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Vanessa Andreatta de Quadros

AGRESSIVIDADE E ANSIEDADE INDUZIDAS PELO ESTRESSE EM PEIXE-ZEBRA: ENVOLVIMENTO DA MONOAMINA OXIDASE, MODULAÇÃO REDOX E ALTERAÇÕES NEUROENDÓCRINAS

Santa Maria, RS
2020

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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciências Biológicas: Bioquímica Toxicológica**

Orientador: Prof. Dr. Denis Broock Rosemberg

Santa Maria, RS
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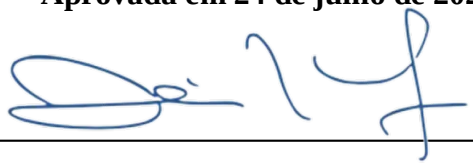

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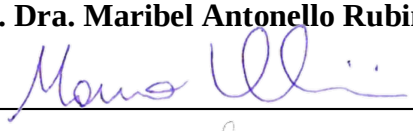

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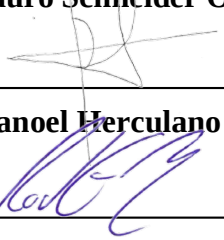

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DEDICATÓRIA

Dedico este trabalho às pessoas mais presentes na minha vida:

Primeiramente a Deus, pela força e coragem para enfrentar os obstáculos desta caminhada.

A minha mãe, pelo exemplo de coragem e amor.

Ao meu pai, o mais generoso e companheiro de todos os pais.

A minha irmã, Fernanda, pelas palavras de incentivo.

Meus maiores PRESENTES SÃO VOCÊS

AMO MUITO TODOS!

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"É para a frente que se anda
É para cima que se olha
E é lutando que se conquista"

(Autor desconhecido)

Imagine uma nova história para sua vida e acredite nela.

(Paulo Coelho)

RESUMO

AGRESSIVIDADE E ANSIEDADE INDUZIDAS PELO ESTRESSE EM PEIXE-ZEBRA: ENVOLVIMENTO DA MONOAMINA OXIDASE, MODULAÇÃO REDOX E ALTERAÇÕES NEUROENDÓCRINAS

AUTORA: VANESSA ANDREATTA DE QUADROS
ORIENTADOR: DENIS BROOCK ROSEMBERG

Os transtornos mentais são considerados pandemias do século XXI que causam inúmeros prejuízos à saúde. Cerca de um terço das pessoas no mundo podem apresentar problemas relacionados à saúde mental. Além disso, sabe-se que muitos transtornos são desencadeados por medo e ansiedade exacerbadas induzidas pelo estresse. Dessa forma, estudar como diferentes agentes estressores podem influenciar respostas bioquímicas e neurocomportamentais em modelos experimentais de laboratório é uma estratégia importante para esclarecer os mecanismos primários envolvido em diversos transtornos mentais. Neste estudo, investigamos a influência de agentes estressores em respostas comportamentais, bem como na atividade da monoamina oxidase (MAO) cerebral, estresse oxidativo e respostas neuroendócrinas em peixe-zebra (*Danio rerio*). No primeiro estudo, avaliamos se a exposição aguda e crônica à substância de alarme (SA) modula o comportamento agressivo (utilizando o teste de agressão induzida pelo espelho) em populações wild-type, (*WT*) e leopardo (*leo)*, bem como se altera a atividade da Z-MAO cerebral. Após a exposição aguda, vimos que a SA aumenta a agressividade e diminui a atividade da Z-MAO. Cronicamente, a SA reduz a agressividade, diminui a atividade locomotora sem alterar a atividade da Z-MAO. Esses dados sugerem que as respostas desencadeadas no comportamento são dependentes do tempo de estresse, demonstrando um possível envolvimento da Z-MAO com a agressividade induzida pelo estresse agudo. No segundo trabalho, avaliamos o envolvimento do estresse repetido nas respostas comportamentais tipo ansiedade (testes do novo tanque e claro/escuro) e parâmetros relacionados ao estresse oxidativo nas duas populações de peixe-zebra. A exposição repetida à SA aumenta o comportamento do tipo ansiedade em *WT* e *leo* em ambos os testes. Além disso, a SA aumenta a atividade enzimática da catalase (CAT), glutationa S-transferase (GST), bem como diminui os níveis de tióis não proteicos (NPSH) em ambas as populações. Somente em *leo* verificamos um aumento da peroxidação lipídica (TBARS). Esses dados sugerem que a SA cronicamente desencadeia respostas ansiogênicas e mudanças nos parâmetros de estresse oxidativo. No terceiro trabalho, investigamos se o estresse crônico previsível (ECP) usando estressores homotípicos, químico (SA), e físico, perseguição com rede (PR), altera as respostas comportamentais e neuroendócrinas em peixe-zebra. Nossos resultados mostram que o ECP-SA, mas não o ECP-PR aumenta o comportamento do tipo ansiedade e os níveis de cortisol em peixe-zebra. Como agudamente ambos os estressores induzem respostas aversivas, sugerimos que o ECP-PR é capaz de induzir uma habituação na resposta de estresse em peixe-zebra. Assim, o ECP induz mudanças comportamentais e neuroendócrinas, dependendo da natureza do estressor. Em suma, os achados descritos nesta tese suportam o uso do peixe-zebra como um organismo modelo atrativo para elucidar os mecanismos do estresse em transtornos mentais, como ansiedade e depressão na neuropsiquiatria translacional.

Palavras chave: Transtorno mental; Medo/ansiedade; Estresse; Peixe-zebra; Pesquisa neurocomportamental.

ABSTRACT

STRESS-INDUCED AGGRESSION AND ANXIETY IN ZEBRAFISH: INVOLVEMENT OF MONOAMINE OXIDASE, REDOX MODULATION, AND NEUROENDOCRINE CHANGES

AUTHOR: VANESSA ANDREATTA DE QUADROS
ADVISOR: DENIS BROOCK ROSEMBERG

Mental disorders are considered XXI century pandemics that cause various health diseases. About a third of people have problems related to mental health worldwide. Furthermore, several disorders are triggered by exacerbated fear and anxiety induced by stress. Thus, studying how different stressors can influence biochemical and neurobehavioral responses in experimental laboratory models is an important strategy to clarify the primary mechanism involved in mental disorders. In this study, we investigated the influence of stressors in behavioral responses, as well as in the monoamine oxidase activity (MAO), oxidative stress, and neuroendocrine responses of zebrafish (*Danio rerio*). In the first study, we evaluated whether acute and chronic alarm substance (AS) exposures modulate aggressive behavior (using the mirror-induced aggression (MIA) test) in wild-type, (*WT*) and leopard (*leo*) populations, as well as whether alter brain Z-MAO activity. After acute exposure, AS increased aggression, and decreased Z-MAO activity. Chronically, AS reduced aggression, decreased locomotor activity, and did not alter Z-MAO activity. These data suggest that such responses caused by stress depend on duration of stressor, demonstrating a possible involvement of Z-MAO with the aggressiveness induced by acute stress. In the second study, we evaluated the involvement of repeated stress in anxiety-like behavior (using the light/dark and novel tank tests) and parameters related to oxidative stress in two zebrafish populations. Repeated exposure to AS increased anxiety-like behavior in *WT* and *leo* in both tests. Moreover, AS increased the catalase (CAT) and glutathione S-transferase (GST) activities, as well as decreased non-protein thiols (NPSH) levels in both populations. Only in *leo* we verify an increased in lipid peroxidation (TBARS). We suggest that AS chronically triggers anxiogenic responses and changes brain oxidative stress parameters. In the third work, we investigated whether predictable chronic stress (PCS) using two homotypic stressors, chemical (AS) and physical, net chasing (NC), alters the behavioral and neuroendocrine responses. PCS-AS, but not PCS-NC, increases anxiety-like behavior and cortisol levels in zebrafish. Because both stressors acutely elicited aversive responses, PCS-NC might trigger habituation to stress response. In general, we suggest that PCS induces behavioral and neuroendocrine changes depending on the nature of the stressor. These novel findings described here support the use of zebrafish as an attractive model organism to elucidate the mechanisms of stress in mental disorders, such as anxiety and depression in translational neuropsychiatry.

Keywords: Mental disorders; fear/anxiety; Stress; Zebrafish; Neurobehavioral research.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS

5-HT	Serotonina
AC	Amígdala central
ATCH	Hormônio adrenocorticotrófico, do inglês “adrenocorticotropic hormone”
CAT	Catalase
CID-10	Classificação Internacional de Doenças
CRF	Fator liberador da corticotrofina hipotalâmico, do inglês “corticotropin-release factor”
ECI	Estresse crônico imprevisível
ECP	Estresse crônico previsível
ERO	Espécies reativas de oxigênio
FAD	Flavina-adenosina-dinucleotídeo
GPx	Glutationa peroxidase
GR	Receptor glicocorticoide
GSH	Glutationa reduzida
H_3NO	3-N-óxido de hipoxantina
HM	Habenula medial
HPA	Hipotálamo-pituitária-adrenal
HPI	Hipotálamo-hipófise-interrenal
ISRS	Inibidores seletivos da recaptação da serotonina
leo	População leopardo
MAO	Monoamina oxidase
MDA	Malondialdeído
MIA	Teste de agressão induzida pelo espelho, do inglês “mirror-induced agression test”
NLET	Núcleo do leito da estria terminal
PR	Perseguição com rede
SA	Substância de alarme
SNC	Sistema nervoso central
SOD	Superóxido dismutase
TAG	Transtorno de ansiedade generalizada
TBARS	Substâncias reativas ao ácido tiobarbitúrico, do inglês “thiobarbituric reactive substances”
TOC	Transtorno obsessivo-compulsivo
WT	População selvagem, do inglês “Wild-type”
Z-MAO	Monoamina oxidase de peixe-zebra

SUMÁRIO

1. APRESENTAÇÃO

Esta Tese aborda assuntos relacionados aos efeitos do estresse sobre parâmetros bioquímicos e comportamentais utilizando o peixe-zebra como organismo modelo. Ela encontra-se estruturada da seguinte forma:

INTRODUÇÃO: Revisão da literatura com caracterização dos temas abordados na tese.

MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO: Serão apresentados na forma de dois artigos científicos e um manuscrito submetido para publicação.

DISCUSSÃO GERAL: Serão apresentadas as interpretações e comentários sobre os artigos e o manuscrito científico.

CONCLUSÃO: Conclusões parciais sobre as hipóteses levantadas, bem como conclusão geral unificando os principais achados da Tese.

PESPECTIVAS: Apresentação das possibilidades de novos estudos a partir dos resultados obtidos.

REFERÊNCIAS: Lista as referências utilizadas na Introdução e Discussão geral da Tese.

2. INTRODUÇÃO

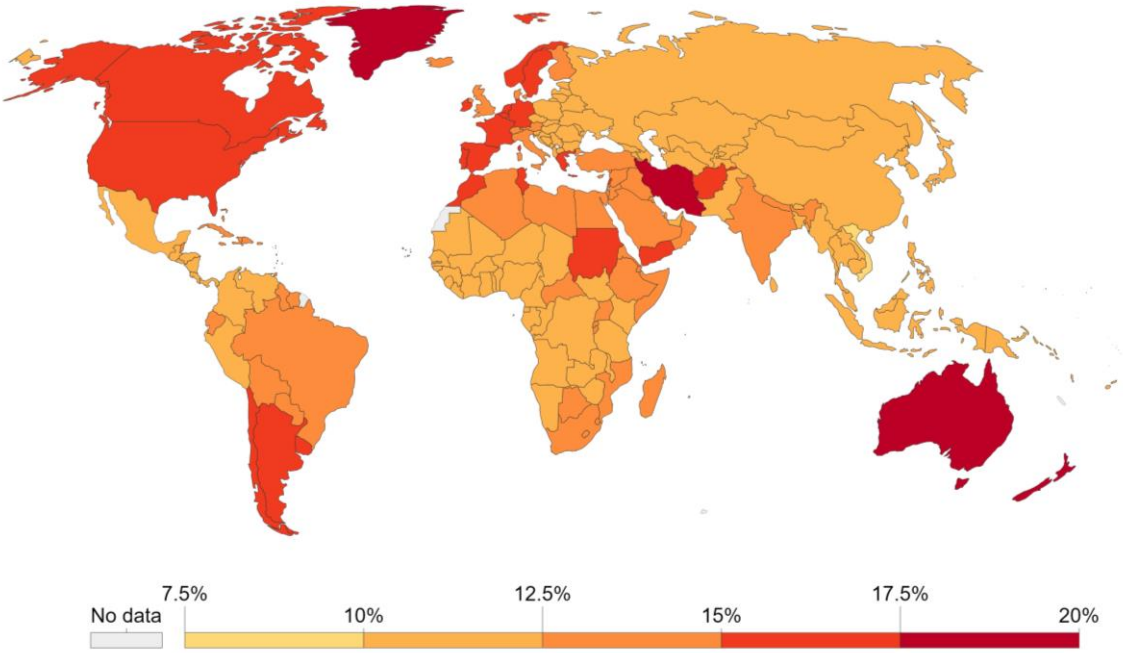
2. 1. TRANSTORNOS NEUROPSIQUIÁTRICOS

2.1.1. Saúde mental

Conforme a Organização Mundial da Saúde, a saúde é um "estado de completo bem-estar físico, mental e social e não apenas a ausência de doença ou enfermidade". Consequentemente, a saúde mental é parte integrante à saúde e ao bem-estar. O que determina a saúde mental e os transtornos mentais inclui uma série de atributos individuais, como por exemplo, capacidade de gerenciar pensamentos, emoções e comportamento, bem como interações entre indivíduos, tais como sociais, culturais, econômicas, políticas e ambientais (WHO, 2013). Somente em 2017, uma pesquisa realizada no 'Global Burden of Disease' conduzida pelo 'Institute for Health Metrics and Evaluation' estima que 792 milhões de pessoas vivam com algum transtorno mental. Isso representa um pouco mais de uma em cada dez pessoas no mundo (10,7%) (IHME, 2017) (Figura 1). Segundo dados da IHME, 14,51 % dos brasileiros apresentam algum tipo de doença mental.

Os transtornos mentais caracterizam-se por alterações no pensamento, emoções e/ou comportamento (LAKE e TURNER, 2017). Apesar de pequenas alterações no organismo serem naturais ao longo da vida, quando causam uma angústia significativa, podem interferir na vida cotidiana, sendo consideradas como uma doença mental ou um transtorno de saúde mental. Neste caso, os efeitos podem ser duradouros ou temporários (WHO, 2017). Aproximadamente 50% dos adultos sofrem de algum tipo de transtorno de doença mental em algum momento de suas vidas e apenas 20% procuram assistência médica. Do total de adultos afetados, mais da metade apresentam sintomas de moderados a graves, e muitos podem desenvolver outros transtornos, tais como transtorno de ansiedade e transtorno depressivo. Esses transtornos têm grande impacto sobre o humor, e tais condições de saúde são diagnosticáveis e diferem-se dos sentimentos comuns de tristeza, estresse ou medo (WHO, 2017).

Figura 1 – Dados de 2017 sobre a prevalência mundial dos transtornos de saúde mental.



Fonte: imagem adaptada de 'Institute for Health Metrics and Evaluation (IHME)', 2017.

Diversos fatores podem contribuir para o desenvolvimento de algum transtorno mental, tais como hereditários (genéticos), fatores físicos (biológicos), psicológicos e ambientais (incluindo fatores sociais e culturais) (DSM-5, 2014). No entanto, convêm ressaltar que não somente os atributos individuais (capacidade de gerenciar pensamentos, emoções, comportamentos e interações), mas os fatores sociais, culturais, econômicos, políticos e ambientais (políticas nacionais, proteção social, padrões de vida, condições de trabalho e apoio comunitário) contribuem para saúde mental (WHO, 2017). Além disso, estresse, desnutrição, infecções perinatais e exposição a perigos ambientais também são fatores que colaboram para os transtornos mentais (APA, 2015).

Apesar de existirem estratégias para a prevenção e tratamento dos transtornos mentais, notamos um aumento significativo nos casos a nível mundial (WHO, 2017). Os transtornos mentais possuem um custo econômico maior quando comparados com outras doenças crônicas. Perda de renda (por mortalidade), perda nas produções, incapacidade ou aposentadoria precoce são custos associados ao diagnóstico e ao tratamento no sistema de saúde. Acredita-se que entre os anos de 2011 e 2030, o prejuízo na economia mundial seja em torno de US$ 16,3 trilhões (TRAUTMANN; REHM; WITTCHEN, 2016). Assim, a compreensão dos mecanismos subjacentes do estresse relacionados ao desenvolvimento de

transtornos mentais se torna importante para elucidar os efeitos comportamentais, neuroquímicos e fisiológicos presentes nesses transtornos.

2.1.2. Estresse

O estresse é parte integrante da vida humana, e o indivíduo pode sofrer constantemente estímulos que culminem em uma resposta biológica (MCEWEN, 2007). Quando submetidos a um determinado evento estressor, nosso corpo reage com diversas adaptações fisiológicas, produzindo uma série de hormônios e produtos químicos. Esse mecanismo envolvido no estresse ocorre devido à necessidade de ajustes no corpo para uma reação física, seja para lutar ou fugir de diversas situações aversivas potencialmente perigosas (KYROU; TSIGOS, 2009). Além disso, o estresse é classificado em dois grupos, conhecidos como eustresse e distresse. Enquanto o eustresse, "estresse bom", refere-se às experiências de duração limitada, que se pode dominar, resultando em uma sensação de realização (KUPRIYANOV; ZHDANOOV, 2014; SELYE, 1976), o distresse, "estresse ruim", trata-se de experiências onde há falha do controle e domínio (SELYE, 1974). Quando tais experiências são severamente prolongadas ou recorrentes, ocasionam esgotamento emocional e físico, suscetíveis ao desenvolvimento de transtornos mentais (MCEWEN, 2007). A origem deste tipo de estresse são os sentimentos de tristeza, abandono, medo, cansaço, bem como ansiedade e para o organismo tentar manter a estabilidade, o sistema biológico desencadeia mudanças fisiológicas e comportamentais conhecidas como alostase (KUPRIYANOV; ZHDANOOV, 2014; SELYE, 1975). O corpo humano é adaptável, porém, alterações alostática pode não ser adaptáveis em um longo período, resultando em um desgaste denominado sobrecarga alostática. No entanto, essa sobrecarga não se mantem por muito tempo sem ocasionar consequências nocivas no organismo (MCEWEN, 2000; MCEWEN, 2013). Portanto, a resposta ao estresse em um curto período é adaptativa e importante para que o organismo tenha uma maior chance de sobrevivência (CHARMANDARI; TSIGOS; CHROUSOS, 2005). Por outro lado, em situações estressoras prolongadas, nosso corpo pode entrar em colapso e o estresse pode ser um fator crítico no desenvolvimento de diversos transtornos mentais (CHARMANDARI; TSIGOS; CHROUSOS, 2005; MCEWEN, 2007).

Cronicamente, o estresse pode causar ansiedade, depressão, disfunção cognitiva e/ou executiva, doença cardiovascular (aterosclerose), doenças metabólicas, doenças degenerativas e transtornos do sono (CHROUSOS, 2009; LUPIEN et al., 2009; MCEWEN, 2007). A exposição crônica a estressores pode prejudicar diversas funções fisiológicas, bem como

afetar o metabolismo energético e prejudicar o crescimento, a reprodução e o sistema imunológico, além de causar alterações no desenvolvimento da personalidade e no comportamento agressivo (KVETNANSKY; SABBAN; PALKOVITS, 2009; KYROU; TSIGOS, 2009; MCEWEN, 2007; ØVERLI et al., 2004; SUMMERS; WINBERG, 2006). Além disso, a exposição repetida a estressores pode desregular o eixo Hipotálamo-Pituitária-Adrenal (HPA), assim como afetar a homeostase celular e diversos sistemas de neurotransmissão, culminando em várias alterações comportamentais, tais como o aumento da ansiedade que pode levar à depressão (CHROUSOS, 2009; POPOLI et al., 2011; SANDI; HALLER, 2015). Porém, sabe-se que a frequência, intensidade e diferença de estressores são fatores que podem influenciar diretamente as respostas comportamentais e bioquímicas relacionadas ao estresse (KIILERICH et al., 2018).

2.1.2.1. Tipo de estressores

O estresse é uma combinação de respostas que envolve sintomas físicos, psicológicos e neurocomportamentais (MCEWEN, 2007). Muitas respostas relacionadas ao estresse podem estar associadas com alguns transtornos de ansiedade, bem como correlacionadas com a modulação em diferentes mecanismos fisiológicos (DSM-5, 2014; ROBICHAUD et al., 2019; WILKINS, 2019). Embora o estresse agudo possa vir a desencadear danos no organismo, estímulos aversivos aplicados cronicamente proporcionam um maior dano ao animal (HAILE; GRANDPRE; KOSTEN, 2001; MARIN; CRUZ; PLANETA, 2007; PASTOR-CIURANA et al., 2014). Apesar do estresse crônico imprevisível (ECI) utilizando estressores heterotípicos (diferentes tipos de estressores) seja mais correlacionado com o surgimento de doenças neuropsiquiátricas (CHAKRAVARTY et al., 2013; PIATO et al., 2011), alguns estudos mostram que o estresse crônico previsível (ECP) utilizando um determinado estressor homotípico (apenas um tipo de estressor) também pode desencadear respostas fisiológicas e neurocomportamentais associadas a transtornos mentais (AL-MOHAISEN; CARDOUNEL; KALIMI, 2000; VYAS; CHATTARJI, 2004; ZHU et al., 2017). Além disso, as respostas aos estressores podem variar conforme o tipo de estresse, a duração, frequência e intensidade do estímulo aversivo utilizado (ZUCCHI et al., 2009). Contudo, a duração e a frequência excessiva ao estresse pode desencadear um processo de habituação e, consequentemente, uma diminuição da resposta fisiológica ao estresse (CRESTANI, 2016; HERMAN, 2013). Embora o ECP possa induzir um processo adaptativo, a exposição a certos estressores homotípicos, como presença de predador, não desencadeia esse efeito (GRISSOM; BHATNAGAR, 2009;

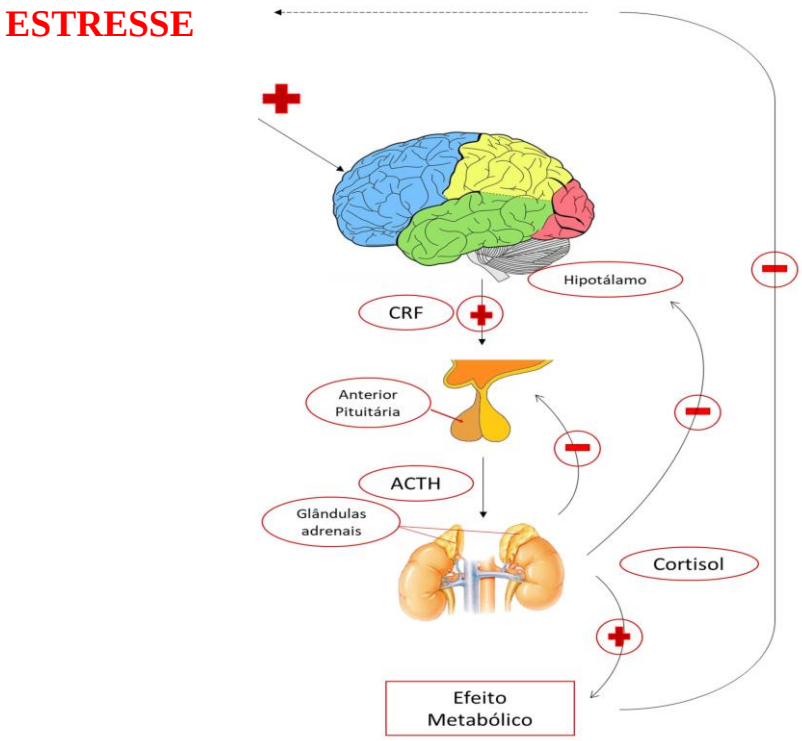
ZUCCHI et al., 2009). Portanto, utilizar estressores homotípicos de naturezas diferentes, é importante para entendermos como o ECP pode afetar respostas comportamentais, bioquímicas, bem como modular o funcionamento do eixo HPA e a consequente ativação das vias relacionadas ao estresse.

2.1.2.2. Cortisol

Em humanos, a ativação do eixo HPA está envolvida na produção e liberação do cortisol como mediador principal de resposta ao estresse (WALKER et al., 2008). O estresse crônico pode desregular a atividade desse eixo, desempenhando um papel em várias doenças neuropsiquiátricas, como a depressão e ansiedade (BROWN; VARGHESE; MCEWEN, 2004). A ativação do eixo HPA inicia-se no hipotálamo, que consequentemente ativa o sistema nervoso central (SNC) e periférico. Uma situação estressante estimula a secreção do fator liberador da corticotrofina hipotalâmico (CRF). Em resposta ao CRF, a pituitária libera o hormônio adrenocorticotrófico (ATCH) na corrente sanguínea. Então, o cortisol é secretado pela glândula adrenal e exerce seus efeitos via receptor glicocorticoide (GR), um fator de transcrição nuclear ativado por ligante (HERMAN et al., 2016) (Figura 2). Porém, quando há níveis aumentados do cortisol no organismo, ocorre um mecanismo de retroalimentação negativa à liberação hormonal, modulando negativamente a liberação de glicocorticoides bem como a sua interação com os receptores dos tecidos-alvo (HERMAN et al., 2016).

Pelo fato da desregulação do eixo HPA estar associada com diversos transtornos neuropsiquiátricos, incluindo transtornos de ansiedade e depressão (BROWN; VARGHESE; MCEWEN, 2004; HOLSBOER, 2000; WALKER et al., 2013), a avaliação de parâmetros neuroendócrinos pode auxiliar na compreensão dos processos adaptativos e fisiopatológicos relacionados ao estresse. Contudo, além do eixo HPA estar envolvido nas respostas neurocomportamentais desencadeadas pelo estresse, outros mecanismos biológicos, tais como a produção de espécies reativas de oxigênio e o estresse oxidativo, podem estar aumentados após eventos estressantes.

