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Luiz Vinicius Costa da Rosa

ANÁLISE DO COMPORTAMENTO SOCIAL DO PEIXE-ZEBRA: UMA ABORDAGEM SOBRE RESPOSTAS DO TIPO ANSIEDADE E NOCIFENSIVAS

Santa Maria, RS
2022

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Tese de doutorado apresentado ao programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, do Centro de Ciências Naturais e Exatas da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção de título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Denis Broock Rosemberg

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Aprovado em 28 de abril de 2022

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A todos colegas cientistas no Brasil, os quais lutam pelo avanço científico frente ao atual cenário de obscurantismo em nosso país.

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“Negar a ciência é muito mais fácil do que aprende-la.”.

(Mario Bunge)

“A ciência consiste em substituir o saber que parecia seguro por uma teoria, ou seja, por algo problemático”.

(José Ortega y Gasset)

Assim como casas são feitas de pedras, a ciência é feita de fatos. Mas uma pilha de pedras não é uma casa e uma coleção de fatos não é, necessariamente, ciência”.

(Jules Henri Poincare)

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

ANÁLISE DO COMPORTAMENTO SOCIAL DO PEIXE-ZEBRA:
UMA ABORDAGEM SOBRE RESPOSTAS DO TIPO ANSIEDADE E NOCIFENSIVAS

AUTOR: LUIZ VINICIUS COSTA DA ROSA
ORIENTADOR: DENIS BROOCK ROSEMBERG

O comportamento social desempenha um papel adaptativo essencial em diversas espécies no reino animal. Em virtude do papel biológico deste comportamento, o uso de modelos de pesquisa em animais sociais, como o peixe-zebra (*Danio rerio*), apresenta-se como uma estratégia para a compreensão dos processos biológicos associados a esse complexo comportamento. Visando elucidar aspectos relacionados ao comportamento social do peixe-zebra, buscamos aqui caracterizar as respostas comportamentais frente a diferentes contextos, abordando situações de estímulos ansiogênicos e nociceptivos. Em um primeiro estudo, desenvolvemos uma nova metodologia de análise tridimensional (3D) da ocupação espacial de cardumes de peixe-zebra e analisamos as alterações comportamentais induzidas pela substância de alarme de coespecíficos (CAS), cafeína (CAF) (substâncias ansiogênicas) ou diazepam (DZP) (substância ansiolítica). As análises 3D comportamentais revelaram que a CAS e a CAF diminuíram o volume do cardume, distância média dos peixes em relação ao ponto centroide do cardume e aumentaram a geotaxia, mas apenas a CAS reduziu a distância média entre peixes quando comparados ao grupo controle (CTRL). Por outro lado, o DZP aumentou o volume do cardume e distância entre peixes. Além dos achados referentes às manipulações farmacológicas, realizamos uma comparação entre análises bidimensionais (2D) e 3D, as quais demostraram diferenças interpretativas dos resultados comportamentais devido às limitações das análises 2D, que subestimam a distribuição dos indivíduos em uma perspectiva dimensional. Desta maneira, o experimento demostrou a importância da otimização das técnicas de análises, afim de representar mais precisamente a ocupação espacial de grupo de animais. Em um segundo estudo, utilizando a metodologia 3D desenvolvida, investigamos os efeitos do comportamento nocifensivo nas respostas sociais no peixe-zebra. Para esta finalidade, analisamos as respostas comportamentais antes e após injeção intraperitoneal (i.p.) de tampão fosfato-salino (PBS) (CTRL), ácido acético 5% (AA), morfina 2,5 mg/kg (MOR) ou ácido acético 5% mais morfina 2,5 mg/kg (AA+MOR) em apenas um indivíduo de cardume de quatro peixes. Além disso, testamos a preferência social por cardumes tratados com PBS, AA ou por aquário vazio. Os dados revelaram que a administração i.p. de AA desequilibra a homogeneidade do cardume, por induzir dispersão do peixe manipulado e agrupamento dos animais não manipulados. Além disso, a coadministração de morfina protegeu dos efeitos comportamentais induzidos por AA. O teste de preferência social revelou uma ampla preferência por cardumes PBS em relação ao AA. Portanto, o segundo estudo mostrou que a nocicepção é capaz de modular a sociabilidade do peixe-zebra, bem como a capacidade de reconhecimento visual de comportamentos nocifensivos. Em suma, os dados dos estudos desenvolvidos nesta tese demostram a plasticidade do comportamento social do peixe-zebra como uma resposta adaptativa defensiva frente a mudanças aversivas. De modo geral nossos achados que poderão servir de suporte para refinar as metodologias de análise comportamental, bem como as de bem-estar animal desta espécie em ambientes laboratoriais

Palavras chave: Peixe-zebra; comportamento social; análise tridimensional; ansiedade, dor, nocicepção.

ABSTRACT

Ph.D. Thesis
Graduate Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

ZEBRAFISH SOCIAL BEHAVIOR ANALYSIS:
AN ANXIETY-LIKE AND NOCIFENSIVE RESPONSES APPROACH

AUTHOR: LUIZ VINÍCIUS COSTA DA ROSA
ADVISOR: DENIS BROOCK ROSEMBERG

Social behavior plays a key adaptative role in several species on animal kingdom. Due to the biological role of this behavior, the use of social animal, such as the zebrafish (*Danio rerio*), represents a strategy to understand the biological processes associated which sociability. Aiming to elucidate how social behavior are affected by different contexts, we characterized the neurobehavioral responses in the presence of modulators of anxiety and nociceptive stimuli. In first paper, we developed a new three-dimensional (3D) analysis methodology of the shoal spatial occupation and investigated the behavioral changes induced byconspecific alarm substance (CAS), caffeine (CAF) (anxiogenic substances) and diazepam (anxiolytic substance). Behavioral 3D analyses revealed that both CAS and CAF decreased the shoal volume, average fish distance to the centroid point, and increased geotaxis, but only CAS reduced the inter-fish distance when compared to control (CTRL). Conversely, DZP group showed increased shoal volume and inter-fish distance. Thus, these data revels the importance of 3D analyses to represent the spatial occupation of zebrafish groups. In a second study, using the 3D developed protocol, we investigated the effects of nocifensive behavior on zebrafish social responses. For this purpose, we analyzed the behaviors before and after an intraperitoneal injection (i.p.) of phosphate-buffered saline (PBS) (CTRL), acetic acid 5% (AA), morphine 2.5 mg/kg (MOR) or acetic acid 5% plus morphine 2.5 mg/kg (AA+MOR) in only one subject from a four-fish shoal. Furthermore, we tested the social preference to PBS-, AA-treated shoals, or empty aquarium. In general, AA administration disrupted the shoal homogeneity, by eliciting dispersion of manipulated fish and clustering of non-manipulated fish. Moreover, morphine coadministration protected against AA-induced behavioral changes. Social preference test revealed a markedly preference to PBS shoals over AA. Therefore, this second study shows that the nociception can modulate zebrafish sociability. Overall, our findings presented here demonstrate the plasticity of the zebrafish social behavior as an adaptative defensive behavior. Altogether, the current thesis can support behavioral analysis methodology refinement, as well as the welfare of this specie in laboratorial facilities.

Keywords: Zebrafish, social behavior, three-dimensional analysis, anxiety, pain, nociception

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

2D – Bidimensional
3D – Tridimensional
5-HIIA – ácido 5 hidroxi indol acético
A_1 – Receptor de adenosina do tipo inibitório A_1
A_{2A} – Receptor de adenosina do tipo facilitatório A_{2A}
A_{2B} – Receptor de adenosina do tipo facilitatório A_{2B}
A_3 – Receptor de adenosina do tipo inibitório A_3
AA – Ácido acético
AA + MOR – Ácido acético e morfina
ASIC – Canais iônicos sensíveis a ácidos
CAF – Cafeína
CAS – Substância de alarme de coespecífico
DOPAC – Ácido 3,4-di-hidroxifenilacético
DZP – Diazepam
$GABA_A$ – Receptor do ácido gama-aminobutírico do tipo A
HPI – Eixo Hipotalâmico-pituitária-intrarrenal
MOR – Morfina
PBS – Tampão fosfato-salino
TLRs – Receptores “tool-like”
TRPs – Receptores de potencial transitórios

SUMÁRIO

1 APRESENTAÇÃO

Esta tese está constituída em diferentes seções mencionadas a seguir. No item **INTRODUÇÃO**, consta uma revisão da literatura sobre os temas abordados nesta tese. A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob forma de um artigo cientifico publicado e um manuscrito científico, os quais se encontram no item **DESENVOLVIMENTO.**

As secções Materiais e Métodos, Resultados e Discussão encontram-se no artigo e manuscrito científico, representando a íntegra deste estudo. Os itens **DISCUSSÃO**, **CONCLUSÃO**, **PERSPECTIVAS**, **REFERÊNCIAS BIBLIOGRÁFICAS** E **ANEXOS** encontram-se no final desta tese. Os itens **DISCUSSÃO**, **CONCLUSÃO** e **PERSPECTIVAS** visam discutir os trabalhos de forma integrada, bem como apresentar uma conclusão geral sobre os achados relacionados ao tema com a perspectivas futuras. As **REFERÊNCIAS BIBLIOGRAFICAS** referem-se somente as citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO** desta tese.

2 INTRODUÇÃO

2.1 COMPORTAMENTO SOCIAL

O comportamento social é um fenômeno complexo que existe em diferentes espécies na natureza (WYNNE-EDWARDS, 1962). Dentre as diversas vantagens adaptativas promovidas por esse comportamento, podemos citar o aumento da eficiência reprodutiva, sucesso em fugas de predadores, transferência de informação, vigilância, bem como o aumento da eficácia no forrageamento (KILLEN et al., 2017; MILLER R. C., 1922; TREHERNE; FOSTER, 1981). Assim como observado em diferentes organismos, o ser humano possui comportamento social inato (FEHR; FISCHBACHER, 2003). O comportamento social do homem é um dos componentes essenciais no sucesso evolutivo da espécie por desempenhar um grande papel na comunicação humana, assim, facilitando a reprodução, a sobrevivência e o bem estar emocional (COHEN; UNDERWOOD; GOTTLIEB, 2000; ROBINSON et al., 2019; YOUNG, 2008). É sabido que o comportamento social pode ser alterado em diversas patologias como depressão, ansiedade, estresse pós-traumático, esquizofrenia, transtorno bipolar e do espectro autista (BARON-COHEN et al., 1999; PERUGI et al., 2001; VAN AMERINGEN et al., 1991; WASHBURN et al., 2016). Em relação aos animais laboratoriais, já é demonstrado que as interações sociais em espécies com comportamento social inato são de fundamental importância para a saúde e bem-estar, e sua ausência leva a diversos efeitos negativos (HAN et al., 2018; HOUSE; LANDIS; UMBERSON, 1988; SHAMS et al., 2017; ZORZO et al., 2019). Situações ambientais que afetam a socialização humana, como por exemplo o distanciamento e isolamento social, podem propiciar o surgimento de transtornos psiquiátricas como ansiedade, depressão e agressividade (CAMPAGNE, 2019; HAWKLEY; CAPITANIO, 2015; LOADES et al., 2020; MATTHEWS et al., 2015). Ainda, indivíduos que sofrem por isolamento, distanciamento social e solidão possuem maior índice de mortalidade (ALCARAZ et al., 2019; HOLT-LUNSTAD et al., 2015; HOUSE; LANDIS; UMBERSON, 1988). Por fim, considerando a importância adaptativa do comportamento social, o uso de espécies sociais como sujeito de pesquisa se mostra uma interessante estratégia de pesquisa para elucidar os mecanismos relacionados a esse e tipo de comportamento.

2.2 USO DO PEIXE-ZEBRA EM ESTUDOS DO COMPORTAMENTO SOCIAL

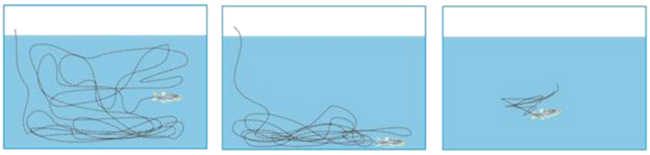
Dentre as espécies amplamente utilizadas em pesquisas de neurociência comportamental e que apresentam como característica um robusto comportamento social está o peixe-zebra (*Danio rerio*) (BUSKE; GERLAI, 2011; ENGESZER et al., 2007b; PRITCHARD et al., 2001). Essa espécie é um pequeno ciprinídeo de água doce nativo da Ásia, que em seu ambiente natural forma pequenos cardumes (3 - 10 peixes por grupo) (ENGESZER et al., 2007a; SURIYAMPOLA et al., 2016), cujo comportamento social é de fácil replicabilidade e reprodutibilidade em condições laboratoriais (BUSKE; GERLAI, 2012; GERLAI et al., 2000; MILLER; GERLAI, 2007). O comportamento social do peixe-zebra desenvolve-se conforme o amadurecimento do indivíduo, demostrando um robusto comportamento social a partir de 30-40 dias pós fertilização (BUSKE; GERLAI, 2011), podendo estar correlacionado com aumento da concentração de dopamina, DOPAC (Ácido 3,4-di-hidroxifenilacético), serotonina e 5-HIIA (ácido 5 hidroxi indol acético) cerebral (BUSKE; GERLAI, 2012). Além disso, é sabido que o antagonismo dopaminérgico reduz a preferência por coespecíficos em peixe-zebra (SCERBINA; CHATTERJEE; GERLAI, 2012), reforçando o papel monoaminérgico sobre o comportamento social em peixe-zebra. Ainda, larvas que possuem atividade neuroendócrina comprometida por mutação em *disc1*, apresentam atividade social prejudicada (EACHUS et al., 2017), demostrando um possível envolvimento do eixo de estresse hipotalâmico-pituitária-inter-renal (HPI), dos peixes teleósteos (WENDELAAR BONGA, 1997) no comportamento social. Desta maneira, sugere-se que o comportamento social é resultante da ativação e sincronização complexa de vias de sinalização molecular distintas em peixe-zebra.

Em relação às vantagens apresentadas pelo peixe-zebra em estudos neurocomportamentais, podemos citar o pequeno tamanho (adultos medem cerca de 3 a 5 cm), o baixo custo e o reduzido espaço para manutenção (LELE; KRONE, 1996). Outra vantagem do peixe-zebra é o complexo comportamento de natação em três direções, que permite reconstruções tridimensionais da locomoção e posicionamento (CACHAT et al., 2011; ROSA et al., 2018), permitindo dessa maneira, uma detalhada análise fenotípica das respostas comportamentais. Ainda, o genoma do peixe-zebra é completamente sequenciado e seus genes possuem um alto grau de similaridade com os genes humanos, bem como também apresenta os principais sistemas de neurotransmissores caracterizados nos mamíferos, o que possibilita seu uso como organismo modelo para o estudo de doenças humanas (HOWE et al., 2013; RICO et al., 2011). Dessa forma, o peixe-zebra convém como uma ferramenta translacional no campo da neurociência comportamental, permitindo a investigação das bases neurológicas dos fenótipos comportamentais.

2.3 COMPORTAMENTO SOCIAL COMO RESPOSTA DEFENSIVA EM PEIXE-ZEBRA.

Estímulos potencialmente aversivos são comumente utilizados para a investigação de fenótipos comportamentais relacionados a respostas defensivas em peixe-zebra (EGAN et al., 2009; QUADROS et al., 2016, 2021). Tais estímulos induzem respostas comportamentais, como o aumento da geotaxia, nado errático, episódios de congelamento, tigmotaxia, escototaxia, aumento da coesão do cardume **(Figura 1)** (CANZIAN et al., 2017; MAXIMINO et al., 2010; ROSEMBERG et al., 2011; SACKERMAN et al., 2010). Baseado nesse princípio, diversas técnicas são realizadas para induzir o comportamento defensivo em ambientes laboratoriais, como por exemplo, a presença de um fator novidade (EGAN et al., 2009), perseguição por rede (MEZZOMO et al., 2019), presença de predador (FONTANA et al., 2018), uso de sinalizadores químicos de alarme (ex. substancia de alarme de coespecíficos - CAS) (QUADROS et al., 2016; SPEEDIE; GERLAI, 2008). Além disso, estímulos aversivos induzem tanto respostas individuais como respostas de grupo em peixe-zebra (CANZIAN et al., 2017; EGAN et al., 2009).

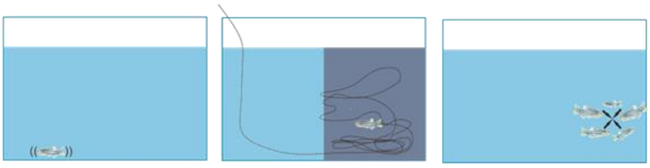
Figura 1. Exemplo de comportamentos defensivos do peixe-zebra.



Exploração normal

Geotaxia
(Preferência pelo fundo)

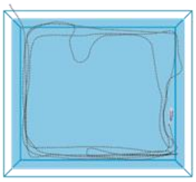
Nado errático
(Nado rápido e sem direção)



Congelamento
(imobilidade e hiperventilação)

Escototaxia
(Preferência por ambientes escuros)

Agrupamento

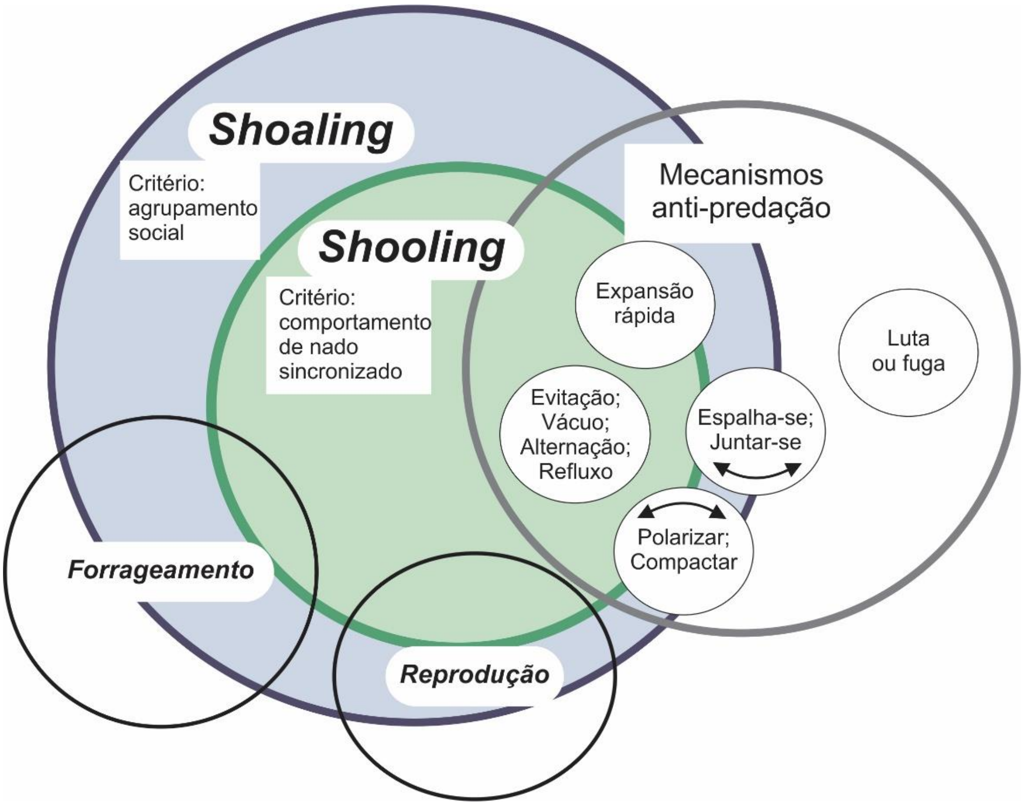


Tigmotaxia
(Preferência pela periferia)

Fonte: Do autor.

Em situações normais ou que o animal adulto possua habituação, o peixe-zebra apresenta uma preferência social inata, formando cardumes com nado não polarizado (KALUEFF et al., 2013; MILLER; GERLAI, 2012). Contudo, em situações estressantes ou potencialmente aversivas, induz-se um aumento da coesão do cardume (*shoaling*) como uma resposta defensiva, podendo chegar a apresentar um nado polarizado e organizado (*schooling*) (BUSKE; GERLAI, 2012; MILLER; GERLAI, 2012; PITCHER, 1986). Este tipo de comportamento confere vantagens anti-predatórias por causar confusão em predadores (BODE et al., 2010) **(Figura 2)**.

Figura 2. Diagrama do papel biológico do "shoaling e schooling" em peixes teleósteos sociais.



Fonte: adaptado de PITCHER., 1986

Além disso, respostas defensivas individuais ou de grupo podem ser induzidas através da manipulação farmacológica de substâncias ansiogênicas, como a CAS e a cafeína (CAF); ou podem ser atenuado por substâncias ansiolíticas como Diazepam (DZP) (CANZIAN et al., 2017; GEBAUER et al., 2011; LADU et al., 2015; ROSA et al., 2018).

