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BIOQUÍMICA TOXICOLÓGICA

Thiele Piber de Souza

# INFLUÊNCIA DO SEXO NAS DIFERENÇAS COMPORTAMENTAIS PROMOVIDAS PELA EXPOSIÇÃO AO ETANOL EM PEIXES-ZEBRA SUBMETIDOS À INTERAÇÃO COM UM OBJETO NÃO FAMILIAR

Santa Maria, RS

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Dissertação apresentada ao curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção do título de **Mestra em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr. Denis Broock Rosemberg

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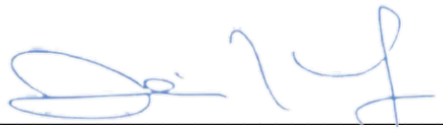

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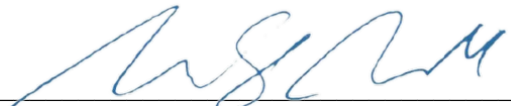

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Santa Maria, RS, Brasil
2021

## DEDICATÓRIA

Agradeço aos meus pais, Dilse e Volnei, pelo apoio incondicional em todos os momentos difíceis dessa jornada. Este trabalho é dedicado a eles.

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## RESUMO

### INFLUÊNCIA DO SEXO NAS DIFERENÇAS COMPORTAMENTAIS PROMOVIDAS PELA EXPOSIÇÃO AO ETANOL EM PEIXES-ZEBRA SUBMETIDOS À INTERAÇÃO COM UM OBJETO NÃO FAMILIAR

AUTORA: Thiele Piber de Souza
ORIENTADOR: Prof. Dr. Denis Broock Rosemberg

Em humanos, o consumo de etanol (EtOH) modula as funções comportamentais de forma bifásica, variando da desinibição à sedação. Os efeitos são dependentes da dose e influenciados por fatores ambientais e individuais, como o sexo biológico. O uso de modelos animais representa uma ferramenta promissora para avaliar se os efeitos do EtOH, sobre os domínios comportamentais, são dependentes do sexo. O peixe-zebra (*Danio rerio*) apresenta os principais sistemas de neurotransmissores envolvidos com as respostas mediadas por EtOH e, devido à sua alta sensibilidade farmacológica, representa um organismo modelo atraente para avaliar os efeitos comportamentais do EtOH nas funções cerebrais. Dessa forma, investigamos se os efeitos agudos de diferentes concentrações de EtOH são dependentes do sexo em peixes-zebra submetidos ao teste de campo aberto (OFT) com a influência de um objeto não familiar. Os animais foram separados por sexo em quatro grupos e expostos de forma aguda ao EtOH (0%, 0,25%, 0,5% e 1,0% v/v) por 1 h. Após a exposição, os peixes foram individualmente inseridos no OFT, o qual continha um objeto não familiar (esfera preta; diâmetro: 1 cm) na área cental do aparato. A atividade comportamental foi registrada por 5 min. Para a análise comportamental, o tanque foi virtualmente dividido em três áreas: periférica, intermediária e central. Como comportamento basal, as fêmeas apresentaram uma resposta exploratória e um padrão de interação com o objeto que refletem um comportamento mais ansioso e tímido em relação aos machos. Fêmeas expostas a 0,5% de EtOH aumentaram as investigações mais rápidas no objeto em comparação aos machos, enquanto a exposição a 1,0% de EtOH reduziu a locomoção em ambos os sexos e aumentou a imobilidade apenas em machos. A análise de componentes principais (PCA) revelou que os componentes que mais representaram as variâncias totais foram os comportamentos semelhante a ansiedade, atividade exploratória e locomoção. De modo geral, nossos achados mostram a existência de um efeito dependente do sexo em peixes-zebra expostos agudamente ao EtOH e testados no OFT com um objeto não familiar. Assim, o presente estudo suporta a investigação dos efeitos neurobiológicos do EtOH sobre fenótipos comportamentais distintos utilizando peixes machos e fêmeas em estudos futuros de neurociência translacional.



**Palavras-chave:** Álcool. Comportamento. Interação com objeto. Sexo biológico. Zebrafish.

## ABSTRACT

### INFLUENCE OF SEX ON BEHAVIORAL DIFFERENCES PROMOTED BY EXPOSURE TO ETHANOL IN ZEBRAFISH SUBMITTED TO INTERACTION WITH A NON-FAMILY OBJECT

