the minimum time window needed to induce the blunted physiological stress response in the individually housed group needs to be investigated by examining fish isolated for shorter time periods.

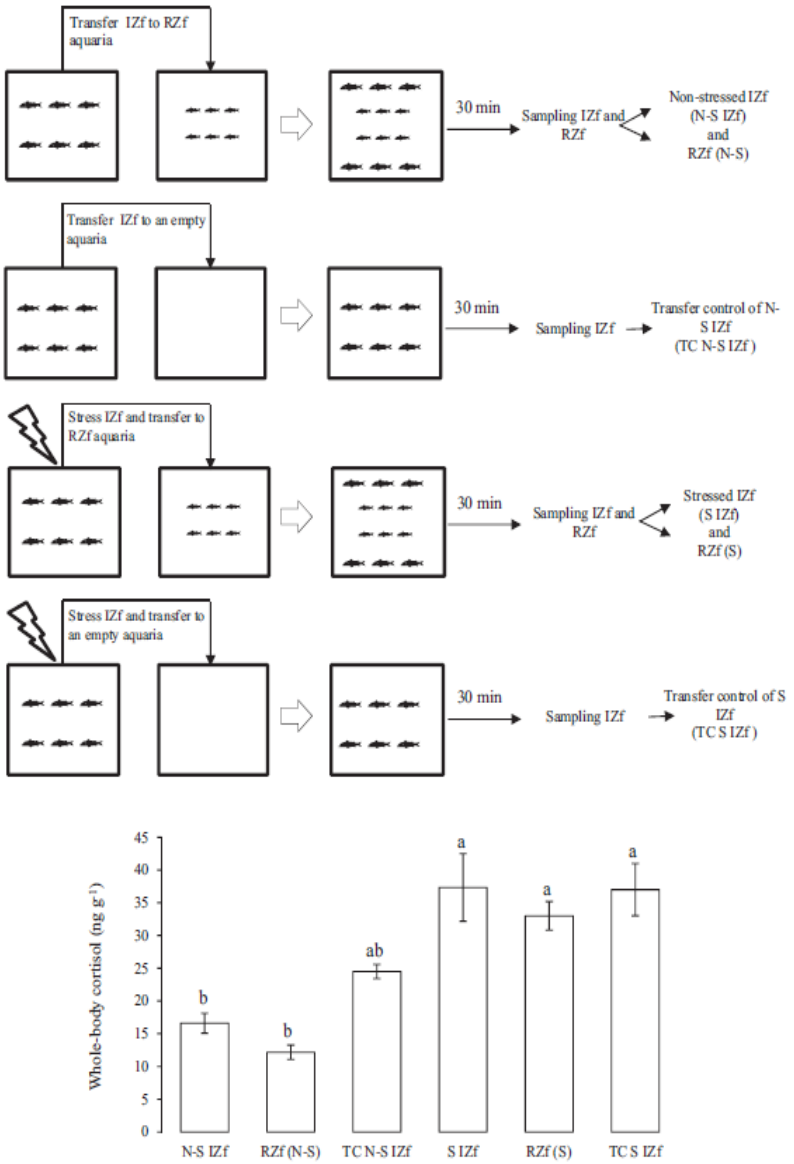

Fig. 3. Experiment 3. Schematic representation of the experimental design and peak cortisol concentrations after introducing stressed fish into a resident shoal and after transfer to an empty tank. Different letters above the histograms indicate differences between the means (ANOVA followed by Tukey's multiple range test, $P < 0.0001$, $F_{5,30} = 16.194$).

Therefore, the results of Experiment 3 and Experiment 4, in which resident zebrafish were stressed only after the introduction of the group of stressed zebrafish, indicate differences in stress perception and maximization of the cortisol response due to chemical and/or behavioral cues from other individuals of the shoal as causal mechanisms. Perceived stress is signaled through the disordered release of substances [8] that both alter behavior and activate the HPI axis [8–10].

The "group effect" on the stress response has been described in other taxa. In birds, the presence of fearful individuals in a group affects the ability of other group members to cope with stress [29]. Group-housed, male striped mice that later become solitary exhibit a trend toward lower corticosterone levels relative to that of those that remain in groups [30]. In humans, group behavior can be affected by the mood and fearfulness of individuals within the group [31–33]. In social animals, such as laying hens, the social transmission of behaviors can affect other members of the group [25,26]. The zebrafish (*D. rerio*) is a highly social animal [19,34], and similar responses can be expected.

Finally, we excluded the possible effects of crowding [25] because fish were housed at a density of 1 fish per 2.7 L, and water quality levels were maintained within the normal/preferred range for the species. We also excluded the possibility that downregulation of the HPA axis [35,36] provoked by an eventual chronic stress caused by the isolation period was responsible for the observed differences because non-stressed control fish after 5, 10, 15 and 30 days of isolation did not show increased cortisol concentrations. In fact, the stress response provoked by shoal deprivation and by novel environment exploration is an acute response [23].

We therefore conclude that the group is able to potentiate stress in fish species, even those that naturally shoal.

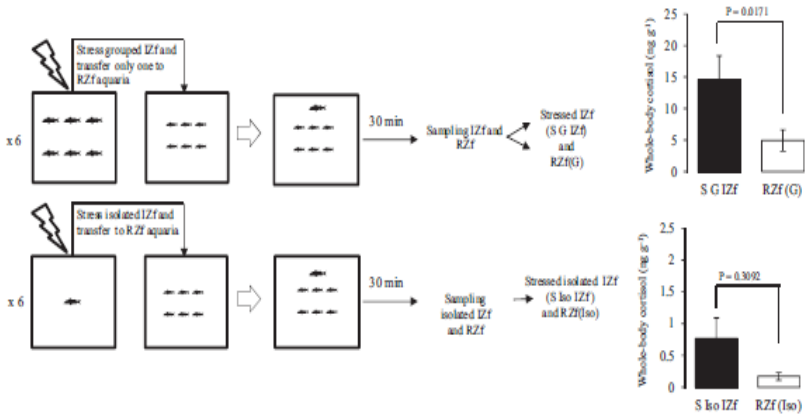

Fig. 4. Experiment 4. Schematic representation of the experimental design and peak cortisol concentrations (mean ± SEM) after transfer to a novel environment in zebrafish either housed individually or in groups. Student *T* test (A, P = 0.0171) and Mann–Whitney (B, P = 0.3092).

Acknowledgments

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3 ARTIGO 2 - FLUOXETINE AND DIAZEPAM ACUTELY MODULATE STRESS INDUCED-BEHAVIOR

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Research report

Fluoxetine and diazepam acutely modulate stress induced-behavior

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HIGHLIGHTS

- Stress increased the locomotor activity and the time spent at bottom of the tank.
- Fluoxetine and diazepam prevented these changes.
- Stress, fluoxetine and diazepam decreased social interaction.
- Stress increases aggressiveness, not reversed by fluoxetine and diazepam.
- The presence of fluoxetine and diazepam in environment may alters behavior of fish.

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ABSTRACT

Drug residue contamination in aquatic ecosystems has been studied extensively, but the behavioral effects exerted by the presence of these drugs are not well known. Here, we investigated the effects of acute stress on anxiety, memory, social interaction, and aggressiveness in zebrafish exposed to fluoxetine and diazepam at concentrations that disrupt the hypothalamic–pituitary–interrenal (HPI) axis. Stress increased the locomotor activity and time spent in the bottom area of the tank (novel tank). Fluoxetine and diazepam prevented these behaviors. We also observed that stress and fluoxetine and diazepam exposures decreased social interaction. Stress also increased aggressive behavior, which was not reversed by fluoxetine or diazepam. These data suggest that the presence of these drugs in aquatic ecosystems causes significant behavioral alterations in fish.

1. Introduction

The zebrafish is considered a good model to study anxiety, stress, and predator–prey relationships. Its hypothalamic–pituitary–interrenal (HPI) axis is well characterized [1,2], and its neuroendocrine system displays robust responses to stress [1,3–5]. The effects of acute and chronic stress have been studied in zebrafish. Acute stress modulates behavior and HPI axis, and induces an imbalance in the antioxidant status in zebrafish [6–8]. In a chronic model of unpredictable stress, the protocol increased anxiety-like behavior, impaired memory and induced neuroendocrine dysfunction [5]. Other protocols have extended the understanding of the effects of chronic stress in zebrafish [9–12]. However, the modulation of stress response by drugs is poorly understood.