2.4 MODULAÇÃO FARMACOLÓGICA DO COMPORTAMENTO SOCIAL DO PEIXE-ZEBRA

É descrito na literatura que diversas substâncias são capazes de alterar o comportamento social em vertebrados (BLANCO-GANDÍA et al., 2015; LÓPEZ-CRUZ et al., 2016; OSBORNE et al., 2017). Em peixe-zebra, drogas que modulam o comportamento tipo ansiedade, como por exemplo o etanol, taurina, CAS, CAF e DZP, também influenciam o comportamento social (CANZIAN et al., 2017; FONTANA et al., 2018; GEBAUER et al., 2011; GIACOMINI et al., 2016; LADU et al., 2015; MÜLLER et al., 2017).

A CAF (1,3,7-trimetilxantina) é um antagonista não seletivo dos receptores adenosina de ação inibitória (A_1 e A_3) e facilitatória (A_{2A}, e A_{2B}) (FRANCIS et al., 2011; FREDHOLM et al., 1999; GUERREIRO; MARIEN; MICHEL, 2011; MÜLLER; JACOBSON, 2011). O antagonismo dos receptores adenosinérgicos é considerado o principal mecanismo responsável pelos efeitos majoritários da cafeína no sistema nervoso central, o qual resulta em diferentes alterações neurofisiológicas. Tais modificações exercem influência na fisiologia de diversos sistemas de neurotransmissão, às quais podem culminar em diferentes aspectos neurocomportamentais (ex: agressividade, atenção, ansiedade e comportamento social)(BOTTON et al., 2017; CUNHA, 2001; LADU et al., 2015; MAHDI et al., 2019; MCLELLAN; CALDWELL; LIEBERMAN, 2016).

Em relação à CAS, essa substância está presente nas células epiteliais em peixes teleósteos, sendo liberada no ambiente quando a pele do peixe é lesionada. A CAS é detectada pelo sistema olfativo dos peixes coespecíficos e exerce uma sinalização química que resulta em respostas comportamentais do tipo “luta ou fuga” dos animais (MOURABIT et al., 2010), e induz a resposta defensivas de grupo (SPEEDIE; GERLAI, 2008). Além disso, é caracterizada por produzir comportamento do tipo medo, pois é capaz de aumentar a expressão do gene *c-fos* na habênula em peixe-zebra (OGAWA; NATHAN; PARHAR, 2014). Por último, o DZP é um benzodiazepínico clássico que influencia a atividade dos receptores inibitórios $GABA_A$, causando um robusto efeito ansiolítico (BANDARA et al., 2020; GEBAUER et al., 2011). Em peixe-zebra, os efeitos da exposição ao DPZ afetam o eixo HPI, reduzindo a liberação de cortisol (DE ABREU et al., 2014).

Desta maneira, a utilização de duas substâncias com ação ansiogênica e um fármaco classicamente utilizado como ansiolítico é de grande valia para a investigação de novos fenótipos comportamentais em uma análise tridimensional, os quais poderão servir como base no estudo de potenciais fármacos ou sobre a influência de contextos ambientais no comportamento social em peixe-zebra.

2.5 COMPORTAMENTO SOCIAL E MUDANÇA DE CONTEXTO

Devido ao comportamento social ser um comportamento complexo que depende da interação de um grupo de indivíduos, aspectos individuais, de contexto social ou ambiental acabam por interferir tanto no comportamento dos indivíduos como do grupo (BHAT; GREULICH; MARTINS, 2015; ENGESZER et al., 2007a; NERI et al., 2019). Um estudo recente mostrou que a presença de coespecíficos modifica a resposta de nado de peixes-zebra expostos à CAF, quando comparado a animais testados isoladamente (NERI et al., 2019), outro estudo demonstrou que a presença de animais estressados em um cardume eleva o nível de cortisol de coespecíficos (GIACOMINI et al., 2015). Além disso, estímulos visuais e olfatórios da presença de coespecíficos alteram as respostas defensivas de congelamento de peixes-zebra expostos à CAS, reforçando a importância de se identificar e caracterizar os fatores que afetam o comportamento social (FAUSTINO; TACÃO-MONTEIRO; OLIVEIRA, 2017). Apesar de já estar descrito na literatura a influência do estímulo social na resposta comportamental frente à exposição de substâncias ansiogênicas, a influência de um indivíduo que apresenta comportamento alterado nas respostas comportamentais dos seus coespecíficos do grupo ainda foram completamente exploradas.

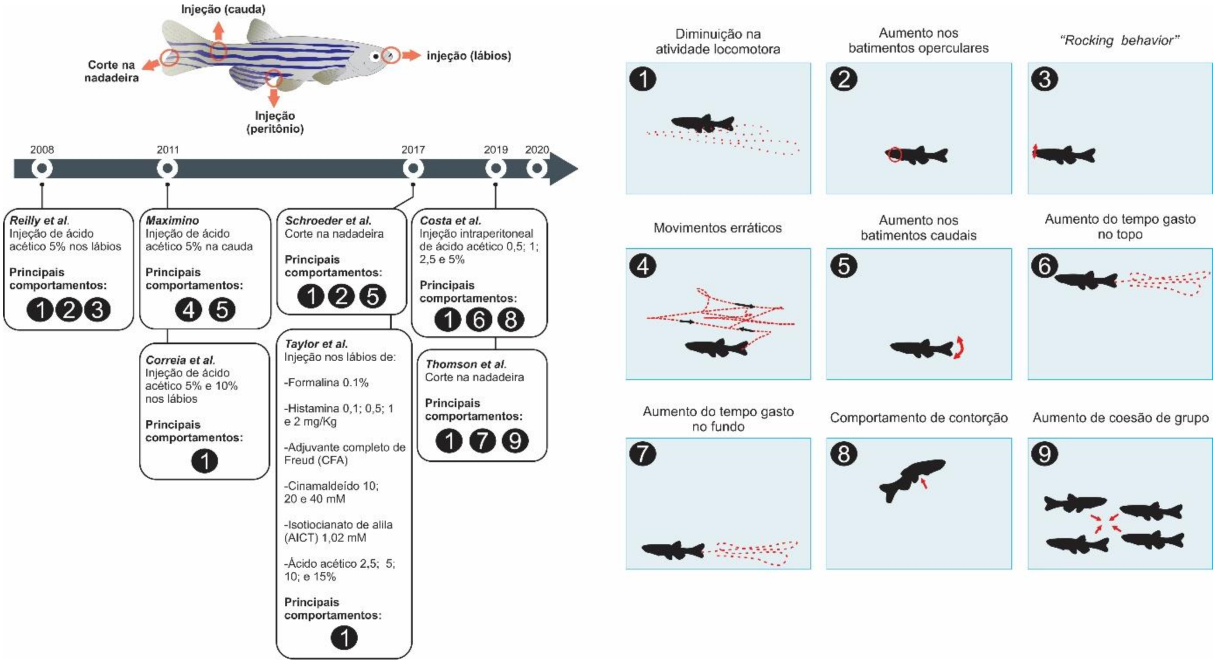
Como sabido, estímulos aversivos causam alterações comportamentais marcantes em peixe-zebra. A nocicepção, transmissão de sinalização nervosa por nociceptores, é um forte estímulo aversivo que gera um rico repertório de respostas comportamentais nocifensivas (comportamento do tipo dor) em peixe-zebra (COSTA et al., 2019; MAXIMINO, 2011; REILLY et al., 2008b). Além disso, já foi demonstrado que a nocicepção aumenta a liberação de cortisol em tilápias (ROQUES et al., 2012) e peixe-zebra (dados do grupo ainda não publicados), o que evidencia o efeito modulatório da dor no comportamento e em marcadores de estresse em peixes teleósteos. Entretanto, a influência desse estímulo em cardumes com a presença de um animal sob efeito nociceptivo não é bem elucidada em peixe-zebra. Assim, o estudo de uma possível alteração no componente social por um estímulo nociceptivo fornecerá achados que ajudaram a aperfeiçoar a análise de bem-estar animal em ambiente laboratoriais e em manipulações experimentais.

2.6 NOCICEPÇÃO EM PEIXE-ZEBRA

A nocicepção é um evento de sinalização que envolve a transmissão de sinais nervosos por nociceptores em fibras nervosas primárias aferentes em resposta a estímulos mecânicos, térmicos ou químicos potencialmente nocivos (BASBAUM et al., 2009; LOESER; TREEDE, 2008). Estes estímulos chegam ao sistema nervoso central, gerando a percepção de dor e desencadeando respostas comportamentais adaptativas, conhecido como comportamentos nocifensivos. Devido a importância biológica da nocicepção como sinal de percepção de estímulos nocivos (DARWIN, 1872; WOOLF, 2010), a caracterização dos fenótipos comportamentais expressos na presença de algógenos é um componente chave para avaliar as respostas comportamentais individuais e de grupo relacionadas à nocicepção (COSTA et al., 2019; THOMSON et al., 2019).

Embora majoritariamente os roedores sejam utilizados na pesquisa da dor (Stevenson et al., 2006; Meotti e Santos, 2010), estudos experimentais utilizando peixe-zebra como sujeito de pesquisa têm ajudado a avaliar os mecanismos evolutivamente conservados subjacentes à nocicepção e seus fenótipos comportamentais correlatos (COSTA et al., 2019; GONZALEZ-NUNEZ; RODRÍGUEZ, 2009; REILLY et al., 2008b). Peixes teleósteos apresentam mecanismos evolutivamente conservados envolvidos na nocicepção em comparação com mamíferos, répteis e aves (REILLY et al., 2008a; SNEDDON, 2002, 2003; SNEDDON; BRAITHWAITE; GENTLE, 2003). Nos humanos, as fibras C estão amplamente distribuídas pelo sistema nervosos compreendendo em torno de 50% das fibras sensoriais (YOUNG; KING, 1973). Entretanto, nos peixes teleósteos as fibras C são encontradas aproximadamente 4%, as fibras A-beta são as mais comuns (53%) seguido por A-delta (33%) e A-alpha (9%) (SNEDDON, 2002). Essa diferença de fibras C entre humanos e peixes pode estar relacionada a um processo evolutivo dos animais terrestres, os quais estão mais expostos às mudanças mais drásticas de temperatura, agente químicos nocivos e maior chance de lesões mecânicas (SNEDDON, 2002). Apesar das diferenças neuroanatômicas, o peixe-zebra é sensível a vários algógenos e substâncias analgésicas, demostrando um rico repertório comportamental (**Figura 3)** (COSTA et al., 2019; MAXIMINO, 2011; REILLY et al., 2008b; SCHROEDER; SNEDDON, 2017). ,3

Figura 3. Figura representativa dos principais fenótipos comportamentais relacionado a nocicepção em peixe-zebra.



Fonte: adaptado de COSTA et al., 2021

Além disso, já foram caracterizados no peixe-zebra receptores relacionados à nocicepção homólogos aos dos humanos, como por exemplo: os canais iônicos receptores de potencial transitórios (TRPs), canais iônicos sensíveis a ácidos (ASICs) e receptores “tool-like” (TLRs) (GAU et al., 2013; WATZKE; SCHIRMER; SCHOLZ, 2007), demostrando o potencial do peixe-zebra como modelo de nocicepção em uma perspectiva translacional. Embora a rápida acessão de estudos que caracterizam as respostas comportamentais e fisiológicas relacionadas a dor em peixe-zebra na atual literatura, a caracterização dos efeitos da nocicepção nos comportamentos sociais em grupos heterogênicos de peixes é pouco compreendida.

3 JUSTIFICATIVA

A utilização de peixe-zebra em estudos neurocomportamentais vem se mostrando uma promissora estratégia na elucidação do comportamento social (BUSKE; GERLAI, 2011, 2012; FAUSTINO; TACÃO-MONTEIRO; OLIVEIRA, 2017; SHAMS et al., 2017), o qual desempenha um papel fundamental na saúde, bem-estar e sobrevivência de diversas espécies, incluído o ser humano (COHEN; UNDERWOOD; GOTTLIEB, 2000; KILLEN et al., 2017). Devido ao papel exercido por esse comportamento para a sobrevivência, torna-se importante o desenvolvimento de estratégias visando avaliar o componente social com o intuito de auxiliar na compreensão de fenômenos (intrínsecos e extrínsecos) relacionados à sociabilidade e ao isolamento frente a diferentes contextos. Assim, o estudo comportamental usando espécies sociais como sujeito de pesquisa se demonstra uma promissora ferramenta experimental para esclarecer os paradigmas desse complexo comportamento numa perspectiva translacional (GERLAI et al., 2000; SOARES et al., 2018; SOARES; GERLAI; MAXIMINO, 2018).

Considerando a complexidade do comportamento social, o qual sofre influência multifatorial, e sua importância biológica, é importante otimizar as técnicas de análises de fenótipos comportamentais existentes, o que pode permitir uma rica e detalhada análise do repertório comportamental da espécie em estudo. Um exemplo de estratégia para aumentar o poder de análise comportamental em peixe-zebra é a exploração de técnicas para avaliação tridimensional do padrão de comportamento (CACHAT et al., 2011; MACRÌ et al., 2017; ROSA et al., 2018). Entretanto, esse tipo de abordagem ainda é pouco explorado com relação à ocupação espacial de cardumes. Em geral, esperamos que este estudo possibilite uma melhor caracterização da resposta espaço-temporal do comportamento social do peixe-zebra, além de fornecer uma nova técnica de fácil utilização e de baixo custo para análise de comportamento social em vertebrados.

A fim de contribuir na compreensão do envolvimento da nocicepção no comportamento social do peixe-zebra, investigamos também o comportamento social de um cardume de peixe-zebra na presença de um indivíduo sob resposta nocifensiva. Além disso, analisamos a preferência social por animais com comportamento tipicamente normais frente a animais com comportamento nocifensivo, investigando uma possível distinção visual entre os tipos de cardumes supracitados.

4 HIPÓTESES

- O uso de uma análise tridimensional do comportamento de um cardume do peixe zebra apresenta sensibilidade a diferentes manipulações do comportamento do tipo ansiedade, o que poderá levar ao aprimoramento do poder de análise dos testes de sociabilidade na espécie;

- A análise comportamental tridimensional pode apresentar discrepâncias nas análises de parâmetros comportamentais em relação às análises bidimensionais, bem como a comparação entre dados das análises bidimensionais de perspectivas visuais diferentes apresentarão discrepâncias entre si;

- A presença de um indivíduo com comportamento nocifensivo altera o comportamento basal de um cardume de peixes-zebras;

- O peixe-zebra é capaz de identificar visualmente animais com comportamento nocifensivo, bem como distingui-los dos animais com comportamento padrão.

5 OBJETIVOS

5.1 OBJETIVO GERAL

Caracterizar os efeitos promovidos por moduladores do comportamento do tipo ansiedade, bem como as alterações no comportamento social do peixe-zebra na presença de peixes com respostas nocifensivas.

5.2 OBJETIVO ESPECÍFICOS

Utilizando peixes-zebra adultos do tipo selvagem, o presente projeto tem como objetivos específicos:

- Desenvolver um método de análise tridimensional de ocupação espacial de cardume que seja acessível e de fácil utilização, a fim de analisar o comportamento de grupo, bem como identificar fenótipos comportamentais que possam ser observados nessa metodologia;
- Explorar as ações promovidas por moduladores de ansiedade sobre o comportamento social do peixe-zebra através de análise tridimensional, e compará-las com dados obtidos por análises bidimensionais;
- Analisar a resposta comportamental de grupo relativo na presença de um animal com comportamento tipo de dor, utilizando uma análise de perspectiva tridimensional;
- Investigar a preferência entre cardumes com comportamento padrão e comportamento do nocifensivo, e uma possível identificação visual entre estes cardumes através do teste de preferência social.

6. DESENVOLVIMENTO

As metodologias utilizadas e os resultados desta tese estão demonstrados na forma de um artigo científico publicado e um manuscrito científico submetido para publicação em periódicos internacionais de relevante fator de impacto na área.

- O artigo científico foi publicado na revista *Progress in Neuro-Psychopharmacology and Biological Psychiatry* n° 102 no ano de 2020 (Qualis Referência 2016-2020 CAPES A1, FI: 5.067) intitulado: "Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses".

- O Manuscrito Científico está submetido à revista *Behavioural Brain Research* (Qualis Referência 2016-2020 CAPES A1, FI: 3.332) e se intitula: "Acetic acid-induced nociception modulates sociability in adult zebrafish: influence on shoaling behavior in heterogeneous groups and social preference".

6.1 ARTIGO CIENTÍFICO

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Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses

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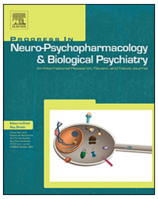

Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses

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ABSTRACT

Social behaviors are key components that play adaptive roles in various species, including humans. The zebrafish (*Danio rerio*) is a social species and the shoaling behavior can be pharmacologically manipulated either by anxiogenic or anxiolytic substances, providing translatable data in neuropsychiatric research. Here, we aimed to characterize the shoaling behavior in zebrafish under different pharmacological manipulations in a three-dimensional (3D) perspective using the spatial coordinates of the fish positions. Temporal and spatial reconstructions of shoal occupancy were performed after exposure to conspecific alarm substance (CAS) and caffeine (CAF) (anxiogenic substances) or diazepam (DZP) (a classical anxiolytic drug). Behavioral 3D analyses and spatiotemporal reconstructions of the shoaling behavior revealed that both CAS and CAF decreased the shoal volume, the average fish distance to the centoid point, and increased shoal geotaxis, but only CAS reduced the inter-fish distance when compared to control (CTRL). Conversely, DZP group showed increased shoal volume and inter-fish distance. Because substantial differences were verified when the shoaling response was analyzed in 3D and 2D perspectives, we reinforce the use of 3D reconstructions of fish positions to assess how different manipulations affect the social behavior of zebrafish. The novel procedure described here represents an easy-to-use, inexpensive, and alternative tool to perform a spatiotemporal reconstruction of the shoal occupancy under different pharmacological manipulations, complementing the existing quantification of locomotion activity of multiple fish.

1. Introduction

Social behavior is a natural phenomenon found in various species that offers adaptive advantages, such as predator avoidance, information transfer, reproductive success, and foraging (Killen et al., 2017; Miller, 1922; Treherne and Foster, 1981). In humans, the social behavior represents a key factor to improve communication, thereby facilitating reproduction, survival, and emotional well-being (Robinson et al., 2019). Abnormal social behavior is a comorbidity of several psychiatric diseases, including anxiety-releated disorders, depression, autism spectrum disorders, schizophrenia, and bipolar disorder (Baron-Cohen et al., 1999; Perugi et al., 2001; Van Ameringen et al., 1991; Washburn et al., 2016). Thus, the use of experimental model organisms with high sociality represents a promising strategy to investigate the social behavior in a translational perspective (Gerlai et al., 2000; Soares et al., 2018a; Soares et al., 2018b).

The zebrafish (*Danio rerio)* is a freshwater teleost that forms small shoals in the wild (3–10 fish per group) (Engeszer et al., 2007; Pritchard et al., 2001) and has been considered a popular animal model in behavioral neuroscience (Fontana et al., 2018a; Kalueff et al., 2014). Notably, the shoaling behavior can be easily reproducible under laboratory conditions (Buske and Gerlai, 2012; Gerlai et al., 2000; Miller and Gerlai, 2007) using a small number of cohorts per group, adhering to the 3Rs principles of ethical animal experimentation (Canzian et al., 2017; Green et al., 2012). Moreover, the zebrafish has evolutionarily conserved genetic and physiological features, as well as displays the major neurotransmitter systems characterized in mammals (Howe et al., 2013; Rico et al., 2011). This species also serves as an emerging tool for translational neuropsychiatric research, allowing a detailed pharmacological investigation of how different compounds act on the

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CNS influencing the social behavior (Buske and Gerlai, 2012; Canzian et al., 2017; Müller et al., 2017). In stressful and potentially dangerous environments, zebrafish increase shoaling as a defensive response (Buske and Gerlai, 2011; Canzian et al., 2017; Fontana et al., 2018b). Furthermore, defensive behaviors can be enhanced by anxiogenic substances, such as conspecific alarm substance (CAS) and caffeine (CAF); or attenuated by anxiolytic drugs, such as diazepam (DZP) (Canzian et al., 2017; Gebauer et al., 2011; Ladu et al., 2015; Rosa et al., 2018).