AUTHOR: Thiele Piber de Souza
ADVISOR: Prof. Dr. Denis Broock Rosemberg

In humans, ethanol (EtOH) modulates behavioral functions in a biphasic manner, ranging from disinhibition to sedation. The effects of EtOH are dose-dependent and influenced by environmental and individual factors, such as the biological sex. The use of animal models represents a promising tool to assess whether EtOH modulates different behavioral domains in a sex-dependent manner. The zebrafish (*Danio rerio*) has the main neurotransmitter systems involved in EtOH-mediated responses and, due to its high pharmacological sensitivity, represents an attractive model system to assess the behavioral effects of EtOH on brain functions. Thus, we investigated whether the acute effects of different EtOH concentrations are sex-dependent in zebrafish submitted to the open field test (OFT) with the influence of a non-familiar object. Animals were separated by sex into four groups and acutely exposed to EtOH (0%, 0.25%, 0.5% and 1.0% v/v) for 1 h. After exposure, fish were individually placed into the OFT, which contained a non-familiar object (black sphere; diameter: 1 cm) fixed in the central area of the apparatus. Behavioral activity was recorded for 5 min. For the behavioral analysis, the tank was virtually divided into three areas: peripheral, intermediate and central. At the baseline, females showed a distinct exploratory activity and interaction pattern with the object, reflecting a more anxious and shy behavior in relation to males. Females exposed to 0.5% EtOH showed increased faster investigation to the object when compared to males, while exposure to 1.0% EtOH reduced locomotion in both sexes and increased immobility only in males. Principal component analysis (PCA) revealed that the components that most represented total variances of behaviors were anxiety-like responses, exploratory activity, and locomotion. Collectively, our new findings show the existence of a sex-dependent effect in zebrafish models acutely exposed to EtOH tested in the OFT with a non-familiar object. Thus, the present study encourages the use of the zebrafish models of EtOH exposure to assess how sex influences distinct behavioral phenotypes in future translational neuroscience research.



**Keywords**: Alcohol. Behavior. Interaction with object. Biological sex. Zebrafish.

## LISTA DE FIGURAS

## LISTA DE ABREVIATURAS

| | |
|---|---|
| CISA | Centro de Informações sobre Saúde e Álcool |
| dpf | Dias após a fertilização |
| EtOH | Etanol |
| GABA | Acido gama-aminobutírico |
| NMDA | N-metil-D-aspartato |
| OFT | Teste do campo aberto (do inglês, *open field test*) |
| PCA | Análise de componentes principais (do inglês, *principal component analysis*) |
| pH | Potencial hidrogeniônico |
| SNC | Sitema nervoso central |
| WT | Linhagem selvagem (do inglês, *wild type*) |

**SUMÁRIO**

# 1 APRESENTAÇÃO

A presente Dissertação está estruturada da seguinte forma: primeiramente são descritas as partes referentes à **introdução**, que contém uma revisão bibliográfica sobre os itens abordados na Dissertação, fundamentando a **justificativa** e os **objetivos** do presente estudo. A seguir, a **metodologia**, os **resultados** e a **discussão** estão apresentados na forma de artigo científico. A **conclusão**, apresenta os principais achados do trabalho realizado nesta Dissertação. Por fim, as **perspectivas** apresentam as possibilidades de novos estudos a partir de resultados obtidos e as **referências bibliográficas** referem-se às citações que aparecem na seção introdução.

# 2 INTRODUÇÃO

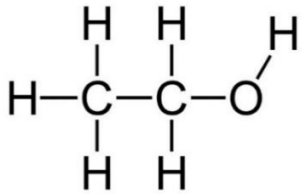
## 2.1 ETANOL: EFEITOS NEUROQUÍMICOS E COMPORTAMENTAIS

O etanol (EtOH) (**Figura 1**), é uma substância amplamente consumida pelos seres humanos (SOUZA; MENANDRO; MENANDRO, 2015). Tradições socioculturais, baixo custo, fácil acesso (SUDHINARASET; WIGGLESWORTH; TAKEUCHI, 2016) e sua ação em reduzir a ansiedade e o estresse, proporcionando a sensação de bem-estar (ROZIN; ZAGONEL, 2012), estão entre os principais fatores para o seu consumo, contribuindo para o desenvolvimento das doenças relacionadas ao uso de álcool (SUDHINARASET; WIGGLESWORTH; TAKEUCHI, 2016). O consumo em excesso reflete uma preocupação de saúde pública, devido às altas taxas de morbidade e mortalidade (WHO, 2014). Por ser uma molécula lipofílica, o EtOH difunde-se nas membranas das células, distribuindo-se rapidamente pela corrente sanguínea até os órgãos e tecidos (EŞEL e DINÇ, 2017). Sua absorção ocorre predominantemente no trato gastrointestinal, entretanto, tecidos como encéfalo e os pulmões, recebem doses iniciais de EtOH mais rapidamente, uma vez que são altamente vascularizados (MULLEN, 1977). Além disso, maiores concentrações de EtOH podem ser encontradas em tecidos como cérebro, fígado coração rins e músculos (CISA, 2020). Por atravessar facilmente a barreira hematoencefálica (EŞEL e DINÇ, 2017; SPANAGEL, 2009), o EtOH produz efeitos deletérios sobre o sistema nervoso central (SNC) que culminam em modificações sobre o comportamento (RIBEIRO; GAIVÃO, 2010; SCHNEIDER et al., 2016; SPANAGEL, 2009).