Drug residue contamination in aquatic ecosystems has been studied extensively [13–19]. There are numerous reports of the presence of drugs and their metabolites in surface water and wastewater in different countries [13,15–17,20–23]. These con-

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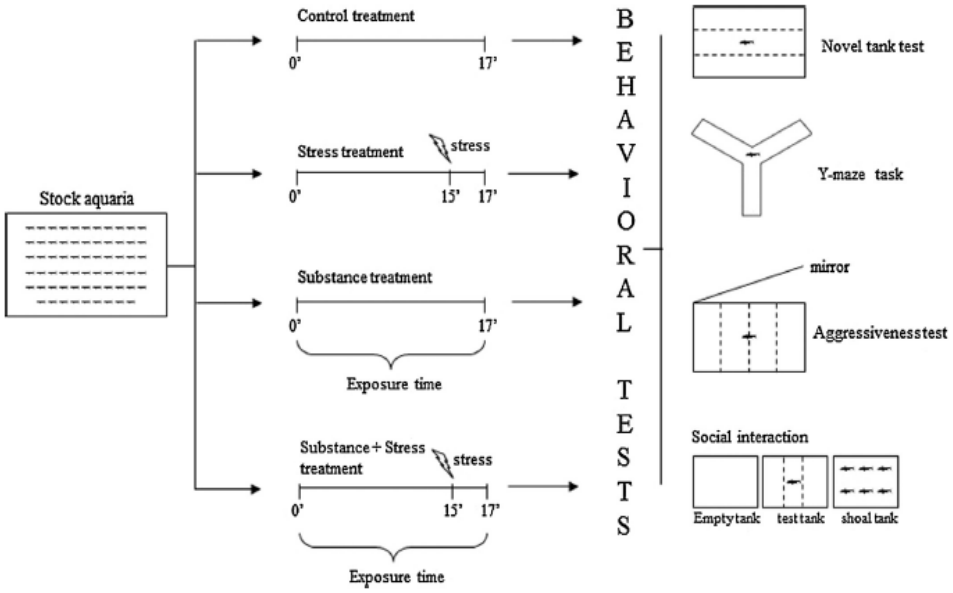

Fig. 1. Schematic view of experimental design.

taminants may negatively affect the human population as well as aquatic organisms [13–18,20,22–24]. Contaminants such as fluoxetine (FLU) and diazepam (DZP) can induce a number of neuroendocrine [25–27] and behavioral [26,28,29] alterations in exposed fish.

Here, we evaluated the effects of acute stress on behavioral parameters in zebrafish exposed to FLU and DZP. Specifically, we assessed anxiety, memory, social interaction, and aggressiveness.

2. Materials and methods

2.1. Ethical note

This study was approved by the Ethics Commission for Animal Use (CEUA) of the Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol #10/2014-CEUA) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Experimental animals

A stock population of 400 mixed-sex (50/50) 180 days-adult wild-type zebrafish (*Danio rerio*), short-fin (SF) strain, were held 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 ± 2 °C; pH 7.0 ± 0.25; dissolved oxygen at 6.5 ± 0.4 mg/L; total ammonia at <0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22 mg/L $CaCO_3$.

2.3. Drugs tested and exposure time

We used FLU (Daforin®, EMS, Brazil) and DZP (União Química, Brazil) at concentrations of 50 μg/L and 16 μg/L, respectively [27]. The animals were exposed to these drugs for 15 min, which is considered a sufficient period to elicit behavioral responses [28].

2.4. Stress protocol

After a 15 days period for habituation to laboratory conditions, fish were randomly distributed into the following groups: experimental fish exposed to FLU (50 μg/L) or DZP (16 μg/L) and untreated fish (control group). Experimental fish were then exposed to treatment for 15 min, and they underwent an acute stress challenge. This acute stress challenge consisted of harassing them with a pen net for 120 s in groups of three fish, except for Y-maze task where fish was stressed alone (Fig. 1). Different sets of fish then underwent the following behavioral tests: novel tank test, y-maze task, social interaction, and aggressiveness.

2.5. Evaluation of behavioral parameters

In all studies, fish behavior was recorded by a Logitech Quickcam PRO 9000 camera and the videos analyzed using ANY-maze® software (Stoelting CO, USA), which tracked animal behavior throughout testing.

2.5.1. Novel tank test

Fish were transferred individually to a test aquarium (24 × 8 × 20 cm; width × depth × height) and filmed for 6 min. The following parameters were analyzed: relative time in the bottom part of the tank (%), absolute turn angle, mean swimming speed (m/s), number of crossings, and total distance traveled (m).

2.5.2. Y-maze task

Fish were tested in a tank with three arms measuring 25 × 8 × 15 cm (length × width × height). Different geometric shapes (squares, circles, and triangles) were used as visual stimuli and placed on the outer wall of each arm, and the remaining area was covered with black plastic. The Y-maze arms were randomly assigned: start arm, in which the fish starts the test, new arm (locked during the initial test, but open during the second test), and the permanently open arm. The Y-maze center is a neutral area, and therefore, it was not counted in the analysis. The task consisted of two phases with a 1-h interval between them. In the first phase

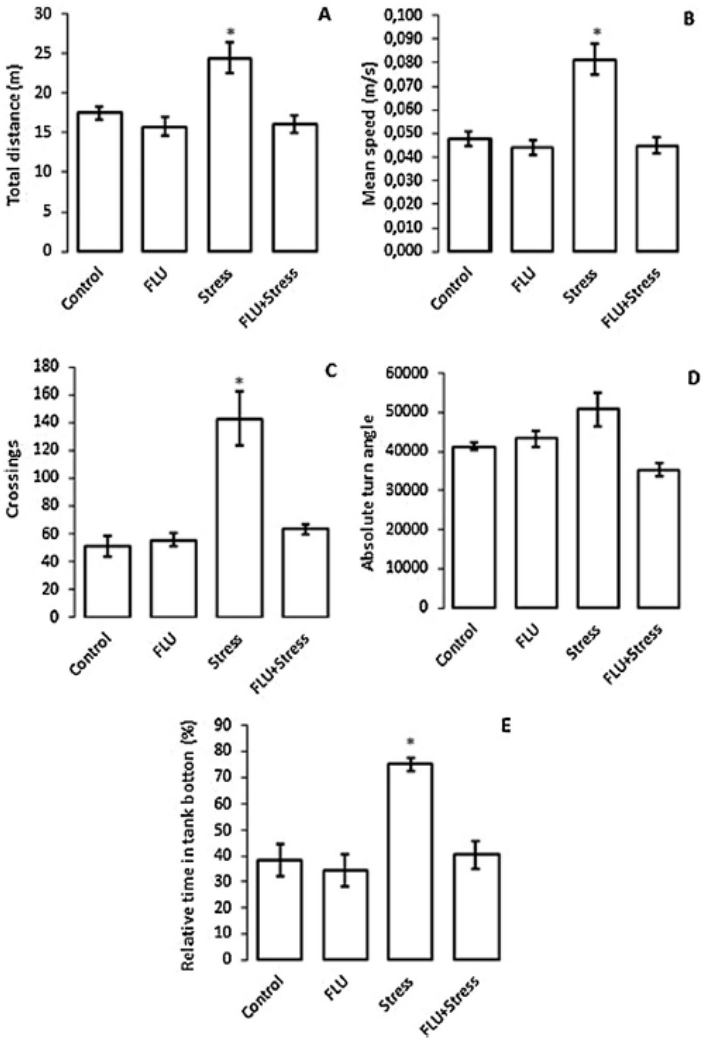

Fig. 2. Novel tank test in zebrafish exposed to fluoxetine and controls. (A) Total distance traveled; (B) mean speed; (C) number of crossings; (D) absolute turn angle, and (E) relative duration at the bottom of the tank. The * indicates statistical difference verified by two-way ANOVA followed by the Tukey test. Data are expressed as mean ± standard error of the mean of 9–12 animals per treatment.

(5-min training), the fish could explore the start and the open arms with the new arm closed. In the second phase, fish were placed in the start arm and were allowed to freely access the three arms. The following parameters were analyzed: total distance, number of intersections, time, distance, and number of entries into the new arm [30].

2.5.3. Social interaction test

In this task, fish were transferred individually to the test aquarium measuring 30 × 15 × 10 cm (width × depth × height). The test tank was positioned between two equal-sized tanks, one without fish and the other containing a group of 15 conspecifics [31]. After transfer, fish were acclimated to the test aquarium for 30 s, and then behavior was recorded for 10 s. Image analysis was done by virtually dividing the test tank into three vertical segments. The first segment is nearest to conspecifics, while the third segment was next to the empty tank. The relative time zebrafish spent in the first segment was calculated as response to social stimuli.

2.5.4. Aggressiveness

Fish behavior when viewing their image in a mirror was used to indirectly quantify aggressiveness [31]. We used this test to verify if stress alters natural aggressive behavior and if FLU and DZP modulate this behavior. A test tank measuring 30 × 15 × 10 cm (length × width × height) was filled with 6 L water, and a mirror (45 × 38 cm) was placed on one side of the tank at an angle of 22.5°. Thus, the left side of the mirror was near the tank and the right side remote from the tank to reflect a closer or more remote image of the fish as it swims by. The interaction of the fish with its own image was recorded for 60 s after two acclimatization periods (30 s and 10 min). For the analysis of the recorded images, the tank was virtually divided into four equal segments to enable quantification of the number of entries into these areas. The entrance and the dura-