Different zebrafish protocols used to assess the social behavior have been described elsewhere (Canzian et al., 2017; Gerlai et al., 2000; Ladu et al., 2015). Most of these protocols are often restricted to plane perspectives (2D), and the analyses of shoal occupancy endpoints and social interaction in a three-dimensional (3D) perspective has not been fully explored yet. Because zebrafish present a three-dimensional (3D) swimming activity when isolated and in groups (Cachat et al., 2011; Maaswinkel et al., 2013; Macrì et al., 2019; Macrì et al., 2017; Rosa et al., 2018; Stewart et al., 2015), the goal of this study was to perform a spatiotemporal characterization of the zebrafish shoaling behavior using a 3D perspective under different pharmacological manipulations. For this purpose, we analyzed the spatial coordinates of fish positions to measure the social behavior under the influence of compounds that are known to modulate anxiety-related behavior. Behavioral responses were measured in 2D (both frontal and top views) and 3D perspectives to investigate a possible existence of substantial inconsistencies of these protocols when shoal occupancy- and social interaction-related endpoints are investigated after exposure to anxiogenic (CAS and CAF) or anxiolytic (DZP) substances. In general, we expect this proof-of-concept study will help complement the existing locomotion measures of shoaling tendency by determining the spatiotemporal reconstructions of shoal occupancy specifically using the spatial coordinates of fish subjected to pharmacological manipulations that positively and/or negatively influence the social behavior.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) with 4–6 months-old (~50:50 male:female ratio, short fin phenotype) were acquired from a commercial supplier (Hobby Aquários, RS, Brazil). Fish were maintained for two weeks in 40-L thermostatic aquariums at a maximum density of 4 fish per liter and kept under constant mechanical and chemical filtration before the experiments to acclimate to the laboratory facility. Tanks were filled with non-chlorinated water at 27 ± 1 °C, pH 7.2, conductivity at 1300–1500 μS.cm^{-1}, dissolved oxygen at 6.0 ± 0.1 mg/L, total ammonia at < 0.01 mg/L, nitrate (NO_3^-) < 50 mg/L, nitrite (NO_2^-) < 0.1 mg/L, alkalinity and hardness at 75 mg/L $CaCO_3$. Illumination was provided by ceiling-mounted fluorescent light tubes on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am and off at 9:00 pm) (Avdesh et al., 2012). Fish were fed thrice daily with commercial flake fish food (Alcon BASIC™, Alcon, Brazil). All experiments were performed using fish obtained from three independent batches. Animals used were naive and maintained in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria (process number 2220181215).

2.2. Conspecific alarm substance (CAS) extraction

Conspecific alarm substance was extracted as described elsewhere (Egan et al., 2009; Quadros et al., 2016). A donor fish was previously anesthetized in 4 °C water, euthanized by decapitation, and the blood remnants were removed using distilled water and cotton swab. The donor fish body was placed in a Petri dish and shallow scrapings were performed along each side of the body (10–15 scrapings) with a scalpel blade. Then, 10 mL of distilled water was added to prepare CAS extract from damaged epithelial cells. To maintain the biological effects of CAS throughout the experiments, all procedures described above were performed on ice. Experiments were carried out using CAS preparations from at least three donor fish in order to ensure data reproducibility.

2.3. Exposure protocol

Four zebrafish per shoal ($n = 7–8$ shoals per group) were acutely exposed in 1 L aquarium to 100 mg/L caffeine (CAF) (Sigma Aldrich, St. Louis, MO, USA) for 15 min, 3.5 mL/L conspecific alarm substance (CAS) for 5 min, or 0.16 mg/L diazepam (DZP) (EMS S/A, Brazil) for 10 min diluted in non-chlorinated water. Another group exposed to non-chlorinated water was run as a control group (CTRL). In groups with less than 15 min exposure (CAS and DPZ), drugs were added after a previous non-chlorinated water exposure in order to ensure a similar manipulation condition and exposure period for all groups (see details in Fig. S1). The number of fish per shoal was chosen based on previous reports (Canzian et al., 2017; Green et al., 2012; Pritchard et al., 2001; Schmidel et al., 2014), which shows a significant shoaling response and fully adhere to the reduction criteria of the 3Rs principles of ethical animal experimentation (Green et al., 2012). Moreover, using four fish per shoal represents a simpleway to calulate the shoal volume by fish coordinates. Times of exposure were chosen based on previous reports, which described robust effects of CAF, CAS, and DZP on zebrafish behavioral activity (Egan et al., 2009; Gebauer et al., 2011; Ladu et al., 2015; Quadros et al., 2016; Rosa et al., 2018).

2.4. Shoaling behavior task

The shoaling behavior task was used to assess social and exploratory activity of zebrafish shoals (Canzian et al., 2017; Green et al., 2012; Schmidel et al., 2014). After the exposure period, each zebrafish shoal was placed in the experimental tank (20 cm length x 15 cm height x 20 cm width), with a 10 cm water column height. Shoaling behaviors were recorded for 6 min using two orthogonal-located cameras (one positioned at 60 cm from the bottom of the apparatus and another located at 60 cm from the back wall of the apparatus). Screenshots were made every 10 s across the trial (totalizing 72 screenshots per shoal). Regarding frontal view images, the apparatus was divided into three equal horizontal areas (bottom, middle, and top) to detect the vertical distribution of fish. For top view images, the same tank was divided into three areas: periphery area (less than 2.5 cm from the edge of the tank), intermediate area (more than 2.5 cm and less 5 cm from the edge of tank) and center area (more than 5 cm from the edge of the tank) to measure the horizontal distribution of fish (Fig. 1). Two experimental tanks were used per group in order to minimize the influence of the experimental apparatus on the behavioral responses.

2.5. Three- and bi-dimensional analysis of shoaling behavior

Screenshots from synchronized videos of frontal and top view perspectives were exported to the Image J 1.49 software for Windows™. To obtain the spatial "x and y" (frontal camera) and "x and z" (top camera) coordinates of fish, we have calibrated the software using the known dimensions of the apparatus (in cm). After obtaining the individual fish coordinates, we calculated the inter-fish distance in both 3D and 2D perspectives (the latter using frontal and top views), shoal volume (3D perspective), as well as the shoal area in both frontal and top views (2D perspectives). Pilot experiments were performed in order to verify whether 3D behavioral endpoints could differ if the "x" coordinate obtained from the top or frontal view is considered for data analyses. Notably, analyses comparing the results from these two "x" coordinates of a shoal did not reveal significant differences in the inter-fish distance and shoal volume (data not shown). Here, 3D shoal behaviors were reconstructed using the "x" coordinate from the top perspective, in which the distance from the water surface to the bottom of the tank was

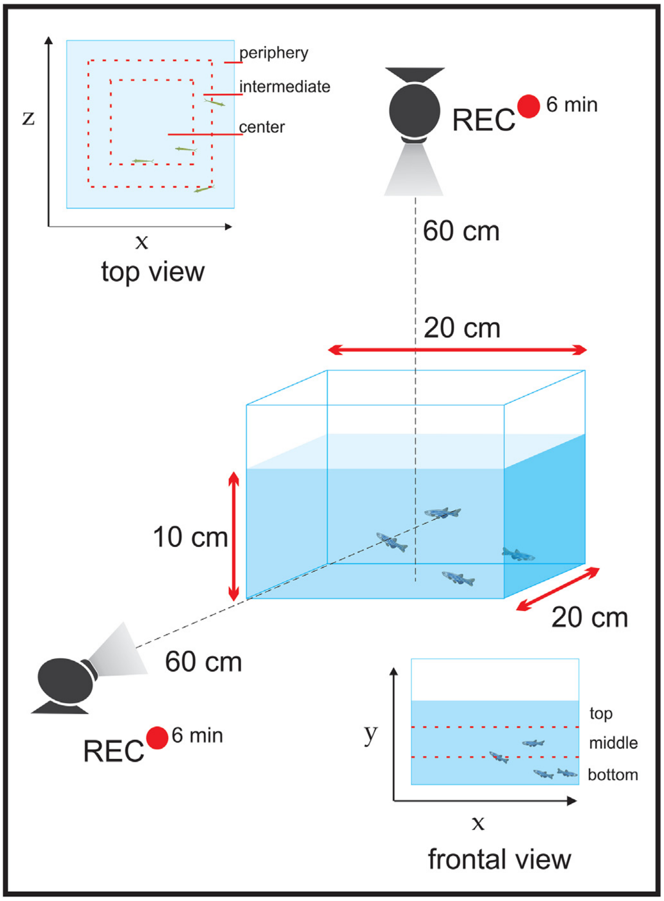

Fig. 1. Behavioral recordings setup and experimental tanks. Tanks were 20 cm × 20 cm × 15 cm (length × width × height) and the water level was 10 cm height. Shoaling behaviors were recorded for 6 min using two orthogonal-located cameras (one positioned at 60 cm from the bottom of the apparatus and another located at 60 cm from the back wall of the apparatus). Behavioral recordings were made at 30 frames/s.

10 cm.

For 3D analyses of the shoaling behavior, the distance between fish was determined as follows:

$$a = \sqrt{(xA - xB)^2 + (yA - yB)^2 + (zA - zB)^2};$$

Where a is the distance between fish A and B; xA, yA, and zA are the coordinates of the fish A; xB, yB, and zB are the coordinates of the fish B. As shown in Fig. 2A, the coordinates used in the equation described above can vary depending on the fish analyzed.

To determine the centroid position of the shoal coordinates x, y, and z on 3D perspective, we calculated the arithmetic means of x, y, and z fish coordinates, as described bellow:

$$xG = \frac{1}{n}\sum_{i=1}^{n} x_i;$$

$$yG = \frac{1}{n}\sum_{i=1}^{n} y_i;$$

$$zG = \frac{1}{n}\sum_{i=1}^{n} z_i;$$

Where xG, yG, and zG are the coordinates of the centroid point of the shoal and x_i, y_i and z_i are the coordinates of fish A, B, C, and D.

Using 3D analyses, the distance between fish to the centroid point of the shoal was determined as follows:

$$g_A = \sqrt{(xG - xA)^2 + (yG - yA)^2 + (zG - zA)^2};$$

Where g_A is the distance between fish A to the centroid point; xG, yG, and zG are the coordinates of the centroid point; xA, yA, and zA are the coordinates of the fish A (see details in Fig. 2B). The coordinates used in the equation described above can vary depending on the fish analyzed.

We also expressed an homogeneity index calculated as follows:

$$I_A = g_A * \frac{(n-1)}{\left(\sum_{i=1}^{n} g_i - g_A\right)}$$

Where I_A is the homogeneity index of the fish A; the g_A is the distance between fish A to the centroid point of the shoal; $\sum_{i=1}^{n} g_i$ is the summation of all distances between fish to the centroid point of the shoal; n is the number of fish in the shoal. The homogeneity index formula was performed to measure the ratio of the distance of each subject (to the centroid point of the shoal) to the average inter-fish distance to the centroid position considering the other three conspecifics (without the influence of the analyzed subject). The higher index value for each shoal position across time was selected to the statistical analysis. Shoals with homogeneous dispersion show index values closer to 1. Values higher than 1 represents fish more distant to the centroid in relation to their conspecifics, while values lower than 1 reflects fish closer to the centroid position of the shoal. The distances between fish to the centroid point of the shoal used in the equation described above can vary depending on the fish analyzed. (Fig. 2B)

Using the 3D perspective, the shoal volume was determined as follows:

$$Volume = \sqrt{\frac{det\begin{vmatrix} 0 & c^2 & d^2 & e^2 & 1 \\ d^2 & 0 & f^2 & b^2 & 1 \\ d^2 & f^2 & 0 & a^2 & 1 \\ e^2 & b^2 & a^2 & 0 & 1 \\ 1 & 1 & 1 & 1 & 0 \end{vmatrix}}{288}},$$

Where a, b, c, d, e and f are the distance between fish (Fig. 2A).

Using the 2D perspective, the distance between fish was calculated as follows:

$a = \sqrt{(xA - xB)^2 + (zA - zB)^2}$ for the top view;

$a = \sqrt{(xA - xB)^2 + (yA - yB)^2}$ for the frontal view.

Where a is the distance between fish A and B; xA, yA and zA are the coordinates of the fish A; xB, yB, and zB are the coordinates of the fish B. Notably, the coordinates used in the equations described above can vary depending on the fish analyzed (Fig. 2C and Fig. 2D).

The shoal area (2D perspectives) was determined as follows:

$$Area = \frac{1}{2} X \left(det \left| \begin{pmatrix} xA & xB \\ zA & yB \end{pmatrix} + \begin{pmatrix} xB & xC \\ zB & yC \end{pmatrix} + \begin{pmatrix} xD & xD \\ zD & yD \end{pmatrix} + \begin{pmatrix} xD & xA \\ zD & yA \end{pmatrix} \right| \right)$$

for the top view;

$$Area = \frac{1}{2} X \left(det \left| \begin{pmatrix} xA & xB \\ yA & yB \end{pmatrix} + \begin{pmatrix} xB & xC \\ yB & yC \end{pmatrix} + \begin{pmatrix} xD & xD \\ yD & yD \end{pmatrix} + \begin{pmatrix} xD & xA \\ yD & yA \end{pmatrix} \right| \right)$$

for the frontal view.

Where x, y, and z are the respective coordinates of fish A, B, C, and D (Fig. 2C and Fig. 2C). Because three arrangements of coordinates are predictable using the shoal area formula, the arrangement with a minor perimeter must be selected for assessing the correct quadrilateral shape (Fig. 2C and Fig. 2D).

All measurements can be performed using spreadsheet application and statistical packages (*e.g.,* Microsoft Excel™) previously configured (see Supplementary Materials for details).

2.6. *Neurophenotyping of zebrafish shoal behavior*

The spatial coordinates of fish obtained by Image J 1.49 software for

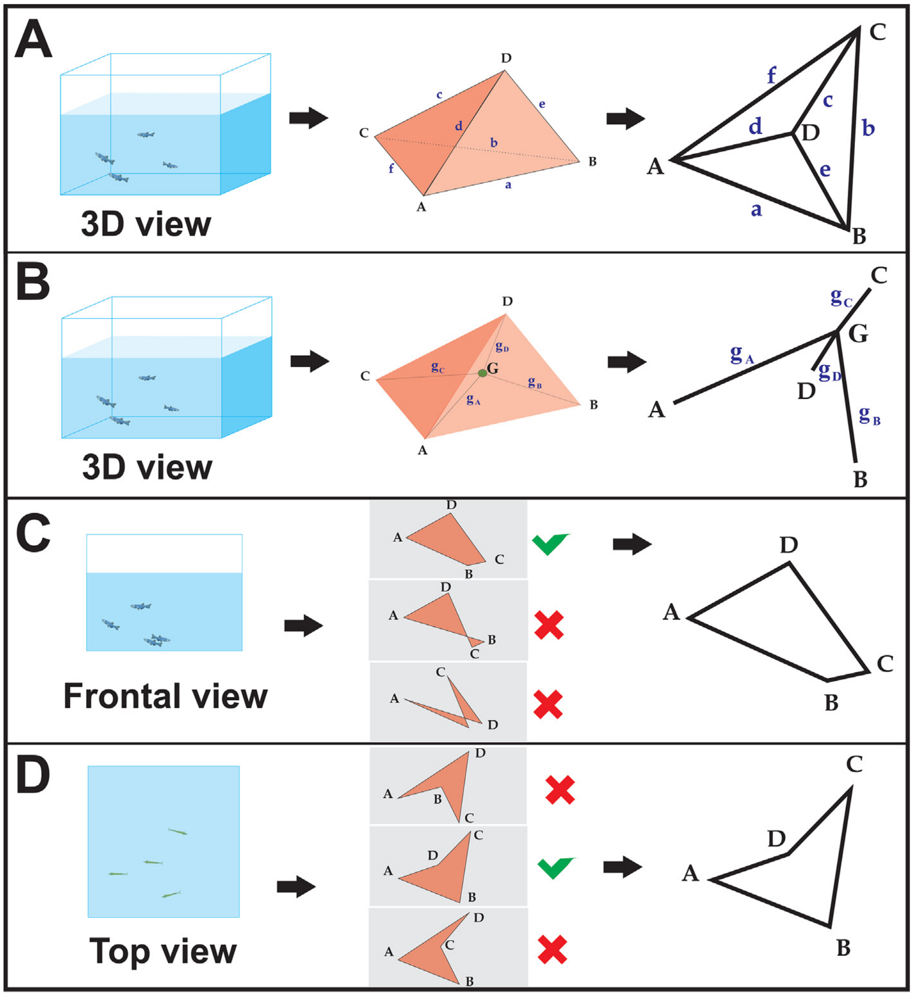

Fig. 2. Three- and bi-dimensional analyses of shoaling behavior. Spatial coordinates *x*, *y*, and *z* of four fish were obtained and represented by A, B, C and D. Inter-fish distance represented by ***a,b,c,d,e*** and ***f*** distances on 3D analysis (A) and fish distance to the centroid point of the shoal *G* represented by **g_A,g_B,g_C and g_D** (B). Because three arrangements of coordinates are predictable on 2D analyses, we selected the arrangement with minor perimeter for assessing the correct quadrilateral shape in both frontal (C) and top view (D).

Windows™ were exported to Graphis 3D graphing software™ for behavioral 3D reconstruction every 20 s of the shoal positions. The exported traces were analyzed based on similarity to each other by three-trained observers (inter-rater reliability > 0.9) on a consensus basis. The representative reconstruction was selected for each experimental group.

2.7. Statistics

Normality of data and homogeneity of variances were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. Shoaling behavioral endpoints were expressed as means ± standard error of the mean (S.E.M.) and analyzed by one- or two-way analysis of variance (ANOVA). Temporal analysis of shoaling behavior was assessed using data exported by minute (six images every 10 s) and repeated measures ANOVA was performed using treatment and time as factors. The area under curve (AUC) of the number of fish per area across time was quantified to express the spatial distribution of shoals, being represented as arbitrary units, as exemplified in Fig. S2. Results were expressed as means ± standard error of means (S.E.M.) and analyzed by one-way analysis of variance (ANOVA). The temporal analysis was made using AUC calculated per minute (see Fig. S2 for details) and repeated measures ANOVA was performed using treatment and time as factors. Differences between groups were further assessed by Student-Newman-Keuls multiple comparisons test whenever appropriate. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Social behavior parameters

Fig. 3 shows the inter-fish distance and the shoal volume (for 3D perspective) or area (for 2D perspectives) after acute CAF, CAS, and DZP exposure. Three-dimensional analysis of behavior (Fig. 3A) revealed that both CAS and CAF decreased the shoal volume ($F_{(3,\ 26)} = 9.226$, $p = 0.0003$), but only CAS reduced the inter-fish distance when compared to CTRL ($F_{(3,\ 26)} = 9.789$, $p = 0.0002$). Conversely, DZP increased both inter-fish distance ($F_{(3,\ 26)} = 9.789$, $p = 0.0002$) and shoal volume ($F_{(3,\ 26)} = 9.226$, $p = 0.0003$).

Using 2D analyses (frontal view) (Fig. 3B), we verified that CAS and CAF decreased the inter-fish distance ($F_{(3,\ 26)} = 9.847$, $p = 0.0002$) and the shoal area ($F_{(3,\ 26)} = 12.55$, $p < 0.0001$), while DZP increased only the shoal area when compared to CTRL ($F_{(3,\ 26)} = 12.55$, $p < 0.0001$). In the top-view perspective, (Fig. 3C), CAS decreased the inter-fish distance and the shoal area, while DZP increased both behavioral parameters ($F_{(3,\ 26)} = 7.918$, $p = 0.0007$ and $F_{(3,\ 26)} = 7.635$, $p = 0.0008$, respectively).

Concerning the temporal analysis of shoaling behavior in the 3D perspective, repeated measures ANOVA yielded significant effects of

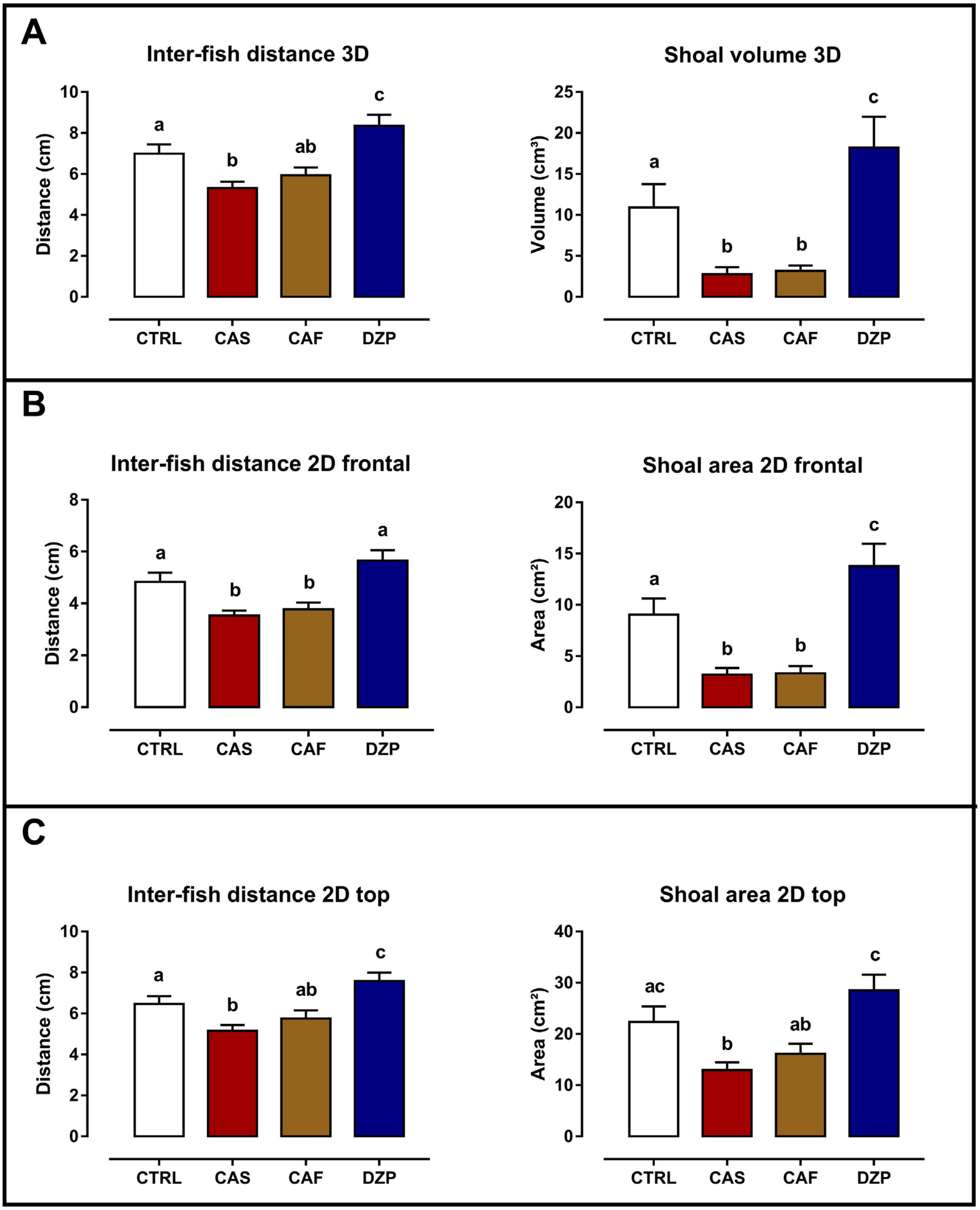

Fig. 3. Effects of acute exposure to CAS, CAF, and DPZ on inter-fish distance and shoal occupation size in 3D (A), 2D frontal (B) 2D top analyses (C). Data are expressed as means ± S.E.M and analyzed by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Different letters indicate statistical differences among groups ($p \leq 0.05$, $n = 7$–8 per group).

treatment on the inter-fish distance (CAS and DZP) ($F_{(3,\ 26)} = 9.792$, $p = 0.0002$) and shoal volume (DZP) ($F_{(3,\ 26)} = 9.225$, $p = 0.0003$). Basically, CAS reduced the inter-fish distance, while DZP increased both inter-fish distance and shoal volume when compared to CTRL (Fig. S3).