Figura 2: Funcionamento do eixo hipotálamo-pituitária-adrenal (HPA). Após ao estresse, a ativação do eixo HPA que se inicia no hipotálamo e estimula a secreção CRF que em resposta a pituitária libera o ATCH na corrente sanguínea. Então, o cortisol é secretado pela glândula adrenal e exerce, por retroalimentação negativa, a mediação as respostas do estresse no organismo.



Fonte: autoria própria

2.1.2.3. Estresse oxidativo

Espécies reativas de oxigênio (ERO) e radicais livres são produzidos durante o metabolismo aeróbico normal. Quando ocorre uma formação excessiva de ERO juntamente com a redução das defesas antioxidantes, ocorre o estresse oxidativo (BARBOSA et al., 2010; MONICZEWSKI et al., 2015; VALKO et al., 2007). O dano oxidativo afeta diversas estruturas celulares, a neurotransmissão, bem como as vias de transdução de sinal (HALLIWELL, 2006; HOVATTA; JUHILA; DONNER, 2010), ocorrendo em várias condições neuropsiquiátricas (RAMMAL et al., 2008; SMAGA et al., 2015). Os sistemas biológicos possuem estratégias para o controle dos efeitos nocivos produzidos pelas ERO e radicais livres. Malondialdeído (MDA) e proteína carbonilada (PC) são produtos que podem ser utilizados como indicadores de dano oxidativo e as defesas antioxidantes enzimáticas, tais como superóxido dismutase (SOD), catalase (CAT) e a glutationa peroxidase (GPx), e as defesas não enzimáticas como tióis não proteicos (NPSH) estão envolvidas no controle desses danos (VALKO et al., 2007). Alterações na atividade da SOD e da CAT podem estar

associadas com transtorno de fobia social (ATMACA et al., 2008), transtorno do pânico (KULOGLU et al., 2002a) e transtorno obsessivo-compulsivo (TOC) (KULOGLU et al., 2002b), corroborando a estreita relação entre doenças neuropsiquiátricas e estresse oxidativo. Além disso, a superprodução de ERO é prejudicial para a atividade de enzimas do SNC (YOUDIM; BAKHLE, 2006). No entanto, a modulação de parâmetros redox e seus efeitos sobre os sistemas de neurotransmissão e padrões comportamentais é complexa e ainda carece de futuros estudos.

2.1.2.4. Sistema serotonérgico

Diferentes neurotransmissores, como serotonina, dopamina e noradrenalina desepenham um papel fundamental nas interações agonísticas, como as respostas relacionadas ao comportamento agressivo (BORTOLATO et al., 2009, p. 2009; HERCULANO; MAXIMINO, 2014; LESCH; MERSCHDORF, 2000; SALLINEN et al., 2009). A monoamina oxidase (MAO) é uma enzima responsável pela degradação de várias aminas biogênicas, contendo na sua estrutura uma coenzima denominada flavina-adenosina-dinucleotídeo (FAD) (NIKOLAC PERKOVIC et al., 2016; ORELAND, 2004). Em mamíferos, existem duas isoformas desta enzima, denominadas MAO-A e MAO-B e ambas realizam a desaminação oxidativa de diferentes neurotransmissores monoaminérgicos e neuromoduladores (BORTOLATO; CHEN; SHIH, 2008; NIKOLAC PERKOVIC et al., 2016; ORELAND, 2004). Ambas as isoformas possuem diferenças em seu peso molecular, propriedades imunológicas e localizações anatômicas, bem como uma sensibilidade distinta a inibidores e diferenças em suas preferências por substrato (BORTOLATO; SHIH, 2011; NIKOLAC PERKOVIC et al., 2016; ORELAND, 2004). Embora ambas as isoformas degradam a dopamina, triptamina e tiramina, a MAO-A é responsável pela degradação da serotonina, norepinefrina e epinefrina, enquanto a MAO-B degrada a β-feniletilamina e benzilamina (BORTOLATO; SHIH, 2011). Estudos recentes mostram que alterações na MAO-A e MAO-B podem estar correlacionadas com alguns comportamentos associados a transtornos neuropsiquiátricos (GODAR et al., 2014). Dessa forma, estudar os efeitos de agentes estressores em diferentes emoções, bem como nas alterações do sistema serotonérgico torna-se importante para compreender as bases neurobiológicas e os fenótipos correlatos à fisiopatologia dos transtornos mentais.

2.1.3. Medo e ansiedade

Medo e ansiedade são respostas desencadeadas quando o organismo se envolve em situações específicas aversivas. Essas respostas podem ser expressas por diversos comportamentos defensivos fazendo com que o animal desenvolva um sistema de "auto-proteção" (BLANCHARD et al., 1993a, 1998). Enquanto o medo refere-se a uma resposta imediata ou uma ameaça iminente, a ansiedade é caracterizada como uma resposta de ameaça potencial ou distante (BLANCHARD et al., 1998; MAXIMINO et al., 2010b). Dessa maneira, estudos vêm sendo desenvolvidos com o propósito de verificar os aspectos comportamentais e neuroquímicos envolvidos nas respostas de medo e ansiedade em diversas espécies (JESUTHASAN, 2012).

Doenças neuropsiquiátricas como fobia social, pânico, estresse pós-traumático, transtorno obsessivo-compulsivo e depressão já estão sendo relacionadas com respostas exacerbadas de medo e/ou ansiedade (CATTELL, 1966; DAVIS et al., 2010; JESUTHASAN, 2012). Assim, o estudo dos aspectos emocionais associados a fatores ambientais e genéticos é de grande utilidade para a compreensão dos fenótipos e dos mecanismos bioquímicos envolvidos em transtornos neuropsiquiátricos, bem como possibilitar a busca por intervenções farmacológicas a fim de obter tratamentos efetivos (ADOLPHS et al., 1995; CRAWLEY, 1989; KALUEFF; TUOHIMAA, 2004; KALUEFF; WHEATON; MURPHY, 2007; MAXIMINO et al., 2013c; RODGERS; POWER; HOPE, 1997).

Os fenótipos associados ao medo e ansiedade em modelos experimentais podem ser diferenciados farmacologicamente. A administração de benzodiazepínicos e citalopram, por exemplo, responde para o tratamento de ansiedade, mas não em situações de medo (HOWLAND, 2016). Além disso, ambas as respostas podem envolver diferentes estruturas cerebrais (BLANCHARD et al., 1993b; DAVIS; WATERS, 1997; GRILLON et al., 2009; MAXIMINO et al., 2013a; MCNAUGHTON; GRAY, 2000). Sabe-se que a habênula medial (HM) e amígdala são responsáveis por regular respostas de medo. Lesões causadas no centro da amígdala reduz o comportamento associado com o medo, indicando que esta região encontra-se ativada nessas condições (AGETSUMA et al., 2010; BLANCHARD; BLANCHARD, 1972; KAPP et al., 1979). Outra estrutura importante que recebe informações do centro da amigdala é o núcleo do leito da estria terminal (NLET), a qual aparentemente não está correlacionada com respostas de medo, mas possui um importante papel nas respostas de ansiedade (DAVIS et al., 2010; HITCHCOCK; DAVIS, 1991). Além disso, reações defensivas envolvem a regulação de neuromoduladores, tais como as monoaminas e peptídeos (MAXIMINO, 2012). A ativação dos receptores de serotonina (5-HT) 5-HT_{1A}- e 5-

HT_2 na região substância cinzenta periaquedutal inibem as respostas de medo, enquanto nos núcleos amigdaloides, a ativação dos receptores do tipo 5-HT_2 e 5-HT_3 aumenta a resposta de ansiedade (GUIMARÃES; CARABREZ; GRAEFF, 2008; HALE; LOWRY, 2011; PAUL et al., 2014). Nesse sentido, respostas de medo e ansiedade parece depender da região do cérebro específica em que a 5-HT atua, bem como o tipo de receptor que é ativado (LIMA-MAXIMINO et al., 2020). Devido às limitações existentes em pesquisas com humanos, tanto em aspectos éticos quanto operacionais, é necessário o aprimoramento de pesquisas básicas utilizando espécies de laboratório para uma abordagem translacional da neurobiologia do medo e ansiedade (JESUTHASAN, 2012).

Embora estudos dos comportamentos relacionados ao medo e ansiedade em roedores sejam amplos, estes apresentam um elevado custo e possuem um baixo rendimento para triagens de novos fármacos (LEUNG; MOURRAIN, 2016). Considerando que o funcionamento de diferentes neurotransmissores é altamente conservado durante o processo evolução, a análise dos mecanismos envolvidos em respostas de medo e ansiedade em modelos animais alternativos pode ter grande relevância para uma melhor compreensão dessas emoções em diferentes transtornos (JESUTHASAN, 2012).

2.1.4. Transtorno de ansiedade

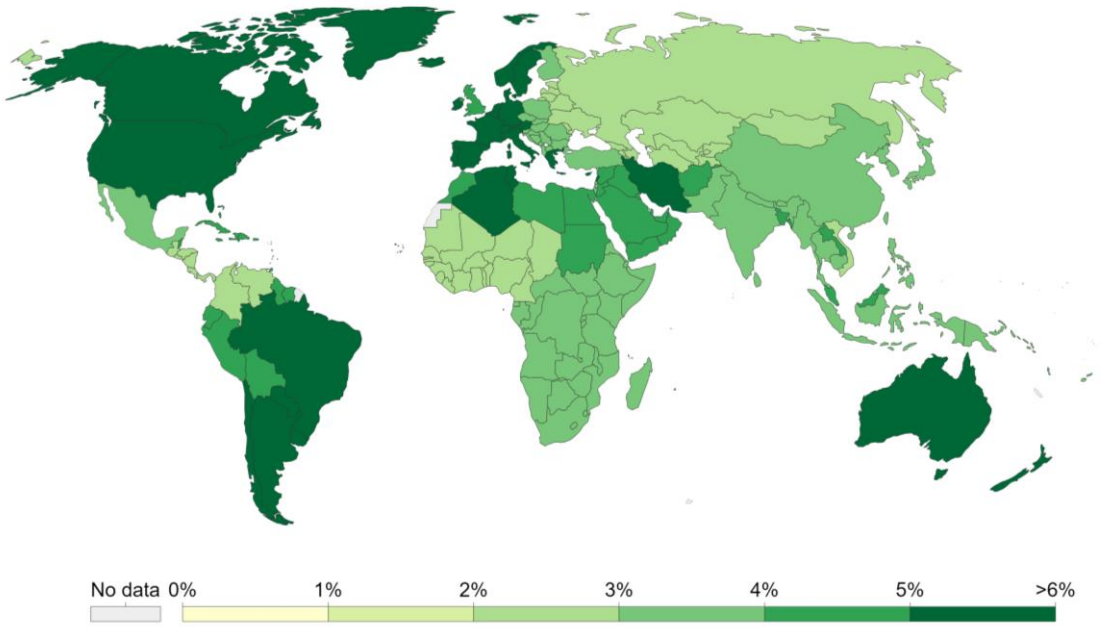
Os transtornos de ansiedade correspondem a um grupo de transtornos mentais que compartilham como características principais medo e ansiedade excessivos (WHO, 2017). Esses transtornos persistem por um longo período afetando o desenvolvimento, e são induzidos com frequência por estresse persistente (DSM-5, 2014). Os transtornos de ansiedade são classificados pela DSM-5 (2014) como: transtorno de ansiedade de separação, mutismo seletivo, fobia específica, transtorno de ansiedade social, transtorno de pânico, agorafobia, transtorno de ansiedade generalizada (TAG), transtorno de ansiedade induzido por substancias/medicamentos, transtorno de ansiedade devido à outra condição médica, transtorno de ansiedade especificado e transtorno de ansiedade não especificado (DSM-5, 2014). Indivíduos com transtorno de ansiedade normalmente são preocupados, apresentam tensão muscular e vigilância em preparação para um perigo futuro, apresentando dificuldade em vivenciar momentos atuais (ROBICHAUD; KOERNER; DUGAS, 2019).

Para cada transtorno de ansiedade, o diagnóstico segue critérios de forma singular. Segundo a Classificação Internacional de Doenças (CID-10), apreensão, tensão motora e hiperatividade autonômica são os sintomas mais frequentes para identificar o transtorno (WHO,

1992). Além disso, indivíduos acometidos com o transtorno de ansiedade apresentam outros problemas de saúde mental, como a depressão (KALUEFF; TUOHIMAA, 2004; ROBICHAUD; KOERNER; DUGAS, 2019). O prejuízo na qualidade de vida dos pacientes acometidos, bem como problemas educacionais e ocupacionais, podem estar relacionados a um aumento de mortalidade devido à doença (KUPFER, 2015).

Somente nos Estados Unidos da América (EUA), os transtornos de ansiedade atingem aproximadamente 40 milhões de adultos e o tratamento pode custar mais de 42 bilhões de dólares por ano (HURRELL; HOUWING; HUDSON, 2017). Os transtornos de ansiedade variam entre 2,5 e 7% por país em todo o mundo. Aproximadamente 284 milhões de indivíduos já apresentaram um tipo de transtorno de ansiedade em 2017, caracterizando o distúrbio mental com maior prevalência (Figura 3). O Brasil é um dos países com maior índice de pessoas com transtorno de ansiedade (6,07%) (RITCHIE; ROSER, 2018). Sabe-se que as mulheres são mais propensas em sofrer distúrbios de ansiedade do que os homens,63% (179 milhões) e 37% (105 milhões) respectivamente (RITCHIE; ROSER, 2018). Tal fato pode ser explicado pelos hormônios presentes no sexo feminino, além das diferenças na sintomatologia, no metabolismo e na resposta à farmacoterapia (JALNAPURKAR; ALLEN; PIGOTT, 2018).

Figura 3 – Dados de 2017 sobre a prevalência mundial dos transtornos de ansiedade.



Fonte: imagem adaptada de 'Institute for Health Metrics and Evaluation (IHME)', 2017.

O tratamento para o transtorno de ansiedade pode ser realizado com farmacoterapia ou psicoterapia, de forma isolada ou combinada. Além disso, benzodiazepínicos (diazepam, por exemplo), para ansiedade aguda e antidepressivos (classe dos inibidores seletivos da recaptação da serotonina – ISRS - como paroxetina, citalopram, sertralina, fluoxetina e escitalopram) para transtornos de ansiedade e depressão, são utilizados no tratamento (JALNAPURKAR; ALLEN; PIGOTT, 2018). Dessa forma, transtornos de ansiedade consistem em uma problemática mundial que afeta diversas funções biológicas, e consequentemente a vida social do indivíduo. Portanto, o uso de animais de laboratório consiste em uma importante estratégia para a busca de uma melhor compreensão das bases neurobiológicas e dos fenótipos associados à ansiedade (CACHAT et al., 2010; GERLAI, 2010, 2011; MAXIMINO et al., 2010; STEWART et al., 2011).

2.1.5. A utilização do peixe-zebra na pesquisa científica

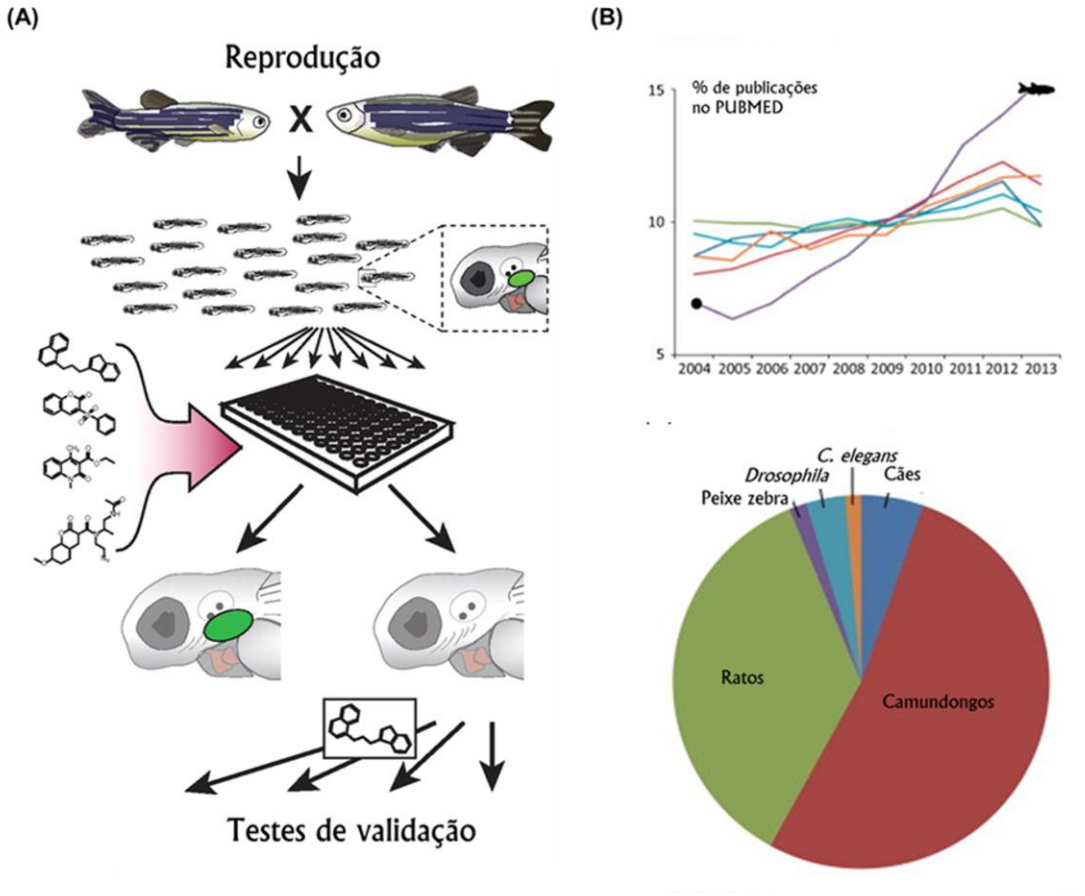
O peixe-zebra (*Danio rerio*) é uma espécie tropical de água doce conhecida popularmente como "paulistinha", pertence à família Cyprinidae e nativo da Ásia (WHITLOCK; WESTERFIELD, 2000). Apresenta diversas vantagens para uso como modelo experimental, dentre elas podemos citar o pequeno tamanho (adultos podem medir de 3 a 5

cm), baixo custo, pequeno espaço para manutenção, grande prole (50 a 200 ovos por dia para cada fêmea em condições otimizadas de reprodução), presença de ovos translúcidos e o rápido desenvolvimento até a fase adulta (aproximadamente 2–3 meses) (DAHM; GEISLER, 2006; LELE; KRONE, 1996).

George Streisinger foi o pioneiro na pesquisa da biologia do peixe-zebra utilizando técnicas de mutagênese sítio dirigidas para estudos relacionados ao desenvolvimento(DAHM; GEISLER, 2006; GRUNWALD; EISEN, 2002; GULATI-LEEKHA; GOLDMAN, 2006; RINKWITZ et al., 2015). O genoma do peixe-zebra já é completamente sequenciado e seus genes possuem um alto grau de similaridade com os genes dos mamíferos (aproximadamente 70%), o que possibilita estudar doenças humanas (HOWE et al., 2013). Devido a esses aspectos, o peixe-zebra combina a relevância de ser um vertebrado com a escala de um invertebrado, o que favorece a elaboração de protocolos de triagens de médio/alto rendimento quando comparado a roedores em estágios pré-clínicos (GOLDSMITH, 2004; LEUNG; MOURRAIN, 2016) (Figura 4).

Estudos em áreas como bioquímica, neuroquímica, e farmacologia e biologia do comportamento já são desenvolvidos com o peixe-zebra (BLASER; KOID; POLINER, 2010; EDWARDS; MICHEL, 2002; EGAN et al., 2009; FONTANA et al., 2016; GERLAI, 2003; MAXIMINO et al., 2011; MEZZOMO et al., 2016; PIATO et al., 2011; ROSEMBERG et al., 2011). O uso do peixe-zebra como organismo experimental está crescendo gradativamente e a espécie é potencialmente útil para estudos dos comportamentos defensivos e para a investigação de fenótipos relacionados ao medo e à ansiedade (CACHAT et al., 2010; GERLAI, 2010, 2011; MAXIMINO et al., 2010b; STEWART et al., 2011).

Figura 4 – Uso do peixe zebra na pesquisa científica. **(A)** Aplicação em testes de triagem de compostos em fase pré-clínica. **(B)** Diagramas representativos do número de publicações utilizando a espécie no Pubmed.



Fonte: Adaptado de Clements & Traver, 2012 e Stewart et al., 2014.

2.1.6. Medo e ansiedade em peixe-zebra

Apesar dos estudos relacionados com modelos que induzem comportamentos do tipo medo e ansiedade em peixe-zebra ainda não serem totalmente elucidados, trabalhos tem mostrado avanços significativos nesta área do conhecimento na última década (EGAN et al., 2009; GUO, 2009; KOKEL; PETERSON, 2008; MAXIMINO et al., 2010b, 2013c). Atualmente, muitas pesquisas neuroquímicas, neuroanatomicas e farmacológicas em peixe-zebra estão sendo desenvolvidas com a finalidade de buscar novos conhecimentos sobre as emoções, como medo e ansiedade (GUO; WAGLE; MATHUR, 2012; JESUTHASAN, 2012). Em peixe-zebra, durante situações estressoras, o eixo hipotálamo-hipófise-interrenal (HHI) é ativado e promove o aumento dos níveis de cortisol, o qual é envolvido na resposta ao estresse (ALSOP; VIJAYAN, 2009; COLLIER et al., 2017; MADARO et al., 2015;

MOMMSEN; VIJAYAN; MOON, 1999). Além disso, o encéfalo possui regiões específicas caracterizadas por respostas a estímulos aversivos, como a amígdala central (AC), responsável pelas respostas de ameaça, medo ou resposta imediata, cujas lesões pode causar redução ou perda do comportamento associada ao medo, bem como a região da habênula, ligada às respostas de ameaça potencial, comportamento emocional e expressão da memória (AMO et al., 2010, p. 20; BLANCHARD; BLANCHARD, 1972; KAPP et al., 1979; MAXIMINO et al., 2013a; MCNAUGHTON; GRAY, 2000; OKAMOTO; AGETSUMA; AIZAWA, 2012). Além disso, estudos recentes mostram à estreita relação da 5-HT no comportamento defensivo em vertebrados, sugerindo que em níveis diferentes, essa monoamina aumenta à ansiedade e diminui o medo em peixe-zebra (LIMA-MAXIMINO et al., 2020). Neste sentido, o efeito dualístico da 5-HT mostra que o aumento agudo da 5-HT aumenta o comportamento tipo ansiedade e inibe respostas de medo, enquanto baixos níveis dessa monoamina diminui os efeitos ansiogênicos e aumenta comportamento de medo (LIMA-MAXIMINO et al., 2020). No entanto, a forma na qual o encéfalo determina essas ameaças, identificando as regiões específicas para respostas de medo e ansiedade, e outros comportamentos ainda são desconhecidas (OLIVEIRA et al., 2013).

2.1.7. Agressividade em peixe-zebra

Interações agonísticas são determinadas por comportamentos que ocorrem em situações competitivas, seja por ameaça, luta por território ou agressão. Embora a agressividade seja adaptativa, esse comportamento pode desencadear estímulos nocivos ou alerta a outros organismos (COMAI et al., 2012; JONES E NORTON, 2015; LESCH E MERSCHDORF, 2000). Após os animais enfrentam uma situação perigosa real ou alguma ameaça potencial, a ativação do sistema nervoso simpático é ativado visando preparar o organismo para um comportamento de "luta ou fuga" (KALIN et al., 1998; KENNEY et al., 2017; LOPEZ-LUNA et al., 2017). No entanto, quando exacerbado, o comportamento agressivo pode ser maladaptativo. Atualmente, pesquisas demonstram a relação intima entre os distúrbios intensos de agressão com algumas doenças neuropsiquiátricas como déficit de atenção/hiperatividade (TDAH), esquizofrenia, doenças de Parkinson, Alzheimer e transtornos de ansiedade (JONES E NORTON, 2015; MAHALINGAIAH et al., 2015; NEUMANN et al., 2010). Apesar dos recentes avanços que descrevem o envolvimento de diferentes sistemas de neurotransmissores no comportamento agressivo, os circuitos estruturais subjacentes à agressão não são totalmente compreendidos (TELESAND

OLIVEIRA, 2016). Uma vez que o peixe-zebra apresenta repostas comportamentais e neuroquímicas conservadas frente a essas emoções, a diferença comportamental e a variação genética entre populações, mostra ser uma ferramenta importante para melhor compreender as bases neurobiológicas do medo, da ansiedade e da agressividade.

2.1.8. Populações de peixe-zebra: selvagem e leopardo

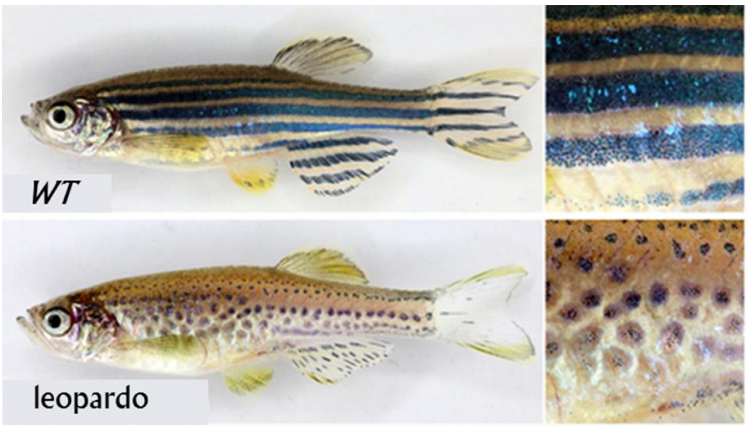
Pesquisas mostram que diferentes populações de peixe-zebra possuem genética e comportamento tipo ansiedade distintos (EGAN et al., 2009; HOWE et al., 2013). Portanto, a seleção de diferentes populações pode ter grande aplicação no design experimental uma vez que a seleção poderia aprimorar a triagem de compostos ansiolíticos (comportamento mais visível em populações ansiosas, como por exemplo, o *leopardo* [*leo*]) e ansiogênicos (populações selvagem [*WT*] cujo comportamento tipo ansiedade é reduzido) (EGAN et al., 2009; MAXIMINO et al., 2013b; QUADROS et al., 2016) (Figura 5).