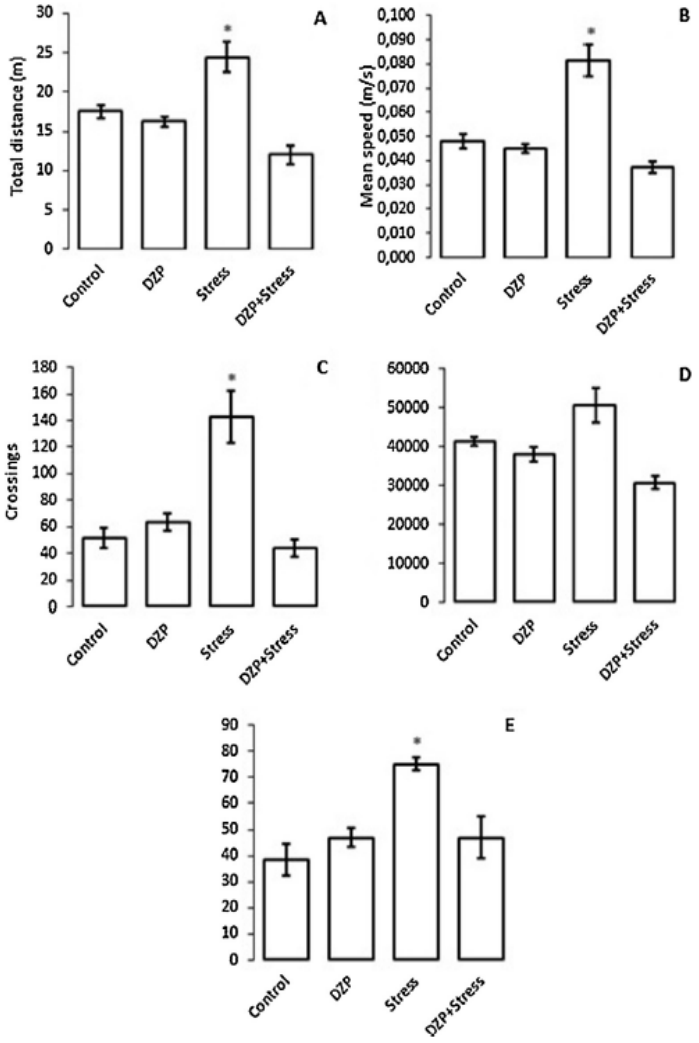

Fig. 3. Novel tank test in zebrafish exposed to diazepam and controls. (A) Total distance traveled; (B) mean speed; (C) number of crossings; (D) absolute angle turn, and (E) induration on the bottom of the tank. The * indicates statistical difference verified by two-way ANOVA followed by the Tukey test. Data are expressed as mean ± standard error of the mean of 9–12 animals per treatment.

tion of staying in segment 1 indicated the preference for proximity to the opponent and therefore aggressiveness.

2.6. Statistical analysis

Data were analyzed by two-way ANOVA followed by the Tukey test. The homogeneity of variance was determined using Hartley's test, and normality was assessed using the Kolmogorov–Smirnov test.

3. Results

All statistical data from behavior tests are shown in Table 1.

3.1. Novel tank test

Stress increased locomotor activity, as analyzed by total distance, average speed, and number of crossings, whereas it did not change the absolute turn angle. In addition, stressed fish spent more time in the bottom area of the tank. FLU and DZP did not affect locomotion *per se*, but prevented all of the stress-induced behavioral changes (Figs. 2 and 3).

3.2. Y-maze task

The acute stress protocol, FLU, or DZP, did not alter memory acquisition in this task (Figs. 4 and 5).

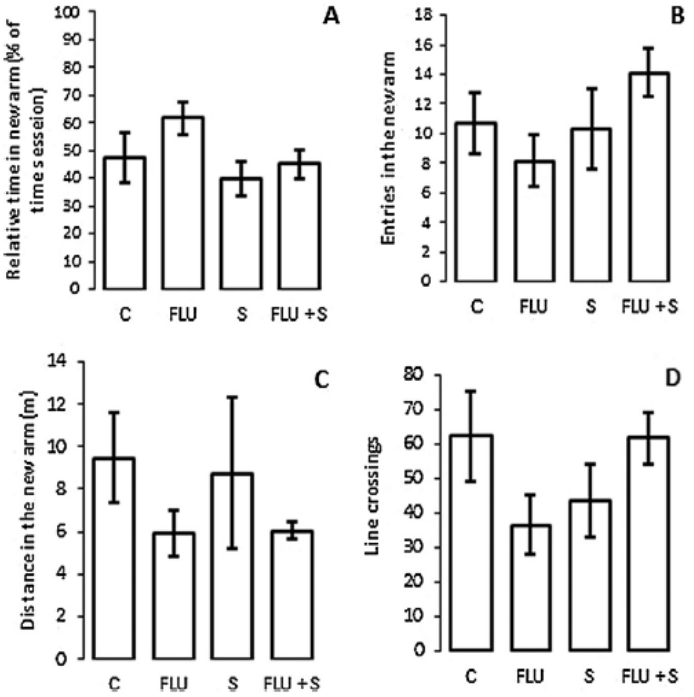

Fig. 4. Zebrafish response to the Y-maze task after training and locomotion parameters during the session and exposure to fluoxetine and stress. (A) Relative time in the new arm (% of shooting session length); (B) entries into the new arm; (C) distance traveled in the new arm and (D) crossings between lines. Data are expressed as mean ± standard error of the mean of 7–10 animals per treatment. Different symbols indicate statistical difference verified by two-way ANOVA followed by the Tukey test.

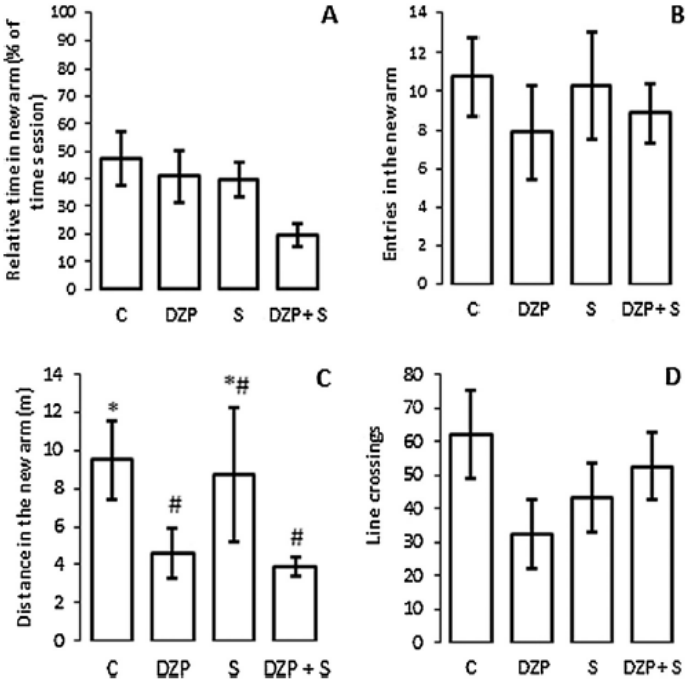

Fig. 5. Zebrafish response to the Y-maze task after training and locomotion parameters during the session and exposure to stress and diazepam. (A) Relative time in the new arm; (B) entries in the new arm; (C) distance traveled in the new arm and (D) crossings between lines. Data expressed as mean ± standard error of the mean of 7–10 animals per treatment.

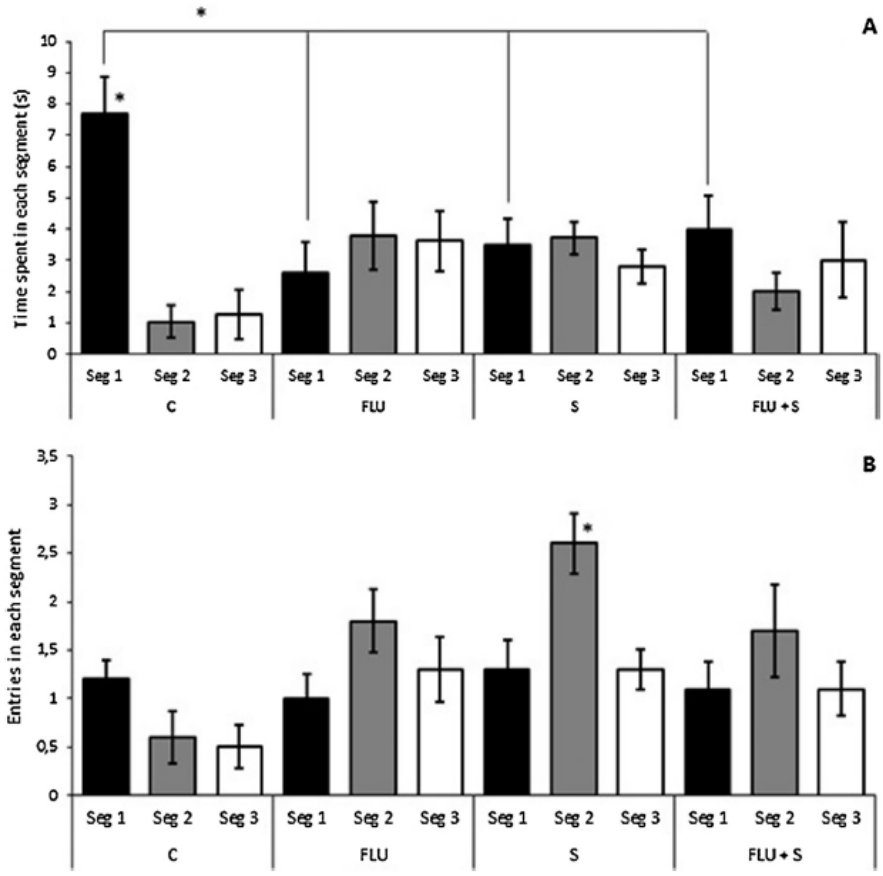

Fig. 6. Zebrafish response to the social interaction test after exposure to stress and fluoxetine. (A) Time in different time segments. The asterisk on the line indicates the time in segment 1 for the control fish was greater than all others that did not differ by Bonferroni test. The asterisk below the line indicates that only the control fish showed significant differences between the duration of time spent in those segments (two-way ANOVA followed by the Tukey test). (B) Entries in the different segments. The asterisk indicates that only in the stressed fish was difference between the time spent in the segments (two-way ANOVA followed by the Tukey test).