3.2. Vertical and horizontal distributions of the shoals

Fig. 4A shows the vertical distributions of the zebrafish shoals. CAS and CAF decreased the number of animals in the top ($F_{(3,\ 26)} = 6.66$, $p = 0.0017$), as well as increased the number of animals in the bottom area ($F_{(3,\ 26)} = 7.013$, $p = 0.0013$). The horizontal distribution of shoals did not reveal significant differences between groups (Fig. 4B).

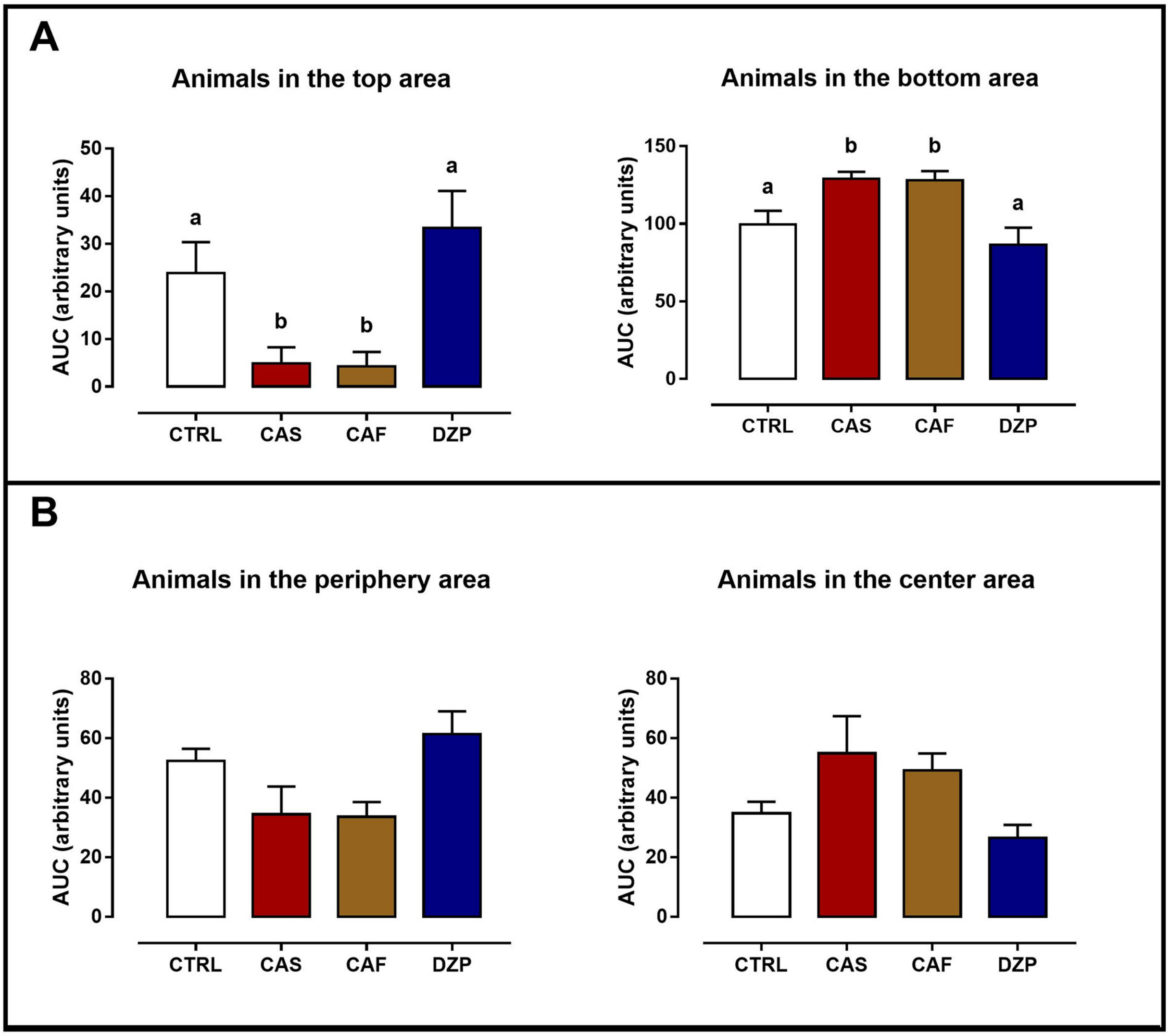

Fig. 4. Effects of CAS, CAF, and DZP on vertical (A) and horizontal (B) distribution of shoals. The area under curve (AUC) of the number of fish per area across time was quantified to express the spatial distribution of shoals, being represented as arbitrary units. Results are expressed as means ± S.E.M. and analyzed by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Different letters indicate statistical differences among groups ($p \leq 0.05$, $n = 7$–8 per group).

Temporal analysis of vertical swimming showed a significant effect of treatment on the number of fish in the top (CAS, CAF, DPZ) ($F_{(3, 26)} = 7.409$, $p = 0.0010$) (Fig. 5A). While CAS- and CAF-treated fish were less distributed in the top area than CTRL, DZP increased the number of animals in the top (Fig. 5A). No effects were observed concerning the number of animals in the bottom area across time (Fig. 5B). Temporal analysis of horizontal distribution revealed significant effects of time ($F_{(5, 130)} = 6.766$, $p < 0.0001$) and treatment (for CAS and CAF groups) ($F_{(3, 26)} = 3.816$, $p = 0.0217$) on the number of animals in the periphery area (Fig. 5C). A significant effect of time was also observed in the number of animal in the center area ($F_{(5, 130)} = 8047$, $p < 0.0001$) (Fig. 5D). Regarding the horizontal distribution of shoal across time, the number of animals in the periphery increased across time, thereby reducing the distribution of fish in the center area.

3.3. Centroid position analysis

Fig. 6A shows the average distance of fish to the centroid point of the shoal. CAS and CAF decreased the respective behavioral endpoint when compared with CTRL ($F_{(3, 26)} = 11.56$, $p = 0.0001$). Two-way ANOVA yielded a significant treatment × position interaction ($F_{(6, 78)} = 3675$, $p = 0.0029$) and post-hoc analyses revealed that both CAS and CAF increased the frequency of the centroid point in the bottom area (Fig. 6B). Moreover, no differences were observed regarding the homogeneity index calculated from the most distant fish to the centroid position of the shoal (Fig. 6C).

4. Discussion

In this study, we explored how modulators of anxiety-like behavior affect the zebrafish shoaling behavior in both 3D and 2D perspectives. Using the individual spatial coordinates, we describe a new procedure to perform a spatiotemporal reconstruction of the social behavior. Our protocol allowed a pharmacological assessment of how anxiogenic and anxiolytic compounds influence the social interaction of fish, shoal occupancy, and dispersion patterns, complementing the zebrafish shoaling activity measurements described previously (Macrì et al., 2019; Macrì et al., 2017). Notably, 3D reconstructions of the shoaling behavior showed a different profile when anxiogenic substances (CAF and CAS) were tested, which could not be detected by classical frontal 2D analysis. Our results corroborate the inconsistencies between 2D and 3D analyses of the swimming behavior (Macrì et al., 2019, Macrì et al., 2017), reinforcing the relevance of assessing the shoaling behavior in a 3D perspective when zebrafish are exposed to different

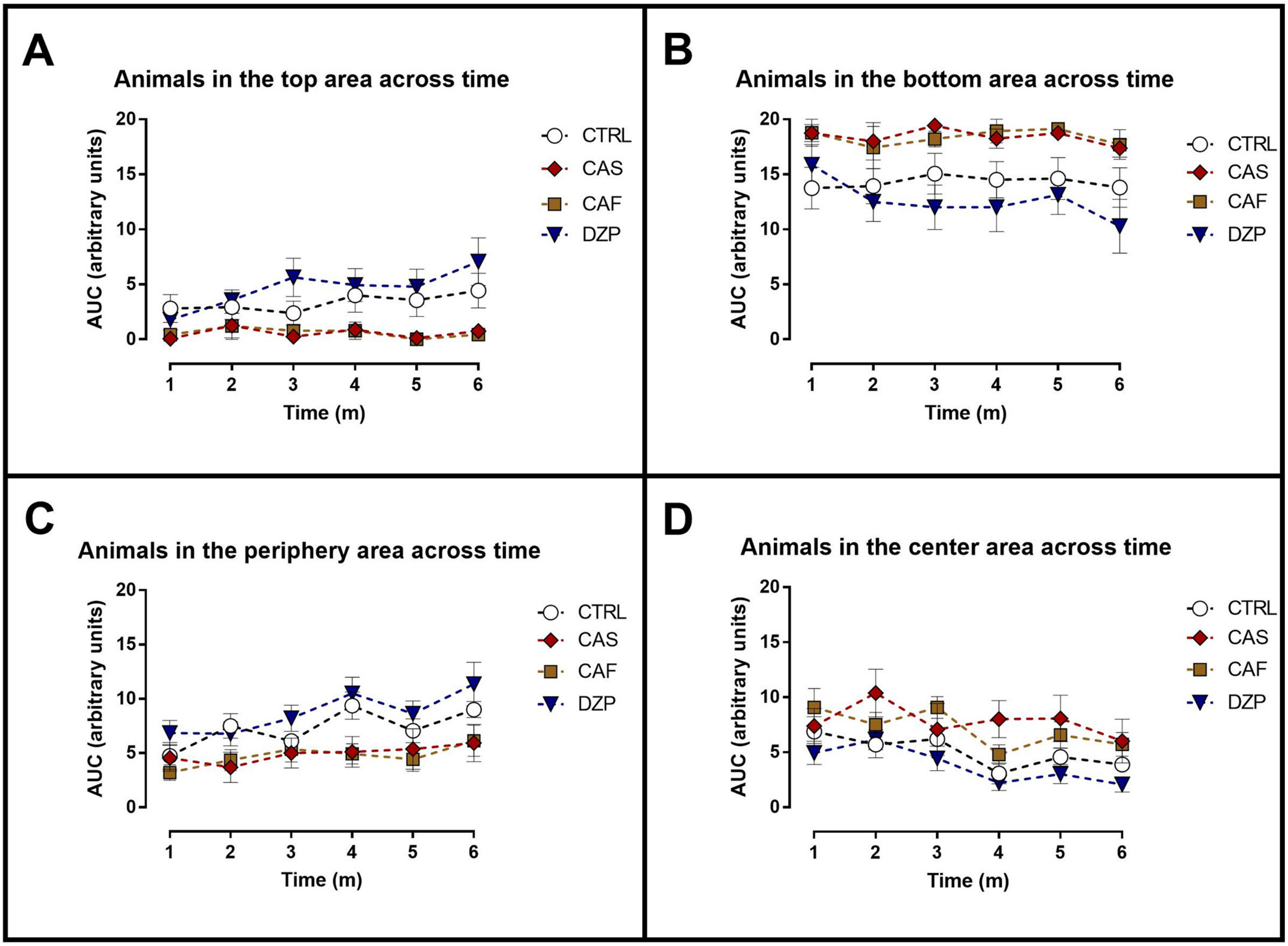

Fig. 5. Number of animals in top (A), bottom (B), periphery (C), and center areas (D) across time. Temporal analysis of behavior was made by calculating the AUC per minute. Data are expressed as means ± S.E.M and analyzed by repeated measures ANOVA followed by Student-Newman-Keuls multiple comparison test ($p \leq 0.05$, $n = 7–8$ per group).

pharmacological manipulations.

The 3D neurophenotyping of zebrafish behavior allows a more precise reconstruction of the swimming traces, providing a complex representation of the locomotion (Cachat et al., 2011; Macrì et al., 2017; Rosa et al., 2018). To date, 3D analyses of zebrafish shoals are often performed by assessing the general locomotion and fish trajectories (Audira et al., 2018; Maaswinkel et al., 2013; Macrì et al., 2019; Macrì et al., 2017). However, the overall pattern of cohesion and dispersion in a group of fish using spatial coordinates has not been fully explored yet. Here, we examined several endpoints to investigate the

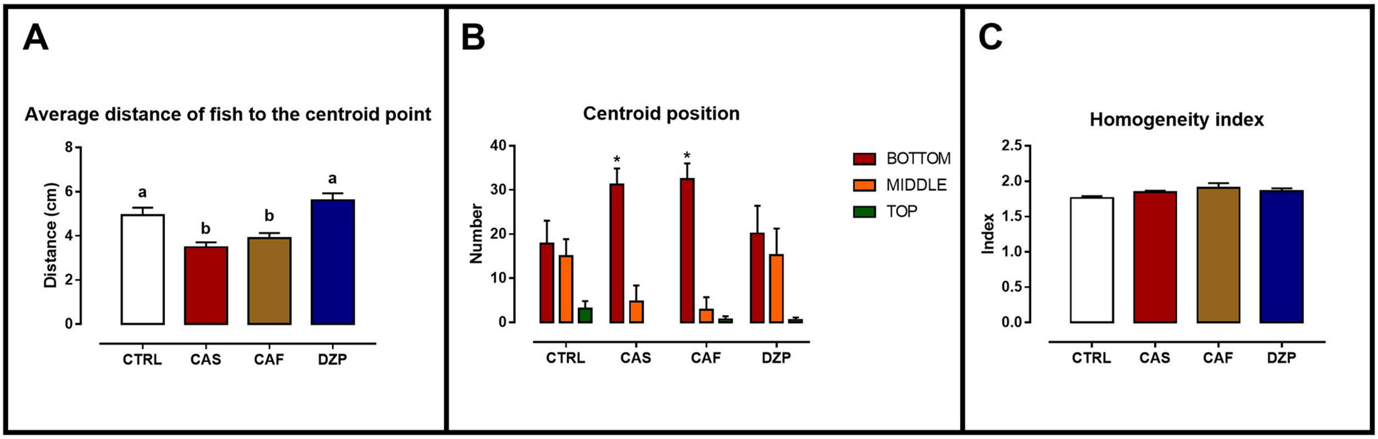

Fig. 6. Average distance of fish to the centroid point (A), centroid position (B), and homogeneity index (C). Data are expressed as means ± S.E.M and analyzed by one-way ANOVA (A and C) or two-way ANOVA (B) followed by Student-Newman-Keuls multiple comparison test whenever necessary. Different letters indicate statistical differences between groups (A and C) and the asterisks indicate differences to the respective value of CTRL (B) ($p \leq 0.05$, $n = 7–8$ per group).

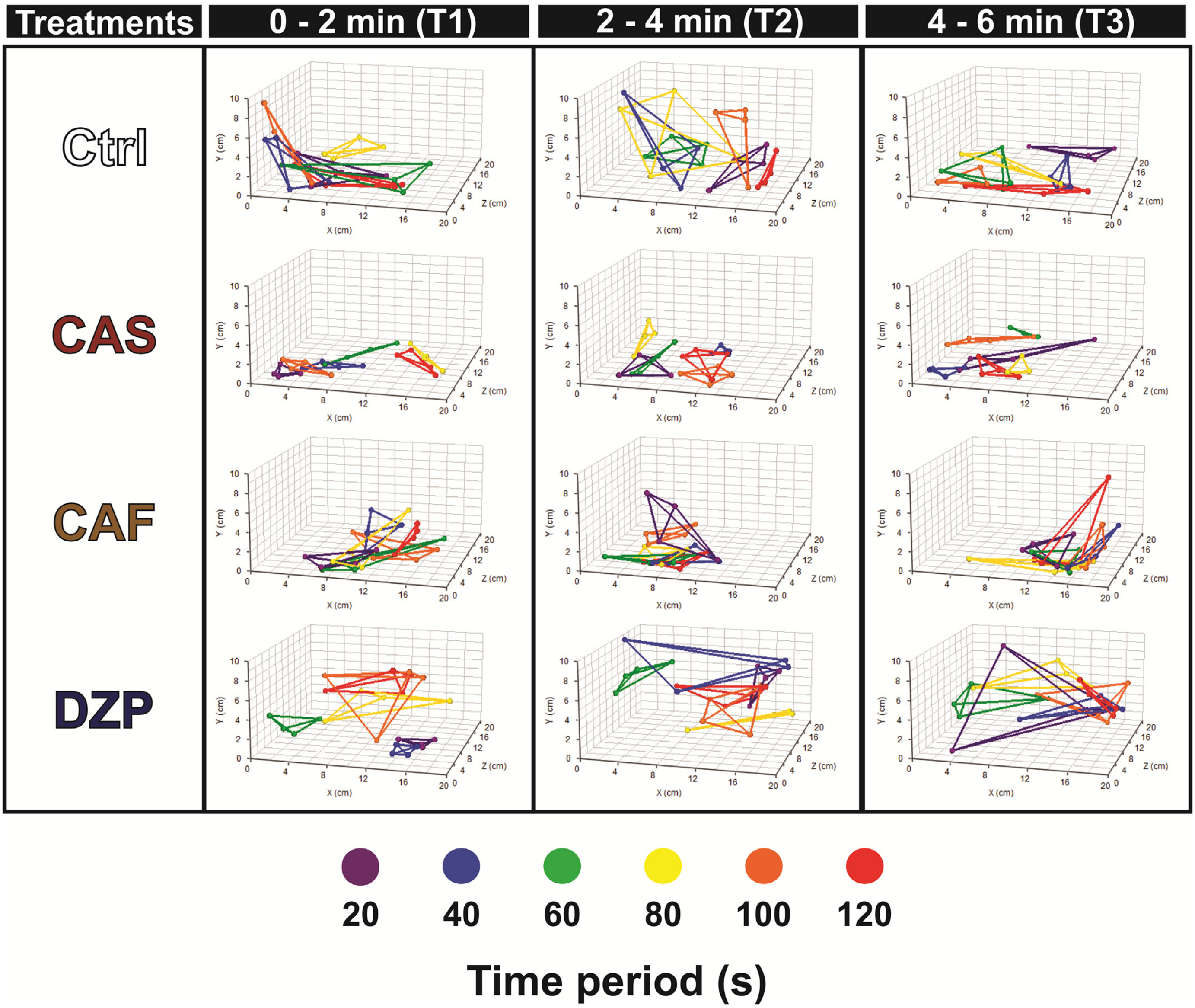

Fig. 7. Representative 3D reconstructions of zebrafish shoal occupancy in the absence or presence of CAS, CAF, and DZP. Different colors represent the shoal position across the trial. Data were obtained by plotting the *x*, *y*, and *z* coordinates obtained from frontal- and top-view recordings of the 6-min trial.

shoaling behavior in the presence of different anxiety modulator compounds. This protocol is inexpensive and can be performed both manually or using freely available softwares to detect fish position after proper calibration. Importantly, the shoal volume is restricted to a four-fish shoal, but the other endpoints described (*e.g.*, inter-fish distance, fish distance to the centroid point, vertical and horizontal distributions of the shoal, and homogeneity index) can be easily assessed using more than four fish in a shoal. Although sophisticated video-tracking softwares to analyze the velocity of fish and general locomotion endpoints exist, this proof-of-concept study highlights the potential utility and feasibility of the spatiotemporal reconstructions of shoal occupancy using *x*, *y*, and *z* coordinates. Thus, we expect that subsequent studies will further advance in this field aiming to enhance both position detection and temporal analyses by applying more rigorous and sensitive tools.