As células de pigmentação possuem importância para a determinação e diferenciação de populações em peixe-zebra. Após o desenvolvimento (aproximadamente 3-6 semanas), o formato e a coloração pigmentar se definem (FROHNHÖFER et al., 2013). A população *WT* possui linhas alternadas de coloração clara/escura, horizontais ao longo do corpo até a cauda, enquanto a população *leo* desenvolve uma série de pontos escuros não uniformes em todo o corpo (FRANKEL, 1979; WATANABE et al., 2006). A pigmentação apresentada pela população *leo* é causada por uma mutação no gene *connexin 41.8* (*Cx41.8*) (HAFFTER et al., 1996; WATANABE; WATANABE; KONDO, 2012), responsável pela codificação da junção de hiato ("gap junction protein α5", GJA5). As junções de hiato são canais intercelulares que permitem a passagem de pequenas moléculas e íons entre células vizinhas e, portanto, importantes em funções de química e acoplamento elétrico (IRION et al., 2014). A mutação no gene *Cx41.8* em *leo,* culmina em uma modificação no padrão de organização dos melanossomos quando comparados aos da população *WT* (WATANABE; WATANABE; KONDO, 2012; MADERSPACHER; NÜSSLEIN-VOLHARD, 2003).

Como uma resposta inata apresentada por diversos vertebrados, o padrão de pigmentação possui um importante papel na obtenção de alimento, na comunicação social e nas respostas anti-predatórias (FUJII et al., 2000; NASCIMENTO; ROLAND; GELFAND, 2003). A modulação das respostas pigmentares pode estar associada com mudanças em parâmetros do comportamento tipo ansiedade, na resposta natural dos indivíduos expostos à ambientes escuros e iluminados (resposta de camuflagem), na interação social

(comportamentos de acasalamento ou agressivos), bem como ser resultado de exposição a compostos químicos ou a agentes estressores.

Figura 5 – Peixes-zebra das populações selvagem (*WT*) e leopardo (*leo*), com detalhes do padrão de pigmentação em listras azuladas ou pontos escuros dispersos.



Fonte: http://www.eb.tuebingen.mpg.de/research/emeriti/research-group-colour-pattern-formation.html (adaptado e acessado em julho de 2016).

2.1.9. Modelos de indução de estresse físico e químico em peixe-zebra

A substância de alarme (SA), feromônio de alarme ou mensageiro químico de alerta foi descrito pela primeira vez em 1938 por Karl von Frisch e denominada *"Schreckstoff"* (SMITH, 1992). A SA é produzida pelas células epiteliais de peixe teleósteos, e liberada em situações de lesão. Ela é detectada pelo sistema olfativo e exerce uma sinalização química que resulta em respostas comportamentais do tipo "luta ou fuga" dos animais (MOURABIT et al., 2010). Essa substância possui um curto período de ação, apresentando duração máxima de 30 minutos (PARRA; ADRIAN; GERLAI, 2009). Uma única exposição à SA é capaz de modular o repertório de comportamento defensivo em peixe-zebra durante 24 horas, sugerindo que o modelo poderia ser útil em estudos relacionados ao estresse pós-traumático e que a SA induz sensibilização de comportamentos defensivos (LIMA et al., 2016).

Embora o composto químico 3-N-óxido de hipoxantina (H_3NO) tenha sido identificado como uma das moléculas presentes na SA, sua caracterização química não é totalmente elucidada (MAXIMINO et al., 2018b; QUADROS et al., 2016; SPEEDIE; GERLAI, 2008). A SA é caracterizada por ser uma mistura complexa, uma vez que possui fragmentos de glicosaminiglicanos e sulfato de condroitina, capazes de induzir respostas comportamentais diferenciadas em peixe-zebra. Além disso, acredita-se que o sulfato de condroitina seja a molécula responsável por ativar um subconjunto de neurônios olfatórios (ARGENTINI, 1976; MATHURU et al., 2012; PFEIFFER, 1982). Quando a substância de alarme de co-específico é liberada na água, o animal exposto realiza movimentos erráticos, podendo permanecer no fundo do aquário em um episódio de congelamento ou, quando em cardume, se aglomeram, além de alterar seu padrão de pigmentação, ficando mais pálidos (CANZIAN et al., 2017; SPEEDIE; GERLAI, 2008). A exposição à SA é bem caracterizada por produzir comportamento do tipo medo, uma vez que é capaz de aumentar a expressão do gene *c-fos* na habênula de peixe-zebra (NATHAN; OGAWA; PARHAR, 2015; OGAWA; NATHAN; PARHAR, 2014). Além disso, estudos mostram que a exposição à SA desencadeia alterações comportamentais em outros modelos animais (BARRETO et al., 2014).

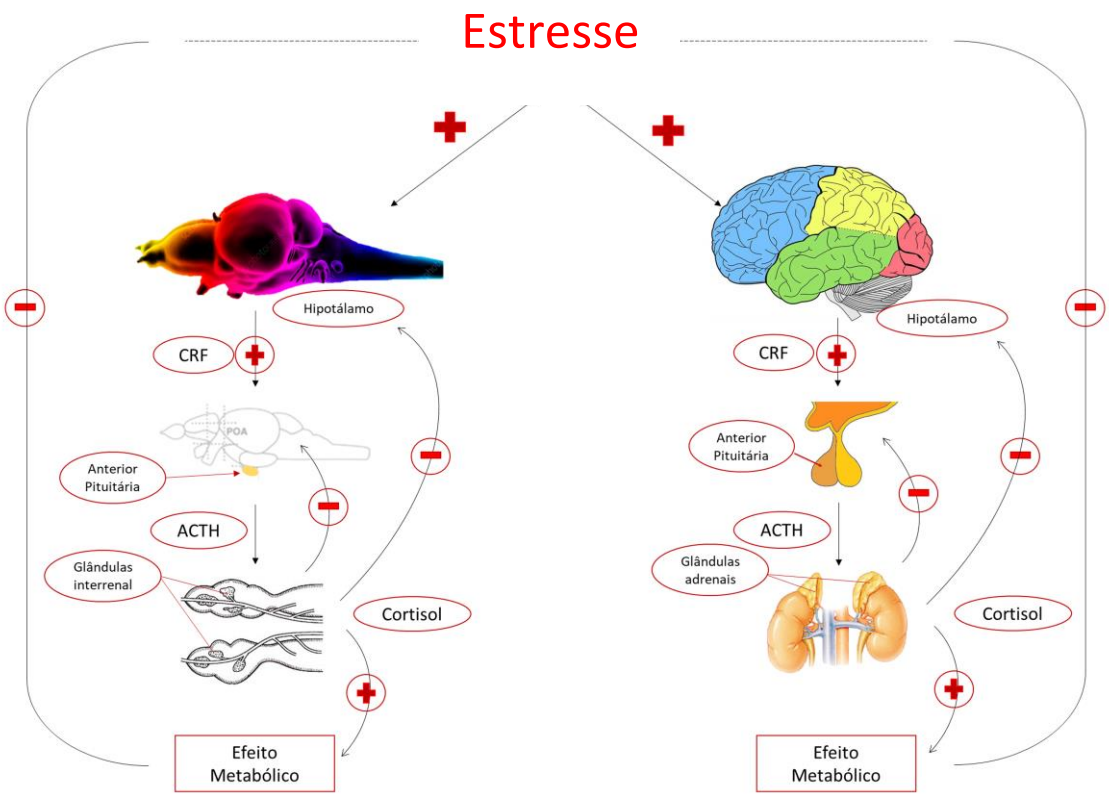
A resposta ao estresse é um importante mecanismo que fornece condições de enfrentar situações adversas, elevando os níveis de cortisol e resultando na mobilização de energia para ações defensivas e comportamento de esquiva (BARTON; MORGAN; VIJAYAN, 2002). A perseguição com rede (PR) reflete um comportamento de esquiva, onde o animal tende a evitar o objeto de perseguição (ABREU et al., 2018). Estudos utilizando PR mostram que agudamente o protocolo aumenta o comportamento do tipo ansiedade, bem como os níveis de

cortisol (ABREU et al., 2014; BARCELLOS et al., 2011). No entanto, os efeitos crônicos induzidos pela repetição da PR em respostas fisiológicas e comportamentais em peixe-zebra ainda não é elucidado. De fato, a validação de modelos comportamentais que analisem as respostas de diferentes populações e estressores que induzam medo e ansiedade, poderia servir como uma importante ferramenta para investigar as causas proximais do estresse em transtornos mentais.

2.1.10. Regulação do estresse em peixe-zebra

Os efeitos desencadeados pelo estresse em peixe-zebra são muito semelhantes aos que ocorrem em humanos e podem ser avaliados por respostas neuroendócrinas após exposição a estímulos estressores (CACHAT et al., 2010). O peixe-zebra possui o eixo HPI, análogo ao eixo HPA de mamíferos (ALSOP; VIJAYAN, 2009), cuja ativação culmina na produção e liberação do cortisol (Figura 6). Como mencionado anteriormente, quando comparado com humanos, o peixe-zebra possui 70% de similaridade genética bem como as respostas relacionados ao estresse evolutivamente conservados, o que favorece a validação desse organismo modelo como ferramenta para estudar as respostas relacionadas ao estresse (ALSOP; VIJAYAN, 2009; HOWE et al., 2013). Pesquisas mostram que após exposição de agentes estressores em peixe-zebra, o cortisol começa a aumentar após 3 minutos, com pico máximo em 15 minutos e retorno aos níveis basais 1 hora após o estresse (BARCELLOS, 2007; RAMSAY et al., 2009). Dessa forma, estudar como a resposta comportamental em diferentes paradigmas comportamentais pode ser influenciada pelo estresse é de grande relevância na pesquisa científica.

Figura 6 – Comparativo entre peixe-zebra e ser humano na regulação do estresse



Fonte: da autora

2.1.11. Testes comportamentais em peixe-zebra

Pesquisas em neurociência translacional utilizando o peixe-zebra vem ganhando espaço para melhor entender o significado do padrão de repertórios comportamentais na espécie (LEUNG; MOURRAIN, 2016). A compreensão dos mecanismos neurológicos juntamente com os fenótipos comportamentais associados à espécie pode fornecer importantes descobertas sobre potenciais biomarcadores e sobre as bases genéticas envolvidas em diversas patologias (RICO et al., 2011). Neste sentido, pesquisadores desenvolveram um catálogo detalhado onde identificaram e caracterizaram os tipos de comportamento existentes no peixe-zebra, desde sua fase larval até adulto (Zebrafish Behavioral Catalogue , ZBC) (KALUEFF et al., 2013). Medo e ansiedade podem ser investigados usando alguns estímulos comumente utilizados na pesquisa comportamental em peixe-zebra. O teste a novidade (teste do novo tanque), é um dos estímulos empregados, e propõe avaliar os perfis de locomoção, motricidade e exploração vertical dos animais (EGAN et al., 2009; GROSSMAN et al., 2010;

MATHUR; LAU; GUO, 2011; MAXIMINO et al., 2010b; ROSEMBERG et al., 2011; SACKERMAN et al., 2010). O teste da escototaxia (teste do tanque claro/escuto) é outro protocolo para verificar respostas de ansiedade pelo conflito gerado na presença de ambientes aversivos (claro) e preferenciais (escuro) (MAXIMINO et al., 2010a). Nesse contexto, o teste tem sido validado como um modelo para a avaliação do comportamento tipo ansiedade na espécie (BLASER; CHADWICK; MCGINNIS, 2010; MAXIMINO et al., 2010a, 2011; ROSEMBERG et al., 2012). Outro estímulo que pode ser utilizado para avaliar respostas que podem ser influenciadas por medo e ansiedade é o teste de agressão induzida pelo espelho (MIA – do inglês, "mirror-induced aggression test") (FONTANA et al., 2016; GERLAI et al., 2000). Em humanos, a agressão intensificada pode estar associada com algumas doenças neuropsiquiátricas, incluindo transtornos de ansiedade (JONES; NORTON, 2015; MAHALINGAIAH; PONNUSAMY; SINGH, 2015; NEUMANN; VEENEMA; BEIDERBECK, 2010). Neste teste, podemos avaliar a relação entre locomoção, agressividade e impulsividade do animal frente a um oponente virtual, sua imagem refletida no espelho (FONTANA et al., 2016; GERLAI et al., 2000)**.** Portanto, estudos referentes à medo e ansiedade em peixe-zebra apresentam um grande potencial para à avaliação dos padrões comportamentais apresentados por indivíduos que possuem características genéticas e fisiológicas distintas(MAXIMINO et al., 2013c).

3. JUSTIFICATIVA

Segundo dados da OMS, 70% da população do Brasil sofre com o estresse, e esta porcentagem pode atingir cerca de 90% da população mundial (WHO, 2013). Conforme o ministério da saúde, o estresse pode ser um dos fatores primários para o desenvolvimento de diferentes psicopatologias. O estresse faz parte da vida cotidiana de todos os indivíduos e sua resposta desencadeia alterações físicas, emocionais e comportamentais (WHO, 2012). Diferentes estressores com relação ao tipo de agente (homotípico ou heterotípico) e/ou duração (estresse agudo ou crônico) são capazes de desenvolver mudanças comportamentais ou bioquímicas, as quais podem se tornar maladaptativas. Dessa forma, o estudo da fisiologia do estresse em modelos animais é fundamental para a compreensão da base mecanística envolvida em diferentes transtornos mentais.

O uso de peixe-zebra vem ganhando espaço em pesquisas de neurociência, com aplicabilidade para o estudo de doenças humanas (FONTANA et al., 2018; STEWART et al., 2012). A avaliação dos fenótipos comportamentais associados a um determinado contexto é uma importante estratégia para a descoberta de potenciais biomarcadores e das bases genéticas envolvidas em diversas patologias, tais como: modelo de doença de Alzheimer (MUSA et al., 2001; JOSHI et al., 2009), de Parkinson (BRETAUD et al., 2004; SARATH BABU et al., 2016), de epilepsias (ESCAYG e GOLDIN, 2010; HORTOPAN e BARABAN, 2011), de esquizofrenia (SEIBT et al., 2010; SEIBT et al., 2011; SEIBT et al., 2012), e de transtornos relacionados ao estresse (PIATO et al., 2011; DAL SANTO et al., 2014; QUADROS et al., 2016; CANZIAN et al., 2017). Considerando a dificuldade de interpretar a resposta comportamental em animais, é importante uma validação que possa corroborar a utilização de um protocolo experimental. Portanto, essa pesquisa servirá de suporte para uma melhor compreensão das respostas comportamentais, neuroquímicas e endócrinas associadas ao efeito promovido por agentes estressores em diferentes populações de peixe-zebra, as quais possuem diferenças basais no comportamento do tipo ansiedade (EGAN et al., 2009; HOWE et al., 2013, QUADROS et al., 2016). Dessa forma, acreditamos que a presente Tese contribuirá para a elucidação dos efeitos neurocomportamentais promovidos por diferentes exposições a agentes estressores em peixe-zebra.

4. HIPÓTESES

- As exposições agudas e repetidas à SA desencadeiam um aumento no comportamento agressivo, aumentando a atividade da monoamina oxidase de peixe-zebra (Z-MAO) após a exposição aguda. As respostas basais são dependentes de população, onde animais “menos ansiosos” podem ser mais ousados e, portanto, mais agressivos.

- A exposição repetida à SA aumenta o comportamento tipo ansiedade e o estresse oxidativo em ambas as populações, bem como os efeitos basais são dependentes de população, visto que *leo* é mais ansioso que *WT*.

- O estresse crônico previsível (ECP), aumenta respostas comportamentais do tipo ansiedade e os níveis de cortisol em peixe-zebra. As respostas ao ECP (presença ou ausência de habituação) podem ser dependentes do tipo de estressor homotípico (PR ou SA).

5. OBJETIVOS

5.1. OBJETIVO GERAL

Investigar a influência do estresse sobre comportamentos relacionadas à agressão e ansiedade, bem como os efeitos em parâmetros neuroquímicos e endócrinos de peixes-zebra.

5.2. OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos promovidos pela exposição aguda e repetida à SA em duas populações de peixe-zebra (*WT* e *leo*) sobre o comportamento do tipo agressivo e atividade da Z-MAO cerebral;

- Avaliar o envolvimento do comportamento do tipo ansiedade e em parâmetros de estresse oxidativo em ambas as populações no estresse repetido à SA;

- Investigar se o estresse crônico previsível à SA e PR altera diferentemente as respostas tipo ansiedade e endócrinas em peixe-zebra e se podem induzir respostas de habituação ao estresse.

6. DESENVOLVIMENTO

As metodologias utilizadas e os resultados desta tese estão demonstrados na forma de dois artigos científicos publicados em periódicos internacionais de relevante fator de impacto na área e um manuscrito.

- O Artigo 1 foi publicado na revista *Progress in Neuro-Psychopharmacology and Biological Psychiatry* (Qualis Referência CAPES A1, FI: 4.315) em 2017 e se intitula: "Modulatory role of conspecific alarm substance on aggression and brain monoamine oxidase activity in two zebrafish populations".

- O Artigo 2 foi publicado na revista *Neurochemistry International* (Qualis Referência CAPES A2, FI: 3.994) em 2019 e se intitula: "Involvement of anxiety-like behaviors and brain oxidative stress in the chronic effects of alarm reaction in zebrafish populations".

- O Manuscrito Científico está submetido à revista *Hormones and Behavior* (Qualis Referência CAPES A1, FI 4.445) e se intitula: "Predictable chronic stress modulates behavioral and neuroendocrine phenotypes of zebrafish: influence of two homotypic stressors on stress-mediated responses".

7. ARTIGOS CIENTIFÍCOS

7.1. ARTIGO CIENTÍFICO 1

Prog Neuropsychopharmacol Biol Psychiatry. 2018 Aug e30; doi:10.1016/j.pnpbp.2018.03.018. Epub 2018 Mar 26.

Modulatory role of conspecific alarm substance on aggression and brain monoamine oxidase activity in two zebrafish populations

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Modulatory role of conspecific alarm substance on aggression and brain monoamine oxidase activity in two zebrafish populations

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ABSTRACT

Aversive conditions can elicit fear and the subsequent activation of the sympathetic nervous system induces 'fight or flight' responses. Previous unpleasant experiences may trigger fear-induced aggression and heightened aggression is a behavioral phenotype associated to various psychopathologies. Since the conspecific alarm substance (CAS) acts as chemical cue that elicits fear in fish species, we evaluated whether acute and chronic CAS exposures modulate aggression in *wild-type* (*WT*) and *leopard* (*leo*) zebrafish using the mirror-induced aggression (MIA) test. Because monoamines influence mood and behavior, we also assessed the effects of CAS on brain *Z*-MAO activity. CAS was isolated from phenotypically similar donor fish and 3.5 mL/L was used for the experiments. In the acute protocol, fish were tested following a single CAS exposure (5 min). The chronic exposure consisted of exposing the animals once daily (5 min) for 7 consecutive days, with a subsequent test on the 8th day. CAS acutely increased aggression and decreased *Z*-MAO activity in both populations. Conversely, chronic CAS exposure reduced aggression and inhibited locomotion without affecting *Z*-MAO. Differently than *WT*, *leo* showed decreased absolute turn angle and increased latency to attack the mirror following the chronic exposure. At baseline conditions, *WT* were more active, aggressive, and had a lower brain *Z*-MAO activity than *leo*. Overall, we suggest a distinct acute and chronic effect of CAS on aggression and a possible involvement of brain *Z*-MAO in aggressive behaviors. Moreover, the use of different zebrafish populations could serve as emergent tools to investigate the neurobehavioral bases of fear-induced aggression.

1. Introduction

Agonistic interaction is any behavior that occur in competitive situations which includes threat, displays, aggression, and submission. Although aggression is adaptive, it may elicit aversion, noxious stimuli or alert to other organisms (Comai et al., 2012; Jones and Norton, 2015; Lesch and Merschdorf, 2000). When animals face a dangerous situation, the sympathetic nervous system activation promotes hyperarousal, which prepare the organism to 'flight or fight' (Kenney et al., 2017; Lopez-Luna et al., 2017). Thus, depending on the situation, this autonomic response may elicit fear-induced aggression following an intense unpleasant experience, which represents a protective behavior against potential threats (Kalin et al., 1998). In humans, intensified aggression also comorbid attention-deficit/hyperactivity disorder (ADHD), schizophrenia, Parkinson's and Alzheimer's diseases, and anxiety disorders (Jones and Norton, 2015; Mahalingaiah et al., 2015; Neumann et al., 2010). Despite the recent advances describing the involvement of different neurotransmitter signaling pathways in aggressive behavior, the neural circuits underlying aggression are not fully understood (Teles and Oliveira, 2016). Due to the relationship between locomotion, aggressive behavior, and impulsivity, aggression may be difficult to define, isolate, and measure using experimental animals (Way et al., 2015).

Different neurotransmitters, such as serotonin, dopamine, and noradrenaline play a key role in agonistic interactions (Bortolato et al., 2009; Lesch and Merschdorf, 2000; Sallinen et al., 2009; Seo et al., 2008). Monoamine oxidase (MAO) is a flavin-adenine dinucleotide (FAD) enzyme that degrades these biogenic amines. In mammals, two isoenzymes (MAO-A and MAO-B) with distinct substrate preferences, immunological properties, anatomical location, and sensitivity to

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inhibitors have been described (Bortolato and Shih, 2011; Nikolac Perkovic et al., 2016; Oreland, 2004). Although both isoenzymes degrade dopamine, tryptamine, and tyramine, MAO-A cleaves serotonin, norepinephrine, and epinephrine, while MAO-B degrades β-phenylethylamine and benzylamine. Associative studies related to MAO-A and aggression have been focused on genetics and a lower MAO-B activity in platelets accompanies aggression in humans (Belfrage et al., 1992; Skondras et al., 2004) suggesting a close relationship between MAO-B and aggressive traits. Thus, laboratory animals are powerful tools to investigate the influence of monoamine degradation and aggression.

Zebrafish (*Danio rerio*) has several features that make it an attractive vertebrate model in neurobehavioral research, such as genetic similarities and neurochemical conservation (Fontana et al., 2018; Rosemberg et al., 2012; Stewart et al., 2014; Wong et al., 2004; Wyatt et al., 2015). Furthermore, zebrafish has integrated projections into a circuit of amygdala-like structures in the telencephalon, as well as hypothalamic structures, serotonergic innervation and monoaminergic systems (Anichtchik et al., 2006; Gayoso et al., 2011; Neumann et al., 2010). Zebrafish express a single MAO isoform (*Z*-MAO) that shares 70% identity with mammalian MAO-A and MAO-B, but several residues of the substrate-binding site are more similar to rodent MAO-A than MAO-B (Aldeco et al., 2011; Howe et al., 2013; Jones and Norton, 2015; Setini et al., 2005).

The influence of monoaminergic neurotransmission in anxiety-like behavior has been postulated in zebrafish. Although acute exposure to fluoxetine – a serotonin reuptake inhibitor – rescues anxious phenotype in *leopard* (*leo*) population, it is inactive in wild-type (*WT*) (Maximino et al., 2013). When compared to *WT*, the *leo* phenotype displays prominent defensive behaviors, showing pronounced bottom dwelling (Egan et al., 2009), higher social cohesion (Canzian et al., 2017), and increased scototaxis (Maximino et al., 2013). These populations present distinct shoaling responses in the presence of conspecific alarm substance (CAS), a chemical cue which elicits fear (Egan et al., 2009; Quadros et al., 2016). Importantly, CAS activates habenular *c-fos* expression in zebrafish, as well as increases blood glucose, hemoglobin, epinephrine, norepinephrine, and extracellular serotonin levels in the brain (Maximino et al., 2014; Ogawa et al., 2014). CAS-exposed zebrafish also show exacerbated freezing and erratic movements, as well as prolonged escaping behavior, suggesting persistence of aversive behavior following a single exposure (Maximino et al., 2018). Additionally, repeated exposure to a same noxious stimulus promotes long-term changes in the CNS, which may impair locomotion, influencing the overall behavioral activity (Wright et al., 2013). Since these behaviors depend largely on the nature and intensity of the noxious stimulus, acute and chronic CAS exposure reflect different contexts, which may modulate aggression in populations with distinct baselines of anxiety (Miczek et al., 2002). Thus, considering the behavioral and physiological responses of CAS-exposed zebrafish, we sought to investigate whether CAS acutely (single exposure) and chronically (repeated exposure over 7 days) modulates aggressive behavior of *WT* and *leo* populations. Additionally, to investigate the effects of CAS on monoamine degradation, we determined *Z*-MAO activity in brain samples.

2. Materials and methods

2.1. Subjects

Adult short fin *wild type* (*WT*) and *leopard* (*leo*) zebrafish (*Danio rerio*) were obtained from a local commercial distributor (Hobby Aquários, RS, Brazil). Animals were 5–7 months-old and a 50:50 (male:female) proportion was used for the experiments. Since the genetic background of fish was unknown, we used the term "population" for the respective phenotypes. Importantly, these populations are expected to be genetically heterogeneous which closely resembles the natural conditions, decreasing the effects of arbitrary genetic drifts (Canzian et al., 2017; Speedie and Gerlai, 2008; Watanabe et al., 2006). Animals were kept in 40-L tanks for at least two weeks prior the experiments with a maximum density of 4 fish per liter. All tanks were filled with non chlorinated water treated with AquaSafe™ (Tetra, VA, USA), kept under mechanical, biological, and chemical filtration, and held at 27 ± 1 °C. Fluorescent light tubes provided a constant room lighting adjusted into an artificial 14 h/10 h light/dark photoperiod (lights on at 7:00 am). All animals were experimentally naïve and fed thrice daily with Alcon BASIC™ flake fish food (Alcon, Brazil). This study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (106/2014).