3.3. Social interaction test

The acute stress protocol and FLU and DZP exposure decreased social interaction, since fish in these treatments spent less time in the segment near to the shoal aquaria (Figs. 6 and 7). However, they showed no preference for any of the segments, as seen in the control group. Stress promoted an increase in the number of entries into segment 2. This effect was not observed in the control and drug-exposed groups.

3.4. Aggressiveness

After 30 s of habituation in the test tank, stressed fish showed more aggressive behavior as compared to control fish. FLU did not alter this behavior. After 10 min of habituation in the test tank, FLU reduced aggressiveness in non-stressed fish but not in stressed fish (Fig. 8). Moreover, DZP did not alter aggressiveness following either habituation time (Fig. 9).

4. Discussion

Here we show that acute stress increases anxiety-like behavior as evidenced by the increased time spent in the bottom area of the tank. We also showed that acute exposure to FLU or DZP exerted an anxiolytic-like effect, reversing the behavioral changes provoked by the acute stress protocol. The acute stress protocol induced locomotor changes that were reversed by FLU and DZP. These drugs, at the concentrations used, did not cause sedation or relevant motor side effects. Thus, the anxiolytic effects of FLU and DZP may be related to the blockade of cortisol responses to acute stress as verified previously in zebrafish [27].

Zebrafish show anxiety-like behavior when stressed [29,4,5,32–37]. This behavior was verified by the increased time spent in the bottom area of the tank. The anxiolytic effect of FLU has been reported in the literature following exposure daily or every 2 days at a concentration of 100 μg/L [4,35,36]. The influence of FLU on the stress neuroendocrine axis has been reported in some studies [2,38]. There are studies reporting that FLU influences the genetic expression of glucocorticoid [35–38] and mineralocorticoid receptors as well as the expression of GABA transporters in the brain, causing attenuation of the stress response [36,37].

The anxiolytic effect of FLU has been reported in rodents [39] and fish [4,40,41]. In addition to modulating serotonin, FLU exerts an anxiolytic effect that modulates neuropeptides and neurosteroids [36]. Although the anxiolytic effect exerted by FLU is well known,

Table 1
Results of two-way analysis of variance (ANOVA) of different behavioral tests.

Behavior test/parameter	Drug	Figs.	Comparison	DF	F-value	P-value	Partial Eta-squared
Novel tank/distance travelled	FLU	2 A	Interaction	1, 34	4.3	0.046	0.112
			Drug effect	1, 34	6.78	0.003	0.285
			Stress effect	1, 34	5.16	0.03	0.132
Novel tank/mean speed	FLU	2 B	Interaction	1, 34	10.86	0.002	0.242
			Drug effect	1, 34	10.69	<0.0001	0.386
			Stress effect	1, 34	12.12	0.001	0.263
Novel tank/crossing number	FLU	2 C	Interaction	1, 34	9.76	0.004	0.223
			Drug effect	1, 34	5.17	0.011	0.233
			Stress effect	1, 34	13.78	0.001	0.288
Novel tank/absolute turn angle	FLU	2 D	Interaction	1, 34	7.06	0.012	0.172
			Drug effect	1, 34	5.17	0.011	0.233
			Stress effect	1, 34	0	0.995	0
Novel tank/time in tank bottom	FLU	2 E	Interaction	1, 34	4.32	0.045	0.113
			Drug effect	1, 34	4.88	0.014	0.223
			Stress effect	1, 34	9.4	0.004	0.217
Novel tank/distance travelled	DZP	3 A	Interaction	1, 34	14.91	<0.0001	0.305
			Drug effect	1, 34	22.51	<0.0001	0.398
			Stress effect	1, 34	0.88	0.354	0.025
Novel tank/mean speed	DZP	3 B	Interaction	1, 34	20.4	<0.0001	0.375
			Drug effect	1, 34	26.52	<0.0001	0.438
			Stress effect	1, 34	8.24	0.007	0.195
Novel tank/crossing number	DZP	3 C	Interaction	1, 34	16.81	<0.0001	0.331
			Drug effect	1, 34	10.14	0.003	0.230
			Stress effect	1, 34	6.98	0.012	0.170
Novel tank/absolute turn angle	DZP	3 D	Interaction	1, 34	7.17	0.011	0.174
			Drug effect	1, 34	14.38	0.001	0.297
			Stress effect	1, 34	0.11	0,74	0.003
Novel tank/time in tank bottom	DZP	3 E	Interaction	1, 34	17.11	<0.0001	0.335
			Drug effect	1, 34	0.02	0.9	0
			Stress effect	1, 34	0.87	0.357	0.25
Y-maze task/relative time in the new arm	FLU	4 A	Interaction	1, 31	0.42	0.521	0.013
			Drug effect	1, 31	1.91	0.177	0.058
			Stress effect	1, 31	3.06	0.09	0.09
Y-maze task/entries in the new arm	FLU	4 B	Interaction	1, 31	2.39	0.132	0.072
			Drug effect	1, 31	0.03	0.865	0.001
			Stress effect	1, 31	3.62	0.066	0.105
Y-maze task/distance traveled in the new arm	FLU	4 C	Interaction	1, 31	0.02	0.875	0.001
			Drug effect	1, 31	2.79	0.105	0.083
			Stress effect	1, 31	0.04	0.845	0.001
Y-maze task/crossings between lines	FLU	4 D	Interaction	1, 31	4.95	0.033	0.138
			Drug effect	1, 31	0.14	0.711	0.004
			Stress effect	1, 31	0.1	0.749	0.003
Y-maze task/relative time in the new arm	DZP	5 A	Interaction	1, 28	0.83	0.369	0.029
			Drug effect	1, 28	3.19	0.085	0.102
			Stress effect	1, 28	3.68	0.065	0.116
Y-maze task/entries in the new arm	DZP	5 B	Interaction	1, 28	0.01	0.941	0
			Drug effect	1, 28	0.51	0.483	0.018
			Stress effect	1, 28	0.16	0.695	0.006
Y-maze task/distance traveled in the new arm	DZP	5 C	Interaction	1, 28	0	0.974	0
			Drug effect	1, 28	5.1	0.032	0.015
			Stress effect	1, 28	0.12	0.736	0.004
Y-maze task/crossings between lines	DZP	5 D	Interaction	1, 28	3.1	0.089	0.1
			Drug effect	1, 28	0.83	0.371	0.029
			Stress effect	1, 28	0.01	0.953	0
Social interaction/time in segment 1	FLU	6 A	Interaction	1, 36	7.36	0.01	0.170
			Drug effect	1, 36	5.03	0.031	0.123
			Stress effect	1, 36	1.89	0.178	0.05
Social interaction/entries in segment 1	FLU	6 B	Interaction	1, 36	0	1	0
			Drug effect	1, 36	0.58	0.449	0.016
			Stress effect	1, 36	0.15	0.704	0.004
Social interaction/time in segment 1	DZP	7 A	Interaction	1, 36	2.17	0.15	0.057
			Drug effect	1, 36	3.06	0.089	0.078
			Stress effect	1, 36	5.75	0.022	0.138
Social interaction/entries in segment 1	DZP	7 B	interaction	1, 36	0.16	0.69	0.004
			Drug effect	1, 36	2.59	0.16	0.067
			Stress effect	1, 36	0	1	0
Aggressiveness test (1′)	FLU	8	Interaction	1, 26	9.69	0.004	0.272
			Drug effect	1, 26	0.71	0.408	0.026
			Stress effect	1, 26	0.29	0.591	0.011
Aggressiveness test (10′)	FLU	8	Interaction	1, 26	0.47	0,5	0.018
			Drug effect	1, 26	0.13	0.720	0.005
			Stress effect	1, 26	2.65	0.116	0.093
Aggressiveness test (1′)	DZP	9	Interaction	1, 26	1.67	0.208	0.06
			Drug effect	1, 26	0.608	0.443	0.023
			Stress effect	1, 26	2.4	0.133	0.085
Aggressiveness test (10′)	DZP	9	Interaction	1, 26	1.41	0.245	0.052
			Drug effect	1, 26	2.94	0.098	0.102
			Stress effect	1, 26	4.62	0.041	0.151

The table summarizes the main effects of and the interaction between drug and acute stress. DF – degrees of freedom.