Regarding the pharmacological effects on shoaling behavior, frontal 2D analyses showed a decreased inter-fish distance in CAF-exposed fish when compared to CTRL, which did not occur in a 3D perspective. These apparent controversial data results from the limitations of frontal perspective analysis, which underestimate the horizontal distribution and overestimate the vertical activity of fish (Fig. 1). Conversely, the 2D top perspective did not show differences in the shoal area between CAF-exposed fish and CTRL, which can result from overestimating fish horizontal dispersion restricting geotaxis assessment (Fig. 1). Although decreasing the size of the test apparatus in one dimension may reduce such influence on 2D analysis, this procedure restricts the swimming activity of fish, which makes it difficult to understand how zebrafish groups distribute both vertically and horizontally.

Here, both CAS and CAF increased geotaxis, decreased the shoal volume, and reduced the average distance of fish to the centroid point. However, only CAS decreased the inter-fish distance, exhibiting a prominent shoaling behavior across the trial in the representative reconstructions. DZP, a benzodiazepine that targets GABA$_A$ receptors (Bandara et al., 2020; Gebauer et al., 2011), evoked robust anxiolytic-like phenotypes by increasing both inter-fish distance and shoal volume, as observed in the spatiotemporal reconstructions of the shoals (Fig. 7). Among the various behavioral endpoints measured, the inter-fish distance is often used to express fish dispersion (Canzian et al., 2017; Green et al., 2012; Schmidel et al., 2014). However, because multiple distances are assigned to each fish (see the spreadsheet provided in Supplementary Materials for details), this endpoint can be influenced by the proximitity/distance of a subject to the other inidividuals of the shoal. Estimating the average distance to the centroid point can decrease such interference by calculating a single distance per fish. Moreover, the analysis of the centroid position of the shoal can also be suitable to estimate the vertical activity of a zebrafish group (Fig. 6B). Changes in the shoal volume reflects altered shoal coesion, which can be influenced by the inter-fish distance, horizontal and vertical distribution of shoal. Because all exposures did not alter the homogeneity index when compared with CTRL, our results support the influence of CAS, CAF, and DPZ in the shoaling response (Fig. 6C).

The anxiogenic substances tested can influence zebrafish behavioral responses differently. For example, CAF elicits anxiety-like phenotypes probably *via* adenosine receptors blockade (Egan et al., 2009; Neri et al., 2019; Rosa et al., 2018). As a naturalistic aversive chemical cue, CAS activates habenular *c-fos* expression (Ogawa et al., 2014), and a role of the serotonergic system with the positive modulation of anxiety- and fear-like phenotypes has been predicted (Lima-Maximino et al., 2020). Although our data clearly reflect the robust effect of anxiogenic susbtances on the social response, which may result from the activation of distinct molecular signaling pathways (Canzian et al., 2017), the exact mechanisms underlying the shoaling behavioral patterns described here still require future scrutiny. Notably, previous data revealed substantial differences on the swimming activity when a single caffeine-treated fish was tested isolated or in the presence of unexposed animals (Neri et al., 2019). In our study, because all fish in the same group were pharmacologically treated, these findings reinforce the existence of social- and context-dependent effects in the zebrafish behavior. Importantly, the homogeneity index described here allows testing the spatiotemporal coordinates of a single-treated fish in relation to other conspecifics in future pharmacological approaches to verify possible interactions of drugs and social environment.

In conclusion, this study reports the 3D spatiotemporal reconstruction of zebrafish shoaling response under the influence of anxiogenic and anxiolytic substances using the spatial fish coordinates across time. While multiple questions remain unclear in this field, our novel findings can be considered as an easy-to-use, inexpensive, and alternative tool to perform a spatiotemporal reconstruction of the shoal occupancy, complementing the existing quantification of locomotion activity of multiple fish (Macrì et al., 2019; Macrì et al., 2017). The inconsistencies observed in the behavioral measurements using 2D and 3D analyses briefly discussed here also suggest a promising new avenue of neurophenotyping methods for zebrafish in behavioral neuropsychopharmacology.

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Author contributions

Conceived and designed the experiments: L.V.R. and D.B.R. Performed the experiments: L.V.R., J.C., and V.A.Q. Analyzed the data: L.V.R., J.V.B., F.V.C., and D.B.R. Contributed reagents/materials/analysis tools: D.B.R. Wrote the paper: L.V.R., F.V.C., and D.B.R. All authors read and approved the final manuscript.

Ethical statement

1) This material has not been published in whole or in part elsewhere;
2) The manuscript is not currently being considered for publication in another Journal;
3) All authors have been personally and actively involved in substantive work leading to the manuscript and are responsible for its content.

Declaration of Competing Interest

None

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5.2 MANUSCRITO CIENTÍFICO

Acetic acid-induced nociception modulates sociability in adult zebrafish: influence on shoaling behavior in heterogeneous groups and social preference.

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Acetic acid-induced nociception modulates sociability in adult zebrafish: influence on shoaling behavior in heterogeneous groups and social preference

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Abstract

Due to the recognition of fishes as sentient beings, the zebrafish (*Danio rerio)* has become an emergent animal model system to investigate the biological processes of nocifensive responses. Here, we aimed to characterize the zebrafish social behavior in a nociception-based context. For this propose, using a three-dimensional analysis of heterogeneous shoals, we investigated the main behavioral responses in two 6-min trials: before (baseline) and after a single intraperitoneal (i.p) injection of 10 μL phosphate-buffered saline (PBS) (control), acetic acid 5% (AA), morphine 2.5 mg/kg (MOR) or acetic acid 5% plus morphine 2.5 mg/kg (AA+MOR) in one subject from a four-fish shoal. The social preference of individuals for tanks with shoals of fish treated with PBS, 5% AA, or an empty aquarium were also tested. We verified that AA administration disrupted the shoal homogeneity by eliciting dispersion of treated fish with simultaneous clustering of non-manipulated fish. Moreover, morphine coadministration protected against AA-induced behavioral changes. The social preference test revealed a clear preference for conspecifics (PBS and AA) over an empty tank, with a prominent preference for PBS- over AA-treated shoals was verified. Overall, our novel findings show that nociception modulates zebrafish sociability with a role of visual recognition of nocifensive behaviors in such response. Although future studies are needed to fully elucidate how nociception influences zebrafish social behavior, our study contributes to improve the welfare assessment of zebrafish shoals under distinct experimental manipulations.

Keywords: zebrafish; pain; nocifensive responses; shoaling behavior; social preference

1. Introduction

Social behaviors play key roles in various species, such as predator avoidance, information transfer, reproductive success, and foraging [1–3]. The expression of social behavior can vary depending on environmental conditions [4–6], promoting adaptive responses in aversive situations [7–11]. Considering the fundamental importance of social behaviors, the use of social species as a research subject is an interesting research strategy to elucidate the mechanisms related to this and the type of behavior in both ecological and translational approaches [12–15].

The zebrafish (*Danio rerio*) is a small freshwater cyprinid with a robust social behavior widely used in neuroscience research [15–17]. Under laboratory conditions, zebrafish shoaling behavior can be easily assessed in groups of 3–10 fish per shoal [13,17,18]. Importantly, pharmacological and non-pharmacological manipulations testing four fish simultaneously in a test tank provide data with high replicability and reproducibility, fully adhering to the 3Rs principles of animal experimentation [7,19,20]. The presence of evolutionarily conserved genetic and physiological features, as well as the expression of the major neurotransmitter systems characterized in humans [21,22], make zebrafish a suitable model organism to investigate how different experimental approaches influence shoaling behavior and social preference. Notably, when exposed to aversive stimuli, zebrafish show aversive responses [23,24], which can also be detected by conspecifics [25].

In normal situations, zebrafish show an innate social preference, forming shoals with non-polarized swimming [26,27]. However, stressful or potentially aversive situations increase shoal cohesion as a protective behavior [7,27–29]. Thus, due to the context-dependent feature of zebrafish social responses, studies using aversive conditions constitute promising tools to provide relevant data to translational, ecological, and animal welfare research.

Nociception is an aversive stimulus that involves the transmission of painful signals by nociceptors in the primary afferent nerve fibers in response to mechanical, thermal, or chemical cues [30,31]. These stimuli then reach the central nervous system (CNS), triggering pain perception and adaptive behavioral responses in acute situations. Similar to traditional rodents models, zebrafish display complex pain-related behaviors (also known as nocifensive behaviors) [32–34] and conserved molecular features involved in nociception (*e.g.*, transient receptor potential (TRP) ion channels, acid-sensing ion channels (ASICs), and toll-like receptors (TLRs)) [35,36]. Studies regarding pain-like responses in zebrafish models are emerging rapidly, but the effects of nociception on zebrafish social behavior are poorly explored. Although the effects of fin clipping on shoal activity, cohesion, and occupancy of one, three, and six fish in a 6-fish shoal have been reported [37], the influence of acetic acid on shoaling behavior and social preference have not been explored yet. Thus, the goal of this study was to investigate how acute pain influences social responses in adult zebrafish. For this purpose, we analyzed the influence of acetic acid on a heterogeneous group of zebrafish. We calculated the spatial coordinates of fish (x, y, and z positions) to measure the social behavior of three healthy zebrafish in the presence of one subject intraperitoneally injected with a 5% acetic acid solution, known to elicit abnormal writing-like behavior in adult zebrafish, which has been closely related to acute visceral pain described for rodents [32,38,39]. Moreover, we assessed how zebrafish interact with phenotypically normal and abnormal fish using the social preference test, aiming to investigate the influence of visual cues to distinct each shoal condition.

2. Materials and Methods

2.1. Animals

Adult zebrafish (*Danio rerio*) with 4–6 months-old (~50:50 male:female ratio, shortfin phenotype), and weighing 0.25 to 0.3 g were purchased from a commercial supplier (Hobby Aquários, RS, Brazil). Fish were maintained for two weeks in 40-L thermostatic aquariums at a maximum density of two fish per liter and kept under constant mechanical and chemical filtration before the experiments to acclimate to the laboratory facility. Tanks were filled with non-chlorinated water at 27 ± 1ºC, pH 7.2, conductivity at 1,300–1,500 μS.cm^{-1}, dissolved oxygen at 6.0 ± 0.1 mg/L, total ammonia at < 0.01 mg/L, nitrate (NO_3^-) < 50 mg/L, nitrite (NO_2^-) < 0.1 mg/L, alkalinity and hardness at 75 mg/L $CaCO_3$. Illumination was provided by ceiling-mounted fluorescent light tubes on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am and off at 9:00 pm) [40]. Fish were fed thrice daily with commercial flake fish food (Alcon BASIC^{TM}, Alcon, Brazil). All experiments were performed using cohorts obtained from three independent batches. Animals used were naive and maintained following the National Institute of Health Guide for Care and Use of Laboratory Animals. All experiments described here were approved by the Institutional Animal Care and Use Committee (protocol number 2220181215).

2.2. Shoaling behavior task

The shoaling behavior task was used to assess the group responses of zebrafish as reported previously [7,19,20,41]. Four zebrafish were simultaneously placed in the experimental tank (20 cm length x 15 cm height x 20 cm width), with a 10 cm water column height. After 1 min of habituation, we recorded a 6-min video using two orthogonal-located cameras (one positioned at 60 cm from the bottom of the apparatus and another located at 60 cm from the back wall of the apparatus) connected to a laptop to assess the baseline behavior of the group [20]. Then, one animal of the shoal was gently handled, anesthetized in cold water

[32], briefly immobilized using a small wet fishing net, and 10 µL of phosphate-buffered saline (PBS) (control), acetic acid 5% (AA), morphine 2.5 mg/kg (MOR) or acetic acid 5% plus morphine 2.5mg/kg (AA+MOR) were administered intraperitoneally (i.p.) using a BD Ultra-fine™ syringe (6 mm needle length and 0.25 mm needle gauge) [32]. After injection, the treated subjects were reintroduced into the experimental tank. After 1 min of habituation, the shoaling behavior was recorded for 6 min [20]. Screenshots were made every 10 s across the trial (totalizing 144 screenshots per shoal), and treated fish was continuously monitored for further data analysis (both at the baseline and after the injection).

Screenshots from synchronized videos of frontal and top view perspectives were further exported to the Image J 1.49 software for Windows^{TM}. To obtain the spatial "*x* and *y*" (frontal camera) and "*x* and *z*" (top camera) coordinates of fish, the software was calibrated using the known dimensions of the apparatus (in cm). Two trained researchers (inter-rater reliability > 0.90) identified the treated subjects aiming to obtain the individual fish spatial coordinates. Then, we performed the 3D analysis of shoal behavior using the protocol described elsewhere [20]. As main endpoints, we measured the inter-fish distance, the inter-fish distance of the injected fish, the inter-fish distance between non-injected fish, nearest neighbor distance of the injected fish, nearest neighbor distance between non-injected fish, and homogeneity index of the injected fish (**Fig. 1A**). The homogeneity index was calculated as the ratio of the distance of injected fish (to the centroid point of the shoal) in relation to the average distance to the centroid position of the other three non-injected conspecifics (**Fig. 1B**). Fish with homogeneous dispersion show index values closer to 1. Values higher than 1 represent fish more distant to the centroid concerning their conspecifics, while values lower than 1 reflect increased proximity to the centroid position of the shoal [20]. Analyses were performed using *n* = 6 shoals per group.

2.3. Social preference test

To assess the social preference, fish (n = 9 per group) were placed individually in the social preference apparatus (25 cm length x 15 cm height x 10 cm width) with the water column adjusted to 10 cm. The experimental tank was placed between two stimuli tanks (15 cm length x 15 cm height x 10 cm width) with transparent walls, allowing visual contact with these apparatuses. The experimental tank was virtually divided into 3 segments, in which two lateral areas (6.25 cm length) were closest to both stimuli tanks, and the third one comprised a center area (12.5 cm length). Stimuli tanks consisted of (i) three PBS-injected zebrafish (PBS); (ii) three non-injected fish (Ø); or (iii) three 5% acetic acid-injected zebrafish (AA) kept in home tank water. Importantly, the left/right position of each stimulus tank was changed between trials to minimize the influence of potential spatial bias on the behavioral responses measured. Fish were acclimated for 30 s in the test tank and behaviors were recorded for 60 s based on the protocol described previously [13]. The time spent in each area, absolute turn angle, and distance traveled were quantified using the ANY-maze^{TM} software (Stoelting, CO, USA) at 30 frames/s.

2.4. Statistics

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett’s tests, respectively. Shoaling behavioral endpoints were expressed as means ± standard error of the mean (S.E.M.) and analyzed by Student *t*-test, one-way analysis of variance (ANOVA), or repeated measures (RM) two-way ANOVA (animals and time as factors). Differences between groups were further assessed by Student-Newman-Keuls multiple comparisons test whenever appropriate. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Three-dimensional analyses of shoaling behavior

Fig. 2 shows the inter-fish distance between fish before and after injections. No significant differences were found after PBS **(Fig. 2A)**, AA **(Fig. 2B)**, MOR **(Fig. 2C)**, and AA+MOR **(Fig. 2D)** administrations when compared to their controls.

When behavioral activities of the fish group were measured, two-way ANOVA showed that PBS did not elicit significant differences between injected and non-injected fish **(Fig. 3A)**. For AA treatment, significant effects of animals x time interaction ($F_{(1, 10)} = 25.37$, $p = 0.0005$) and animals ($F_{(1, 10)} = 12.67$, $p = 0.0052$) were found. Basically, AA increased the inter-fish distance of the injected fish compared to their conspecifics, while untreated animals (non-injected fish) showed a significant reduction of the respective behavioral endpoint **(Fig. 3B)**. Both MOR **(Fig. 3C)** and AA+MOR **(Fig. 3D)** did not alter the inter-fish distance of injected and non-injected animals.

The effects of treatments on the nearest neighbor distance were also verified **(Fig. 4)**. PBS administration **(Fig. 4A)** did not change the respective behavior of fish, while the injection of AA showed significant effects of animals x time interaction ($F_{(1, 10)} = 63.7$, $p < 0.0001$), animals ($F_{(1, 10)} = 18.65$, $p = 0.0015$), and time ($F_{(1, 10)} = 38.77$, $p < 0.0001$). Post-hoc analyses revealed that AA increased the nearest neighbor distance in injected fish when compared to their baseline and non-injected conspecifics **(Fig. 4B)**. Moreover, although MOR alone did not change the nearest neighbor distance **(Fig. 4C)**, the AA+MOR group showed a significant effect of time ($F_{(1, 10)} = 63.7$, $p < 0.0001$), in which an increased nearest neighbor distance was verified for after the injection **(Fig. 4D)**. Regarding the homogeneity index, no differences were found in PBS **(Fig. 5A)** and MOR **(Fig. 5C)** compared to their respective baseline. However, both AA ($t = 8.055$, $df = 10$, $p < 0.0001$) **(Fig. 5B)** and AA+MOR ($t = 2.884$, $df = 10$, $p = 0.0163$) **(Fig.**

5D) showed a substantial increased homogeneity index of injected fish in relation to their controls.

The comparative behaviors analyzed after the treatments are shown in **Fig. 6.** Although the inter-fish distance did not vary between groups, both inter-fish distance ($F_{(3, 20)} = 9.148$, $p = 0.0005$) and nearest neighbor distance ($F_{(3, 20)} = 18.85$, $p < 0.0001$) of the injected fish showed a significant increase in the AA group, which were antagonized by AA+MOR treatment. AA increased the homogeneity index of the injected fish compared to the PBS group, while MOR attenuated such effect ($F_{(3, 20)} = 39.04$, $p < 0.0001$).

3.2. Social preference

Fig. 7 shows the influence of stimulus fish treated with PBS or AA on social preference. Zebrafish show high preference to either PBS ($t = 7.519$, $df = 16$, $p < 0.0001$) (**Fig. 7A**) or AA ($t = 5.656$, $df = 16$, $p < 0.0001$) (**Fig. 7B**) over an empty aquarium (Ø). However, a remarkable preference to PBS over AA shoal was verified ($t = 7.621$, $df = 16$, $p < 0.0001$) (**Fig. 7C**) and no changes were observed when motility endpoints were measured (distance traveled and absolute turn angle) for each experimental condition **(data not shown)**.

4. Discussion

The recognition of injured conspecifics is important to minimize predation risk and disease contagion, which constitute adaptative features to survival [42–44]. Although the ability of zebrafish to recognize chemical cues and stress responses from other conspecifics has been reported previously [25,45,46], the modulatory role of nociception on group responses and social preference still needs to be fully elucidated. Here, using heterogeneous shoals, we investigated for the first time how a group of zebrafish respond to an individual subjected to a painful stimulus, as well as the potential influence of visual perception on social preference. In general, our findings show that non-manipulated zebrafish tend to avoid fish displaying nocifensive behaviors by increasing shoaling response. Importantly, such findings may be associated, at least in part, with visual recognition of aberrant behavioral phenotypes since animals also revealed a clear preference for PBS over AA-treated fish in the social preference test.

Social behaviors promote positive effects on well-being by facilitating detection of potential predators, foraging, and mating opportunities [14,29,47], as well as reducing the experience of a stressful event [48]. In their natural environment, zebrafish exhibit robust shoaling behaviors, varying from smaller 4–12 fish shoals to larger (up to ~300 fish) in slowly-flowing water and faster-flowing rivers, respectively [49,50]. Aversive conditions are known to elicit defensive behaviors, in which zebrafish increase bottom-dwelling, erratic movements, and immobility [23,46,51]. Similarly, social behavior can also be affected by numerous environmental factors, including potentially dangerous situations [17,46]. Like humans, zebrafish respond to the perceived social support of the shoal and show improved attenuation of stress when conspecifics are present [52,53]. Therefore, using the heterogeneous shoaling paradigm (*i.e.*, in which only one fish is experimentally manipulated with a certain drug) [54] associated with the social preference task may provide interesting insights into how zebrafish behaviors vary in the presence of conspecifics showing abnormal nocifensive phenotypes.

Recent evidence demonstrates that a single intraperitoneal injection of AA induces aberrant behavioral responses in zebrafish (*e.g.*, abdominal constriction-like responses, decreased activity, and stereotyped circling behavior in the top of the tank) [32]. Importantly, such behaviors may persist for at least 30 min, being prevented by classic analgesic drugs, such as morphine [32]. Here, using a 3D analysis of shoaling behavior [20], intragroup analyses revealed that AA increased both the inter-fish distance and nearest neighbor distance of the injected fish in relation to their baseline and other conspecifics. Notably, a marked reduction of the inter-fish distance was verified in non-manipulated animals in the presence of AA-treated fish, suggesting increased shoaling response. Because zebrafish is a social species [17], the social buffering may provide relevant social support to an individual from the negative impact of aversive conditions. Thus, the "group effect" observed here in the presence of an injured conspecific can be predicted, similarly to what as occur in other taxa, such as other fishes [55], birds [56], rodents [57,58] and even humans [57,59,60].