2.2. CAS extraction and exposure protocols

CAS was prepared as described previously (Egan et al., 2009). Briefly, donor fish were promptly euthanized by decapitation without anesthesia to avoid chemical interference. Then, zebrafish bodies were further washed twice with distilled water, and the excess of blood was carefully removed with a swab in order to avoid a possible contamination of the extract. CAS was isolated by damaging epidermal cells with 10–15 superficial shallow slices on one side of the fish body with a razor blade. Fish was further placed into a Petri dish and both sides of the body were washed with 20 mL of distilled water. The dish was shaken gently to fully cover lacerated portions of the animals. Fig. 1A depicts a schematic representation of CAS extraction from donor zebrafish.

Zebrafish were exposed individually to 3.5 mL/L skin extract preparation in 500 mL tanks. In the acute protocol, fish were subjected to a single 5-min exposure period (Egan et al., 2009; Quadros et al., 2016; Speedie and Gerlai, 2008). Chronic CAS exposure consisted of exposing animals for 5 min once daily for 7 consecutive days, with a subsequent test on the 8th day (Fig. 1B). This protocol was chosen to verify the influence of repeated exposure to the same chemical cue on behavioral and biochemical parameters avoiding potential acute effects. Control groups were handled in a similar manner, except that only distilled water was added to the tank. Importantly, *WT* and *leo* were exposed to the skin extract isolated from phenotypically similar donor fish.

2.3. Behavioral experiments

All behavioral tests were recorded between 09:00 am and 4:00 pm. Zebrafish behaviors were recorded at 30 frames/s for 6 min using appropriate video-tracking software (ANY-maze™, Stoelting CO, USA). We took all the precautions to obtain representative data and to minimize handling stress. Throughout the experiments, fish were carefully manipulated between the test tanks. Each experimental group comprised individuals from at least two batches and the tank water was replaced after a single trial. Notably, the manipulation, water quality, and illumination were kept uniform throughout the experiments.

2.4. Measurement of aggressive behavior

Aggressive behavior of zebrafish was assessed using the mirror-induced aggression (MIA) test (Fontana et al., 2016; Gerlai et al., 2000). After the exposure periods, fish were individually placed in the test apparatus (25 cm length × 15 cm height × 6 cm width) filled with 1.5 L nonchlorinated water. An inclined mirror (22.5°) was placed in one wall of the tank, in which one vertical edge of the mirror was touching the side of the tank and another edge was further away (Fontana et al., 2016; Gerlai et al., 2000). All other tank sides were covered with opaque partitions in order to reduce environmental cues and to allow two simultaneous recordings. Tanks were virtually divided into two areas related to their proximity to mirror (close and far) (Fontana et al., 2016) and the following endpoints were measured: distance traveled and absolute turn angle (locomotor parameters), number and duration

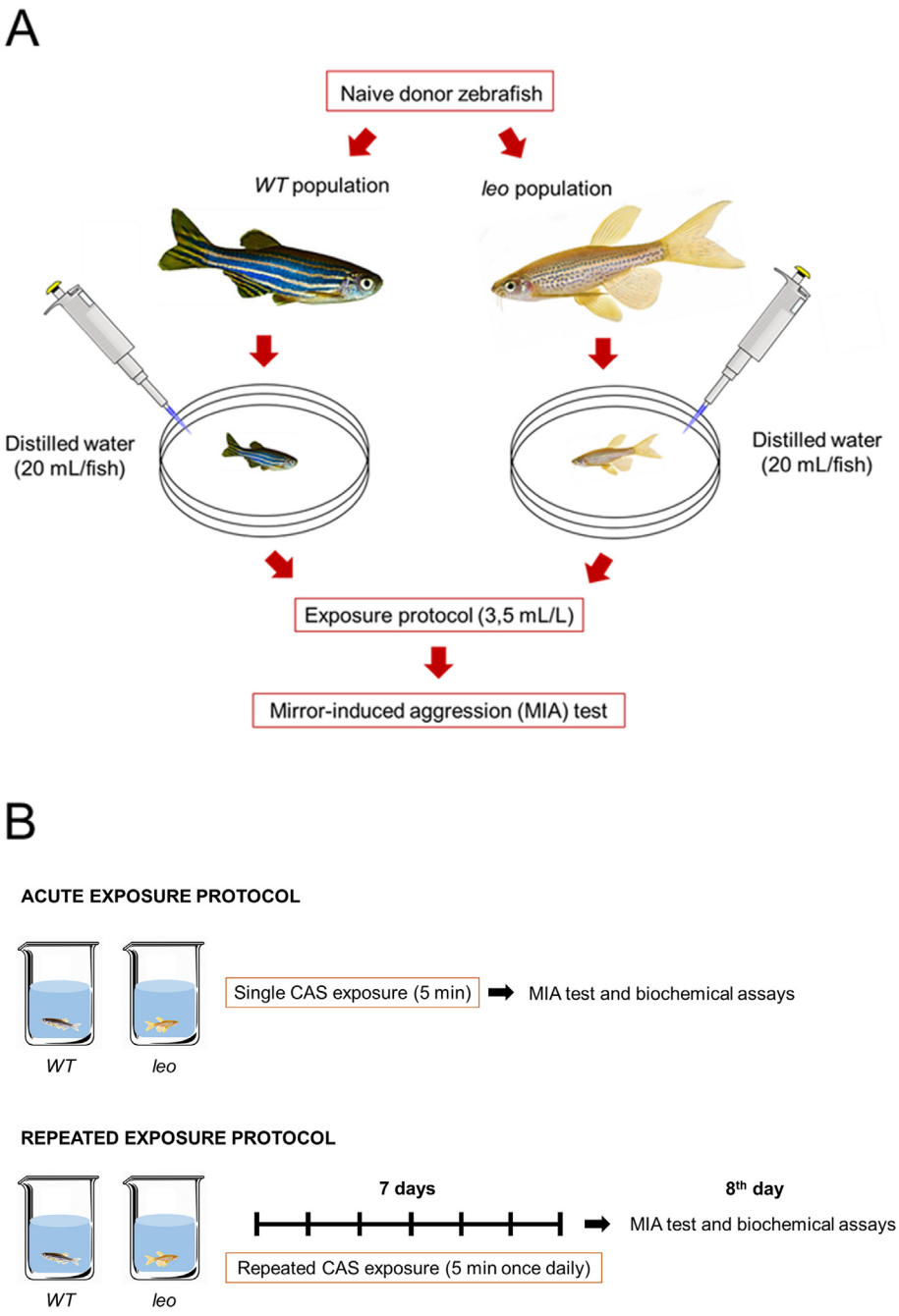

Fig. 1. Schematic representation of the experimental design. (A) CAS extraction from naïve donor zebrafish. The skin extract was prepared on ice and 3.5 mL/L were used. (B) Acute and repeated CAS exposure protocols.

of aggressive episodes, and latency to attack the mirror (aggressive behaviors). Aggressive episodes represent the number of events in which fish attack the opponent image presenting the respective phenotypes: fin erection, accompanied by undulating body movements, fast swimming, and biting towards the mirror in the close area (Fontana et al., 2016; Gerlai et al., 2000; Kalueff et al., 2013). Two trained experimenters blind to the experimental condition (inter-rater reliability > 0.85) coded the aggression towards mirror manually.

2.5. Determination of Z-MAO activity

Monoamine oxidase (Z-MAO) activity was determined as reported previously (Krajl, 1965). After behavioral experiments, zebrafish were anesthetized in water at 4 °C and later euthanized by decapitation. Four zebrafish brains were pooled and homogenized in 1 mL of buffer solution containing 16.8 mM Na_2HPO_4 and 10.6 mM KH_2PO_4, pH 7.4, isotonized with sucrose. Samples were centrifuged at 1.000 ×*g* for 5 min and the supernatants were kept on ice for the experiments. Samples (100–150 µg protein) were mixed with 460 µL of assay buffer (168 mM Na_2HPO_4 and 10.6 mM KH_2PO_4, pH 7.4, isotonized with KCl) and preincubated at 37 °C for 5 min. The reaction started by adding 110 µM kynuramine hydrobromide (Maximino et al., 2013) in a final volume of 700 µL. After 30 min, 10% trichloroacetic acid (300 µL) was added and the tubes were kept on ice. Incubation time and protein concentration were chosenin order to ensure the linearity of the reactions. Samples were further centrifuged at 16.000 ×*g* for 5 min and 800 µL of supernatant was mixed with 1 mL of 1 M NaOH. The fluorescence intensity was measured spectrofluorimetrically (excitation at 315 nm and

emission at 380 nm). The amount of 4-hydroxyquinoline produced was estimated from a corresponding standard fluorescence curve of 4-hydroxyquinoline. Specific activity was expressed as nmol 4-OH quinoline/min/mg protein.

2.6. Protein quantification

Protein was measured at 595 nm using the Coomassie blue reagent and bovine serum albumin as standard (Bradford, 1976). All experiments were performed in duplicate.

2.7. Statistical analyses

Data normality and homogeneity of variances were analyzed using Kolmogorov–Smirnov and Bartlett's tests, respectively. Since all data were normally distributed and homoscedastic, results were expressed as a mean ± standard error of the mean (S.E.M.) and differences between control and CAS groups were determined using unpaired Student's *t*-test. The significance level was set at $p \leq .05$.

3. Results

3.1. Zebrafish populations display differences in basal locomotion, aggression, and brain Z-MAO activity

Basal locomotor activities and aggression-related behaviors significantly differ between *WT* and *leo* (Fig. 2). Overall, *WT* were more active ($t_{(df=46)} = 2.171$, $p = .0176$ for distance traveled and $t_{(df=46)} = 1.803$, $p = .0390$ for absolute turn angle) and more aggressive ($t_{(df=46)} = 2.520$, $p = .0076$ for aggressive episodes and $t_{(df=46)} = 1.882$, $p = .0331$ for aggression/distance ratio) than *leo*. The duration of aggressive behavior and the latency to attack the mirror did not differ between groups ($p > .05$) (data not shown). Moreover, a higher brain *Z*-MAO activity was verified in *leo* population ($t_{(df=10)} = 3.349$, $p = .0037$) (Fig. 3).

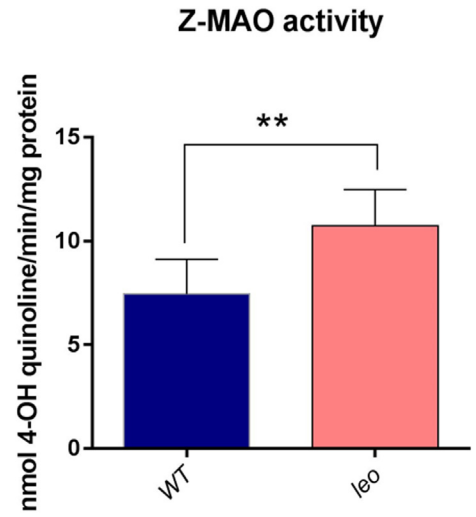

Fig. 3. Basal *Z*-MAO activity in brain tissues of *WT* and *leo*. Data are represented as mean ± S.E.M. and analyzed by unpaired Student's *t*-test. (**$p \leq .01$, $n = 6$ per group).

3.2. Acute CAS exposure increases aggression without changing locomotion

Acute CAS exposure did alter neither the distance traveled nor the absolute turn angle in both *WT* (Fig. 4A) and *leo* (Fig. 4B). Both number and duration of aggressive episodes increased in *WT* ($t_{(df=18)} = 2.159$, $p = .0223$ and $t_{(df=18)} = 2.655$, $p = .0081$ respectively) (Fig. 4C) and in *leo* ($t_{(df=18)} = 3.821$, $p = .0037$; $t_{(df=18)} = 2.327$, $p = .0159$,

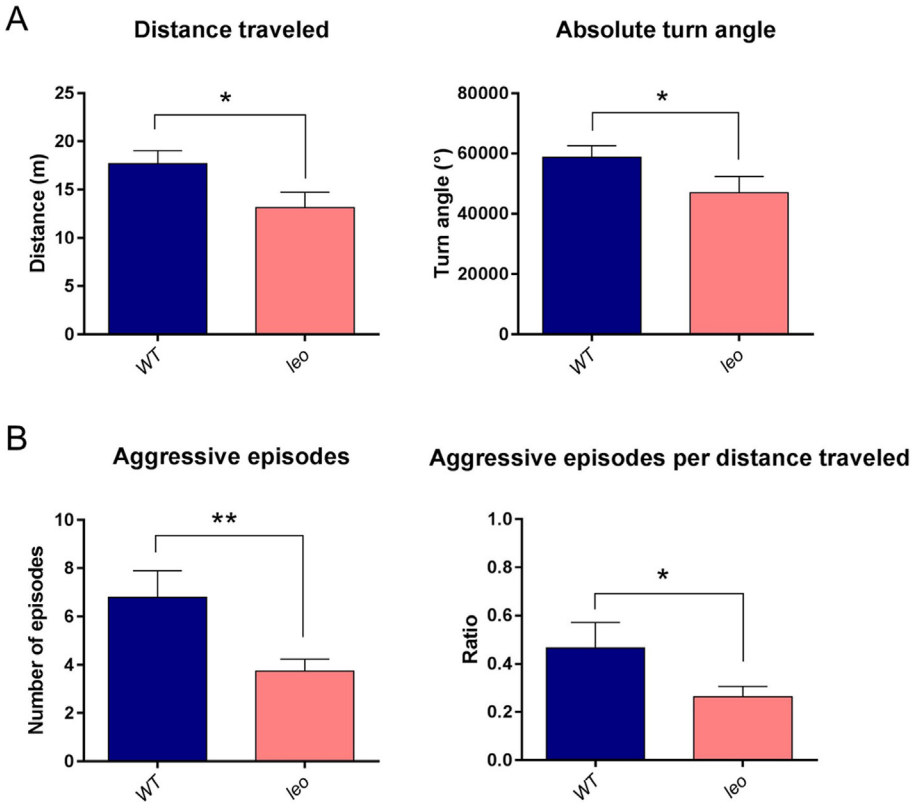

Fig. 2. Behavioral differences of *WT* and *leo* populations in the MIA task. (A) Locomotor parameters. (B) Aggressive behaviors. Data are represented as mean ± S.E.M. and analyzed by unpaired Student's *t*-test. (*$p \leq .05$, **$p \leq .01$, $n = 24$ per group).

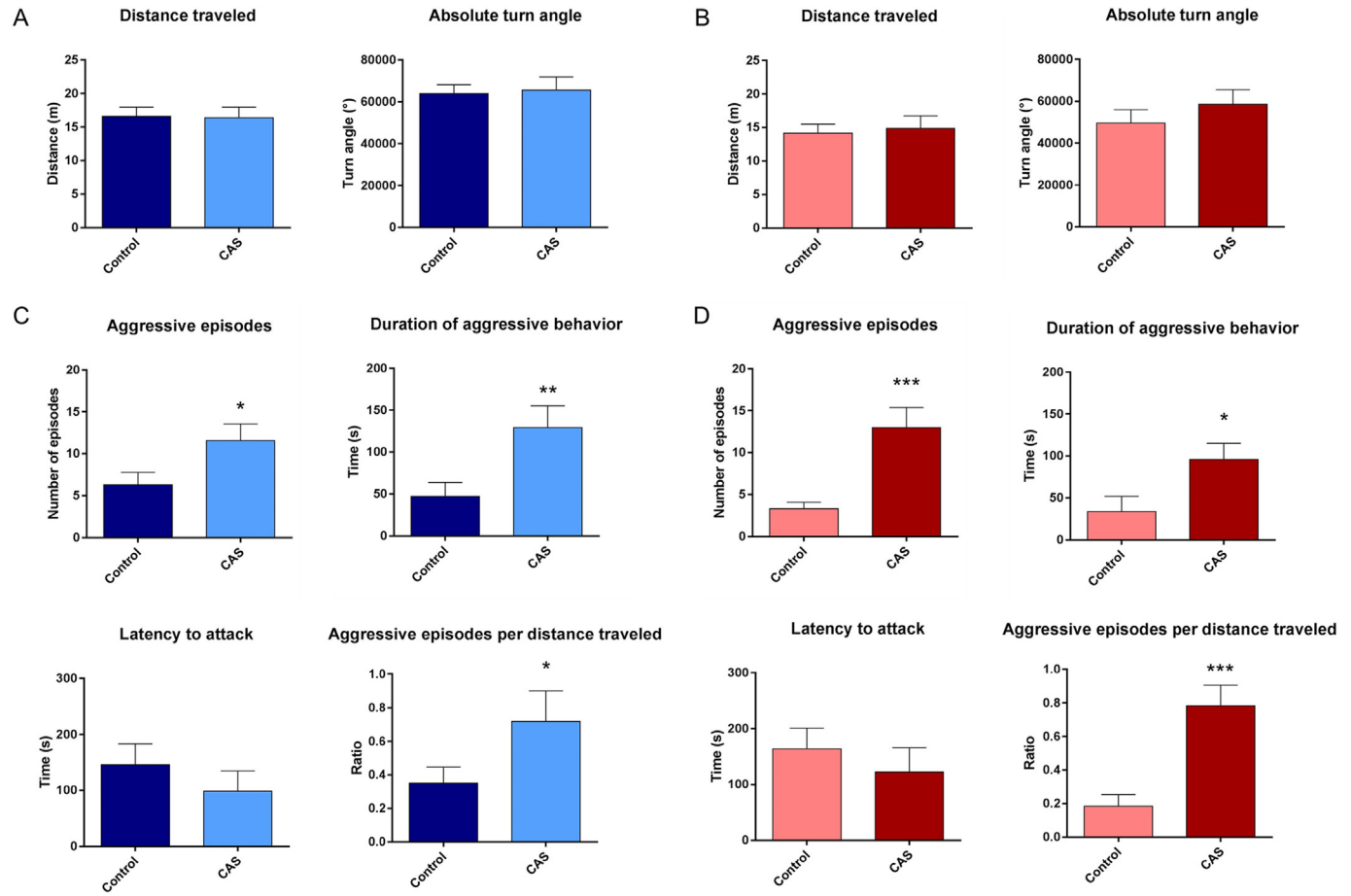

Fig. 4. Acute effects of CAS in *WT* (A and B panels) and *leo* (C and D panels) populations subjected to the MIA test. Animals were exposed to CAS (5 min) and the behavioral activity was further recorded for 6 min. (A) Locomotor parameters in *WT*. (B) Aggressive behaviors in *WT*. (C) Locomotor parameters in *leo*. (D) Aggressive behaviors in *leo*. Data are represented as mean ± S.E.M. and analyzed by unpaired Student's *t*-test. (*$p \leq .05$, **$p \leq .01$, ***$p \leq .005$, $n = 10$ per group).

respectively) (Fig. 4D). Although CAS did not alter the latency to attack the opponent image in both *WT* and *leo* ($p > .05$), the aggression/distance ratio increased in these populations ($t_{(df=18)} = 1.779$, $p = .0460$ for *WT*; $t_{(df=18)} = 3.403$, $p = .0016$ for *leo*) (Fig. 4D).

3.3. Chronic CAS exposure decreases locomotion and reduces aggression

Chronic CAS exposure reduced the distance traveled in *WT* ($t_{(df=26)} = 4.455$, $p = .0001$) (Fig. 5A) and *leo* ($t_{(df=26)} = 2.613$, $p = .0074$) (Fig. 5B). However, only *leo* showed a decrease in the absolute turn angle ($t_{(df=26)} = 3.846$, $p = .0003$) (Fig. 5B). Moreover, CAS reduced the duration of aggressive episodes in *WT* population ($t_{(df=26)} = 2.643$, $p = .0069$) (Fig. 5C). In *leo*, CAS decreased the number ($t_{(df=26)} = 3.274$, $p = .0015$) and duration of aggressive episodes ($t_{(df=26)} = 2.635$, $p = .0070$), as well as increased the latency to attack the mirror ($t_{(df=26)} = 1.802$, $p = .0416$) (Fig. 5D). No changes were observed in the aggression/distance ratio ($p > .05$) (Fig. 5D).

3.4. Acute, but not chronic CAS exposure, decreases Z-MAO activity

Fig. 6 shows the effects of acute and chronic CAS exposure in brain *Z*-MAO activity. Acute exposure reduced the enzyme activity in *WT* ($t_{(df=4)} = 2.402$, $p = .0371$) (Fig. 6A) and *leo* ($t_{(df=4)} = 3.354$, $p = .0142$) (Fig. 6B) when compared to their respective controls. However, chronic CAS exposure did not significantly change brain Z-MAO activity ($p > .05$).

4. Discussion

In this report, we show that CAS modulates aggression and brain *Z*-MAO activity in two zebrafish populations. Baseline endpoints revealed that *WT* were more active and aggressive than *leo*, showing a lower brain *Z*-MAO activity. Although the acute CAS exposure increased aggressive behaviors and decreased *Z*-MAO activity, the chronic exposure reduced locomotion and aggression without affecting brain *Z*-MAO. Moreover, chronic CAS exposure impaired motor patterns and increased the latency to attack the mirror in *leo* population. Since these behavioral phenotypes reinforce distinct acute and chronic effects of CAS on zebrafish populations, we suggest a key role of monoamines in fear-induced aggression.

Alarm cues serve as signals from which individuals identify potential threats in their environment. In fish species, CAS acts as a chemical messenger released when their epithelial cells are damaged, triggering aversive responses (Egan et al., 2009; Pereira et al., 2017; Quadros et al., 2016; Sanches et al., 2015). When a visual predator cue or CAS are present, zebrafish shoals exhibit a stronger social cohesion, as well as increased bottom dwelling and escaping responses (Canzian et al., 2017; Oliveira et al., 2017). Previous data investigated the effects of CAS on physiological responses and even aggression in other species. For example, CAS decreases the swim activity of piauçu fish (*Leporinus macrocephalus*) (Barbosa Júnior et al., 2012), exerts a biphasic locomotion effect in matrinxã (*Brycon cephalus*) (Ide et al., 2003), as well as reduces the aggression of the freshwater cichlid *Pelvicachromis taeniatus* when facing a heterospecific sympatric competitor (Meuthen et al., 2016). Moreover, alarm reactions significantly increased opercular

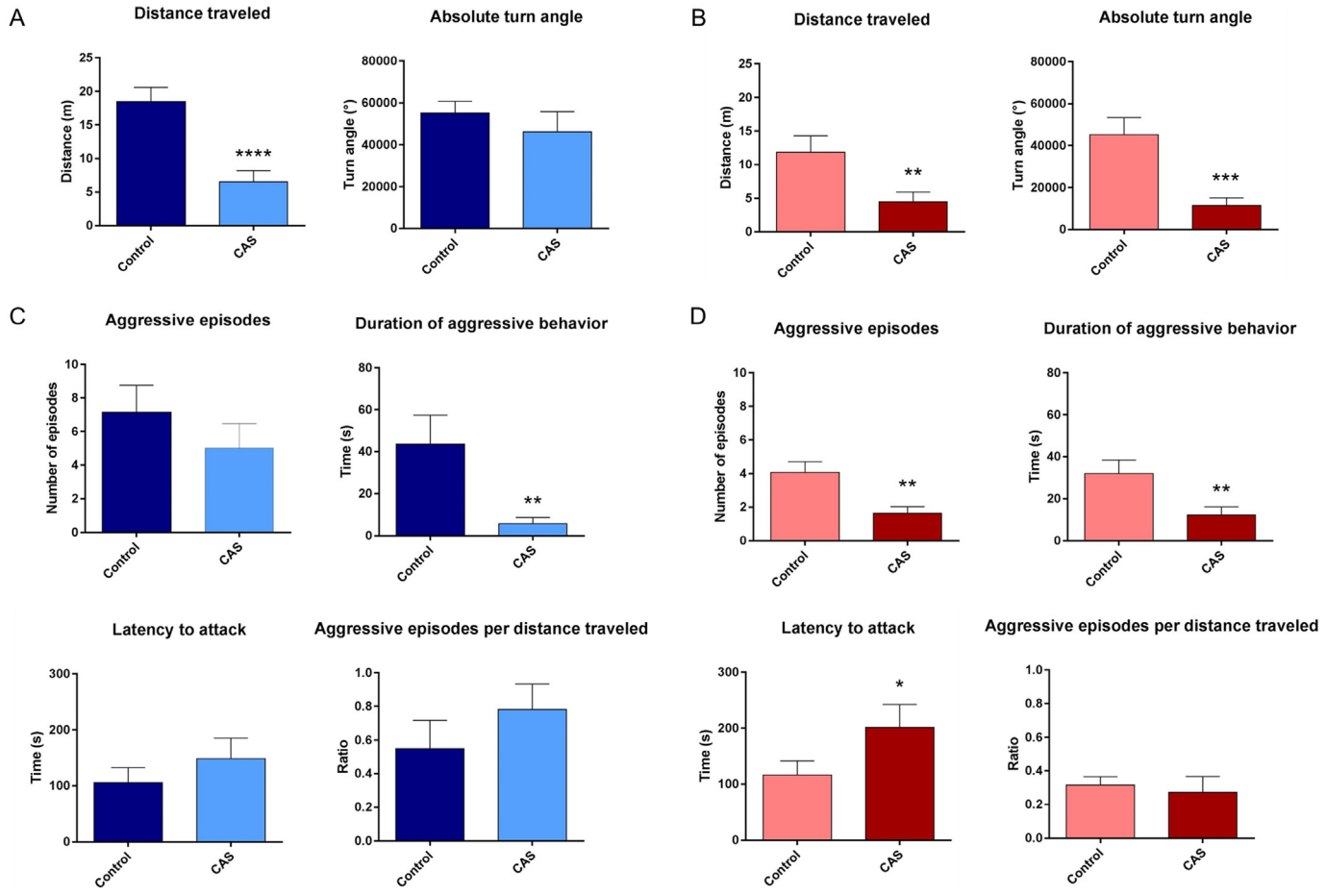

Fig. 5. Effects of repeated CAS exposure in *WT* (A and B panels) and *leo* (C and D panels) populations subjected to the MIA test. Animals were exposed to CAS (5 min) once daily for 7 days and the behavioral activity was recorded for 6 min (day 8). (A) Locomotor parameters in *WT*. (B) Aggressive behaviors in *WT*. (C) Locomotor parameters in *leo*. (D) Aggressive behaviors in *leo*. Data are represented as mean ± S.E.M. and analyzed by unpaired Student's *t*-test. (**$p \leq .01$, ***$p \leq .005$, ****$p \leq .001$, $n = 14$ per group).

movements in rainbow darter (*Etheostoma caeruleum*) and decreased the ventilation rate in piau fish (Barbosa Júnior et al., 2010; Gibson and Mathis, 2006). Since CAS-mediated responses apparently depend on context and species-intrinsic factors, studies aiming to assess how alarm cues modulate behavioral and biochemical parameters in fish species are relevant (Sanches et al., 2015).