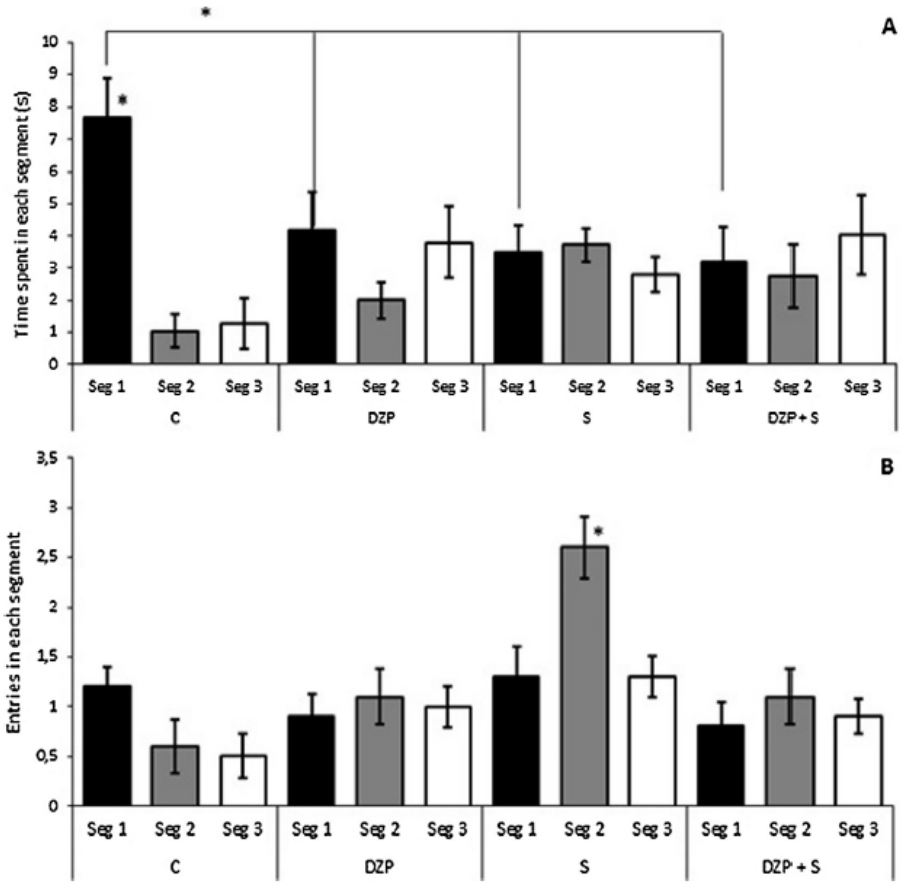

Fig. 7. Zebrafish response to social interaction test after exposure to stress and diazepam. (A) Time in different segments. The asterisk on the line indicates that the time spent by the control fish in the segment 1 was greater than that of to all others that did not differ by Bonferroni test. The asterisk below the line indicates that only the control fish showed differences between the time spent in those segments (two-way ANOVA followed by the Tukey test). (B) Entries into the different segments. The asterisk indicates that only in the stressed fish was there a significant difference between the duration of time spent in the segments (two-way ANOVA followed by the Tukey test).

the exact mechanisms by which this drug blocks the biological response of cortisol in response to stress are not yet clear. On the other hand, the anxiolytic effect of DZP in zebrafish is well established from studies using light/dark and novel tank tests [28,42].

We showed that acute stress and exposure to FLU and DZP immediately before training did not interfere with memory acquisition as evidenced by results of the Y-maze task. However, other studies have reported different influences of FLU on memory acquisition. For example, perinatal exposure to environmental concentrations of FLU modifies memory processes in the cuttlefish *Sepia officinalis*, altering learning and consolidation [43]. Subcutaneous administration of FLU increases consolidation recovery but not acquisition memory in mice [44]. Similarly, studies have reported amnesic effects of DZP that compromise acquisition memory [45,46], object recognition [46], and spatial memory [47] in humans [45] and rodents [46,47]. In young chicks, the effect of DZP on GABA receptors is dose-dependent; low doses increase memory and high doses inhibit memory [48]. The fact that we do not have evidence of memory changes in this study can be attributed to the time interval between exposure to stress and the memory test, which was greater than 60 min, during which time cortisol levels have a tendency to decline to pre-stress levels [27,49]. It was not possible to correlate behavior with cortisol levels in this study.

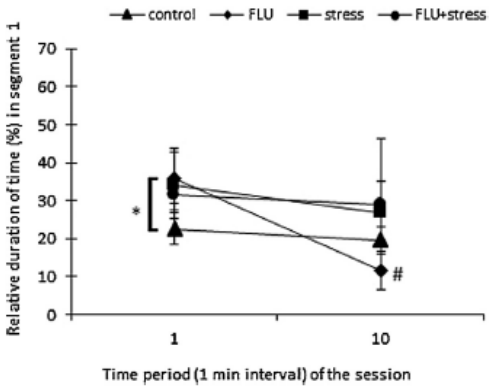

Fig. 8. Zebrafish response to the aggressiveness test after exposure to stress and fluoxetine. Stressed fish spent more time in the segment nearest to the mirror image than did controls in time interval 1 (two-way ANOVA followed by the Tukey test), but not in the 10-min time interval (two-way ANOVA followed by the Tukey test). The # symbol indicates that fish exposed to fluoxetine spent more time in the segment nearest to the mirror image at time interval 1 than they did in time interval 10.

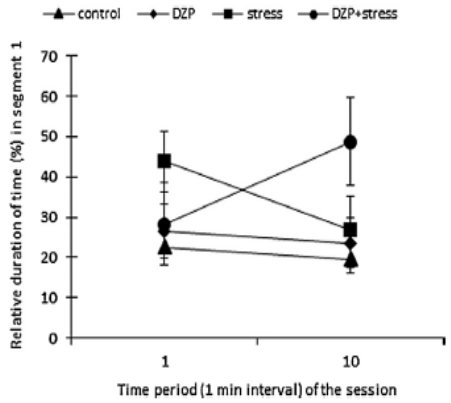

Fig. 9. Zebrafish response to aggressiveness test following exposure to stress and diazepam. The # symbol indicates that fish exposed to fluoxetine spent more time in the segment nearest to the mirror image at time interval 1 than at time interval 10. The time spent in the segment nearest to the mirror image did not differ between both time intervals (two-way ANOVA followed by the Tukey test).

Zebrafish live in shoal communities and exhibit social behavior from the beginning of life. This form of interaction is important to minimize the risk of predation. This species displays a natural tendency to swim close to conspecifics in preference to an empty tank [50]. Here, we showed that fish exposed to acute stress reduce their social interactions. Stress can alter the expression of corticotropin-releasing hormone (CRH) receptors in the hippocampus and amygdala, and thus, can promote behavioral changes related to socialization and aggression in mice [51] and fish [52]. In addition, neurotransmitters and neuropeptides are related to stress, social interaction, and aggressiveness in humans [53], rodents [54], and fish [55]. In fact, FLU increases the expression of the neuropeptides isotocin and vasotocin, promoting an increase in social interaction and decrease in anxiety and aggressiveness in zebrafish [55]. Similarly, chronic FLU treatment normalizes behavioral and biochemical changes in mice with social aversion after chronic stress [56]. Nevertheless, we showed that acute exposure to FLU and DZP eliminated the preference for proximity to conspecifics both in stressed and non-stressed fish. We attribute this effect to fish's altered perception of their relation to the shoal. Considering the importance of fish agglomeration near the shoal, this lack of interaction can lead to vulnerability that affects reproduction and survival.

Stressed fish showed more aggressive behavior compared to non-stressed fish. FLU reduced aggressive behavior in non-stressed fish, but it did not exert this effect on stressed ones. Moreover, DZP did not alter the aggressive behavior. Aggressiveness increased in *Rhesus* monkeys after stress [57]. In rainbow trout, CRH interfered with the levels of 5-HT and dopamine, thereby inhibiting aggressive behavior [52].

In aquatic ecosystems, response to acute stress is important for reproduction, osmoregulation, and predator avoidance [58–60]. On the other hand, the modulation of behavior by FLU and DZP may impair the balance of aquatic ecosystems, although the concentrations used in this study are higher than those measured in the environment. However, there are critical points at which the release of urban effluents may give rise to concentrations greater than those reported in the natural environment [61].

One limitation of this study is that we cannot directly extrapolate these results to the aquatic environment, where fish are chronically exposed to xenobiotics since early development, and in this study, fish were briefly exposed to the drugs. However, this does not lessen the importance of our results, since data about the effects of these drugs modulating acute stress-induced behavior, are very scarce.

Considering the evidence pointing to the presence and persistence of psychotropic drug residues or their active metabolites in water [15,23], as well their action on the CNS, it is essential to assess their effects on aquatic ecosystems and the human populations that may use contaminated water sources.

Acknowledgments

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4 MANUSCRITO SUBMETIDO - ENVIRONMENTAL AND PHARMACOLOGICAL MANIPULATIONS BLUNT THE STRESS RESPONSE OF ZEBRAFISH IN A SIMILAR MANNER

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Environmental and pharmacological manipulations blunt the stress response of zebrafish in a similar manner

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Running title: Environmental and pharmacological manipulations of stress in fish

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Here we provide evidence that both pharmacological and environmental manipulations similarly blunt the cortisol release in response to an acute stressor in adult zebrafish. Different groups of fish were maintained isolated or group-housed in barren or enriched tanks, and then exposed or not to diazepam or fluoxetine. Acute stress increased cortisol levels in group-housed zebrafish maintained in barren environment. Single-housed zebrafish displayed a blunted cortisol response to stress. Environmental enrichment also blunted the stress response and this was observed in both isolated and group-housed fish. The same blunting effect was observed in zebrafish exposed to diazepam or fluoxetine. We highlighted environmental enrichment as an alternative and/or complimentary therapeutic for reducing stress and as a promoter of animal welfare.

Keywords: zebrafish, environmental enrichment, stress, HPA axis.

Introduction

According to the regulatory agencies of research procedures and animal welfare[1], experimental animals must be housed in environments with space and complexity to allow their normal behavioral expression. Thus, several studies have been conducted using environmental enrichment in behavioral experiments in rat[2,3], mice[4,5], human[6] and fish[7,8].

One of the most used fish models is the teleost zebrafish (*Danio rerio*). Zebrafish have been used in several research areas due to genetic homology with humans[9], as a model organism in neuroscience and behavioral studies, as well as to test candidate drugs[10-12]. The effects of many psychiatric drugs have been widely studied in relation to the zebrafish stress response[13-15].

Zebrafish exhibit social behavior since early stages of life[16], preferring swimming in shoals[17]. In fact, behavioral and endocrine responses may differ according to the housing conditions and thus be influenced by group and/or isolation[18-20], as well as environment complexity.