The homogeneity index reflects whether zebrafish are closer to the centroid position of the shoal (*i.e.*, how homogeneous are the fish when compared to their group mates) [20]. Both PBS and MOR did not change the homogeneity index of the injected fish, while AA and AA+MOR showed increased values compared to their respective baseline. Although these findings corroborate the negative role of the algogen in disrupting the homogeneity of the shoal when treated fish are present, intergroup analyses of behavior revealed protective effects of morphine coadministration on the behavioral endpoints measured. Notably, the increased homogeneity index of the injected fish in AA and AA+MOR groups may explain why the inter-fish distance of shoal did not vary between the respective groups. In fact, the manipulated fish showed a significant dispersion of their conspecifics, while non-manipulated animals tend to increase clustering in the tank. These data reinforce the use of individual endpoints analyses in heterogeneous groups [20,61]. Lastly, our 3D data analysis clearly supports that healthy fish are able to discriminate the presence of a single individual with signs of discomfort (*i.e.*, more specifically pain). Furthermore, since morphine has analgesic properties in many species, including zebrafish [32,62,63], alleviating pain-related phenotypes serves as a strategy to modulate group behavior when injured fish are present in a shoal.

Visual discrimination is a key factor that allows zebrafish to identify different environmental contexts, such as the presence of predators [64,65], objects [66], and potential individuals to interact socially [13,16,67]. The social preference task is a behavioral paradigm that allows zebrafish to distinguish the presence and absence of conspecifics in different apparatuses without the influence of potential chemical/physical cues on behavior [13,68,69]. As expected, zebrafish show a robust preference for conspecifics over an empty aquarium, since they spent more time close to fish treated either with PBS or AA. However, when subjects were challenged in the presence of PBS- and AA-treated fish, a markedly preference for PBS group was verified, supporting that visual discrimination of nocifensive responses may indeed elicit aversion, playing a role in the social responses measured here.

Although our experimental approach help elucidate how nociception modulates social behavior, many questions still remain open. Despite the main nocifensive responses do not differ in male and female zebrafish [32], assessing potential sex-dependent effects in heterogeneous shoals can be relevant in future experiments. Moreover, since the perception of conspecifics experiencing stressful situations modulates cortisol release in zebrafish [25], the influence of nociception on the activation of the stress axis in non-manipulated zebrafish should not be ruled out and merits further scrutiny. Although the protocol of fish detection used here is partly laborious, which requires trained researchers to identify manually the manipulated fish, this strategy is inexpensive allowing the quantification of spatial positions of fish using freely available software. Furthermore, this proof-of-concept methodology that uses spatial coordinates for 3D analysis of the shoal may support the development of software able to analyze both occupancy and locomotor parameters in a real-time manner, thereby improving data reliability.

In conclusion, we demonstrate that pain exerts a modulatory effect on zebrafish sociability. Using heterogeneous groups of fish, we verified that AA elicits aversion in non-manipulated animals possibly due to the visual recognition of nocifensive responses. Overall, these results may help improve the welfare assessment of zebrafish shoals under distinct experimental manipulations. This strategy is key to controlling and checking potential adverse effects during treatments, as well as to describing earlier humane endpoints to ensure fish welfare.

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Figure Captions

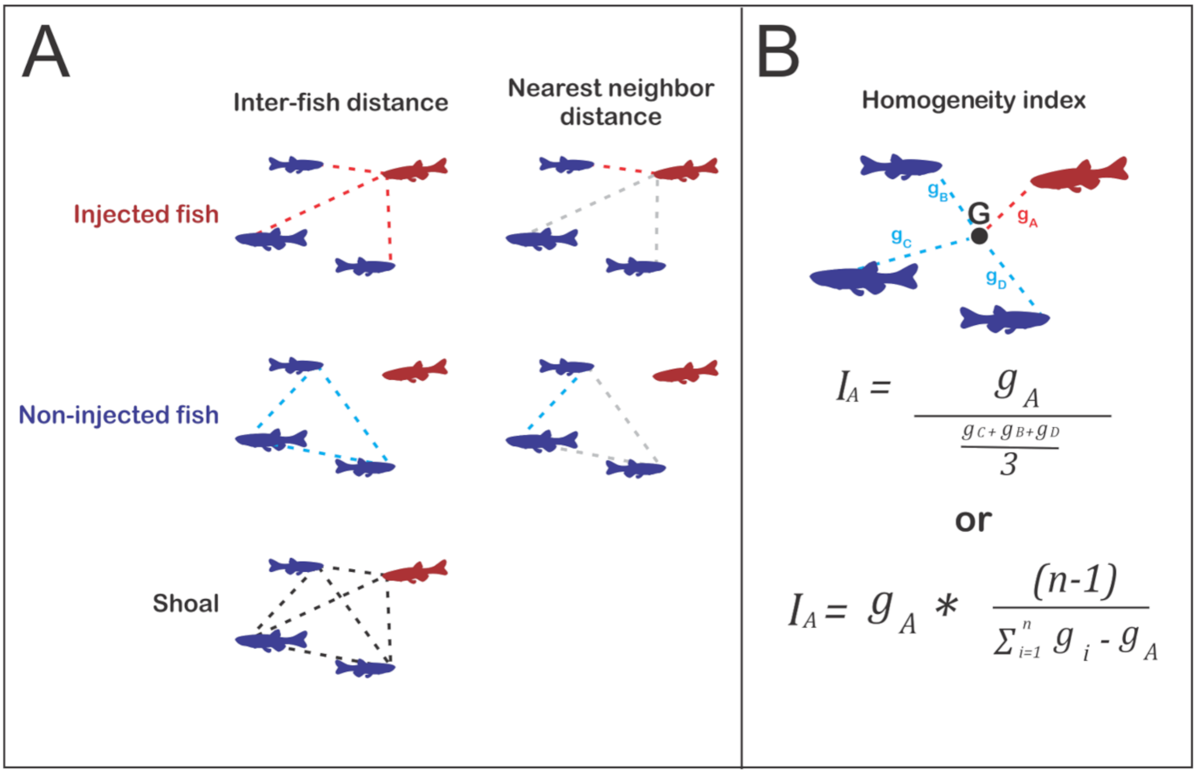

Fig. 1. Schematic diagram showing the main endpoints analyzed in the shoaling behavior task. Dashed lines represent the distances used to measure inter-fish distance and distance to the nearest neighbor distance in injected fish (red), non-injected fish (blue), and inter-fish distance of shoal (black) **(A)**. The homogeneity index of the injected fish was assessed by calculating the distances to the centroid point of shoal G represented by $\boldsymbol{g_A}, \boldsymbol{g_B}, \boldsymbol{g_C}$ and $\boldsymbol{g_D}$ and mathematical formula used is shown **(B).**

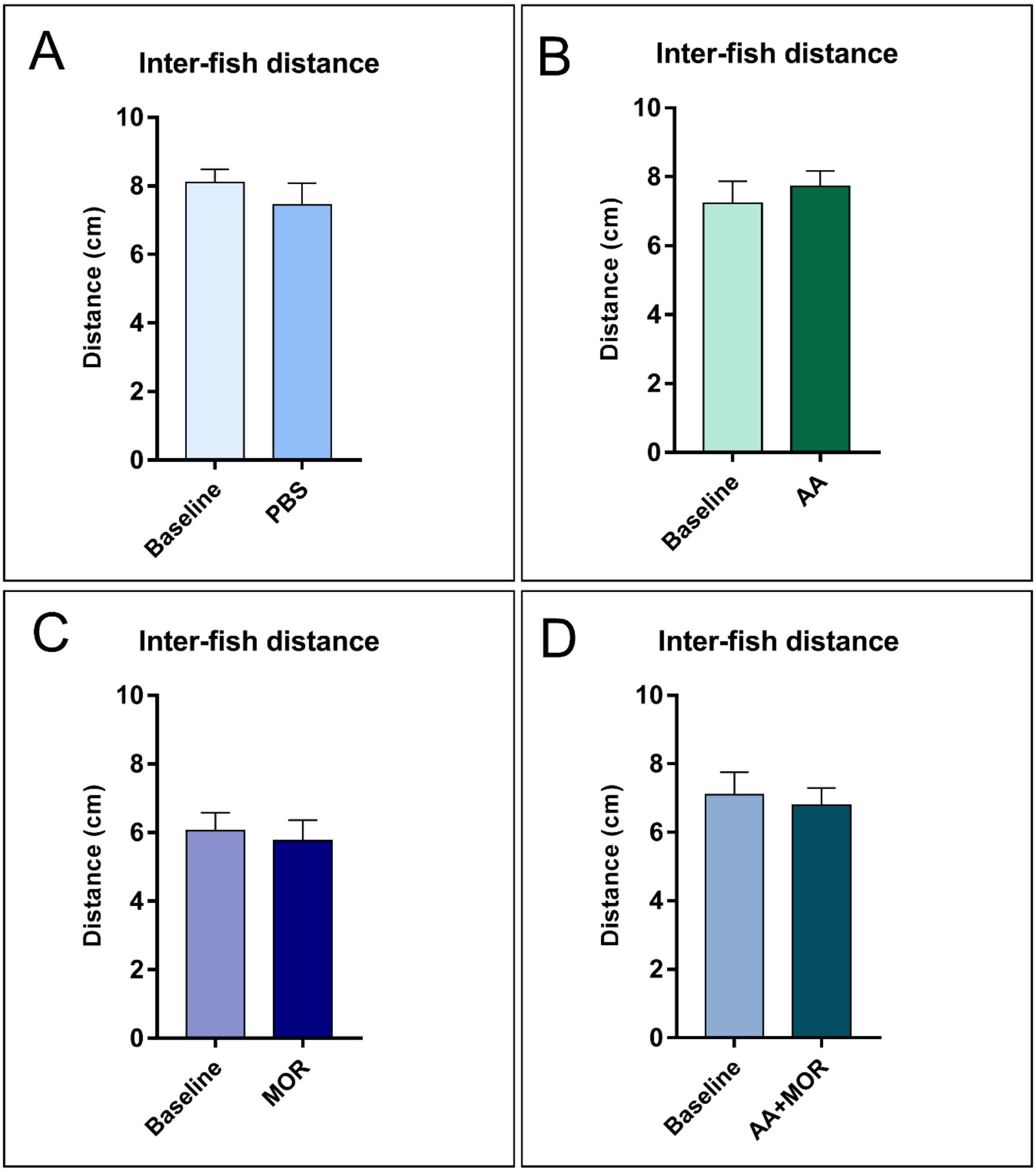

Fig. 2. Inter-fish distance of the shoal before (baseline) and after intraperitoneal injection of PBS **(A)**, AA **(B)**, MOR **(C)**, and AA+MOR **(D)**. Data are expressed as means ± S.E.M and analyzed by Student *t*-test. (*n* = 6 shoals per group).

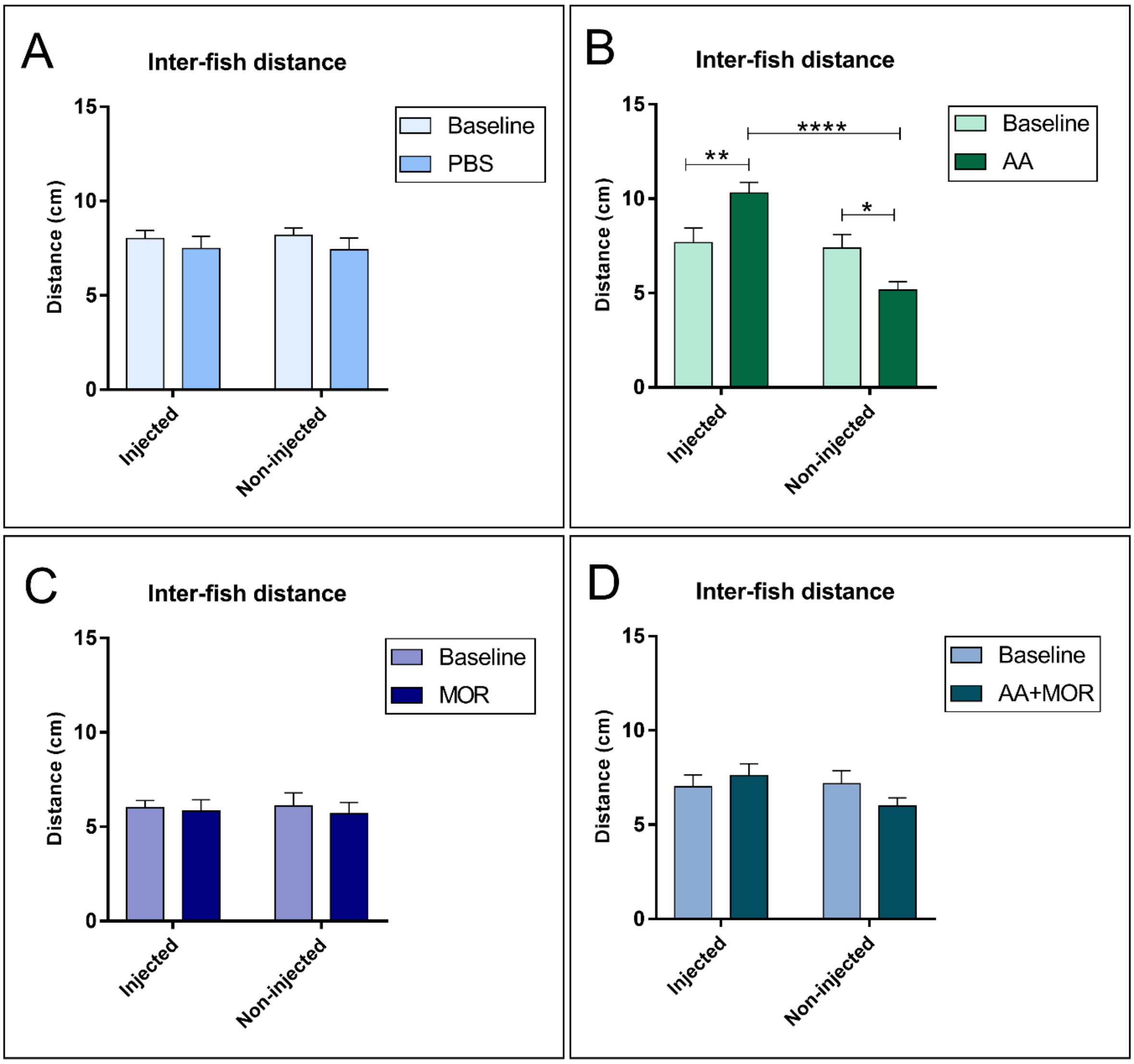

Fig. 3. Inter-fish distance of the injected fish and non-injected fish before (baseline) and after intraperitoneal injection of PBS **(A)**, AA **(B)**, MOR **(C)**, and AA+MOR **(D)**. Data are expressed as means ± S.E.M and analyzed by repeated measures ANOVA followed by Student-Newman-Keuls multiple comparison test. The asterisks indicate statistical differences between groups (*$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$, $n = 6$ shoals per group).

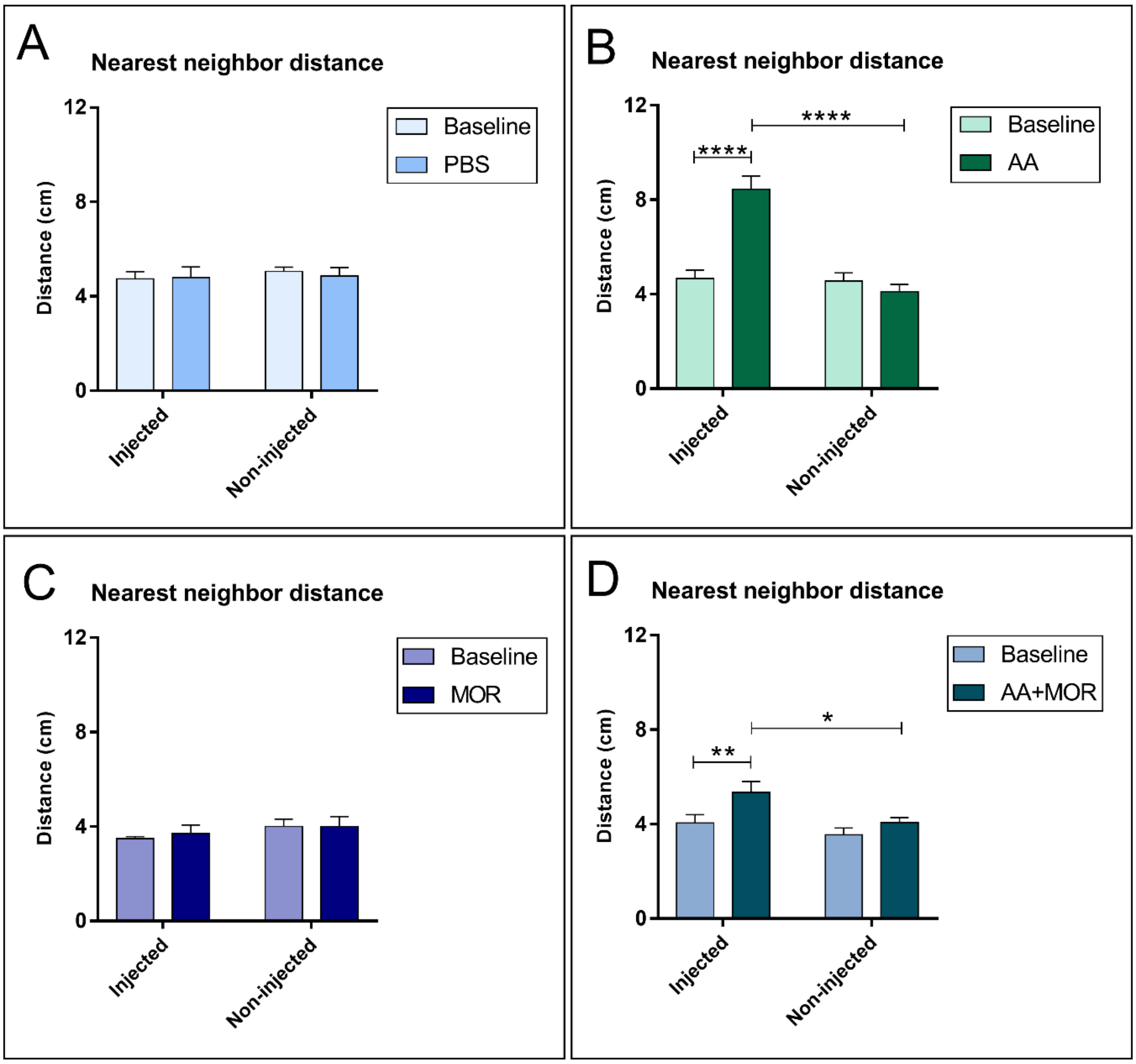

Fig. 4. Nearest neighbor distance of the injected fish and non-injected fish before (baseline) and after intraperitoneal injection of PBS **(A)**, AA **(B)**, MOR **(C)**, and AA+MOR **(D)**. Data are expressed as means ± S.E.M and analyzed by repeated measures ANOVA followed by Student-Newman-Keuls multiple comparison test. The asterisks indicate statistical differences between groups (*$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$, $n = 6$ shoals per group).

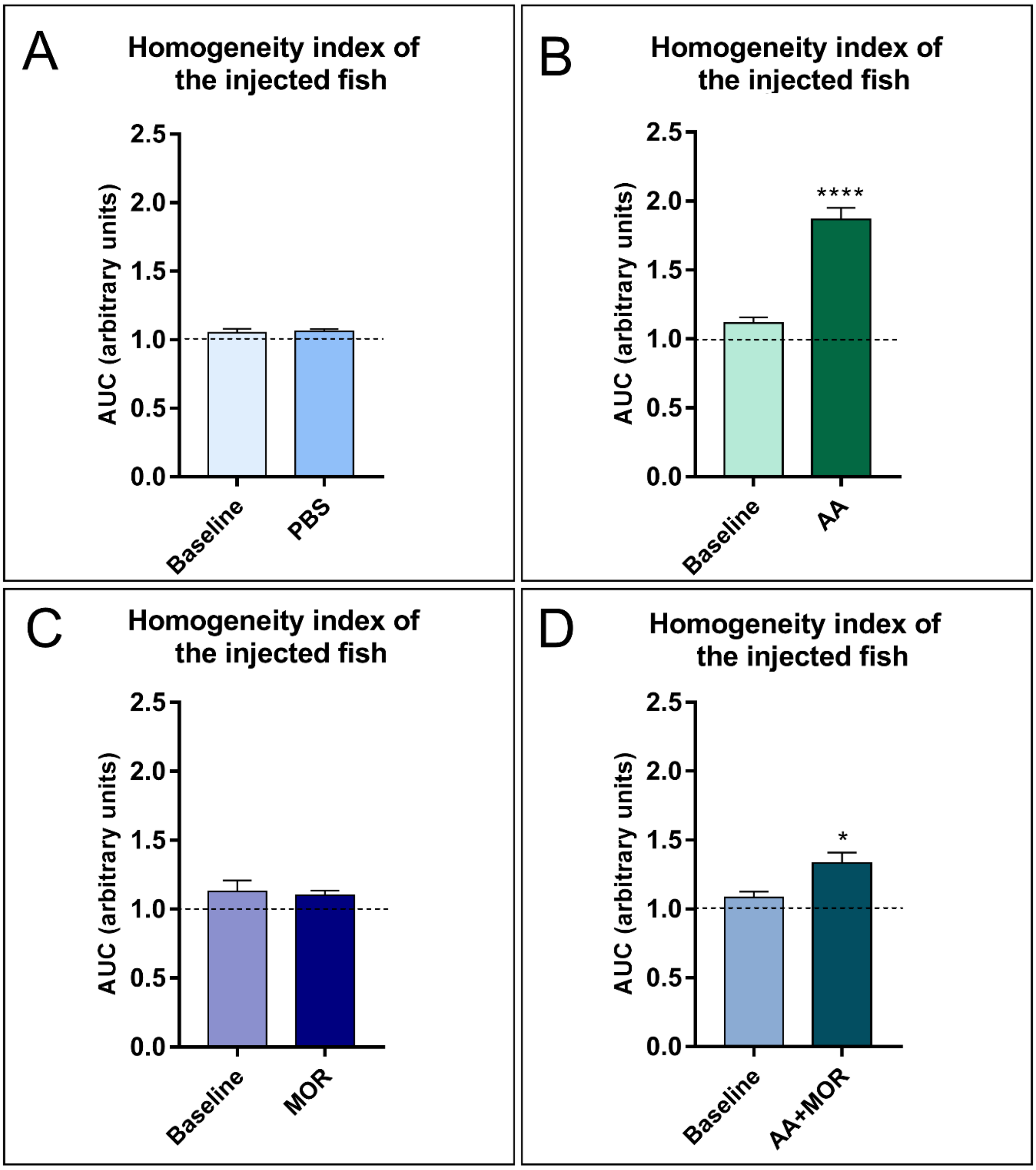

Fig. 5. Homogeneity index of the injected fish before (baseline) and after intraperitoneal injection of PBS **(A)**, AA **(B)**, MOR **(C)**, and AA+MOR **(D)**. Data are expressed as means ± S.E.M and analyzed by Student *t*-test. The asterisks indicate statistical differences between groups (*$p \leq 0.05$, ****$p \leq 0.0001$, $n = 6$ shoals per group).