Using the MIA test to assess aggressive displays towards mirror (Fontana et al., 2016; Gerlai et al., 2000), we observed significant baselines differences, in which *leo* were less active and aggressive than *WT*, corroborating with a more "anxious" profile (Egan et al., 2009). Acute CAS exposure increased aggressive behavior without changing locomotion, whereas the chronic protocol reduced aggression and locomotor activity of both populations. Despite the behavioral responses observed in the acute protocol seem maladaptive, these data can be explained by some factors. After a single CAS exposure, zebrafish show increased catecholamine and blood glucose levels, as well as an exacerbation of fear-like behaviors (e.g., freezing, hyperventilation, erratic movements, geotaxis, scototaxis) (Maximino et al., 2014). Due to the activation of sympathetic autonomic nervous system and the metabolic changes observed previously, these phenotypes suggest that zebrafish display primary and secondary physiological responses following CAS exposure. CAS also increases habenular *c-fos* expression, which reflects the activation of a brain structure homologous to the mammalian amygdala (Ogawa et al., 2014). Although zebrafish form larger shoals naturally, this species displays agonistic interactions when a single fish is isolated with another conspecific to establish territoriality and dominance hierarchies (Oliveira et al., 2011). Since a virtual opponent appeared in the mirror to a fish individually placed in the tank, a positive effect of CAS on fear-induced aggression is predictable. However, considering the aggressive episodes per distance traveled ratio, the chronic exposure affects locomotor activity culminating in reduced aggression. Since *leo* showed reduced absolute turn angle, decreased aggressive episodes, and increased latency to attack the mirror, a complex relationship of activity, aggression, and impulsivity may drive the MIA test phenotypes in zebrafish populations we measured here. Repeated exposure protocols induce long-term changes in the CNS, which often result in behavioral differences when compared to those observed following a single exposure to a specific stimulus (Grissom and Bhatnagar, 2009). Thus, we suggest that chronic CAS exposure elicits tertiary responses causing deleterious effects, which may culminate in locomotor deficits (Barton, 2002). Similarly, rats exposed repeatedly to a same aversive chemical cue (e.g., cue cat odor stimuli) showed decreased crossings in the open field test, suggesting hypolocomotion (Wright et al., 2013). Consequently, the effects of CAS depend on the time of exposure and affects motor patterns in *leo*, reflecting subtle behavioral differences when compared to *WT*.

Monoamines (e.g., serotonin and dopamine) influence mood and behavior, thereby exerting a modulatory role in aggression (Alia-Klein et al., 2008; Godar et al., 2011; Rosell and Siever, 2015). Thus, MAO activity represents an important mechanism to control the monoamine levels in the CNS (Nikolac Perkovic et al., 2016). Mutations in human MAO-A gene resulting in MAO deficiency have been associated to antisocial behavior and aggression (Godar et al., 2016; Takahashi et al., 2011), while a lower MAO-A activity in individuals facing social

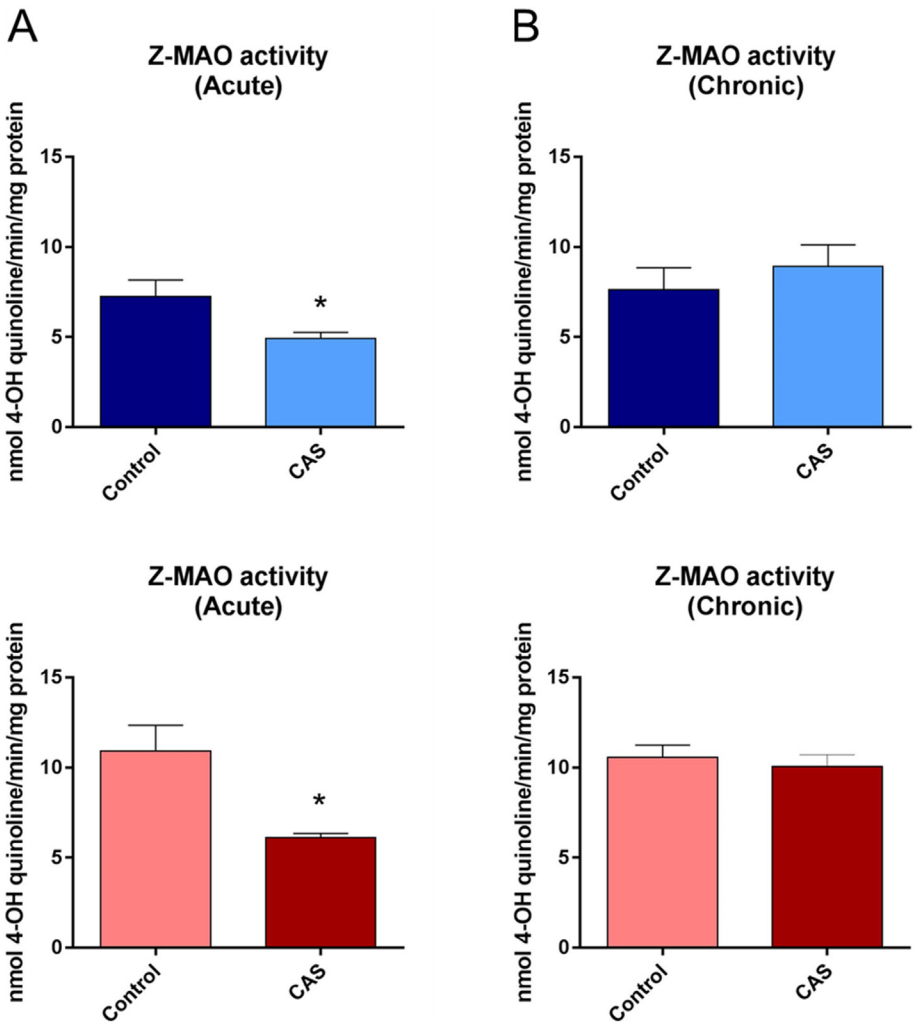

Fig. 6. *Z*-MAO activity in brain tissues of *WT* (A) and *leo* (B) populations following acute and repeated CAS exposure. Data are represented as mean ± S.E.M. and analyzed by unpaired Student's *t*-test. (*$p \leq .05$, **$p \leq .01$, $n = 3$ per group).

exclusion or high provocative situations correlates aggression (Gallardo-Pujol et al., 2013; McDermott et al., 2009). MAO-A knockout mice display aggressive behavior and elevated serotonin, norepinephrine, and dopamine levels in the brain, whereas MAO-B knockout mice do not exhibit aggressive phenotypes (Shih and Chen, 1999). However, studies associate platelet MAO-B activity with psychopathy- and aggression-related personality traits, showing a complex relationship of both isoforms in aggressive behaviors (Nikolac Perkovic et al., 2016). Since zebrafish express a single MAO isoform, this species may be a suitable model organism to investigate the relationship of brain monoamine catabolism and aggression. Decreased *Z*-MAO activity presumably reduces the oxidative metabolism of monoamines, increasing their levels in the brain. As reported previously, *leo* showed increased baseline *Z*-MAO activity, supporting their higher serotonin turnover (Maximino et al., 2013). Although the chronic exposure did not affect brain *Z*-MAO of *WT* and *leo*, CAS acutely decreased *Z*-MAO activity, which may contribute to the increased serotonin levels in the brain following a single exposure (Maximino et al., 2014). Thus, we suggest that monoamines play a role, at least partially, in CAS-mediated aggression. Nonetheless, future studies are needed to clarify the role of *Z*-MAO activity in zebrafish behavioral neurophenotypes.

5. Conclusion

In summary, our novel findings show that CAS acutely elicits fear-induced aggression and decreases brain *Z*-MAO activity in *WT* and *leo* zebrafish populations. However, the reduced aggressive behavior could be associated to a decreased locomotion following the chronic exposure, which did not change *Z*-MAO activity. Collectively, these results highlight the potential use of zebrafish populations to assess the neurobiological bases of fear-induced aggression in vertebrates.

Competing interests

The authors declare that no competing interests exist.

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7.2. ARTIGO CIENTIFÍCO 2

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Involvement of anxiety-like behaviors and brain oxidative stress in the chronic effects of alarm reaction in zebrafish populations

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ABSTRACT

Aversive conditions elicit anxiety responses that prepare the organism to an eventual threat. Nonetheless, prolonged anxiety is a pathological condition associated with various neuropsychiatric disorders. Here, we evaluated whether the conspecific alarm substance (CAS), a chemical cue that elicits aversion, influences anxiety-like behaviors and modulates brain oxidative stress-related parameters in *wild-type* (*WT*) and *leopard* (*leo*) zebrafish following a repeated exposure protocol. CAS exposure was performed for 5 min, once daily for 7 consecutive days. In the 8th day, animals were tested in the light/dark and novel tank tests and their brains were further dissected for biochemical analyses. CAS chronically induced anxiogenic-like states in *WT* and *leo* populations when their behaviors were analyzed in the light/dark and novel tank tests. CAS also increased catalase (CAT) and glutathione S-transferase (GST) activities, as well as non-protein thiol (NPSH) content in *WT* and *leo*, but only *leo* had increased thiobarbituric reactive substance (TBARS) levels in the brain. At baseline conditions, *leo* was more 'anxious' when compared to *WT*, displaying lower CAT activity and carbonylated protein (CP) levels. Overall, CAS chronically triggers anxiety-like behavior in zebrafish populations, which may be associated with changes in oxidative stress-related parameters. Furthermore, the use of different zebrafish populations may serve as an interesting tool in future research aiming to investigate the neurobehavioral bases of neuropsychiatric disorders in vertebrates.

1. Introduction

Anxiety- trauma- and stressor-related disorders constitute serious public health problems worldwide (Kessler et al., 2005; Riaza Bermudo-Soriano et al., 2012). Patients with such disorders may present exacerbated fear and/or anxiety, which differ according to the proximity of the threat (Baldwin et al., 2010, 2005; Smaga et al., 2015). Thus, measuring fear/anxiety-like behaviors in animal models represents an interesting strategy to understand how aversive conditions affect defensive behaviors and help elucidate the mechanisms underlying various neuropsychiatric disorders (Atmaca et al., 2008; Kalueff et al., 2007; Kuloglu et al., 2002b, 2002a; Maximino et al., 2010b; Ozdemir et al., 2009).

Although reactive oxygen species (ROS) are produced during aerobic metabolism, their excessive formation associated with reduced antioxidant defenses culminates in oxidative stress (Moniczewski et al., 2015; Rocha et al., 2003). Oxidative damage affects cellular structures, neurotransmission, as well as transduction signaling pathways (Halliwell, 2006; Hovatta et al., 2010), and correlates with various neuropsychiatric conditions (Rammal et al., 2008; Smaga et al., 2015). Cellular antioxidant defenses, such as glutathione, thioredoxin, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase activities modulate ROS levels, thereby maintaining redox status (Hagedorn et al., 2012). Mounting evidence shows that SOD and CAT activities in the blood are stimulated in patients with social phobia (Atmaca et al., 2008), panic disorder (Kuloglu et al., 2002b), and

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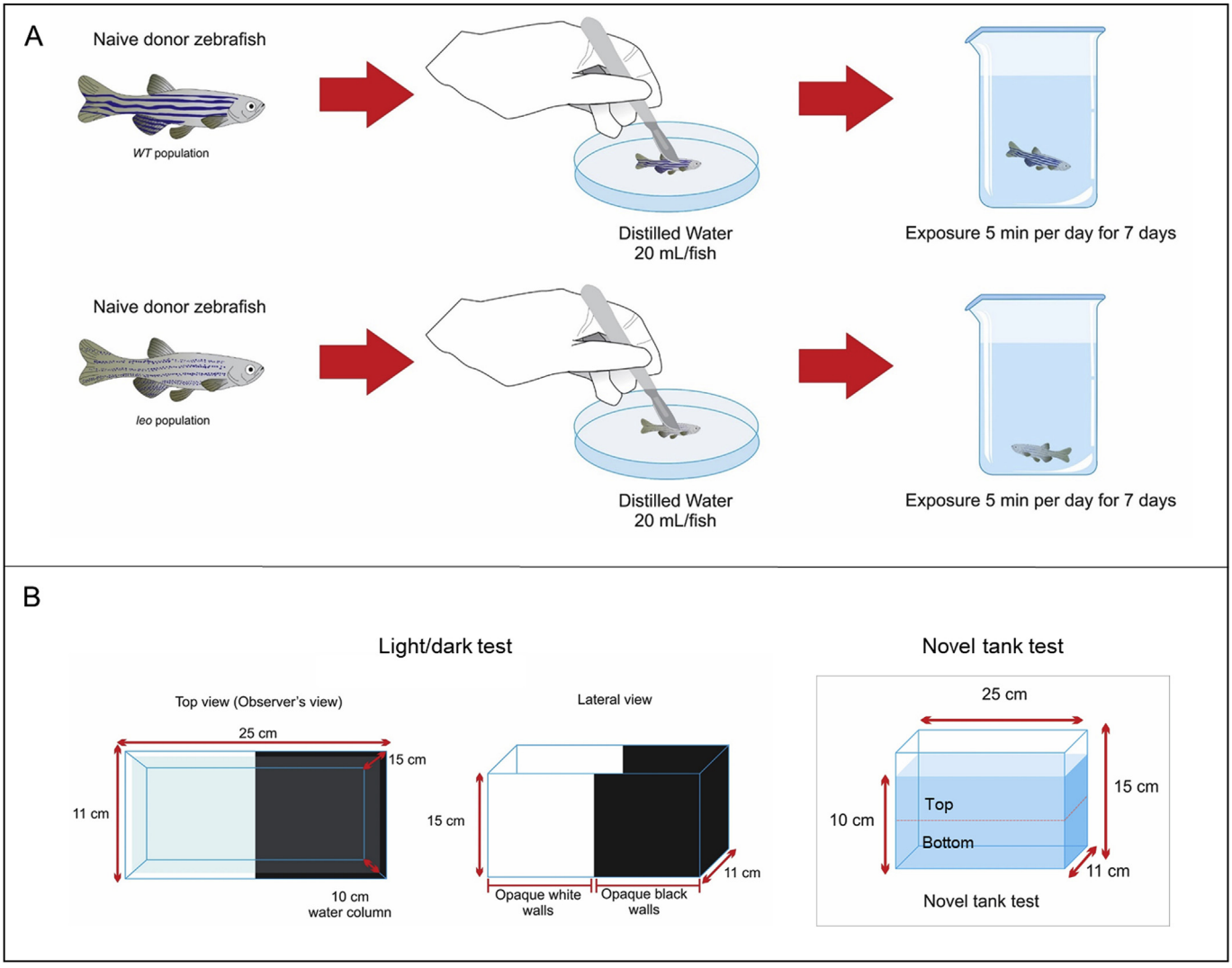

Fig. 1. Schematic representation of experimental procedures and apparatus. (A) CAS extraction from naïve *WT* and *leo* donors and exposure protocol. The skin extract was prepared on ice and 3.5 mL/L were used for the experiments. (B) Experimental apparatuses. The light/dark tank consisted of a glass apparatus divided into two equally sized dark and lit areas. The compartments were delimited by opaque plastic self-adhesive films in black or white colors externally covering walls and floor. The novel tank was divided into two areas (top and bottom) to assess vertical exploration.

transient-compulsive disorder (Kuloglu et al., 2002b, 2002a), suggesting a close relationship between neuropsychiatric diseases and oxidative stress. Therefore, measuring behavioral phenotypes and brain oxidant status in animal models help investigate evolutionarily conserved mechanisms involved in the pathophysiology of anxiety- trauma- and stressor-related disorders.

The zebrafish (*Danio rerio*) is a well-established model organism in biochemical and neurobehavioral research (Fontana et al., 2018; Kalueff et al., 2014; Rosemberg et al., 2012; Stewart et al., 2014). The high degree of genetic conservation when compared to the human genes (~70%) (Howe et al., 2013) and the well-characterized behaviors (Kalueff et al., 2013) provide relevant insights into the genetic bases involved in anxiety-related disorders (Egan et al., 2009; Shin and Fishman, 2002). Moreover, behavioral endpoints that reflect anxiety-like responses have been described in both *wild-type* (*WT*) and *leopard* (*leo*) populations (Egan et al., 2009; Maximino et al., 2013; Quadros et al., 2018, 2016). As compared to *WT*, *leo* spends more time in the bottom area of the tank, displays increased shoaling, and prominent scototaxis, suggesting a more 'anxious' behavior (Canzian et al., 2017; Egan et al., 2009; Maximino et al., 2013). Both *WT* and *leo* present different responses when exposed to the conspecific alarm substance (CAS), a chemical cue that elicits defensive behaviors (Quadros et al., 2018, 2016). CAS increases habenular *c-fos* expression and promotes sympathetic activation, which stimulates adrenaline release and increases blood glucose levels (Maximino et al., 2014; Ogawa et al., 2014). In zebrafish, CAS exposure evokes freezing, erratic movements, escaping behavior (Maximino et al., 2018a) and stimulates aggressive behavior following a single exposure, suggesting activation of 'fight-or-flight' responses (Quadros et al., 2018). Conversely, repeated CAS promotes long-term changes in the CNS by inducing depressant-like behaviors without changing aggression (Quadros et al., 2018). When animals face aversive conditions, biochemical and physiological alterations occur to maintain homeostasis (Chrousos, 2009; Miller and Raison, 2016; Mocelin et al., 2018). However, maladaptive responses following prolonged aversive situations may play a role in anxiety-related disorders and correlated neurochemical mechanisms (Hassan et al., 2016; Hovatta et al., 2010; Mocelin et al., 2015). Therefore, we sought to investigate whether repeated CAS exposure (7 days) modulates defensive behaviors in *WT* and *leo*, as well as to explore the putative involvement of oxidative stress in CAS-mediated responses.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) of the short fin *wild type* (*WT*) and *leopard* (*leo*) populations were obtained from a local supplier (Hobby Aquários, RS, Brazil). Animals were 4–6 months-old and a 50:50 (male:female) proportion was used for the experiments. Because the genetic bases were not fully elucidated, we used of the term "population" to define both zebrafish phenotypes tested here. Animals were separated by their respective phenotypes at a maximum density of four fish per liter. Fish were allocated in 40 L tanks filled with non-chlorinated water and maintained under mechanical, biological, and chemical filtration (temperature and pH set at 27 ± 1 °C and 7.2 ± 0.1, respectively) for 2 weeks before the experiments to acclimate to laboratory conditions. Illumination was provided by fluorescent lamps under a photoperiod cycle (14h/10h, light/dark respectively). All animals used were fed thrice daily with commercial flake fish food (Alcon BASIC™, Alcon, Brazil). The experiments fully adhered to the National Institute of Health Guide for Care and Use of Laboratory. All protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (106/2014).

2.2. Conspecific alarm substance extraction and exposure protocol

CAS was extracted from donor fish previously euthanized, as described elsewhere (Egan et al., 2009; Quadros et al., 2016; Maximino et al., 2018b), by damaging the epithelial cells with a razor blade (10–15 shallow slices on each side of the body). Fish were then washed with 20 mL distilled water in a Petri dish kept on ice, avoiding blood contamination into solution. For chronic CAS exposure, zebrafish were exposed individually to 1.75 mL skin extract preparation into 500 mL tanks for 5 min once daily during 7 consecutive days and tested on the 8th day (Quadros et al., 2018) (Fig. 1A). *WT* and *leo* populations were exposed to CAS obtained from their phenotypically similar donor fish. Control groups were handled in a similar manner, except that only distilled water was added to the tank.

2.3. Behavioral measurements

All behavioral tests were performed between 09:00 a.m. and 4:00 p.m. Behavioral activities were registered in a single 6 min session and the apparatus was positioned on a stable surface. Throughout the experiments, animals were handled carefully to minimize stress and the water was changed after each trial. Behaviors were recorded using a webcam connected to a laptop at 30 frames/s, and subsequently analyzed using appropriate video-tracking system (ANY-maze™, Stoelting CO, USA). All tests were performed using two independent batches.

2.4. Behavioral tests

2.4.1. Light/dark test

Anxiety-like behaviors were determined using the light/dark test as described previously (Maximino et al., 2010a, 2010c; Quadros et al., 2016). The apparatus consisted of a rectangular tank (15 × 10 × 25 cm, height, depth and length respectively), divided in two equally-sized dark and lit areas (Fig. 1B). The compartments were covered externally by opaque black or white self-adhesive films in the walls and floor of the apparatus. Experimental tanks were filled with 2 L non-chlorinated water and animals (n = 12–14 per group) were placed individually in the lit area of the apparatus before starting the test. Behaviors were analyzed for 6 min and the following endpoints were measured: time spent in lit area, number of transitions to the lit area, latency to enter the dark area, and number of risk assessments. Risk assessment episodes were computed manually by two trained observers (inter-rater reliability > 0.85) blinded to the experimental condition. This behavior was counted when fish approximate the lit area without crossing the pectoral fin, associated with a fast return to the dark compartment (Kalueff et al., 2013; Maximino et al., 2014).

2.4.2. Novel tank test

Locomotor and vertical exploratory activity of fish, which reflects habituation of novelty stress, were analyzed in rectangular tanks (25 cm × 15 cm × 11 cm length, height and width respectively), filled with 1.5 L of non-chlorinated water and divided into two equal horizontal areas (bottom and top) (Egan et al., 2009; Rosemberg et al., 2011, 2012) (Fig. 1B). Animals were individually placed in the test tanks and their behaviors further analyzed for 6 min. We measured the distance traveled and absolute turn angle (locomotor-related endpoints), time spent in top area, transitions to top area, and the latency to enter the top (vertical activity parameters). Both erratic movements and freezing (aversive behavioral responses) were computed manually by trained observers (inter-rater reliability > 0.85) blinded to the experimental condition. Freezing was defined as complete immobility associated to increased opercular beat rate for at least 2 s, while erratic movements were considered sharp changes in direction or velocity and repeated rapid darting behavior (Kalueff et al., 2013).

2.5. Biochemical parameters

2.5.1. Tissue preparation

After CAS exposure, fish were previously anesthetized in water at 4 °C and subsequently euthanized by decapitation. Brains were dissected on ice and transferred to microtubes for storage at −80 °C. For each sample, two brains were pooled, homogenized in 500 μL of 50 mM Tris-HCl buffer, pH 7.4, and posteriorly centrifuged at 3000 × g for 10 min at 4 °C. Supernatants were removed for biochemical assays. All tests were run in duplicate.

2.5.2. Antioxidant enzymatic defenses

Superoxide dismutase (SOD) activity was assessed based on the inhibition of superoxide radical reaction with adrenaline (Misra and Fridovich, 1972). Samples (20–30 μg protein) were incubated with 50 mM glycine-NaOH buffer, pH 10.0, in the presence of 1 mM adrenaline. The rate of adenochrome formation was measured on a microplate reader and data were expressed as U SOD/milligram protein.

Catalase (CAT) activity was evaluated based on the rate of H_2O_2 decrease at 240 nm ultraviolet spectrophotometry (Aebi, 1984). Samples (0.01 mL, 20–30 μg protein) were mixed with 1 mL of 50 mM potassium phosphate buffer, pH 7.0 and 0.05 mL H_2O_2 (0.3 M) was used as substrate. Results were expressed as micromole/min/milligram protein.

Glutathione S-transferase (GST) activity was measured using 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in ethanol, 10 mM reduced glutathione, 20 mM potassium phosphate buffer, pH 6.5, and 20 μL of samples (40–60 μg protein) (Müller et al., 2017; Nunes et al., 2017). Enzyme activity was determined by variations in absorbance at 340 nm using the molar extinction coefficient of 9.6 mM/cm (Habig et al., 1974). GST activity was measured according to the amount of enzyme required to catalyze 1 mol CDNB conjugate with GSH/min at 25 °C. Results were expressed in nanomole GS-DNB/min/milligram protein.

2.5.3. Non-protein thiol content quantification

Non-protein thiol (NPSH) levels were determined as described previously (Ellman, 1959). Briefly, 100 μL of sample (60–80 μg of protein) was mixed in 100 μL of TCA 10% and then centrifuged at 3000 × g for 10 min. Later, 30 μL of the supernatant was mixed with 10 μL of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) (0.01 M) dissolved in ethanol. The intense of yellow color developed was measured at 412 nm after 30 min in a microplate reader. Results were expressed as nanomole NPSH/milligram protein.

2.5.4. Measurement of lipid peroxidation and protein carbonylation

Lipid peroxides were estimated by thiobarbituric reactive substance (TBARS) production (Draper and Hadley, 1990). Briefly, 160 µL of 10% Trichloroacetic acid (TCA) was added in 80 µL of samples (80–100 µg protein) and subsequently centrifuged at 10,000 × *g* for 10 min. Later, 100 µL of the supernatant was added to an equal volume of 0.67% thiobarbituric acid (TBA, 4,6-dihydroxypyrimidine-2-thiol) and heated for 30 min at 100 °C. TBARS levels were determined using malondialdehyde (MDA) as standard in a microplate reader at 532 nm. Data were expressed as nanomole MDA/milligram protein.