However, to our knowledge, there are no studies regarding the effects of environmental enrichment, psychotropics and their association (environmental enrichment plus psychotropics) in different housing conditions on the zebrafish stress response. Hence the question: do environmental complexity and psychotropics modulate the stress neuroendocrine axis in different fish housing conditions? To answer this question, our strategy was to evaluate the acute stress response in single- or group-housed fish maintained in environmentally enriched or barren tanks, and exposed or not to diazepam or fluoxetine for a 15-day period.

Results

Waterborne concentrations of FLU and DZP: Concentrations of FLU and DZP declined at 15 days after exposure compared to the nominal concentration. They remained above the range of ecologically relevant concentrations. Neither FLU nor DZP were detected in control water samples (Figure 1 A, B).

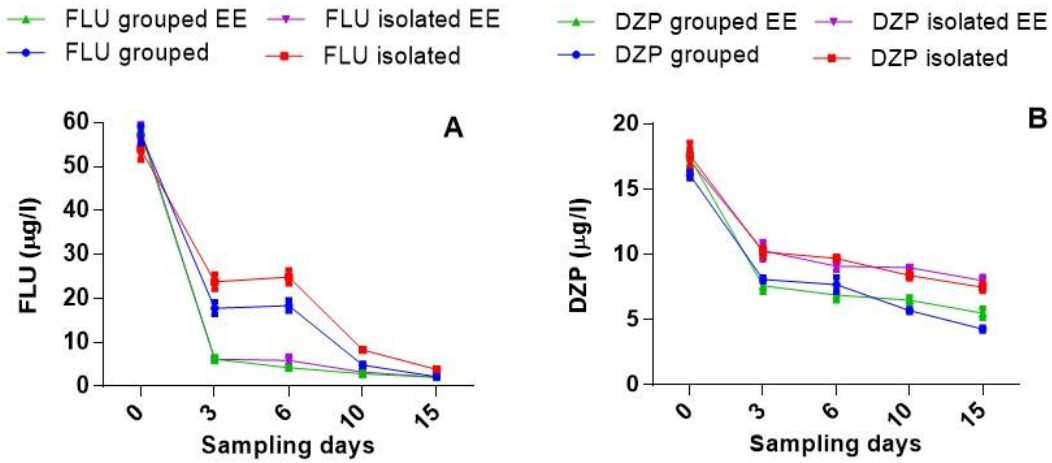

Figure 1. Time course of FLU (A) and DZP (B) degradation during 15-day period. EE – environmental enrichment. Data were expressed as mean ± S.D. of three water samples in which time point.

Housing condition: The basal cortisol levels were similar in all groups. Acute stress increased cortisol levels in group-housed zebrafish maintained in standard environment (barren tanks). Single-housed zebrafish displayed a blunted cortisol response to stress (Figures 2 C, D).

Environmental enrichment: Environmental enrichment blunted the cortisol response to stress in both isolated and group-housed fish. The same effect was observed in zebrafish exposed to diazepam or fluoxetine (Figures 2 C, D).

Drug exposure: Both diazepam and fluoxetine exposure blunted the cortisol response to stress in isolated and group-housed fish. The effects of diazepam in blunting the cortisol response were greater when combined with environmental enrichment (Figures 2 C, D). The 4-way ANOVA yielded significant main effects and interaction effects for housing, environment, stress and drug. Statistical data is shown in supplementary materials.

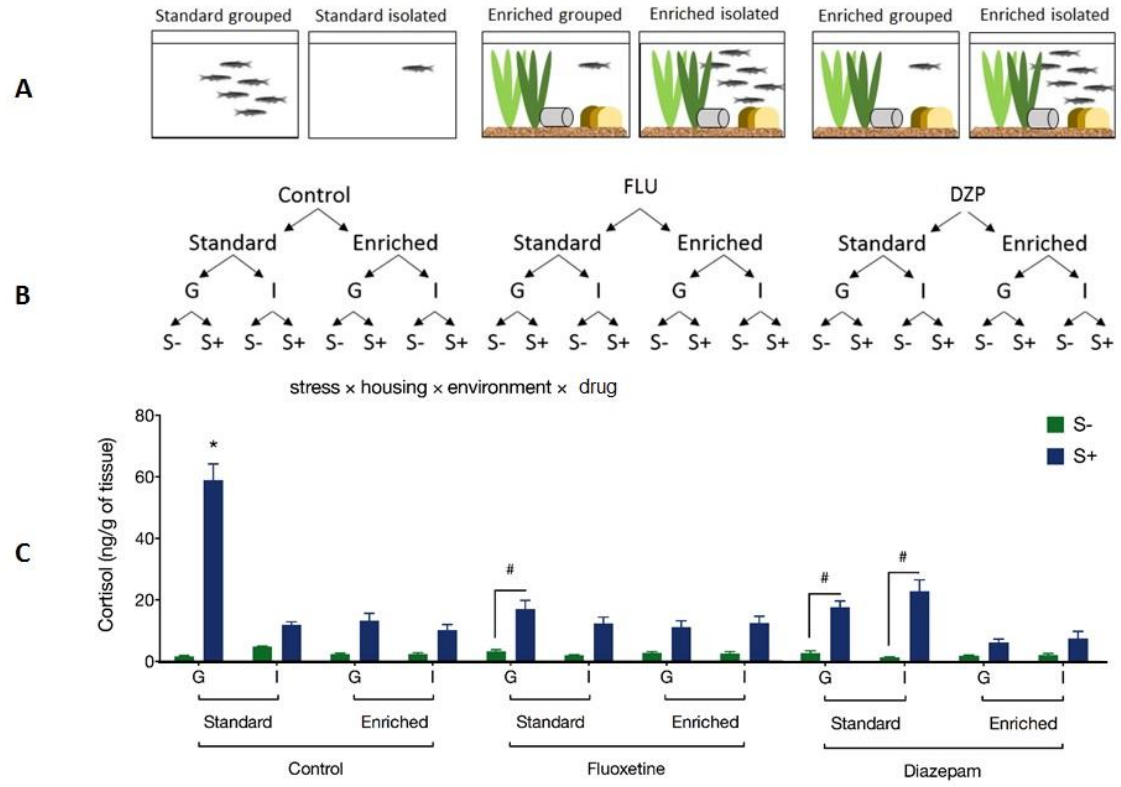

Figure 2. **Study design and results**. (A) Aquaria setup; (B) schematic representation of the experimental design; Effects of acute stress on whole-body cortisol levels in zebrafish housed in groups (G) or isolated (I) in standard or enriched tanks and exposed or not to fluoxetine or diazepam (C). Four-way ANOVA followed by Tukey's multiple comparison test. *p<0.05 compared to all others groups; #p<0.05 compared to the depicted groups. The drawing in the panel A were drawn by LB.

Discussion

Here we provide evidence that both pharmacological and environmental manipulations similarly blunt the cortisol release in response to an acute stressor in adult zebrafish.

We achieve this conclusion since fish housed in environmental enriched tanks as well those exposed to fluoxetine or diazepam presented lower cortisol concentrations that fish housed in barren tanks. We also show that the environmental enrichment is capable to abolish the difference in cortisol concentrations between isolated and grouped zebrafish housed in barren tanks verified in our previous work[19]. Furthermore, cortisol levels after acute stress in fish housed in environmental enriched tanks are similar to unstressed fish.

We hypothesized that the stress-blunting effect of environmental enrichment occurs by providing fish a sense of safety or security in a natural environment with refuge alternatives. The difference between barren versus enriched is in the context in which the fish are housed, as the enriched environment is a natural environment that offers wellness with plants, sand and stones that can serve as protection from threats and interaction; while the barren aquarium does not meet these conditions making fish more vulnerable and therefore with a more responsive stress response – they remain on alert state.

In addition, after 15 days, the response to acute stress observed in fish housed in an enriched environment is similar to fish exposed to diazepam or fluoxetine. Although the inoculation of these drugs was made at the begin of the experimental period, the concentrations of fluoxetine measured at the end of the exposure period are sufficient to block the cortisol response to acute stress in zebrafish[13]. The lower concentrations measured reflect a combined effect of uptake by the fish[21-24], adsorption to organic matter and photodegradation[25-26]. In the end of the exposure on day 15, drug

concentrations remained above the range of ecological relevance[27-30]. Regarding DZP here show that the concentration at the end of exposure period blocked the response to acute stress. The decrease in the stress response by pharmacological agents (diazepam and fluoxetine) may represent the fish being susceptible to a possible lack of response when its needed, as the ability to promote ionic[31], metabolic[32] and behavioral[15] necessary adjustments of the stress response.

Acute stress increases anxiety-like behavior, reduces social interaction and increases aggression in zebrafish that are modulated by fluoxetine and diazepam[15]. The similarity between the effect caused by environmental enrichment and exposure to drugs is thus reinforced by behavioral studies[2-5,7].

The anxiolytic effect of environmental enrichment has been demonstrated in different species[2-5,7]. The preference by interaction with conspecific is abolished in fish isolated in enriched environment[8], and in fish submitted to the acute exposure to fluoxetine and diazepam[15].

Some studies show relationship between environmental enrichment and serotonin. In fact, environmental enrichment increases the expression of 5-HT receptors in mice[5] and increase in threshold of pain[33-34].