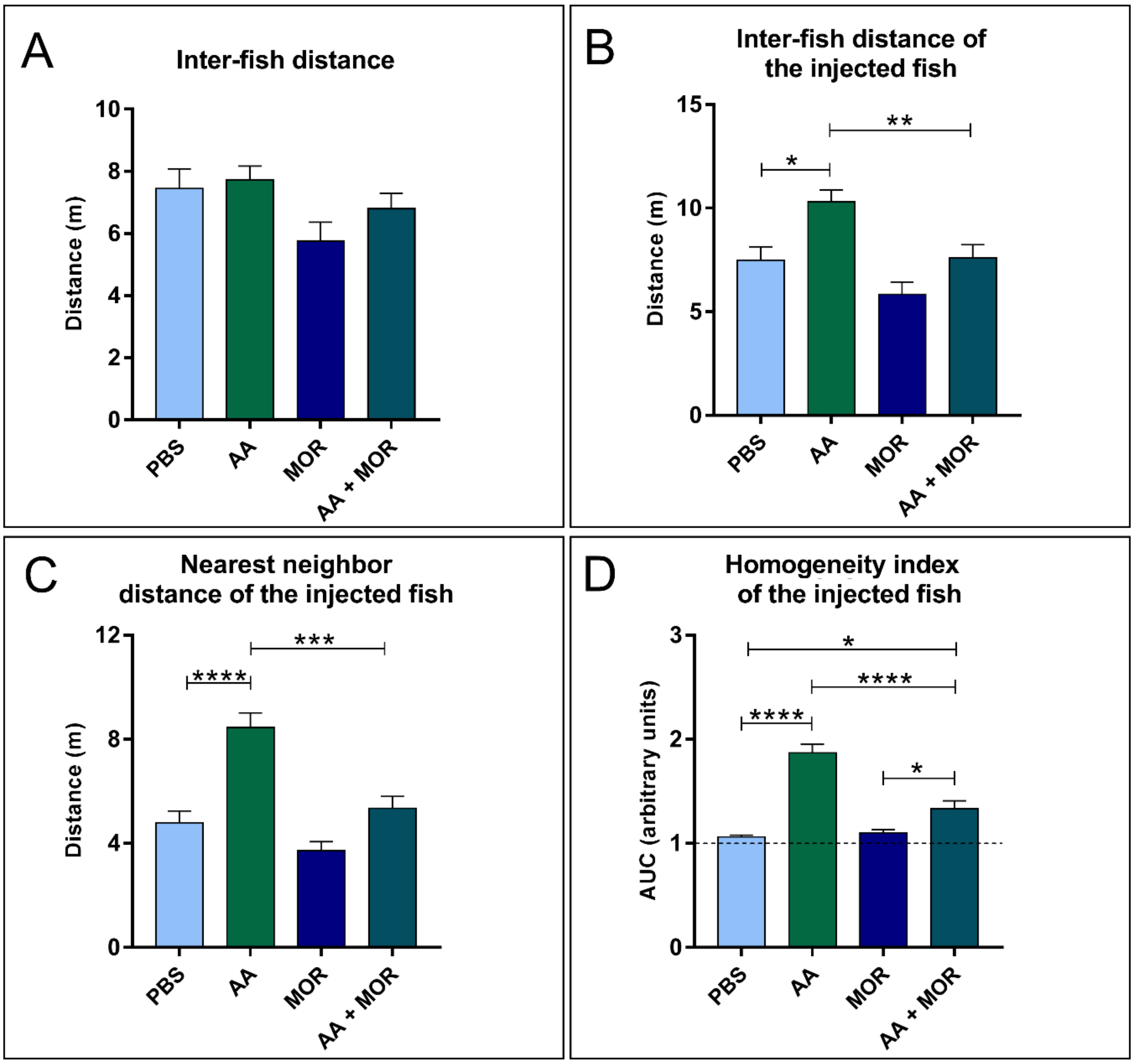

Fig. 6. Comparison of the main endpoints measured after different experimental manipulations: inter-fish distance of shoal **(A)**, inter-fish distance of the injected fish **(B)**, nearest neighbor distance of the injected fish **(C)**, and homogeneity-index of the injected fish **(D)**. Data are expressed as means ± S.E.M and analyzed by one-way ANOVA followed by Student-Newman-Keuls multiple comparison test. The asterisks indicate statistical differences between groups (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.0001$, ****, $p \leq 0.0001$, $n = 6$ shoals per group).

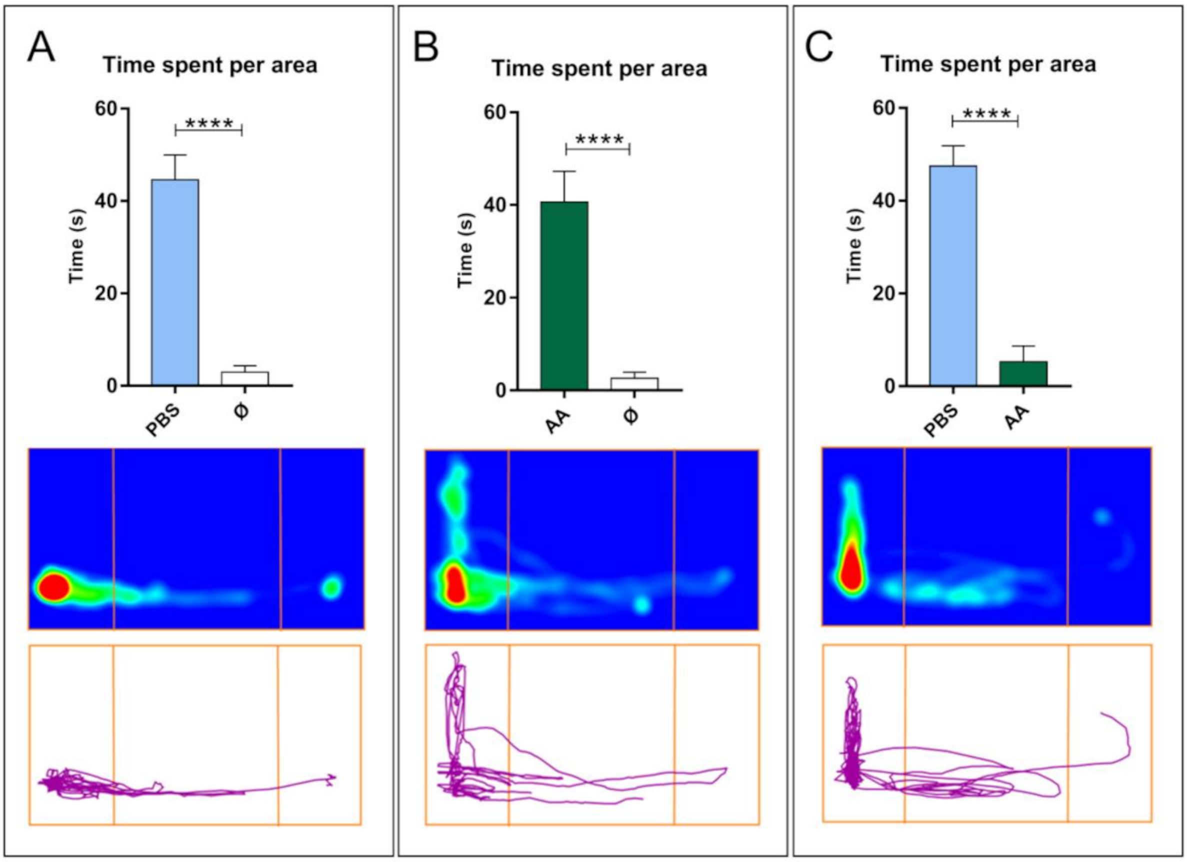

Fig. 7. Time spent per area and representative plots of social preference task in PBS vs Ø **(A)**, AA vs Ø **(B)**, and PBS vs AA **(C)** trials. Data are expressed as means ± S.E.M and analyzed by Student *t*-test. The asterisks indicate statistical differences among groups (****$p \leq 0.0001$, $n = 9$ per group). Red color in occupancy plots indicate the regions in which animals spent more time across the 1 min-trial.

7 DISCUSSÃO

Sabe-se que o comportamento social é fator determinante para sobrevivência e bem estar de diversas espécies do reino animal, incluindo a espécie humana (COHEN; UNDERWOOD; GOTTLIEB, 2000; FEHR; FISCHBACHER, 2003; TREHERNE; FOSTER, 1981). Devido a sua importância biológica, faz se necessária a constante otimização dos métodos científicos de análises sociocomportamentais em modelos animais com grande potencial translacional. No primeiro estudo, desenvolvemos um método que utiliza coordenadas espaciais de cada indivíduos do cardume para uma reconstrução tridimensional das posições e ocupação espacial do cardume dentro da área de teste. Através desta metodologia, mostramos como moduladores da resposta do tipo ansiedade afetam o comportamento de cardume, influenciando as interações sociais entre peixes, ocupação do cardume e coesão de grupo. Utilizando análises de diferentes perspectivas, mostramos e discutimos as discrepâncias nas análises de significância estatística entre as técnicas 3D e 2D, revelando uma diferença no perfil ansiogênico das substâncias CAF e CAS nas reconstruções tridimensionais, nas quais não foram detectadas nas análises bidimensionais. No geral, verificamos a importância de utilização de técnicas de análise espacial mais refinadas que representam mais fidedignamente o nado e a posição dos animais.

Apesar da metodologia requerer um certo grau de labor, o protocolo desenvolvido é de custo baixo podendo ser realizado tanto manualmente ou com ajuda de softwares livres (gratuitos). Além do mais, esta metodologia conceitual que utilizada as coordenadas *x*, *y*, e *z* para uma reconstrução tridimensional do cardume poderá futuramente auxiliar no desenvolvimento de um software, ou extensão de software existentes, capazes de analisar tanto parâmetros posicionais como locomotores em tempo real, assim potencializando o poder de análise do componente social em modelos animais com movimentação tridimensional. Embora a metodologia desenvolvida seja dependente da utilização de quatro animais, desenvolvemos uma análise da localização do ponto centroide, bem como a distância dos peixes em relação a esse ponto, permitindo uma análise com maior número de animais. Além disso, utilizando os dados do ponto centroide do cardume, elaboramos um índice de homogeneidade capaz de identificar animais com posicionamento discrepante em relação aos companheiros de grupo.

A despeito de muitas questões estarem abertas, as novas abordagens descritas neste estudo permitirão elucidação das respostas promovidas por diferentes contextos experimentais sobre o comportamento social do peixe-zebra. Ainda, através de medidas de distância entre peixes, volume de cardume, ponto centroide do cardume, índice de homogeneidade, será possível obter informações sobre a influência de fenômenos intrínsecos e extrínsecos do comportamento social, bem como a análise de social em cardumes heterogênicos.

Devido à importância biológica da dor como sinalização frente a estímulos nocivos, que desencadeia resposta adaptativas importantes para a sobrevivência das espécies complexas (BASBAUM et al., 2009; DARWIN, 1872; WOOLF, 2010) e da importância da nocicepção no bem estar animal de pesquisa (CARBONE; AUSTIN, 2016), optamos pela investigação dos efeitos do evento nociceptivo em um contexto social em um segundo estudo. Desta maneira, fazendo-se uso da metodologia desenvolvida de análise tridimensional de cardume, procuramos caracterizar a resposta comportamental do peixe-zebra frente um contexto de nocicepção e a influência de indivíduos reproduzindo comportamentos nocifensivos em seus coespecíficos.

A nocicepção em peixe-zebra já é descrita em literatura (COSTA et al., 2022), com diversas metodologias que são capazes induzir este tipo de resposta, como por exemplo a injeção de algógenos na cauda (MAXIMINO, 2011) e lábios (REILLY et al., 2008b), bem como metodologias que utilizam de danos mecânicos na nadadeira caudal (SCHROEDER; SNEDDON, 2017). Nosso laboratório de pesquisa desenvolveu uma metodologia de indução à nocicepção através da injeção do ácido acético 5% (AA) via intraperitoneal (COSTA et al., 2019). Diferentemente das outras metodologia que as respostas comportamental observadas são compartilhadas com outros fenótipos comportamentais (ex: ansiedade, medo, agressividade) (COSTA et al., 2019), a metodologia da indução a nocicepção por injeção de AA intraperitoneal desencadeia uma resposta comportamental estereotipada unicamente observada nesta metodologia até então (COSTA et al., 2019). Assim, optamos por esta metodologia neste estudo visando avaliar uma resposta unicamente de cunho nociceptivo. A resposta comportamental observada na metodologia escolhida é caracterizada pela contorção abdominal, a qual resulta numa mudança postural para a posição curvada (COSTA et al., 2019). Ainda, esta alteração sobre a curvatura corporal é revertida com a coadministração de analgésicos, sendo que a ação reversora da morfina 2,5 mg/kg (MOR) é inibida pela coadministração de nalaxona 5mg/kg, caracterizando a resposta comportamental de caráter nosifensiva (COSTA et al., 2019).

Nossos dados demostraram que animais induzidos à nocicepção afetam a interação do cardume, levando aos animais não induzidos do grupo ao aumento da coesão social, enquanto o animal com comportamento nocifensivo permanecia isolado em relação ao resto do grupo. Estas alterações foram comparadas tanto antes quanto depois dos respectivos tratamentos, bem como em comparação entre os diferentes tratamentos. Além disso, nossos dados demostraram que as alterações comportamentais sociais tanto do animal injetado como dos coespecíficos que lhe acompanhavam no cardume foram amenizados pela coadministração de MOR. Estes dados corroboram com os achados da caracterização do protocolo previamente desenvolvido pelo nosso grupo de pesquisa em que coadministração de morfina amenizavam ou aboliam os efeitos induzidos pelo AA, dependendo do parâmetro comportamental analisado (COSTA et al., 2019).

Além das análises tridimensionais, nosso experimento de preferência social demostrou uma preferência pelo lado com coespecíficos em relação **à** ausência de estímulo social. Entretanto, quando ambos lados apresentavam cardumes, um lado com cardume padrão e outro com um cardume com comportamento nocifensivo, foi observado uma ampla preferência dos animais testados por cardumes com comportamento padrão. Estes dados, além de corroborarem com os achados do primeiro experimento do segundo estudo, sugerem uma possível capacidade identificação visual do comportamento nocifensivo reproduzido pelos coespecíficos, já que a única interação possível entre os animais teste com os animais estímulos era de cunho visual. Entretanto, os aspectos que regem esta possível identificação necessitam de estudos futuros para ser melhor elucidados, bem como a capacidade do peixe-zebra de identificar outros padrões comportamentais relacionado a nocicepção.

O peixe-zebra é uma espécie que apresenta uma boa acuidade visual (BILOTTA; SASZIK, 2001; GESTRI; LINK; NEUHAUSS, 2012; TAPPEINER et al., 2012) e esta acuidade visual permite a identificação visual de coespecíficos (ENGESZER; RYAN; PARICHY, 2004), predadores (GERLAI; FERNANDES; PEREIRA, 2009; MCKEE; MCHENRY, 2020), ambiente (COGNATO et al., 2012) e objetos (STEFANELLO et al., 2019). Estudos que utilizaram imagens digitais de peixe-zebra demonstraram que a preferência pela imagem de coespecíficos sofre influência conforme o tamanho, cor e forma do estímulo visual (FERNANDES et al., 2015; SAVERINO; GERLAI, 2008), reforçando a hipótese da capacidade discrição de características visuais de coespecíficos. Dentro das vantagens sociais de uma boa acuidade visual está a vigilância frente a ataque de predadores (PITCHER, 1986). Além disso, estudos anteriores demostram a influência da visão do peixe-zebra na coesão do cardume, já que a distância entre animais reflete sobre a área dos pontos cegos do cardume nos quais são vulneráveis a ataques de predadores (PITA et al., 2015). Com a redução de pontos cegos, aumentaria a probabilidade de visualização de predadores por um ou mais ínvidos e a visualização das respostas comportamentais destes indivíduos por seus coespecíficos desencadearia um efeito cascata comportamental em todo cardume, assim reduzindo drasticamente a chance de predação (PITA et al., 2015). Devido estes achados que demostram a capacidade do peixe-zebra de discrição visual do ambiente, hipotetizamos que esta espécie poderia possuir a capacidade de identificação visual de fenótipos comportamentais aberrantes de coespecíficos, e esta identificação resultaria em uma série de respostas comportamentais.

Enfim, conjecturamos que este indivíduo com comportamento nocifensivo poderia se apresentar como fator de risco para o resto do cardume, sendo potencial atrativo para predação (ELVIDGE; BROWN, 2012; WISENDEN; THIEL, 2002) ou um potencial fonte de contaminação por patologia (SARABIAN; CURTIS; MCMULLAN, 2018). Entretanto, estudo futuros serão necessários para elucidar estes aspectos sociocomportamentais.

8 CONCLUSÕES PARCIAIS

Através dos novos resultados obtidos pelos estudos desenvolvidos durante o período de doutorado, podemos afirmar que:

- A metodologia de análise tridimensional desenvolvida mostrou-se uma alternativa de análise de ocupação espacial viável, replicável e de fácil acesso financeiro;

- A análise tridimensional aperfeiçoou a análise de ocupação espacial do cardume, revelando discrepâncias observacionais entre análises bidimensionais, bem como permitiu análise de diferentes parâmetros comportamentais como análise de ponto centroide e homogeneidade do cardume;

- Exposição ao diazepam promoveu um robusto efeito do tipo ansiolítico, aumentando a dispersão dos peixes do cardume e redução da geotaxia. Por outro lado, a exposição à cafeína e à substância de alarme de coespecíficos promoveram efeitos do tipo ansiedade, aumentando a coesão do cardume e aumento da geotaxia, sendo que estes efeitos foram mais proeminentes na exposição à substância de alarme de coespecíficos;

- A presença de um animal induzido ao comportamento nocifensivo pela administração intraperitoneal de ácido acético 5% gerou uma resposta de grupo, resultante da dispersão animal manipulado, concomitante agrupamento dos animais não manipulados. Tais respostas comportamentais foram atenuadas com a coadministração de morfina 2,5 mg/kg;

- O peixe-zebra possui uma inata preferência por aproximação de coespecíficos. Entretanto, quando testada a preferência entre cardumes com comportamento normal e cardumes reproduzindo comportamento nocifensivos, os animais testados apresentaram uma proeminente preferência de aproximação de cardumes com comportamento normal, sugerindo uma capacidade do peixe-zebra de identificação visual de fenótipos comportamentais aberrante de coespecíficos.

9 CONCLUSÃO FINAL

Em geral, os estudos demostram a alteração do comportamento social do peixe-zebra frente a estímulos distintos, suportando a plasticidade do comportamento social como comportamento adaptativo defensivo. A metodologia elaborada revelou que a perspectiva visual da análise do nado do peixe-zebra pode influenciar a interpretação de dados relativos ao comportamento social em um contexto aversivo, podendo levar a inobservância de fenótipos comportamentais nas perspectivas bidimensionais. Observamos que uma alteração fenotípica relacionada à nocicepção em um único indivíduo é capaz refletir na interação de coespecíficos não apenas ao indivíduo afetado, mas também a interações entre os demais animais do cardume. Além do mais, estas alterações são passiveis de identificação visual, o que pode contribuir nas respostas observadas de coesão do cardume. Por fim, finalizando, os estudos descritos nesta tese proporcionam achados que poderão servir de suporte para refinar os métodos de análise comportamental social em estudos futuros, bem como na manutenção e bem-estar desta espécie em ambiente laboratorial.