Carbonylated protein (CP) levels were measured based on the protocol described elsewhere (Yan et al., 1995). Initially, 200 µL of protein was added to 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N hydrochloric acid and incubated for 1 h in dark. Later, 0.15 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8 containing SDS 3.0%), 0.5 mL of heptane (99.5%) and 0.5 mL of ethanol (99.8%) were added, mixed for 40 s, and centrifuged for 15 min at 3000 × *g*. Proteins were isolated, suspended twice in ethanol/ethyl acetate (1:1) and mixed with 0.25 mL of denaturing buffer. Data were measured colorimetrically at 370 nm and calculated using the molar extinction coefficient of 22,000 M/cm. Protein carbonylation was expressed as nanomole carbonylated proteins/milligram protein.

2.6. Protein quantification

Protein was determined using the Coomassie blue method and bovine serum albumin was used as standard (Bradford, 1976). Samples were run in duplicate and the absorbance was measured at 595 nm.

2.7. Statistical analyses

Normality of data and homogeneity of variances were analyzed using Kolmogorov–Smirnov and Bartlett's tests, respectively. Results were expressed as means ± standard error of mean (S.E.M) and analyzed by two-way analysis of variance (ANOVA) (considering population and treatment as factors), followed by Student–Newman–Keuls multiple comparison test whenever necessary. Differences among groups were set at $p \leq 0.05$ level.

3. Results

3.1. Repeated CAS exposure increases anxiety-like behaviors in WT and leo populations

In the light/dark test (Fig. 2), two-way ANOVA yielded significant effects of population and treatment for the time spent in lit area ($F_{1,48} = 10.35$, $p = 0.0023$ and $F_{1.48} = 57.51$, $p < 0.0001$ respectively), transitions to the lit area ($F_{1,48} = 5.773$, $p = 0.0202$ and $F_{1.48} = 61.55$, $p < 0.0001$ respectively), and latency to enter the dark area ($F_{1,48} = 6.485$, $p = 0.0141$ and $F_{1.48} = 28.01$, $p < 0.0001$ respectively). Repeated CAS exposure reduced the time spent in the lit area and the number of transitions to the respective compartment in both *WT* and *leo*. At the baseline, *WT* population spent more time in the lit area, showing fewer transitions to the lit compartment and higher latency to enter the dark area than *leo*. Furthermore, *leo* displayed more risk assessment episodes than *WT*, but CAS only increased such behavior in *WT* population ($F_{1,48} = 15.53$, $p = 0.0003$ for the interaction term).

In the novel tank test (Fig. 3), although *leo* spent more time in bottom than *WT* at the baseline, CAS reduced the time spent in top for both populations ($F_{1,25} = 21.47$, $p < 0.0001$ for the interaction term). A significant population and treatment interaction for the transitions to top area was observed ($F_{1,25} = 7.321$, $p = 0.0121$), in which only *WT* showed decreased vertical activity following repeated CAS exposure. Moreover, CAS increased the latency to enter the top area in both populations ($F_{1,25} = 55.92$, $p < 0.0001$ for the treatment term). Two-way ANOVA revealed significant effects of population, in which *leo* showed more erratic movements than *WT* at the baseline ($F_{1,25} = 5.737$, $p = 0.0244$). No differences were observed in distance traveled, absolute turn angle, and freezing episodes among groups (data not shown).

3.2. CAS chronically alters antioxidant parameters and lipid peroxidation

Fig. 4 shows the effects of repeated CAS exposure on antioxidant responses and oxidative stress biomarkers. Although SOD activity did not differ among groups (Fig. 4A), significant effects of population ($F_{1,18} = 8.514$, $p = 0.0092$) and treatment ($F_{1,18} = 6.488$, $p = 0.0202$) were observed for CAT activity. CAS increased CAT activity only in *leo*, which showed lower enzyme activity than *WT* at the baseline (Fig. 4A). For GST activity (Fig. 4A) and NPSH levels (Fig. 4B), we verified significant effects of treatment ($F_{1,12} = 33.11$, $p < 0.0001$ and $F_{1,20} = 20.47$, $p = 0.0002$ respectively), where both populations showed a markedly increase in these antioxidant responses following CAS exposure. Fig. 4C shows the effects of CAS on lipid peroxidation and protein carbonylation. We observed higher TBARS levels in CAS-exposed *leo* ($F_{1,12} = 10.5$, $p = 0.0071$ for the interaction term). Although CAS did not induce protein carbonylation, CP levels were lower in *leo* than *WT* at the baseline ($F_{1,15} = 4.703$, $p = 0.0466$ for the population term).

4. Discussion

In this study, our novel data showed that repeated CAS exposure induces anxiogenic-like behaviors and modulates oxidative stress parameters in *WT* and *leo* populations. Basically, in the light/dark test, CAS reduced the frequency of entries in the lit area, but decreased the latency to enter the dark area and increased risk assessments only in *WT*. In the novel tank test, CAS decreased the time spent in top area and increased the latency to enter the top, while only *WT* population showed reduced transitions to top area after CAS exposure. Although CAS increased CAT and GST activities, as well as NPSH levels, in both populations, only *leo* showed pronounced lipid peroxidation following the exposure period. At baseline conditions, *leo* displayed a more 'anxious' phenotype, with reduced CAT activity and CP levels in the brain. Overall, we suggest that repeated CAS exposure induces anxiogenic-like behaviors in zebrafish, accompanied by changes in oxidant status in the brain.

In ostariophysans, CAS plays a key role as a chemical cue of predation risk (Maximino et al., 2018a; Speedie and Gerlai, 2008). This substance is released when epithelial cells are ruptured, triggering robust alert responses when fish face a potential threat (Egan et al., 2009; Quadros et al., 2016; Sanches et al., 2015; Speedie and Gerlai, 2008). CAS elicits anxiogenic-like behaviors since it markedly increases social cohesion, geotaxis, and *c-fos* expression in habenula (Canzian et al., 2017; Ogawa et al., 2014; Oliveira et al., 2017; Quadros et al., 2016). Moreover, CAS increases blood glucose, ephinephrine, and serotonin levels in the brain, suggesting activation of sympathetic nervous system (Maximino et al., 2014). We have previously showed that CAS acutely increases aggression in *WT* and *leo*, but induces depressant-like phenotypes following a repeated exposure protocol (Quadros et al., 2018). Here, we hypothesized that CAS would chronically induce anxiogenic-like effects causing an imbalance of oxidant status in the brain, in such a way that differences in zebrafish populations could be predicted using two behavioral tasks with a good correlation and similar sensitivity to zebrafish anxiety-like states *in vivo* (Kysil et al., 2017). Our data confirmed that *leo* has a more 'anxious' behavior than *WT* and, after repeated exposure, both populations showed increased scototaxis and reduced vertical activity in the light/dark and novel tank tests, respectively. However, only *WT* had decreased latency to enter the dark area and increased risk assessment episodes in the light/dark test, as well as showed fewer transitions to top area in the novel tank test, reinforcing a stronger sensitivity of the respective population following

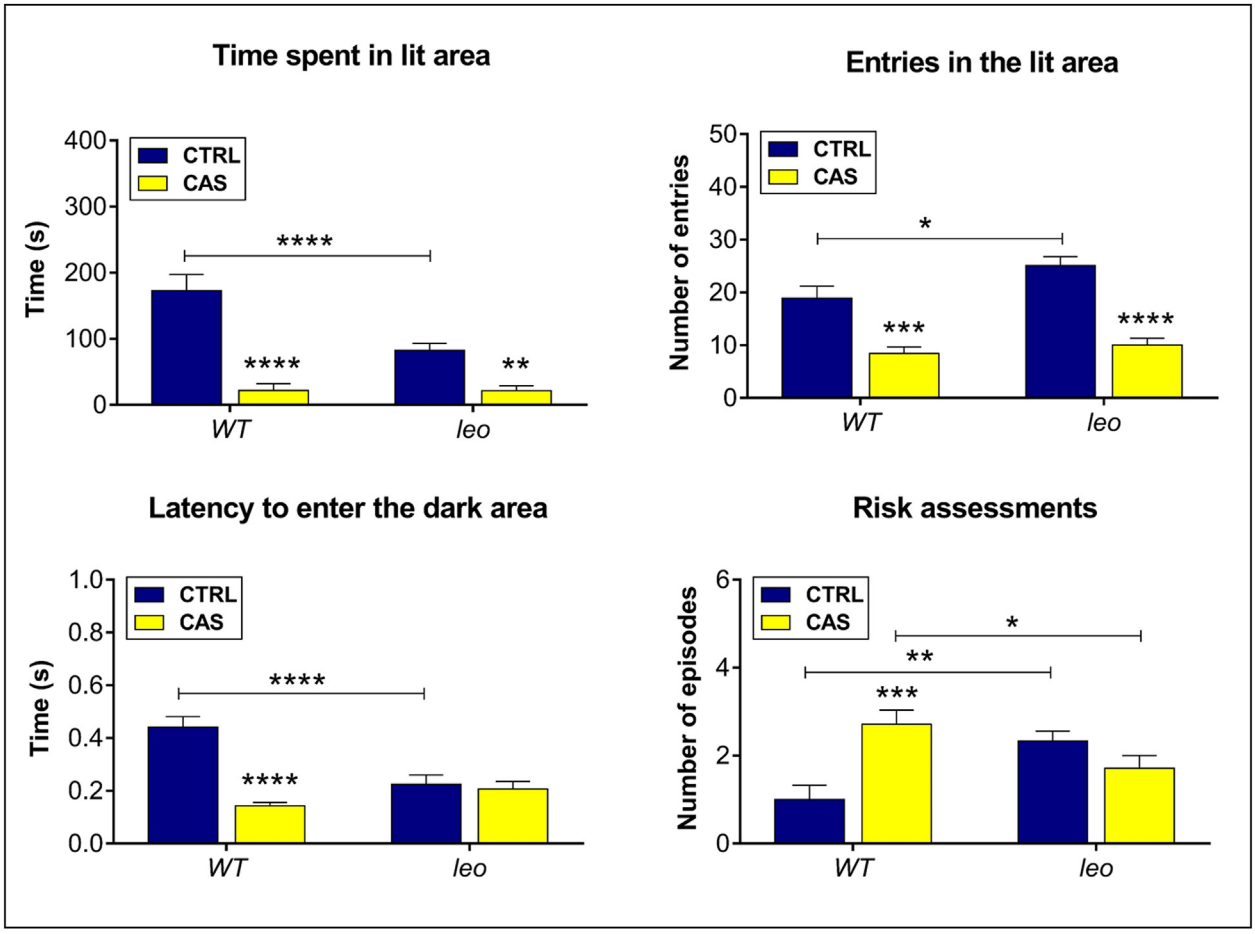

Fig. 2. Repeated CAS exposure induces anxiety-like states in *WT* and *leo* populations in the light/dark test. Animals were exposed to CAS (5 min daily for 7 consecutive days) and their behavioral activities were further recorded for 6 min in the 8th day. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA followed by Student–Neuman–Keuls multiple test whenever appropriate (*$p < 0.01$; **$p < 0.01$; ***$p < 0.005$, ****$p < 0.001$; $n = 12–14$ per group).

chronic CAS exposure. Although one could attribute the absence of some effects in *leo* to a floor effect, anxiogenic-like states in *WT* and *leo* were observed in both light/dark and novel tank tests using the protocol described here. Importantly, a single CAS exposure increases shoaling in *WT*, but not in *leo* (Canzian et al., 2017), while caffeine, another anxiogenic substance, elicits anxiety-like behaviors in *leo* by increasing bottom-dwelling and whole-body cortisol levels depending on the concentration tested (Rosa et al., 2018). These set of data reinforce the utility of zebrafish populations when different behavioral domains are assessed. Although we did not perform a relationship between genes and behavior, our results suggest that genetic variations between populations may contribute, at least partially, to the behavioral differences observed when aversive stimuli are presented.

Aversive situations trigger an innate response that activates transduction signaling pathways aiming to restore homeostasis (Chrousos and Gold, 1992; McEwen, 2007). Thus, the maintenance of redox status is extremely important for brain physiology since the imbalance between oxidants and antioxidant defenses culminates in oxidative stress (Langley and Ratan, 2004; Zhang and Yao, 2013). Prolonged stressful conditions are maladaptive, culminating in neurochemical impairments that affect behavioral responses. For example, increased ROS may damage various biomolecules (*e.g.*, lipids, proteins, and DNA), thereby affecting cellular viability, which are often associated with neurodegenerative diseases (Behl and Moosmann, 2002; Gutowicz, 2011; Niedzielska et al., 2016), and anxiety-related disorders (Guney et al., 2014; Hovatta et al., 2010; Niedzielska et al., 2016).

To investigate whether changes in redox status could influence CAS-induced behaviors, we measured oxidative stress-related parameters in the brain following a 7-day exposure protocol. Physiologically, SOD and CAT play a synergic role to neutralize superoxide anion and hydrogen peroxide, respectively (Gutteridge, 1984; Halliwell, 2006), while GST catalyzes the conjugation of the reduced glutathione (GSH) to xenobiotic substrates for the purpose of detoxification (Dringen et al., 2000; Pisoschi and Pop, 2015). Although the increased NPSH levels, CAT, and GST activities in CAS-exposed *leo* suggest compensatory non-enzymatic and enzymatic mechanisms aiming to regulate homeostasis, these effects do not protect against the lipid peroxidation in this population. Although further experiments are needed to understand how anxiety-like behaviors correlate with oxidant processes following chronic CAS exposure, the differences in enzymatic antioxidant defenses at baseline level observed here could reflect a higher susceptibility of oxidative damage in *leo* and/or genetic variability between populations.

5. Conclusion

In conclusion, our novel data show that repeated CAS exposure induces anxiety-like behaviors and modulates oxidative stress-related biomarkers in zebrafish. Some behavioral and biochemical parameters were substantially different in *WT* and *leo*, both at baseline and after CAS exposure, suggesting population-dependent responses. Furthermore, the anxious behavior following stressful situations could be related to changes in oxidant status in the brain, since we demonstrated a modulatory effect of CAS on oxidative stress-related endpoints. Although additional empirical data are necessary to elucidate the mechanisms underlying alarm responses in zebrafish, we reinforce the growing utility of this aquatic species in modeling anxiety-related

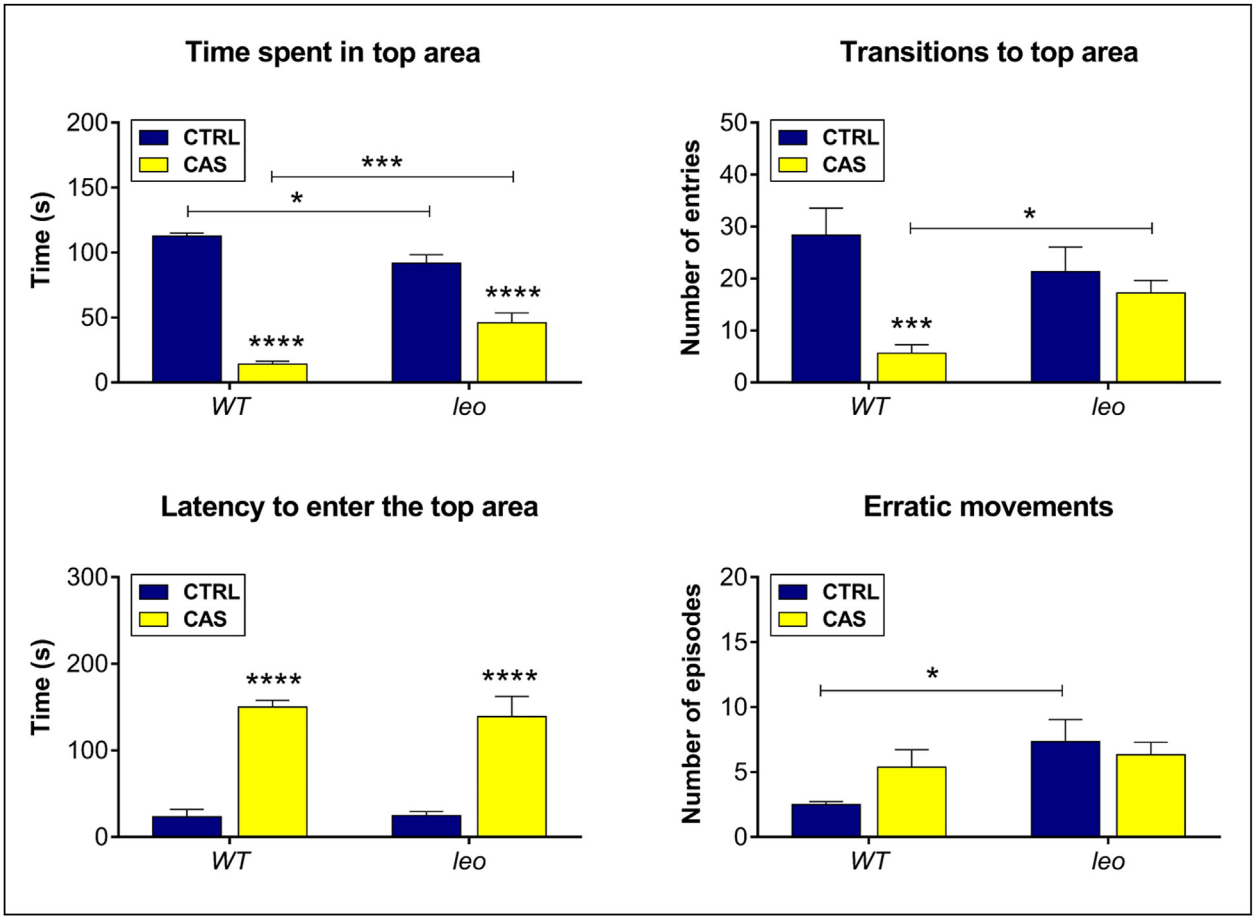

Fig. 3. Repeated CAS exposure modulates exploratory activities of *WT* and *leo* measured in the novel tank test. Animals were exposed to CAS (5 min daily for 7 consecutive days) and their behavioral activities were further recorded for 6 min in the 8th day. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA followed by Student–Neuman–Keuls multiple test whenever appropriate (*$p < 0.05$; ***$p < 0.005$, ****$p < 0.001$; n = 7–9 per group).

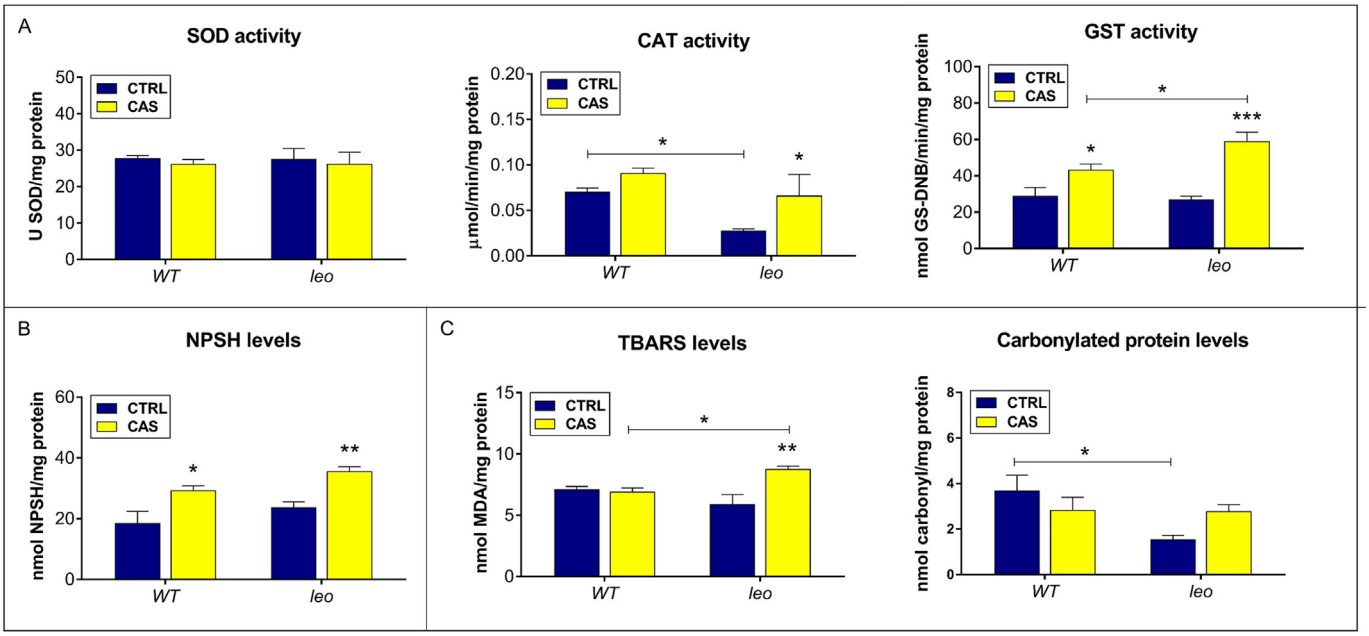

Fig. 4. Antioxidant parameters and biomarkers of lipid and protein damage in the brain of *WT* and *leo* populations following repeated CAS exposure. (A) Enzymatic antioxidant defenses (SOD, CAT, and GST activities); (B) Non-enzymatic antioxidant responses (NPSH amounts); (C) TBARS and CP levels. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA followed by Student–Neuman–Keuls multiple test whenever appropriate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; n = 4–6 per group).

disorders. Thus, future studies aiming to correlate oxidative stress with changes in neurotransmitter signaling pathways will bring novel opportunities to understand how conspecific alarm cues affects brain functions.

Conflicts of interest

The authors declare that no competing interests exist.

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7.3 MANUSCRITO 3

Predictable chronic stress modulates behavioral and neuroendocrine phenotypes of zebrafish: influence of two homotypic stressors on stress-mediated responses

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Abstract

The zebrafish (*Danio rerio*) has been considered a suitable model organism to assess the evolutionarily conserved bases of behavioral and neuroendocrine responses to stress. Depending on the nature of the stressor, prolonged stress may elicit habituation or evoke long-term changes in the central nervous systems (CNS) often associated with various neuropsychiatric disorders. Conspecific alarm substance (CAS) and net chasing (NC) constitute chemical and physical stressors, respectively, which cause aversive behaviors and physiological changes in fishes. Here, we investigate whether the predictable chronic stress (PCS) using two homotypic stressors differently modulates behavioral and physiological responses in zebrafish. PCS-CAS or PCS-NC were performed for 14 days, 2-times daily, while locomotion, exploratory activity, anxiety-like behaviors, and whole-body cortisol levels were measured on day 15. PCS-CAS reduced distance traveled, the number of transitions and time in top area, as well as increased the latency to enter the top in the novel tank test. In the light/dark test, CAS-exposed fish showed decreased time spent in lit area, shorter latency to enter the dark area, and increased risk assessments. PCS-CAS also increased whole-body cortisol levels in zebrafish. Although PCS-NC reduced the latency to enter the dark area, whole-body cortisol levels did not change. Moreover, because CAS and NC acutely promoted anxiogenesis and increased cortisol levels, our data suggest habituation to stress following PCS-NC. Overall, our novel findings demonstrate that PCS induces behavioral and physiological changes in zebrafish depending on the nature of the stressor.

Keywords: stress; physical stressor; aversive chemical cue; cortisol; behavioral responses.

Introduction

The zebrafish (*Danio rerio*) is a promising model organism in genetics, pharmacology, toxicology, and behavioral neuroscience research (Fontana et al., 2018; Stewart et al., 2012). Although the central nervous system (CNS) of teleost fishes shows anatomical differences when compared to that of mammals, the zebrafish brain presents evolutionarily conserved structures with similar functions (Lillesaar et al., 2007). When compared to humans, zebrafish present around 70% of genetic identity (Howe et al., 2013), as well as evolutionarily conserved stress-related responses (Alsop and Vijayan, 2009) and well-characterized behavioral neurophenotypes (Kalueff et al., 2013). Moreover, the hypothalamic-pituitary-interrenal (HPI) axis activation in teleost fishes promotes cortisol release, showing homologous functions to the mammalian hypothalamic-pituitary-adrenal (HPA) axis (Alsop and Vijayan, 2009). Prolonged stress may impair the HPA axis activity, playing a role in various neuropsychiatric disorders, such as depression and anxiety (Brown et al., 2004).

Cortisol is a steroid hormone associated with stress, which can be easily measured in animal models (Johnstone et al., 2012). Moreover, stressful situations increase cortisol levels, thereby altering metabolic processes that culminate in fight or flight responses (Egan et al., 2009; Mommsen et al., 1999). Cortisol binds to the glucocorticoid receptor, which regulates glucose metabolism homeostasis, immune function, and behavioral responses (Mommsen et al., 1999), thereby playing an adaptive role during stress (Fuzzen et al., 2010).

Chronically, stress may induce depression- and/or anxiety-like states (McEwen, 2004). Repeated exposure to stressors can dysregulate the HPA axis, as well as modulate glutamatergic, noradrenergic, dopaminergic, serotonergic, and GABAergic neurotransmitter systems, culminating in various behavioral changes (Chrousos, 2009; Popoli et al., 2011; Sandi and Haller, 2015). Aversive behaviors in zebrafish can be modeled using the conspecific alarm substance (CAS) exposure and the net chasing (NC) protocol (Barcellos et

al., 2011; Egan et al., 2009; Mathuru et al., 2012; Quadros et al., 2018, 2016). In Ostariophysians, CAS elicits robust biochemical, neurochemical and behavioral phenotypes evoking 'fight-or-flight' responses (Maximino et al., 2018a; Ogawa et al., 2014; Quadros et al., 2016). Moreover, a 7-day CAS exposure changes locomotion, exploration, aggression, induces anxiety-like behaviors, and modulates oxidative stress-related biomarkers (Quadros et al., 2019, 2018). Although NC acutely increases cortisol levels and causes defensive behaviors in zebrafish (Abreu et al., 2014; Barcellos et al., 2011) it is unclear whether this protocol influences physiological and behavioral responses after a repeated exposure.