The environmental manipulation can be seen as a non-pharmacological approach to reduce the withdrawal symptoms[2,35] and depressive-like behavior in rats[2,5]. In addition, environmental enrichment restores serum corticosterone and BDNF levels[4,36] in the hippocampus of mice with Rett syndrome[4] and rats submitted the prenatal morphine exposure[36]. In humans, environmental enrichment might be considered as a complementary treatment for autism[6,37] and affective disorders and anxiety[3-5,36].

Given the translational importance of zebrafish, we highlighted environmental enrichment as an alternative and/or complimentary therapeutic for reducing stress and as a promoter of animal welfare.

Methods

Fish

A stock population of 372 mixed-sex (50/50) adult wild-type zebrafish (*Danio rerio*), short-fin (SF) strain, was randomly distributed, isolated or in kept in groups of six fish in barren or enriched tanks and exposed for 15 days to diazepam or fluoxetine. A control group was submitted to the same experimental conditions but without pharmacological treatment (see the scheme in Figure 2B).

This experimental setup was approved by the Ethics Commission for Animal Use of the Universidade de Passo Fundo, Brazil (Protocol #09/2014) and followed the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Drug exposure

We used fluoxetine (Daforin®, EMS, Brazil) and diazepam (União Química, Brazil) at 50 µg/L and 16 µg/L, respectively based on previous results from[13]. We exposed fish to these drugs for 15 days. This period is considered a sufficient time to elicit different responses among isolated and grouped housed zebrafish[19].

Housing

10 L tanks were equipped with biological filters, under constant aeration with air stone and under 14-10 h light-dark regime. We covered all the tanks to prevent water

evaporation and to avoid fish jumping out of the tanks. Barren tanks consist of tanks containing only water while enriched tanks received sand and gravel as a bottom substrate, caps for refuge and natural plants (two branches of *Cabombaceae* and *Pontederiaceae*). See the figure 2A.

Water temperature was maintained at 27 ± 1ºC; pH 7.0 ± 0.2; dissolved oxygen at 6.3 ± 0.3 mg/L; total ammonia at <0.01 mg/L; total hardness at 6 mg/L; and alkalinity at 22 mg/L $CaCO_3$. We fed fish twice a day with TetraMin (Tetra, Melle, Germany). During the experimental period, we did not change the water or remove wastes.

Stress Protocol

After the 15-day period, we applied an acute stress challenge in all fish by persecution with a pen net for 120 s. 15 minutes after the stress, fish were captured and immediately frozen in liquid nitrogen and stored at -20°C until cortisol extraction. In order to prevent a possible stress response induced by manipulation, the time elapsed between capture and killing was less than 10 s. The 15-min time interval following stress is when cortisol level peaks[38].

Whole-body cortisol determination

The extraction and measurement of cortisol from zebrafish have been described in detail by[39]. Briefly, each zebrafish was weighed, and a pool of three fish were minced and placed into a disposable stomacher bag with phosphate buffered saline. The contents were transferred to test tube and ethyl ether was added. The tube was vortexed and centrifuged and then frozen at liquid nitrogen and the unfrozen portion (ethyl ether containing cortisol) was decanted. The ethyl ether was transferred to a new tube and completely evaporated under a gentle stream of nitrogen, yielding a lipid extract

containing the cortisol. The extract was stored at −20 °C until the ELISA was conducted on the samples suspended with 1 ml of PBS buffer.

Whole-body cortisol was measured in duplicate samples of tissue extract with a commercially available enzyme-linked immunosorbent assay kit (EIAgen™ CORTISOL test, BioChem ImmunoSystems). The specificity of the test was evaluated by comparing the parallelism between the standard curve and serial dilutions of the tissue extracts in PBS. The standard curve constructed with the human standards ran in parallel to that obtained using serial dilutions of zebrafish tissue extracts. In the linear regression test, a high positive correlation was found between the curves. The intra-assay coefficient of variation was 3.33–3.65%

Waterborne Concentrations of FLU and DZP

Sample Collection

To determine the concentrations of FLU and DZP in the water, 0.2-L samples were collected from tanks and stored in amber glass bottles on days 0, 3, 6, 10, and 15. For each analysis, triplicate water samples were analyzed. All samples were filtered using 0.22-μm filters before the extraction.

Solid Phase Extraction (SPE)

For FLU extraction, the SPE Strata-X cartridges were conditioned with 10 mL methanol followed by 10 mL HPLC-grade water. Water samples (50 mL) containing FLU were slowly passed through the SPE cartridges at a flow rate of approximately 10 mL/min. After extraction, the cartridges were kept wet, and serial washes with 10 mL of HPLC-grade water were performed before they were vacuum-dried for approximately 10 min.

Samples were eluted with 2 mL methanol acidified with acetic acid 0.1% and collected in disposable glass tubes. The DZP was injected after filtering.

LC-MS/MS Analysis

Analyses were performed using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer (Japan) with a binary pump. The analytical column was an XR-ODS III (150 × 2 mm, 2.2 µm particle size). The mobile phase consisted of: (A) water with 0.1% formic acid and (B) methanol. For the gradient elution, the percentage of (B) changed linearly as follows: 0 min, 5%; 2.0 min, 30%; 4.0 min, 95%; and 5.0 min, 5%; for re-equilibration after each analysis. The flow rate used was 0.3 mL/min and the injection volume was 10 µL. Column temperature was set at 40°C. The MS/MS analysis was performed using electrospray ionization (ESI), with the source in the positive-ion mode, and selected reaction monitoring (SRM) acquisition. The transition with the highest intensity was selected for quantification and the transition with the second highest intensity was used as confirmation. Quantification was performed using an external standard method with a ten-point calibration curve. Linearity was confirmed using the Anderson-Darling normality test[40-41] homogeneity of variances using the Cochran's test[42], and independence of residues using the Durbin-Watson test[43-44]. Regression parameters were estimated by ordinary least squares.

Statistical analysis

Cortisol data were analyzed by four-way ANOVA followed by Tukey's multiple comparison test. FLU and DZP concentrations at 0 and 15 days after exposure were compared using a two-way ANOVA followed by the Tukey's post hoc test. The homogeneity of variance was determined using Hartley's test, and normality was

assessed using the Kolmogorov–Smirnov test. Differences were considered significant at $p<0.05$. Effect sizes were determined as partial Eta squared. The data are expressed as mean + standard error of mean (S.E.M).

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Author contributions statement

A.C.V.V.G., M.S.A. and L.J.G.B. conceptualize the experiments, wrote the manuscript, and prepare the figures. D.G. and R.Z. conducted experimental procedures. A.L.P. analyzed the results. G.K. measured cortisol concentrations. M.T.F. and N.S. measured the drug concentration in the water.

Conflict of interest statement

There are no conflict and competing financial interests to declare.

5 DISCUSSÃO

As respostas comportamentais e endócrinas podem variar de acordo com as formas de alojamento dos peixes e podem ser moduladas pelo enriquecimento ambiental e por fármacos ansiolíticos. Peixes-zebra alojados individualmente apresentaram redução na resposta de cortisol a um estressor agudo em relação aos indivíduos alojados agrupados. Essa diferença na resposta entre peixes isolados e grupos é abolida pelo enriquecimento ambiental e pela exposição à fluoxetina e diazepam. O estresse agudo promove efeito ansiogênico, reduz a interação social, aumenta a agressividade e não interfere na aquisição de memória. A exposição aguda à fluoxetina e diazepam reverte o comportamento de ansiedade desencadeado pelo estresse, reduz a interação social, mas não interfere na aquisição de memória e no comportamento de agressividade desencadeado pelo estresse.

Nossa hipótese é que a resposta do cortisol ao estresse é reduzida em peixes-zebra alojados individualmente, pois depende apenas de sua própria percepção do estressor, enquanto que em peixes agrupados, a resposta ao estresse pode ser aumentada por estímulos químicos e/ou comportamentais dos demais indivíduos do grupo. Nesse contexto concluímos que o grupo é capaz de potencializar o estresse em peixes, mesmo nas espécies que vivem naturalmente em cardume. O "efeito de grupo" na resposta ao estresse tem sido descrito em aves (NICOL, 1995; HOPPITT; BLACKBURN; LALAND, 2007; HAAS et al., 2012), roedores (SCHOEPF; SCHRADIN, 2013) e humanos (BARSADE, 2002; SY; COTE; SAAVEDRA, 2005; OLSSON; NEARING; PHELPS, 2007). Os peixes-zebra são animais altamente sociáveis e sincronizados (MILLER; GERLAI, 2007; 2011) e, portanto, respostas similares podem ser esperadas.

A partir dos resultados de Abreu et al. (2014) verificamos que a exposição aguda aos fármacos fluoxetina e diazepam bloqueia a resposta ao estresse agudo e, diante disso, surgiram algumas questões: **Será que esses fármacos poderiam modular a resposta ao estresse agudo em peixes-zebra alojados individualmente ou em grupo? A resposta ao estresse é alterada em ambiente enriquecido?**

Nesse estudo evidenciamos que o "efeito de grupo" foi abolido pelas manipulações ambiental e farmacológica. Ambas as manipulações similarmente embotaram a liberação de cortisol em resposta a um estressor agudo em peixe-zebra adulto. Chegamos a essa conclusão, uma vez peixes alojados em tanques enriquecidos e

também aqueles expostos à fluoxetina ou diazepam apresentaram concentrações de cortisol mais baixos que peixes alojados em tanques estéreis. Além disso, os níveis de cortisol após estresse agudo em peixes alojados em tanques enriquecidos são semelhantes aos de peixes não estressados.