10 PERSPECTIVAS

O desenvolvimento de uma metodologia em que permite uma análise mais fidedigna da ocupação espacial dos cardumes de peixe-zebra (ROSA et al., 2020) e análise da interferência dos comportamentos nocifensivos nas interações sociais (**Manuscrito Científico**), apresenta achados significativos para futuras investigações relativas ao comportamento ao social desta espécie emergente nas pesquisas neurocomportamentais.

Elucidar como fatores intrínsecos e extrínsecos afetam o comportamento de animais sociáveis é determinante para entendermos as variáveis e os mecanismos que regem este complexo comportamento no qual é essencial para sobrevivência de diversas espécies, bem como otimizar as técnicas de manutenção e manipulação experimental de animais sociáveis em ambiente laboratoriais. Desta maneira, desenvolver novos protocolos experimentais relacionadas ao comportamento social que visam avaliar mudanças ambientais e fisiológicas é uma estratégia para expansão de novos estudos. As técnicas desenvolvidas aqui permitem uma análise individual e conjunta de fenótipos comportamentais, permitindo assim, a análise dos efeitos de substâncias, alterações ambientais e simulação de eventos naturais nos comportamentos sociais em uma perspectiva tanto ecológica como translacional. Além do mais, a analise tridimensional não é restritiva a espécie em estudo, podendo ser adaptada para estudos em outros animais como movimentação tridimensional.

Finalmente, em uma perspectiva de longo prazo, fica a consolidação de uma linha consistente de pesquisa dentro da Universidade Federal de Santa Maria, bem como a metodologia que aprimora a análise social em peixe-zebra. Assim a implementação deste campo de pesquisa certamente trará benefícios não apenas na utilização do peixe-zebra como um modelo alternativo/complementar para estudos neurocomportamentais, mas também, contribuirá para a formação e aperfeiçoamento de recursos humanos.

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ANEXO A – COMPORVANTE DE SUBMISSÃO DO MANUSCRITO CIENTÍFICO

BEHAVIOURAL BRAIN RESEARCH

Role: Author

Username: dbrosemberg@gmail.com

← Submissions Being Processed for Author

Page: 1 of 1 (1 total submissions)

Results per page 10

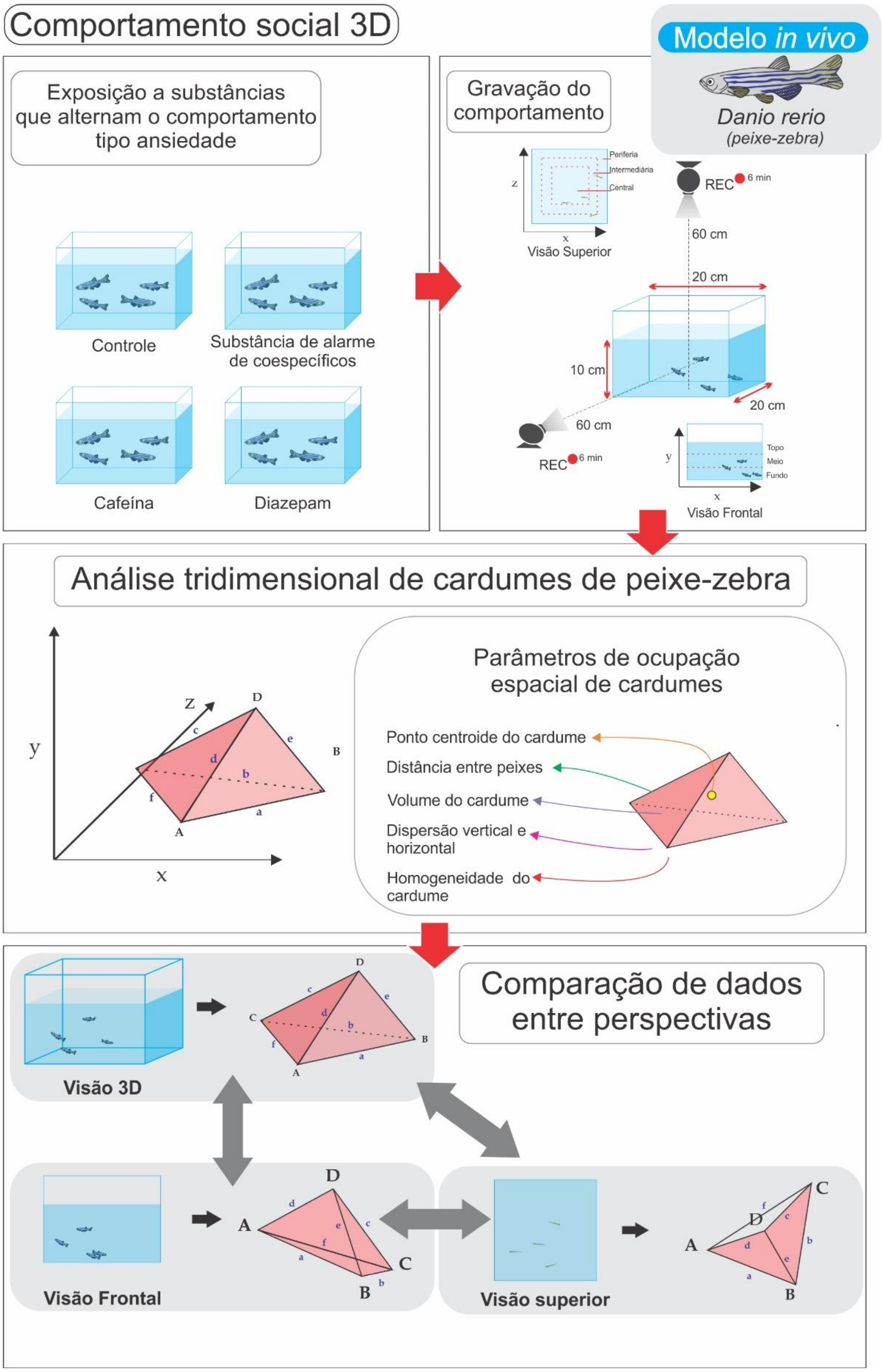
Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	BBRES-D-22-00228	Acetic acid-induced nociception modulates sociability in adult zebrafish: influence on shoaling behavior in heterogeneous groups and social preference	Mar 15, 2022	Mar 24, 2022	Under Review

Page: 1 of 1 (1 total submissions)

Results per page 10

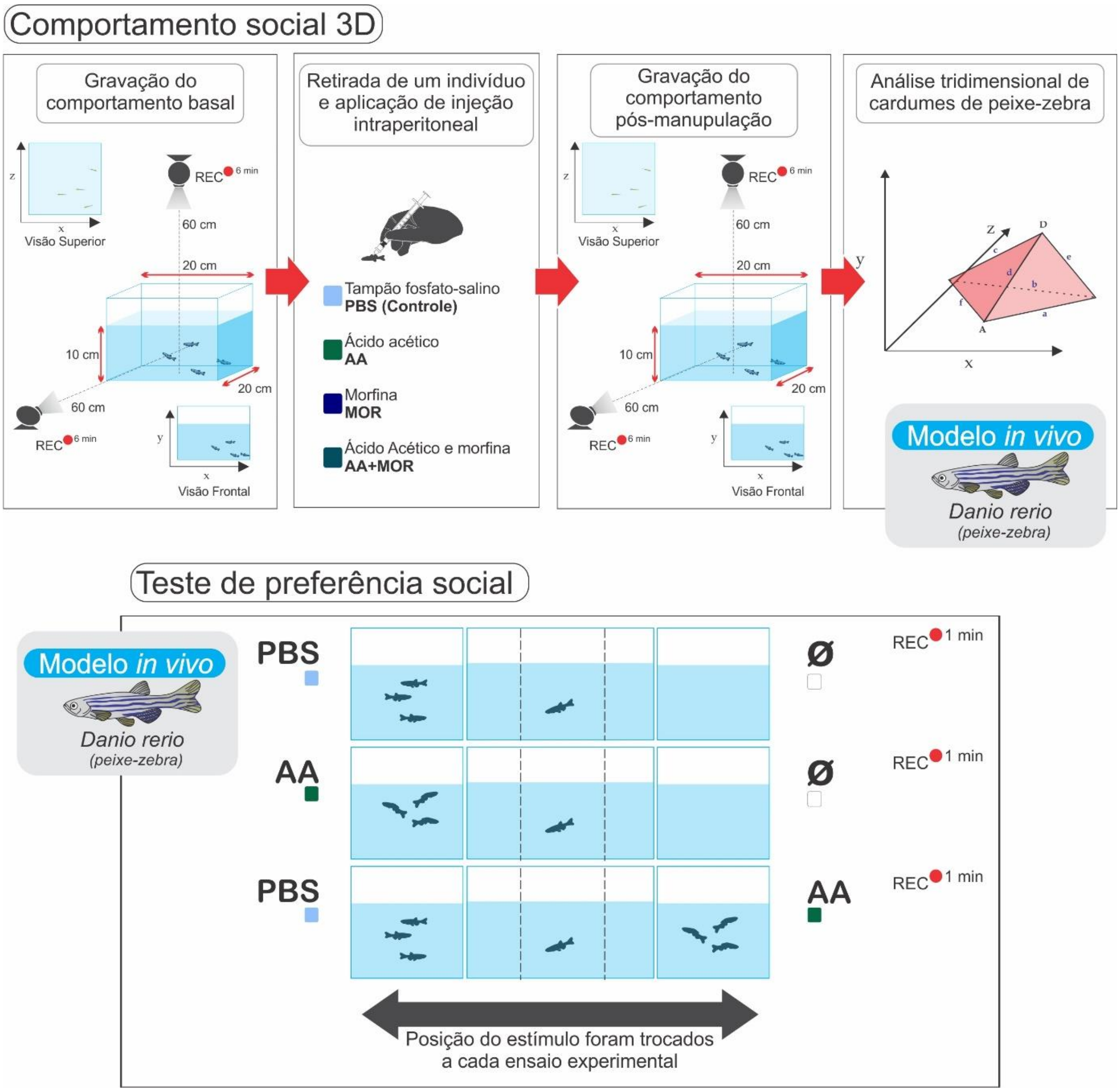
ANEXO B – MAPA CONCEITUAL DOS EXPERIMENTOS ARTIGO 1

Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses

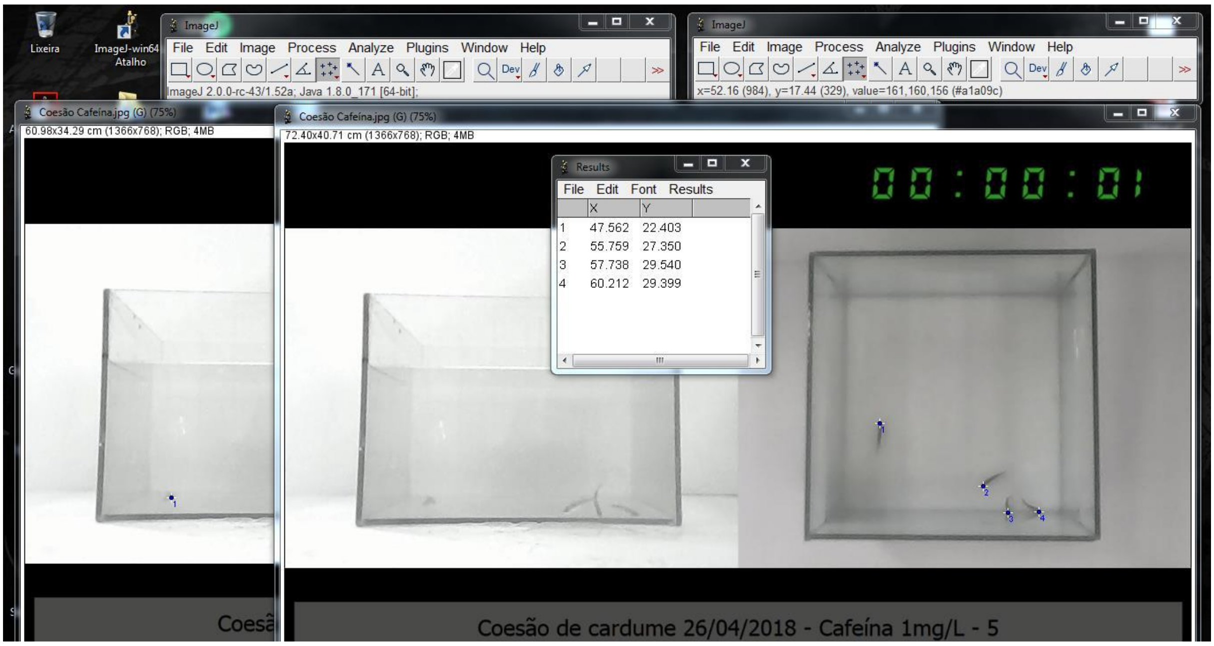


ANEXO C – MAPA CONCEITUAL DOS EXPERIMENTOS MANUSCRITO 1

Acetic acid-induced nociception modulates sociability in adult zebrafish: influence on shoaling behavior in heterogeneous groups and social preference.



ANEXO D – IMAGEM REPRESENTATIVA DO SOFTWARE IMAGE J 1.49 SOFTWARE FOR WINDOWS^{TM} UTILIZADO PARA OBTENÇÃO DAS COORDENADAS ESPACIAIS DAS POSIÇÕES DOS PEIXES NO CARDUME.



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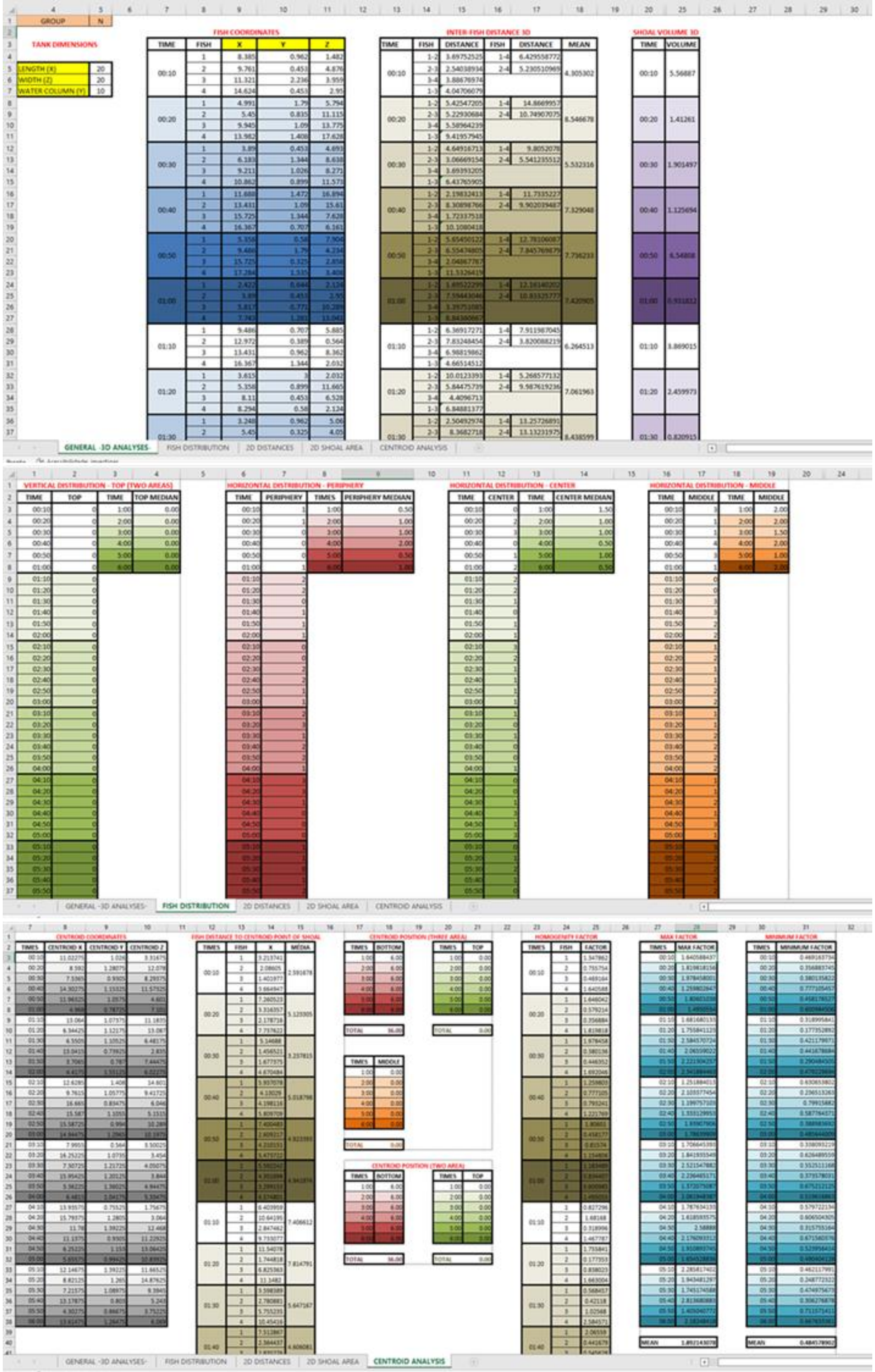
x=52.16 (984), y=17.44 (329), value=161,160,156 (#a1a09c)

60.98x34.29 cm (1366x768); RGB; 4MB

72.40x40.71 cm (1366x768); RGB; 4MB

	X	Y
1	47.562	22.403
2	55.759	27.350
3	57.738	29.540
4	60.212	29.399

ANEXO E – IMAGEM REPRESENTATIVA DA TABELA USADA PARA ANÁLISE DAS COORDENADAS 3D DOS CARDUMES DE PEIXE-ZEBRA



Download em: https://ars.els-cdn.com/content/image/1-s2.0-S0278584620302736-mmc4.xlsx

ANEXO F– PRODUÇÕES CIENTÍFICAS EM COLABORAÇÃO

Artigos produzidos em parceria durante o período do Doutorado:

1. CANZIAN, JULIA ; FRANSCESCON, FRANCINI ; MÜLLER, TALISE E. ; STEFANELLO, FLAVIA V. ; SOUZA, THIELE P. ; **ROSA, LUIZ V.** ; ROSEMBERG**,** DENIS B. . Stress increases susceptibility to pentylenetetrazole-induced seizures in adult zebrafish. EPILEPSY & BEHAVIOR, v. 114, p. 107557, 2021.

2. COSTA, FABIANO V.; ROSA, LUIZ V. ; QUADROS, VANESSA A. ; DE ABREU, MURILO S. ; SANTOS, ADAIR R. S. ; SNEDDON, LYNNE U. ; KALUEFF, ALLAN V. ; ROSEMBERG, DENIS B. . The use of zebrafish as a non-traditional model organism in translational pain research: the knowns and the unknowns. Current Neuropharmacology, v. 19, p. 476-493, 2021.

3. QUADROS, VANESSA A. ; **ROSA, LUIZ V.** ; COSTA, FABIANO V. ; KOAKOSKI, GESSI ; BARCELLOS, LEONARDO J.G. ; ROSEMBERG, DENIS B. . Predictable chronic stress modulates behavioral and neuroendocrine phenotypes of zebrafish: Influence of two homotypic stressors on stress-mediated responses. COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY C-TOXICOLOGY & PHARMACOLOGY, v. 247, p. 109030, 2021.

4. COSTA, FABIANO V. ; **ROSA, LUIZ V.** ; QUADROS, VANESSA A. ; SANTOS, ADAIR R.S. ; KALUEFF, ALLAN V. ; ROSEMBERG, DENIS B. . Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. BEHAVIOURAL BRAIN RESEARCH, v. 359, p. 570-578, 2019.

5. FERREIRA, LUANA MOTA ; **DA ROSA, LUIZ VINÍCIUS COSTA** ; MÜLLER, TALISE ELLWANGER ; DE MENEZES, CHARLENE CAVALHEIRO ; MARCONDES SARI, MARCEL HENRIQUE ; LORO, VANIA LUCIA ; NOGUEIRA, CRISTINA WAYNE ; ROSEMBERG, DENIS BROOCK ; CRUZ, LETÍCIA . Zebrafish exposure to diphenyl diselenide-loaded polymeric nanocapsules caused no behavioral impairments and brain oxidative stress. JOURNAL OF TRACE ELEMENTS IN MEDICINE AND BIOLOGY, v. 53, p. 62-68, 2019.

6. QUADROS, VANESSA A. ; **ROSA, LUIZ V**. ; COSTA, FABIANO V. ; MÜLLER, TALISE E. ; STEFANELLO, FLAVIA V. ; LORO, VANIA L. ; ROSEMBERG, DENIS B. . Involvement of anxiety-like behaviors and brain oxidative stress in the chronic effects of alarm reaction in zebrafish populations. NEUROCHEMISTRY INTERNATIONAL, v. 129, p. 104488, 2019.

Capítulo de livro produzido em parceria durante o período do Doutorado:

1. COSTA, FABIANO V. ; **ROSA, LUIZ V**. ; KALUEFF, ALLAN V. ; ROSEMBERG, DENIS B. . Nociception-related behavioral phenotypes in adult zebrafish. The Neurobiology, Physiology, and Psychology of Pain. 1ed.Londres: Elsevier, 2021, v. 34, p. 387-393.