Maladaptive responses related to stress may play a role in anxiety-related disorders and correlated physiological mechanisms (Campos et al., 2013; McEwen et al., 1995). Although the unpredictable chronic stress (UCS) has been associated with neuropsychiatric disorders and elevated glucocorticoid levels in experimental models (Antoniuk et al., 2019; Joëls et al., 2004; Marcon et al., 2018), the predictable chronic stress (PCS) can also negatively affect physiological and neurobehavioral responses (Vyas and Chattarji, 2004). In rodents, PCS increases seizure susceptibility, promotes oxidative stress, hippocampal neurodegeneration, and increases corticosterone levels (Al-Mohaisen et al., 2000; Zhu et al., 2017). However, the main effects of stress depend on the intensity, duration, and/or predictability (Zucchi et al., 2009) since repeated stress can also facilitate adaptive processes, resulting in habituation to stress (Grissom and Bhatnagar, 2009). Although PCS might induce adaptation (Zucchi et al., 2009), repeated exposure to strong homotypic stressors, as predation, does not elicit such effect (Grissom and Bhatnagar, 2009). Because CAS and NC constitute aversive stimuli from different types, the use of chemical and physical stressors may help elucidate how distinct homotypic stressors modulate behavioral and physiological responses. Here, we sought to investigate whether the repeated exposure to different stressors (physical and chemical) could reflect adaptive and/or maladaptive responses in zebrafish. Therefore, we analyzed the effects

of PCS using NC and CAS as stressors on anxiety-like behaviors (using the novel tank and light/dark tests) and neuroendocrine responses (by measuring whole-body cortisol levels). Moreover, we tested the influence of acute stress responses in stress-related phenotypes to investigate potential differences between single and repeated exposures to each homotypic stressor used.

Materials and Methods

Experimental design

To evaluate whether the PCS protocol using both physical and chemical stressors differently modulates aversive behaviors and whole-body cortisol levels in zebrafish, we compared the effects of CAS and NC on behavioral and neuroendocrine responses. Fish cohorts were stressed for 14 days, 2-times daily, with CAS or NC as homotypic stressors. The respective period and frequency of exposure to the stressors chosen was based on a previous report, which shows prominent effects of stress on defensive behaviors of zebrafish following UCS (Piato et al., 2011). A non-stressed control group remained undisturbed in the same room during the exposure period. To avoid acute effects of the stressors on the parameters tested, behaviors were assessed in the novel tank diving test or in the light/dark test on day 15 and whole-body cortisol levels were measured at the same period. **Fig. 1** shows a schematic representation of the experimental procedures used to analyze the effect of stressors on behavioral responses.

Animals

Adult zebrafish (*Danio rerio*) (short fin *wild type* (*WT*) population, 50:50 male: female proportion, 4-6 months-old aged) were obtained from a local commercial supplier (Hobby Aquários, RS, Brazil). Before the experiments, animals were kept for 2 weeks at a maximum

density of 4 fish per liter in 40 L tanks for acclimatization. Tanks were filled with non-chlorinated water, which was maintained under physical, biological and chemical filtration, at a temperature of 27 ± 1°C. Fish were fed 2-times daily with Alcon BASIC™ flakes (Alcon, Brazil) until apparent satiety and kept in a monitored room with artificial photoperiod adjusted to 14h/10h, light/dark, respectively (lights on at 7:00 am). This study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and all protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (106/2014).

Predictable chronic stress with conspecific alarm substance – PCS-CAS

CAS extraction was performed as described elsewhere (Egan et al., 2009; Quadros et al., 2016). Briefly, naïve donor fish were previously anesthetized in water at 4°C and euthanized by decapitation. Fish were then washed with distilled water and blood was removed with a cotton swab. Donor fish bodies were placed in a petri dish containing 10 mL/fish of distilled water and shallow cuts were performed along each side of the fish (10–15 cuts) in order to damage the epidermal cells, where CAS is stored (**Fig. 1A**). For the PCS-CAS protocol, zebrafish (n = 11–14 per group) were exposed individually to 3.5 mL/L skin extract preparation in 0.5 L tanks for 5 min, 2-times daily (10:00 am and 3:00 pm) for 14 consecutive days. In order, to ensure data reproducibility, CAS preparation was made 2-times daily in a final volume to expose all experimental cohorts. Because CAS extract from a single donor fish was used to expose 4 subjects, a total of 112 animals were used as donors in the PCS-CAS protocol. Additionally, to minimize potential interference of handling, the same experimenter performed the CAS exposure throughout the experimental period. Fish were tested on day 15 to verify the influence of PCS on behavioral endpoints (**Fig. 1B**) and

immediately euthanized following the trial. We also analyzed whether PCS-CAS modulates stress responses by measuring whole-body cortisol levels.

Predictable chronic stress with net chasing – PCS-NC

To induce physical stress, animals (n = 11–14 per group) were individually placed in circular plexiglass tanks (30.5 cm diameter and 6 cm water column) and pursued with a net for 2 min, which elicits stress-related responses in zebrafish (Abreu et al., 2014; Mocelin et a., 2015; Oliveira et al., 2013). For PCS-NC, stress was performed 2-times daily at the same time period (10:00 am and 3:00 pm) for 14 days (**Fig. 1B**). To minimize potential experimental variations, the same trained experimenter executed the NC throughout the 14-day period (circular clockwise movements with the net at a regular speed of approximately 40 turns per minute) (Mocelin et al., 2015). On day 15, behavioral activities were assessed, and fish were euthanized immediately following the experiments. Moreover, whole-body cortisol levels were measured.

Acute effects of the homotypic stressors

To verify the acute effects of CAS and NC on behavioral (n = 12 per group) and neuroendocrine responses (n = 5 per group), we run another set of experiments in which zebrafish were subjected to a single exposure to the homotypic stressor (3.5 mL/L CAS for 5 min or NC for 2 min) as described elsewhere (Egan et al., 2009; Quadros et al., 2016; Oliveira et al., 2013; Abreu et al., 2014). In the acute CAS exposure, a total of 3 fish were used as donors of the skin extract.

Behavioral assays

Behavioral tests were recorded between 9:00 am and 4:00 pm. The experimental tanks were placed on a stable surface and all procedures were recorded for a single 6 min trial. Behavioral activities of individual zebrafish were recorded with a webcam connected to a laptop and then analyzed offline at a rate of 30 frames/s using appropriate video tracking software (ANY-maze^{TM}, Stoelting CO, USA) by data analysts blinded to the experimental condition of fish. Precautions were taken to obtain representative data and to minimize handling stress. Throughout the experiments, fish were carefully handled to minimize handling stress. To obtain representative data, two independent batches of fish were used, as well as two experimental apparatuses per group. Moreover, fish were handled and tested in the same room, with uniform and constant water quality and illumination throughout the experiment.

Novel tank diving test

The novel tank diving test is a protocol used to evaluate locomotion and vertical exploration of zebrafish (Egan et al., 2009; Rosemberg et al., 2012; Wong et al., 2010). After the exposure periods, fish were placed in the novel tank apparatus (25 cm × 15 cm × 11 cm length, height and width respectively), divided in two horizontal areas (top and bottom). Tanks were filled with 2 L of non-chlorinated water and the exploratory behavior was recorded for 6 min. As locomotion-related endpoints, distance traveled (m), absolute turn angle (◦/s), and maximum speed (m/s) were quantified. The latency to enter the top area (s), the number of entries and time spent in top area were measured to quantify the vertical activity of fish, which has been commonly associated with anxiety-like responses (Kalueff et al., 2013).

Light/dark test

The light/dark test was performed to evaluate anxiety-like behavior based on the protocols described previously (Maximino et al., 2010a, 2010b; Quadros et al., 2016). The apparatus (25 cm × 15 cm × 11 cm length, height and width respectively) was divided in two equally-sized black and white compartments, which were covered externally by black or white opaque self-adhesive films on the walls and floor. For behavioral analyses, tanks were filled with 2 L of non-chlorinated water and animals were placed individually in the lit area of the apparatus before starting the test. Each subject was analyzed for 6 min and the following behavioral endpoints were measured: time spent in lit area, transitions to the lit area, latency to enter the dark area and risk assessment. Risk assessment episodes (Kalueff et al., 2013; Maximino et al., 2014) were computed manually by two trained observers (inter-rater reliability > 0.85) blinded to the experimental condition using the video recordings.

Whole-body cortisol extraction and quantification

Whole-body cortisol levels were determined based on the protocol described previously (Sink et al., 2008). On day 15, another cohort of animals was quickly euthanized by immersion in liquid nitrogen to determine the influence of PCS in cortisol levels. Whole-body cortisol was extracted as described elsewhere (Barcellos et al., 2007; Oliveira et al., 2013). Fish were weighed, minced, and placed in 2 mL conical, plastic centrifuge tubes of phosphate buffered saline (PBS, pH 7.4). Homogenates were then transferred to a 10 mL tube and 5 mL of ethyl ether was added. Tubes were kept under constant agitation in a vortex for approximately 1 min, centrifuged for 10 min at 3000 rpm, and frozen in liquid nitrogen. The non-frozen portion, containing ethyl ether together with the cortisol, was decanted and transferred to another tube and evaporated. Finally, the lipid extract containing the cortisol was stored at -20°C until analyses. The accuracy of cortisol detection was tested by calculating the recoveries of samples enriched with known amounts of cortisol (50, 25 and

12.5 ng mL^{-1}). Mean detection accuracy of the fortified samples was 94.3% and all values of cortisol were adjusted for recovery with a cortisol value equation = measured value × 1.0604. Tissue extracts were suspended in 1 mL of PBS buffer and cortisol levels were measured in duplicate using an enzyme-linked immunosorbent assay kit (EIAgen^{TM} CORTISOL test, BioChem ImmunoSystems) (Sink et al., 2008). Samples (n = 5) were run on 96-well plates to analyze the accuracy and subsequently calculate the intra-assay and inter-assay coefficient of variation (CV) (ranging 5–9% and 7–10%, respectively). Another set of experiments were performed after the single exposure to the homotypic stressors. Fish were euthanized 15 min following NC or CAS exposure, which represents the time necessary to reach the cortisol peak in zebrafish (Abreu et al., 2014). Importantly, for both PCS and acute protocols, we did not use the same subjects tested in the behavioral experiments to investigate the effect of stress on neuroendocrine responses. The strategy was chosen to avoid a potential influence of the behavioral procedures in cortisol levels (Kysil et al., 2017).

Statistical analyses

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's test, respectively. Because data were normally distributed and homoscedastic, results were expressed as mean ± standard error of mean (S.E.M) and analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test whenever appropriate. Sample sizes were chosen based on pilot experiments and previous reports (Canzian et al., 2019, Quadros et al., 2019). The significance was set at $p < 0.05$.

Results

The effects of PCS-CAS and PCS-NC on locomotion and vertical exploration were measured in the novel tank diving test. PCS-CAS reduced the distance traveled when

compared to control ($F_{2,38}$ = 3.392, p = 0.0441) (**Fig. 2A**). Although both PCS-CAS and PCS-NC did not alter the absolute turn angle and maximum speed (**Fig. 2A**), PCS-CAS decreased the time spent in top area ($F_{2,38}$ = 9.825, p = 0.0004) and increased the latency to enter the top area ($F_{2,38}$ = 6.363, p = 0.0041) (**Fig. 2B**). PCS-CAS group also showed fewer entries in the top area than control ($F_{2,38}$ = 10.16, p = 0.0003).

In the light/dark test, both PCS-CAS and PCS-NC decreased the latency to enter the dark area when compared to control ($F_{2,32}$ = 15.43, p <0.0001). However, only PCS-CAS increased the time spent in dark area ($F_{2,32}$ = 3.224, p = 0.0530), and the number of risk assessment episodes ($F_{2,32}$= 22.35, p <0.0001). No significant changes were observed in the transitions to lit area among groups (**Fig. 3**). Although PCS-CAS increased whole-body cortisol levels ($F_{2,17}$ = 15.31, p = 0.0002), PCS-NC did not induce significant changes among groups (**Fig. 4**). Conversely, the two homotypic stressors acutely promoted anxiogenesis in both novel tank and light/dark tests (**Table 1**) paralleling increased whole-body cortisol levels (**Table 2**).

Discussion

Here, our novel findings show that PCS modulates anxiety-like responses and whole-body cortisol levels in zebrafish. While PCS-CAS impaired locomotion and induced robust anxiogenic-like behaviors, PCS-NC reduced the latency to enter the dark area in the light/dark test. Moreover, increased cortisol levels were verified only in PCS-CAS group, supporting differences on behavioral and physiological responses after PCS, depending on the nature of the homotypic stressor. Because both CAS and NC acutely promoted anxiogenic-like responses and increased whole-body cortisol levels, we suggest the occurrence of habituation to stress following PCS-NC, but not PCS-CAS.

In fishes, stressful events activate the HPI axis (Madaro et al., 2015) and the resulting downstream cascade facilitates cortisol release into the bloodstream (Mommsen et al., 1999). This phenomenon depends on various factors, such as stressor chronicity, intensity, frequency, and the ability to predict or cope with the situation (Kiilerich et al., 2018). Similar to humans, cortisol is the primary circulating glucocorticoid in fish species. In mammals, if cortisol facilitates the organism to cope with stress, a negative feedback occurs, downregulating the biochemical cascade involved in cortisol release from adrenal glands (Mommsen et al., 1999). Importantly, the HPI activation can be downregulated with repeated exposure to homotypic stressors, triggering habituation responses (Grissom and Bhatnagar, 2009; Thompson and Spencer, 1966). Habituation to stress is a form of nonassociative learning, which may reflect the decrease in physiological responses following repeated homotypic stressor when compared to the larger responses observed in an acute exposure (Grissom and Bhatnagar, 2009). Because strong aversive stimuli may not cause significant habituation, the intensity and nature of stressor may play a role in behavioral and neuroendocrine responses of zebrafish subjected to the PCS protocol.

As a chemical cue, CAS triggers robust aversive responses and elicits defensive behaviors in Ostariophysians (Egan et al., 2009; Maximino et al., 2018a; Quadros et al., 2016; Speedie and Gerlai, 2008). In zebrafish, CAS enhances social cohesion, bottom dwelling, and escaping responses (Canzian et al., 2017; Quadros et al., 2019). CAS can also sensitize anxiety-like behavior in a time-dependent manner and aversive behaviors are also observed for at least 7 days following a single CAS exposure using a conditioned place avoidance task (Lima et al., 2016; Maximino et al., 2018b). Importantly, CAS increases habenular *c-fos* expression and stimulates adrenaline release, increasing blood glucose levels (Maximino et al., 2014; Ogawa et al., 2014). Because the ventral habenula in zebrafish is homologous to the mammalian lateral habenula, CAS is known to elicit strong aversive behaviors, triggering

sympathetic activation (Maximino et al., 2014; Quadros et al., 2019). These physiological responses increase defensive behaviors and cortisol levels, reinforcing the activation of the HPI axis after a single CAS exposure, as observed here. Conversely, NC represents a physical stressor, reflecting another stressful situation possible by mimicking the pursuit predation (Abreu et al., 2018). Acutely, NC robustly increases whole-body cortisol levels reflecting a stressful condition, as well as induces anxiogenic-like effects (Abreu et al., 2014) and lipid peroxidation in brain tissue (Pancotto et al., 2018). Here, we observed that NC acutely, but not chronically, effectively induces anxiogenesis and elevates whole-body cortisol levels. Thus, since the main behavioral endpoints and cortisol levels were at the baseline after the 14-day protocol, our data may reflect a possible habituation to stress following PCS-NC.

To corroborate this hypothesis, we demonstrate how zebrafish respond when challenged to different homotypic stressors depending on the stress duration. In general, CAS exposure was more effective than NC to modulate anxiety. Acutely, although both stressors facilitate cortisol release, the aversive effects were more prominent in CAS than those observed in NC group. Similarly, we showed that PCS-CAS was more effective than PCS-NC to evoke anxiogenic-like phenotypes in zebrafish since PCS-CAS elicited robust aversive responses in both novel tank and light/dark tests, paralleling the increased whole-body cortisol levels. Moreover, the hypolocomotion observed in PCS-CAS group reinforces that changes in locomotor activity may be associated, at least partially, to the impaired vertical exploration in the novel tank diving test. Disruption of habituation to repeated stress may be related to stress-related disorders, such as depression and/or anxiety (Grissom and Bhatnagar, 2009), Therefore, the lack of habituation in PCS-CAS group is supported due to the robust stress-related phenotypes verified after 14 days of exposure, reflecting maladaptive responses. Although there is a clear association between anxiety-related behaviors and cortisol levels in

some psychiatric conditions, further studies aiming to identify the neural mechanisms underlying behavioral and neuroendocrine responses to repeated stress are needed.

Conclusion

In summary, our novel findings show that PCS-CAS, but not PCS-NC, triggers prominent stress responses by eliciting anxiety-like behaviors and markedly increasing whole-body cortisol levels in zebrafish. Since both physical and chemical stressors positively modulates anxiety and cortisol release after the acute protocol, the effects on anxious behavior following PCS could be related to a distinct neuroendocrine regulation of the HPI axis, where PCS-NC shows a putative habituation to stress after 14 days. Although the precise mechanisms describing how CAS and NC influence behavioral and stress responses still remain to be elucidated, this proof-of-concept study shows the susceptibility of zebrafish to PCS varying according to the nature of the homotypic stressor.

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Competing interests

The authors declare no competing interests.

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Figure captions

Fig. 1. Schematic representation of the PCS experimental procedures and behavioral tests. **(A)** CAS extraction from naïve donor and exposure protocol. The skin extract was prepared on ice and 3.5 mL/L were used for the experiments. **(B)** Experiment protocol (PCS-CAS/PCS-NC) and novel tank and light/dark test apparatus. Apparatus were divided in two areas: novel tank test (top and bottom) and light/dark (dark and lit areas) (PCS-CAS: predictable chronic stress – conspecific alarm substance; PCS-NC: predictable chronic stress – net chasing).

Fig. 2. PCS-CAS, but not PCS-NC, affects locomotion and vertical activity of zebrafish. **(A)** Locomotion-related behaviors. **(B)** Vertical exploration, which can reflect anxiety-like responses. Data are expressed as means ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test as post-hoc whenever necessary. Different letters indicate statistical differences between groups ($p < 0.05$; PCS-CAS: predictable chronic stress – conspecific alarm substance; PCS-NC: predictable chronic stress – net chasing). Experiments were performed using $n = 13–14$ per group.

Fig. 3. PCS-CAS induces anxiogenic-like behaviors in the light/dark test. Data are expressed as means ± S.E.M and analyzed by one-way ANOVA, followed by Tukey's test as post-hoc whenever necessary. Different letters indicate statistical differences between groups ($p < 0.05$; PCS-CAS: predictable chronic stress – conspecific alarm substance; PCS-NC: predictable chronic stress – net chasing). Experiments were performed using $n = 11–12$ per group.

Fig. 4. Effect of PCS-CAS and PCS-NC on whole-body cortisol levels. Data are expressed as means ± S.E.M and analyzed by one-way ANOVA followed by Tukey's test as post-hoc. Different letters indicate statistical differences among groups ($p < 0.05$; PCS-CAS: predictable chronic stress – conspecific alarm substance; PCS-NC: predictable chronic stress – net chasing). Experiments were performed using $n = 5$ per group.

Table 1. Acute effects of CAS and NC on locomotion and anxiety-like behaviors of zebrafish measured in the novel tank diving test (NTT) and in the light/dark test (LDT). Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA followed by Tukey's test whenever necessary. *P* values lower than 0.05 are marked in bold and distinct letters indicate statistical differences between groups (*n* = 12 per group). Degrees of freedom (DF) and F values are shown.

Tasks	Behavioral endpoints	Groups			Statistics		
		Control	CAS	NC	DF (n,d)	F	*p* value
NTT	Distance traveled	20.58 ± 2.63	19.66 ± 2.04	19.78 ± 2.18	2,33	0.048	0.954
	Absolute turn angle	67393 ± 7309	64555 ± 9110	60930 ± 3527	2,33	0.212	0.810
	Maximum speed	197.2 ± 21.26	0.268 ± 0.04	0.309 ± 0.07	2,33	0.179	0.837
	Time spent in top area	**92.82 ± 6.95 [a]**	**22.68 ± 8.23 [b]**	**51.46 ± 10.26 [b]**	**2,33**	**16.85**	**< 0.0001**
	Entries in the top area	**39.25 ± 4.40 [a]**	**5.75 ± 1.66 [b]**	**33.92 ± 3.66 [a]**	**2,33**	**27.35**	**< 0.0001**
	Latency to enter the top area	**28.42 ± 5.69 [a]**	**133.2 ± 13.52 [b]**	**64.91 ± 12.23 [a]**	**2,33**	**23.25**	**< 0.0001**
LDT	Time spent in lit area	**197.2 ± 21.26 [a]**	**95.33 ± 13.76 [b]**	**128.3 ± 20.65 [b]**	**2,33**	**7.585**	**0.0019**
	Transitions to lit area	**21.00 ± 2.33 [a]**	**10.75 ± 1.10 [b]**	**20.92 ± 2.83 [a]**	**2,33**	**7.123**	**0.0027**
	Latency to enter the dark area	**13.17 ± 0.86 [a]**	**3.25 ± 0.41 [b]**	**8.83 ± 1.59 [c]**	**2,33**	**21.48**	**< 0.0001**
	Risk assessment	**1.25 ± 0.33 [a]**	**2.92 ± 0.45 [b]**	**2.33 ± 0.26 [ab]**	**2,33**	**5.684**	**0.0076**

Table 2. CAS and NC acutely increase whole-body cortisol levels in zebrafish. Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA followed by Tukey's test. Distinct letters indicate statistical differences between groups (n = 5 per group). Degrees of freedom (DF) and F values are shown.

Groups	Whole-body cortisol levels (ng/g)	Statistics		
		DF (n,d)	F	*p* value
Control	1.56 ± 0.22 [a]			
CAS	5.20 ± 0.40 [b]	2,12	18.56	0.0002
NC	5.10 ± 0.69 [b]			

Figure 1

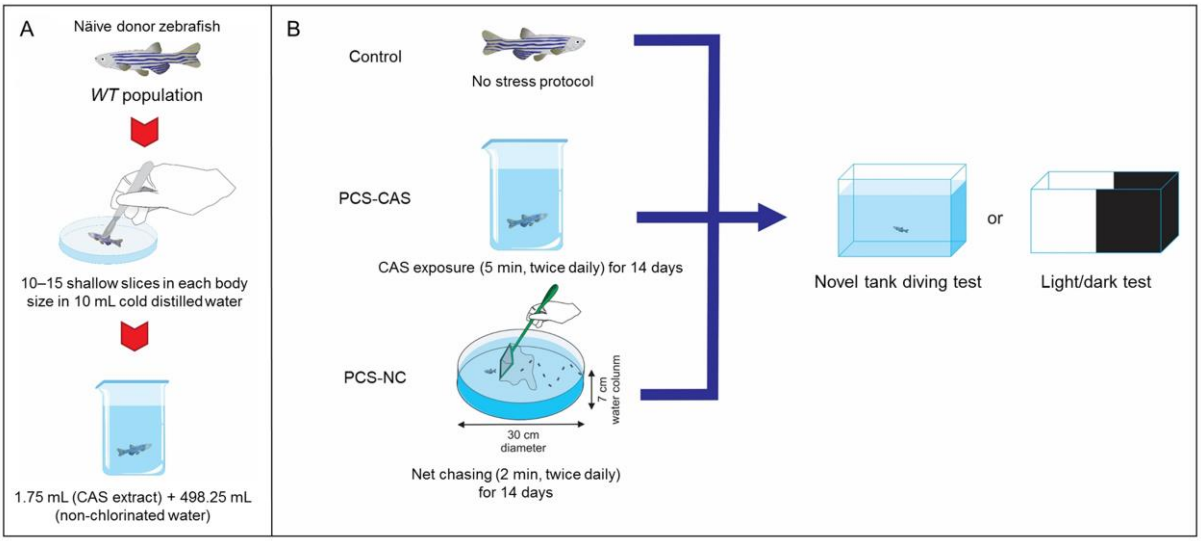

A
Näive donor zebrafish
WT population
10–15 shallow slices in each body size in 10 mL cold distilled water
1.75 mL (CAS extract) + 498.25 mL (non-chlorinated water)
B
Control
No stress protocol
PCS-CAS
CAS exposure (5 min, twice daily) for 14 days
PCS-NC
7 cm water colunnn
30 cm diameter
Net chasing (2 min, twice daily) for 14 days
or
Novel tank diving test
Light/dark test