Nossa hipótese é que o efeito de embotamento da resposta ao estresse causado pelo enriquecimento ambiental ocorre por propiciar ao peixe uma sensação de segurança em um ambiente natural com alternativas de refúgio e desenvolvimento de estratégias de enfrentamento. Além disso, após 15 dias, a resposta ao estresse agudo observada nos peixes alojados num ambiente enriquecido é semelhante à de peixes expostos à fluoxetina ou diazepam. Embora a inoculação destes fármacos tenha sido feita no início do período experimental, as concentrações de fluoxetina medidas no término do período de exposição são suficientes para bloquear a resposta do cortisol ao estresse agudo em zebrafish (ABREU et al., 2014). Quanto ao diazepam mostramos que a concentração no final do período de exposição também foi suficiente para bloquear a resposta ao estresse agudo.

Considerando a modulação da resposta ao estresse agudo pela fluoxetina e diazepam outras questões foram levantadas: **O estresse agudo interfere no comportamento de peixes-zebra? Poderiam esses fármacos modular diferentes comportamentos em peixe-zebra?**

Nós mostramos que o estresse agudo aumenta comportamento tipo ansiedade e que a exposição aguda à fluoxetina ou diazepam exerce efeito tipo ansiolítico, revertendo as mudanças comportamentais provocadas pelo protocolo de estresse agudo. Peixes-zebra exibem comportamento tipo ansiedade quando estressados (SACKERMAN et al., 2010; EGAN, et al., 2009; PIATO et al., 2011, MAXIMINO et al., 2010; BLASER; ROSEMBERG, 2012; PARKER et al., 2012; WONG et al., 2010; ZIV et al., 2013). Por outro lado, o efeito da fluoxetina e diazepam em impedir o comportamento tipo ansiedade após o estresse agudo pode estar relacionado com o bloqueio da resposta de cortisol como verificado por Abreu et al. (2014) em protocolo semelhante. Embora o efeito ansiolítico da fluoxetina (ZIV et al., 2013; ADZIC et al., 2013; EGAN, et al., 2009; BARBOSA JUNIOR et al., 2012; MARGIOTTA-CASALUCI, et al., 2014; WONG et al., 2010; WONG; OXENDINE; GODWIN, 2013) e diazepam (BENCAN et al., 2009; GEBAUER et al., 2011; LEVIN, 2011), sejam bem

conhecidos os mecanismos exatos pelos quais esses fármacos bloqueiam a resposta ao estresse ainda não são claros.

Em relação aos efeitos sobre a memória, estudos evidenciam os efeitos da fluoxetina sobre aprendizagem e consolidação da memória (FLOOD; CHERKIN, 1987; POI et al., 2013) bem como efeitos amnésicos do diazepam (WHITE; SIMSON; BEST, 1997; BUFFET-JERROTT; STEWART, 2002; ORZELSKA et al., 2015). O fato de não termos evidenciado alterações de memória nesse estudo pode ser atribuído ao intervalo de tempo entre a exposição ao estresse e o teste de memória, o qual foi superior à 60 minutos, tempo no qual os níveis de cortisol têm uma tendência a declinar aos níveis de pré-estresse (ABREU et al., 2014; RAMSAY et al., 2006).

Peixes-zebra são espécies de cardume que exibem comportamento social desde o início da vida (GERLAI, 2003), mas também exibem agressividade para conseguir acesso ao alimento, às fêmeas e dominância (ARIYOMO; CARTER; WATT, 2013). Aqui, nós mostramos que os peixes expostos ao estresse agudo reduzem o comportamento social e aumentam a agressividade. O estresse pode alterar a expressão do CRH no hipocampo e amígdala, (BACKSTRÖM et al., 2011; VEENIT; RICCIO; SANDI, 2014) bem como de neurotransmissores e neuropeptídios relacionados ao estresse, interação social e agressividade em seres humanos (MEYER-LINDENBERG et al., 2011), roedores (LUKAS et al., 2011) e peixes (BRAIDA et al., 2012). Por outro lado, a fluoxetina aumenta a expressão dos neuropeptídeos isotocina e vasotocina, promovendo aumento na interação social, diminuição da ansiedade e agressividade em peixes-zebra (BRAIDA et al., 2012). No entanto, nós mostramos que a exposição aguda à fluoxetina e diazepam elimina a preferência pela proximidade com os coespecíficos, tanto em peixes estressados quanto em não estressados. Atribuímos esse efeito à alteração na percepção do peixe em relação ao cardume. Considerando a importância da aglomeração em cardume, essa falta de interação pode levar à vulnerabilidade que afeta a reprodução e sobrevivência. Em relação à agressividade a fluoxetina reduziu o comportamento agressivo em peixes não-estressados, mas não exerceu efeito sobre os estressados; o diazepam não alterou o comportamento agressivo.

Nos ecossistemas aquáticos, a resposta ao estresse agudo é importante para a reprodução, osmorregulação, e evitar predadores (BARTON; IWAMA, 1991; WENDELAAR BONGA, 1997; BARTON; MORGAN; VIJAYAN, 2002). Por outro

lado, a modulação do comportamento por fluoxetina e diazepam pode prejudicar o equilíbrio dos ecossistemas aquáticos, embora as concentrações usadas neste estudo sejam mais elevadas do que as identificadas no ambiente. No entanto, existem pontos críticos nos quais a liberação de efluentes urbanos podem originar concentrações maiores do que as relatadas no ambiente natural (VERLICCHI et al., 2012). No entanto, isto não diminui a importância dos nossos resultados, uma vez que os dados sobre os efeitos de fármacos na modulação destes comportamentos induzidos pelo estresse agudo são muito escassos.

Em relação ao efeito do enriquecimento ambiental sobre os níveis de cortisol uma questão poderia ser levantada. **O enriquecimento ambiental suprime o eixo? Esse efeito não seria prejudicial?** Estudos tem demonstrado que o enriquecimento ambiental não interfere na magnitude da resposta de cortisol (NASLUND et al., 2013; WILKES et al., 2013), indicando que não há supressão do eixo, mas sim possibilita uma maior taxa de recuperação frente aos estressores (POUNDER et al., 2016).

A semelhança entre o efeito provocado por enriquecimento ambiental e pela exposição aos fármacos é, portanto, reforçada por estudos comportamentais (HAJHEIDARI; MILADI-GORJI; BIGDELI, 2015; GOES; ANTUNES; TEIXEIRA-SILVA, 2015; VARMAN; RAJAN, 2015; KONDO et al., 2015; MANUEL et al., 2015). Peixes-zebra preferem ambientes enriquecidos a ambientes sem a complexidade do habitat natural (SCHROEDER et al., 2014; COLLYMORE; TOLWANI; RASMUSSEN, 2015) assim como são atraídos para locais onde há presença de fármacos, inclusive fluoxetina e diazepam (ABREU et al., 2016). Ambientes enriquecidos exercem efeitos ansiolíticos em diferentes espécies (HAJHEIDARI; MILADI-GORJI; BIGDELI, 2015; GOES; ANTUNES; TEIXEIRA-SILVA, 2015; VARMAN; RAJAN, 2015; KONDO et al., 2015; MANUEL et al., 2015) e eliminam a preferência pela interação com coespecíficos em peixes isolados (COLLYMORE; TOLWANI; RASMUSSEN, 2015) da mesma forma que peixes submetidos à exposição aguda à fluoxetina e diazepam (GIACOMINI et al., 2016).

Dada a importância translacional de peixes-zebra, destacamos que o enriquecimento ambiental é uma alternativa terapêutica complementar para reduzir o estresse e como promotor do bem-estar animal. Do ponto de vista ecológico, a presença de fármacos na água pode interferir no comportamento natural diminuindo a reatividade dos peixes frente à estressores bem como interferir nas relações intraespecíficas.

6 CONCLUSÃO

Peixes alojados individualmente apresentam redução na resposta ao estresse agudo; o grupo potencializa o estresse. O estresse agudo aumenta o comportamento tipo ansiedade e agressividade, reduz a preferência por coespecíficos e não interfere na aquisição de memória. Fluoxetina e diazepam reduzem o comportamento tipo ansiedade e a preferência por coespecíficos; não interferem na memória e não revertem a agressividade desencadeada pelo estresse agudo. As manipulações farmacológica e ambiental modulam a resposta ao estresse eliminando a diferença entre peixes isolados e agrupados.

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ANEXO A - PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

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

PARECER Nº 009/2014

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 "**Modulação neuroendócrina por fármacos e enriquecimento ambiental",** registro na CEUA Nº 009/2014, de responsabilidade do pesquisador **Leonardo José Gil Barcellos**.

Com o objetivo de comparar a resposta ao estresse entre peixes isolados e em grupos e avaliar a modulação dessa resposta, 4.096 peixes zebra com idade de 6 meses serão expostos a diferentes concentrações de fluoxetina, diazepam, vasotocina, isotocina e enriquecimento ambiental; e serão avaliados em 4 experimentos: 1. Resposta ao estresse agudo; 2. desafio de estresse agudo em isolados e grupo de peixe-zebra; 3. transferência para um ambiente novo de peixes-zebras isolados e agrupados; 4. efeito da introdução de peixes estressados em um cardume de peixe-zebra residentes. Em cada experimento, os peixes serão coletados para análise do cortisol tecidual. O experimento será realizado no Laboratório de Fisiologia de Peixes do Hospital Veterinário, da Faculdade de Agronomia e Medicina Veterinária (FAMV), Universidade de Passo Fundo (UPF), Campus Passo Fundo.

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 B - PARECER DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

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