Figure 2

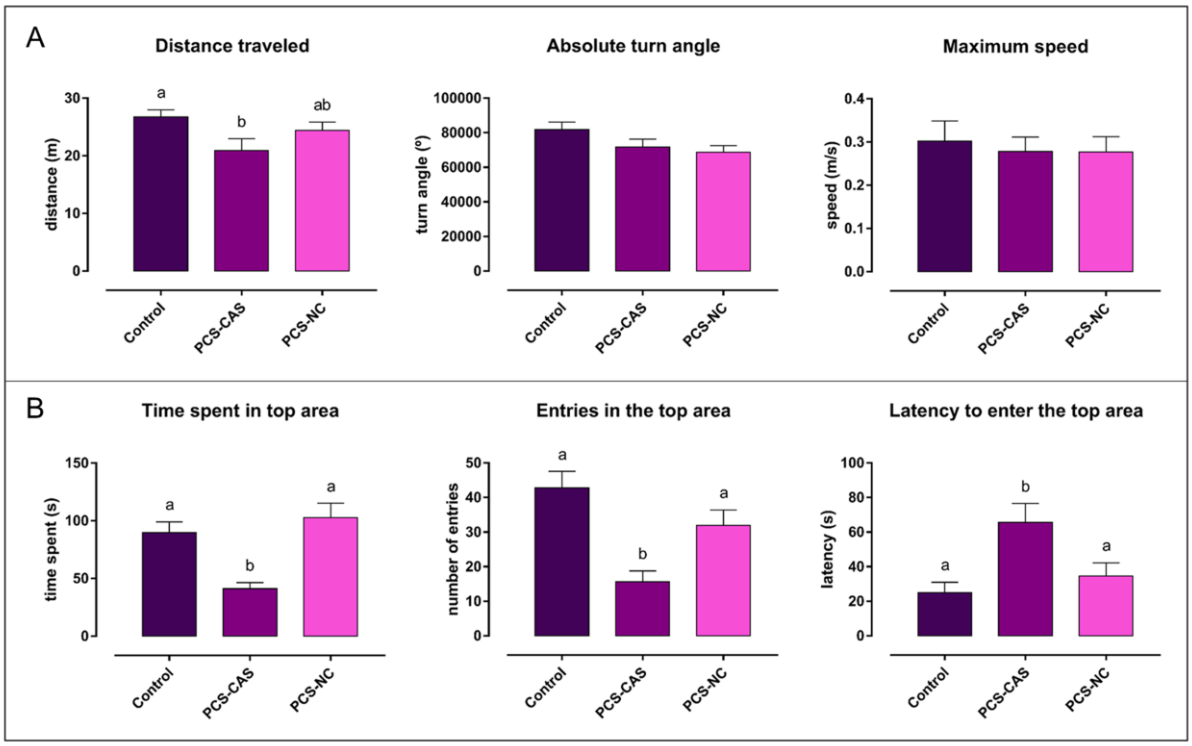

A
Distance traveled
distance (m)
30
20
10
0
a
b
ab
Control
PCS-CAS
PCS-NC
Absolute turn angle
turn angle (°)
100000
80000
60000
40000
20000
0
Control
PCS-CAS
PCS-NC
Maximum speed
speed (m/s)
0.4
0.3
0.2
0.1
0.0
Control
PCS-CAS
PCS-NC
B
Time spent in top area
time spent (s)
150
100
50
0
a
b
a
Control
PCS-CAS
PCS-NC
Entries in the top area
number of entries
50
40
30
20
10
0
a
b
a
Control
PCS-CAS
PCS-NC
Latency to enter the top area
latency (s)
100
80
60
40
20
0
a
b
a
Control
PCS-CAS
PCS-NC

Figure 3

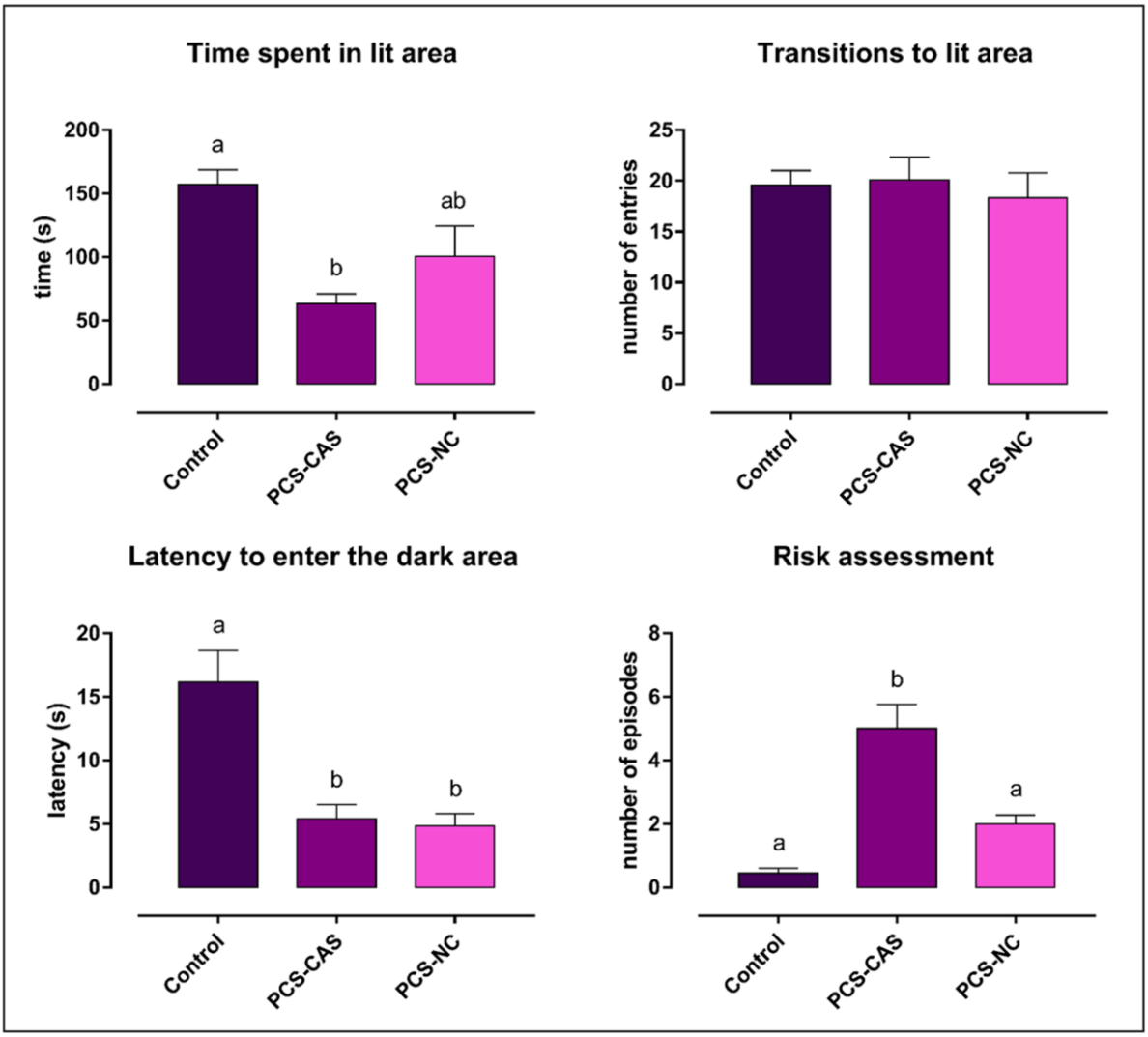
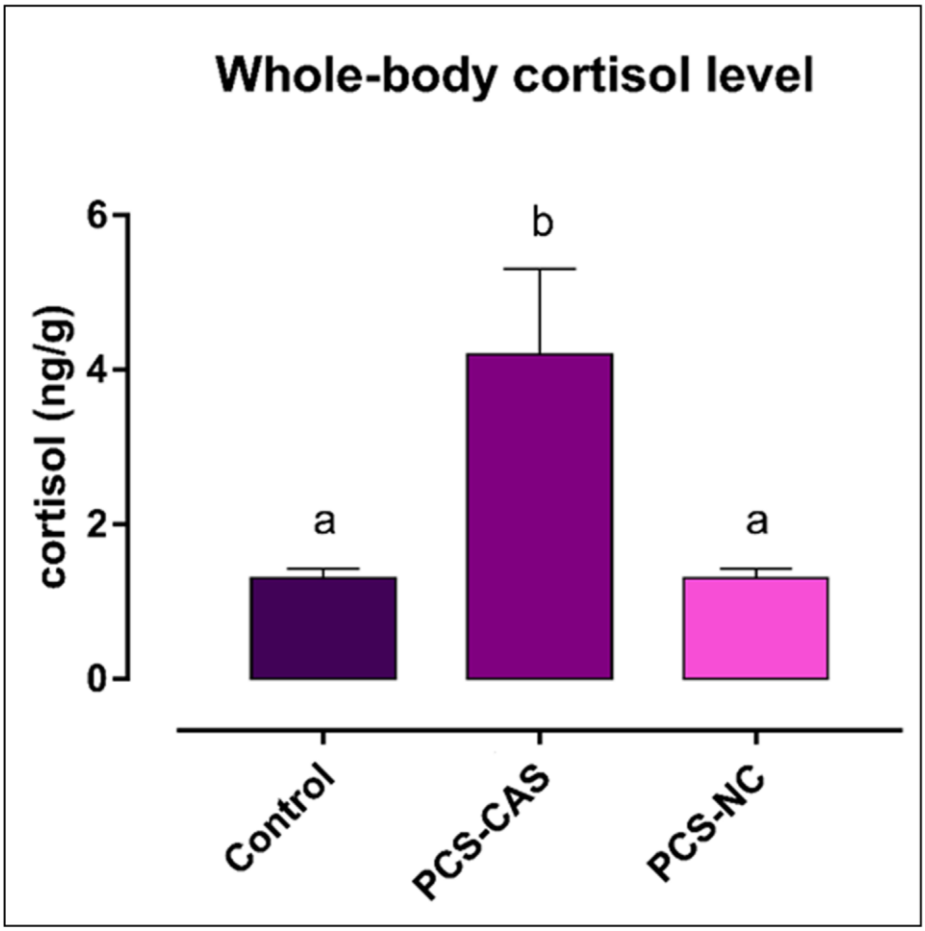

Time spent in lit area
time (s)
200
150
100
50
0
a
b
ab
Control
PCS-CAS
PCS-NC
Transitions to lit area
number of entries
25
20
15
10
5
0
Control
PCS-CAS
PCS-NC
Latency to enter the dark area
latency (s)
20
15
10
5
0
a
b
b
Control
PCS-CAS
PCS-NC
Risk assessment
number of episodes
8
6
4
2
0
a
b
a
Control
PCS-CAS
PCS-NC

Figure 4


Whole-body cortisol level
cortisol (ng/g)
6
4
2
0
a
b
a
Control
PCS-CAS
PCS-NC

8. DISCUSSÃO

Sabe-se que o estresse, bem como a ansiedade são considerados problemas mundiais (KESSLER et al., 2005; RIAZA BERMUDO-SORIANO et al., 2012). Estudos mostram que pacientes que apresentam algum transtorno mental, apresentam emoções exacerbadas como medo e/ou ansiedade desencadeadas por eventos estressantes ao decorrer da vida (BALDWIN et al., 2005, 2010; SHIN; LIBERZON, 2010; SMAGA et al., 2015). Diferentes tipos de estressores, podem estar associados com o desenvolvimento de algum transtorno neuropsiquiátrico (CHAKRAVARTY et al., 2013; SHIN; LIBERZON, 2010; VYAS; CHATTARJI, 2004). A investigação de como o estresse pode alterar as respostas bioquímicas e comportamentais em peixe-zebra é uma estratégia importante para esclarecer o mecanismo primário de diferentes transtornos mentais (STEENBERGEN 1et al., 2011). Dessa forma, trabalhamos com protocolo de estresse agudo e crônico utilizando estressores naturalísticos (SA e PR) para compreendermos como situações aversivas naturais podem influenciar nas respostas comportamentais, bem como os aspectos relacionados ao sistema serotonérgico, estresse oxidativo e endócrino.

Apesar dos efeitos do estresse sempre terem sido objeto de interesse em nosso grupo de pesquisa, os questionamentos sobre o papel do estresse agudo e crônico previsível, utilizando estressores naturalísticos, instigaram nossos estudos envolvendo sua influência em modelos de transtornos psiquiátricos em peixe-zebra. Portanto, como primeiro estudo dessa tese, nós mostramos pela primeira vez a interação da Z-MAO no comportamento agressivo, após a exposição aguda e repetida à SA em duas populações (*WT* e *leo*) de peixe-zebra. Como resultados, verificamos que a população *WT* é mais ativa e agressiva do que a população *leo*, bem como apresenta maior atividade da Z-MAO. Além disso, mostramos que a exposição a SA agudamente, em ambas as populações, aumentou os fenótipos agressivos e diminuiu a atividade da Z-MAO, e cronicamente reduziu a atividade locomotora e agressividade, não alterando a atividade da Z-MAO. Pesquisas já demonstraram o efeito da SA nas respostas fisiológicas e fenótipos comportamentais em outras espécies, diminuindo a atividade locomotora (BARBOSA JÚNIOR et al., 2012) bem como reduzindo a agressividade (MEUTHEN et al., 2016). Além disso, a atividade da MAO exerce influência sobre o humor e o comportamento, dessa forma exercendo uma relação com a agressão (ALIA-KLEIN et al., 2008; GODAR et al., 2011; ROSELL; SIEVER, 2015). Estudos mostram que uma mutação no gene da MAO-A, que desencadeia uma deficiência da MAO em humanos, tem sido associado com comportamento antissocial e agressivo (GODAR et al., 2016; TAKAHASHI et al., 2011). Portanto, pela atividade da MAO representar um importante mecanismo no

controle dos níveis de monoaminas no SNC (NIKOLAC PERKOVIC et al., 2016), sugerimos que as monoaminas em peixe-zebra, assim como em humanos, desempenham um papel importante na agressão mediada pelo estresse (SA).

O estresse altera o comportamento agressivo em protocolos agudo e crônico, desencadeando assim alterações na atividade da Z-MAO agudamente. Comportamentos aversivos podem afetar o SNC, alterando parâmetros bioquímicos através do desequilíbrio no sistema antioxidante (YOUDIM; BAKHLE, 2006). Com isso, hipotetizamos que as respostas ao estresse poderiam estar associadas com modulações no sistema redox. Portanto, o segundo artigo científico mostrado nesta Tese objetivou verificar se o estresse crônico à SA altera parâmetros de estresse oxidativo e comportamento do tipo ansiedade nas duas populações de peixe-zebra. Para avaliar o comportamento tipo ansiedade, verificamos o tempo de permanência no fundo, o tempo de latência para entrada na área do topo, o número de cruzamentos entre as áreas, bem como a atividade locomotora no teste do novo tanque (EGAN et al., 2009; KALUEFF et al., 2013; ROSEMBERG et al., 2011, 2012). Pela primeira vez, mostramos uma possível relação da modulação de parâmetros de estresse oxidativo cerebral com o comportamento do tipo ansiedade após a exposição repetida à SA em *WT* e *leo*. No basal, a população *leo* mostrou ser mais ansiosa que a *WT*, dados que corroboram com alguns estudos mostrando que *leo* demonstra ser mais ansiosa que outras populações (MAXIMINO et al., 2013). Além disso, após a exposição à SA, ambas as populações apresentaram um aumento da escototaxia e uma redução da exploração vertical nos testes claro/escuro e novo tanque, respectivamente. Após a exposição a SA a população *WT* apresentou menor latência para a entrada na área escura, bem como aumentou a avaliação de risco do aparato claro/escuro. Além disso, *WT* diminuiu a transição ao topo no teste do novo tanque, o que sugere que essa população é mais sensível após a exposição crônica à SA, uma vez que não observamos diferença no tratamento na população *leo* nesses parâmetros. Após a exposição a SA, a atividade da CAT, GST e níveis de NPSH aumentaram em ambas as populações, porém somente em *leo* desencadeou um aumento da peroxidação lipídica após a exposição. Em condições basais, a atividade da CAT e níveis de proteína carbonilada foi menor em *leo* quando comparado com *WT*, sugerindo mais uma vez que *leo* possui fenótipos mais ansiosos. Podemos concluir com esses resultados, que a variação genética entre as duas populações, embora não avaliada neste estudo, pode ser fundamental para compreender as diferenças encontradas nas respostas comportamentais frente a diferentes estímulos aversivos.

Em Ostariophysans, a SA desencadeia efeito ansiogênico, além de aumentar a interação social, geotaxia e expressão da *c-fos* habenular (CANZIAN et al., 2017; OGAWA;

NATHAN; PARHAR, 2014; OLIVEIRA et al., 2017; QUADROS et al., 2016). Sabe-se que a SA aumenta a glicose circulante, epinefrina e níveis de serotonina cerebral, sugerindo a ativação do sistema nervoso simpático (HERCULANO; MAXIMINO, 2014). Portanto, situações aversivas desencadeiam respostas comportamentais que possuem um papel fundamental para o organismo, que visa restaurar a homeostase (CHROUSOS; GOLD, 1992; MCEWEN, 2007). Em situações estressoras prolongadas, o organismo desenvolve condições maladaptativas que culmina em alterações no SNC, as quais afetam progressivamente diversas respostas neurocomportamentais (MCEWEN et al., 1995). Importantemente, diversos transtornos neuropsiquiátricos já são correlacionados com alterações no sistema redox, consecutivamente com o aumento de ERO (BEHL; MOOSMANN, 2002; GUTOWICZ, 2011). Dentre esses transtornos, destaca-se o transtorno de ansiedade (GUNEY et al., 2014; HOVATTA; JUHILA; DONNER, 2010; NIEDZIELSKA et al., 2016), um os maiores problemas mundiais vivenciados na atualidade (KESSLER et al., 2005; RIAZA BERMUDO-SORIANO et al., 2012). Em nossos achados, após a exposição crônica à SA, verificamos o aumento da atividade da CAT e Glutationa S-transferase (GST) e uma diminuição nos níveis de tióis não proteicos (NPSH). Acreditamos que após um estímulo aversivo prolongado, o organismo reage com mecanismos compensatórios não enzimático e enzimático para regular a homeostase. Sugerimos que a exposição crônica à SA induz comportamento do tipo ansiedade e altera paramentos relacionados ao estresse oxidativo em peixe-zebra. Dessa forma, reforçamos a crescente utilidade desta espécie aquática na modelagem de transtornos relacionadas à ansiedade.

O estresse crônico pode desencadear doenças como depressão e transtorno de ansiedade (MCEWEN, 2004). A exposição repetida a agentes estressores pode desregular diversos sistemas de neurotransmissão, bem como o eixo HPA (CHROUSOS, 2009; POPOLI et al., 2011; SANDI; HALLER, 2015). Estudos mostram as respostas comportamentais e bioquímicas envolvidas no ECI (CHAKRAVARTY et al., 2013; MARCON et al., 2016; PIATO et al., 2011; ZIMMERMANN et al., 2016), que incluem aumento da ansiedade e níveis de cortisol, bem como expressão dos marcadores pró-inflamatórios cox-2 e il-6. Porém, pouco se sabe sobre os efeitos do ECP no comportamento e parâmetros bioquímicos em peixe-zebra. Portanto, no terceiro estudo, avaliamos se o ECP com diferentes tipos de estressores altera as respostas comportamentais e endócrinas em peixe-zebra. Para isso, empregamos o protocolo de ECP usando dois tipos de estressores homotípicos naturais, a SA (EGAN et al., 2009; QUADROS et al., 2016) e PR (MEZZOMO et al., 2019; OLIVEIRA et al., 2013). Para a avaliação da resposta do tipo ansiedade, utilizamos os testes do tanque

claro/escuro (MAXIMINO et al., 2010a, 2010b; QUADROS et al., 2016) e do novo tanque (EGAN et al., 2009; ROSEMBERG et al., 2012; WONG et al., 2010), além de mensurar os níveis de cortisol de corpo inteiro (SINK; LOCHMANN; FECTEAU, 2008). Neste trabalho, verificamos que o ECP-SA aumenta o comportamento do tipo ansiedade, bem como os níveis de cortisol. Apesar do ECP-PR não alterar esses parâmetros, nós verificamos que, assim como a exposição à SA, a PR agudamente induz respostas relacionadas ao estresse, corroborando estudos prévios (ABREU et al., 2018; ABREU et al., 2014). Em peixes, o eixo HPI é responsável por regular a cascata para a liberação do cortisol na corrente sanguínea (MOMMSEN; VIJAYAN; MOON, 1999). Como ocorre em humanos, o cortisol é o primeiro glicocorticoide a ser liberado como resposta a reações estressoras. Porém, essa cascata depende de alguns fatores, como intensidade do estressor, frequência, cronicidade e a habilidade que o organismo tem de responder frente ao estresse (KIILERICH et al., 2018). No entanto, em situações de estresse prolongado, alguns estressores homotípicos, podem desregular o eixo HPI, desencadeando respostas de habituação (GRISSOM; BHATNAGAR, 2009; THOMPSON; SPENCER, 1966). Alguns estressores homotípicos não levam ao processo de habituação, por exemplo a predação (GRISSOM; BHATNAGAR, 2009; ZUCCHI et al., 2009). Baseado em nosso estudo, mostramos que após 14 dias de estresse à SA, os robustos comportamentos aversivos foram conservados, bem como o aumento dos níveis de cortisol. Interessantemente, estudos mostram que a não habituação do organismo por um estresse repetido, pode estar relacionado com transtornos associados ao estresse como depressão e transtorno de ansiedade (GRISSOM; BHATNAGAR, 2009). Além disso, a SA pode desencadear sensibilização em peixe-zebra (LIMA et al., 2016), bem como induzir respostas aversivas em um protocolo de aversão condicionada ao lugar até 7 dias após uma única exposição (MAXIMINO et al., 2018). A diferença entre os comportamentos do tipo ansiedade mostrados aqui observados após a exposição aos estressores pode refletir uma regulação neuroendócrina do eixo HPI, onde animais submetidos ao ECP-PR se habituam ao estresse crônico de 14 dias. Portanto, sugerimos a uma associação entre o comportamento do tipo ansiedade e as respostas endócrinas em condições psiquiátricas comuns, uma vez que mostramos que o ECP-SA, aumenta o efeito ansiogênico, bem como os níveis de cortisol em peixe-zebra.

De modo geral, os resultados obtidos nesta tese reforçam a importância de estudar modelos relacionados a doenças neuropsiquiátricas associadas ao estresse. Compreender os mecanismos relacionados ao estresse torna-se imprescindível para esclarecer aspectos neurobiológicos envolvidos em diferentes transtornos mentais. Aqui, nós demonstramos que o

estresse é capaz de exercer diferentes respostas, alterando parâmetros relacionados aos sistemas serotonérgico, estresse oxidativo e endócrino em peixe-zebra, com uma nítida relação com as respostas comportamentais observadas. Dessa forma, o uso do peixe-zebra como organismo modelo alternativo, constitui uma ferramenta emergente para explorar como os transtornos mentais associados podem estar associados com o estresse na pesquisa em neuropsiquiatria translacional.

9. CONCLUSÕES

Resumidamente, como conclusões parciais da presente Tese, podemos afirmar que:

- Os animais de ambas as populações apresentaram características de medo e ansiedade após serem expostos a SA, bem como aumentaram a agressividade em peixe-zebra. Esses efeitos observados foram concomitantes com uma atividade diminuída da enzima Z-MAO. A redução da locomoção observada na exposição crônica serve como justificativa para a redução da agressividade, também observada. Além disso, os efeitos basais são dependentes de população, uma vez que a Z-MAO não difere após a exposição à SA em ambas as populações.
- O comportamento do tipo ansiedade é observado em ambas as populações após a exposição crônica à SA. Além disso, as alterações observadas nos parâmetros oxidativo estreitam a relação no envolvimento do sistema redox com alguns transtornos mentais, tais como ansiedade. Contudo, a diferença entre populações, reforçam nossas hipóteses sobre a influência genética no comportamento de populações de peixe-zebra em diferentes contextos.
- O cortisol aumentou após o ECP-SA, refletindo em uma resposta metabólica em peixe-zebra, bem como foi observado um aumento do comportamento do tipo ansiedade. Por outro lado, a ausência de efeitos significativos após o ECP-PR pode sugerir uma resposta de habituação nos animais, visto que ambos os estressores agudamente induzem respostas de estresse.

Com isso, da mesma forma que ocorre em humanos, as respostas mediadas pelo estresse aumentam o comportamento tipo da ansiedade, com o potencial envolvimento do sistema serotonérgico, estresse oxidativo e endócrino em peixes-zebra. Dessa forma, nossos resultados irão servir de suporte para estudos mais avançados com o intuito de elucidar o mecanismo de ação associados a diferentes transtornos psiquiátricos associados e situações de estresse.

10. PERSPECTIVAS

Após a verificação dos efeitos do estresse em nosso trabalho com peixe-zebra, tais como agressão e Z-MAO (QUADROS et al., 2018), ansiedade e estresse oxidativo (QUADROS et al., 2019), bem como ECP e cortisol (Manuscrito Científico), a busca pela elucidação do mecanismo envolvido em transtornos mentais associados com o estresse torna-se ainda mais importante.

Compreender como o estresse atua nos transtornos mentais é determinante para entendermos as vias primárias de muitas doenças. Dessa forma, buscar novos protocolos relacionados ao estresse que visam avaliar testes comportamentais e bioquímicos em modelos de transtornos neurocomportamentais é uma estratégia para a expansão de novos estudos. A SA é um ótimo estressor, e pouco se sabe dos efeitos crônicos no SNC. Portanto, investigar o papel da SA no estresse irá contribuir para elucidar emoções como medo e ansiedade em peixe-zebra, que poderiam desenvolver alguns transtornos mentais. Com isso, através de análises mais profundas do estresse, a investigação dos sistemas serotonérgico, dopaminérgico e noradrenérgico, também é uma importante perspectiva deste estudo.

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ANEXO I – CARTA DE SUBMISSÃO DO MANUSCRITO CIENTÍFICO

Hormones and Behavior

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ANEXO II - PRODUÇÕES CIENTÍFICAS EM COLABORAÇÃO

Artigos produzidos em parceria durante o período do Doutorado:

1. ROSA, LUIZ V.; COSTA, FABIANO V.; CANZIAN, JULIA; BORBA, JOÃO V.; **QUADROS, VANESSA A**.; ROSEMBERG, DENIS B. Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses. PROGRESS IN NEURO-PSYCHOPHARMACOLOGY & BIOLOGICAL PSYCHIATRY, v. 102, p. 109957, 2020.

2. MEZZOMO, NATHANA J.; FONTANA, BARBARA D.; MÜLLER, TALISE E.; DUARTE, TÂMIE; **QUADROS, VANESSA A**.; CANZIAN, JULIA; POMPERMAIER, ALINE; SOARES, SUELEN M.; KOAKOSKI, GESSI; LORO, VANIA L.; ROSEMBERG, DENIS B.; BARCELLOS, LEONARDO J.G. Taurine modulates the stress response in zebrafish. HORMONES AND BEHAVIOR, v. 109, p. 44-52, 2019.

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Selenite Prevents Paraquat-Induced Neurotoxicity in Zebrafish. MOLECULAR NEUROBIOLOGY, v. 6, p. s12035-017-0441, 2017.

9. ROSA, LUIZ VINÍCIUS; ARDAIS, ANA PAULA; COSTA, FABIANO VARGAS; FONTANA, BARBARA DOTTO; QUADROS, **VANESSA ANDREATTA**; PORCIÚNCULA, LISIANE OLIVEIRA; ROSEMBERG, DENIS BROOCK. Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR, v. 165, p. 1-8, 2017.

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