O EtOH se caracteriza por gerar um efeito bifásico sobre o organismo (FILLMORE; WEAFER, 2004; HENDLER et al., 2013), afetando o comportamento social e, dependendo do volume ingerido, pode facilitar ou dificultar as interações interpessoais (CISA, 2004; OSCAR-BERMAN et al., 1997). A ingestão de pequenas quantidades induz efeitos ansiolíticos, promove estados de humor positivos (HENDLER et al., 2013) e estimula a desinibição (HARRISON et al., 2017), proporcionando ao indivíduo a sensação de relaxamento em relação ao ambiente; enquanto doses maiores diminuem a resposta a estímulos e reflexos, causando dificuldade na fala, sedação e sonolência (HENDLER et al., 2013; SPANAGEL, 2009). Assim, por melhorar o desempenho social, através da diminuição do estresse e da ansiedade, o EtOH é comumente consumido por indivíduos que sofrem de fobia social (CARRIGAN; RANDALL, 2003). A fobia social é um distúrbio que pode anteceder o aparecimento do alcoolismo (MERIKANGAS; ANGST, 1995), e pelo EtOH atenuar essa característica, o uso abusivo de

EtOH pode ser estimulado (BITTENCOURT; OLIVEIRA; SOUZA, 2005). Contudo, a dependência ao EtOH ainda possui o envolvimento de fatores genéticos, de sexo, idade, além de aspectos sociais e culturais (SAMOCHOWIEC et al., 2014; WALL; LUCZAK; HILLER-STURMHÖFEL, 2016).

Figura 1 – Representação da fórmula estrutural do EtOH.



Fonte: https://staticserver2.com/edu/static/ms/800/etanol.jpg

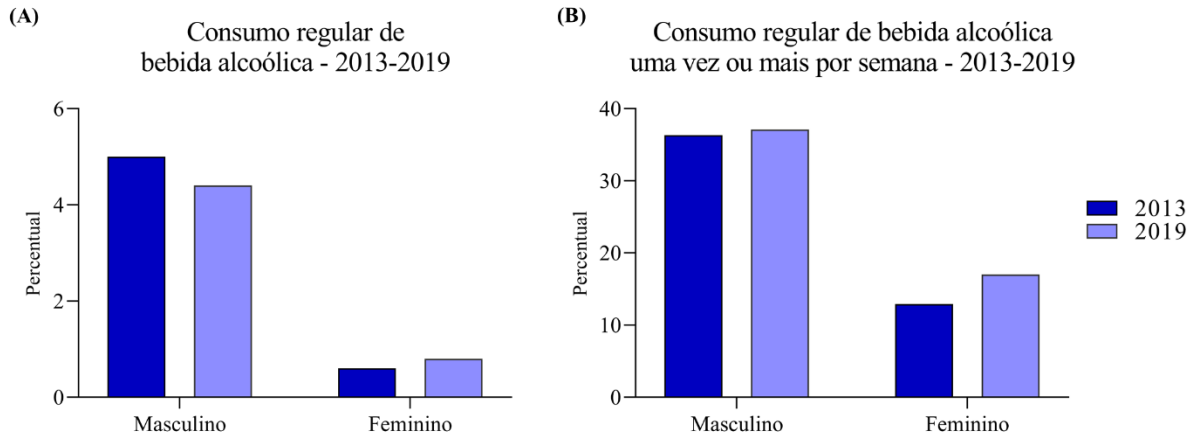
No SNC, o EtOH modula as vias de transdução de sinais inibitórias e excitatórias, assim como o sistema de recompensa através do aumento da liberação de dopamina e serotonina (CHASTAIN, 2006), favorecendo a sensação de bem-estar durante seu consumo (CISA, 2004; OSCAR-BERMAN et al., 1997). O EtOH possui ação depressora por estimular a neurotransmissão mediada pelo ácido gama-aminobutírico (GABA) (BREESE et al., 2006), resultando no relaxamento e na sedação do corpo (CISA, 2012). Concomitantemente, o consumo agudo de EtOH diminui a neurotransmissão glutamatérgica no cérebro (CHASTAIN, 2006; ROBERTO; VARODAYAN, 2017), afetando a memória (CHASTAIN, 2006). Com o uso repetido de EtOH, ocorre um aumento no número de receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) em resposta à redução da atividade do glutamato (CHASTAIN, 2006). Assim, na abstinência, ocorre uma hiperestimulação glutamatergica podendo levar à ocorrência de crises convulsivas e, até mesmo, danos cerebrais irreversíveis (CHASTAIN, 2006). No entanto, os efeitos da ingestão podem variar, não apenas de acordo com a quantidade e a frequência, mas também conforme as características genéticas, como o sexo biológico (CISA, 2012).

## 2.2 A INFLUÊNCIA DO SEXO BIOLÓGICO NOS EFEITOS DO ETANOL

Apesar dos homens ainda serem os maiores consumidores de ácool, segundo o Centro de Informações sobre Saúde e Etanol (CISA, 2020), o sexo feminino encaminha-se para a

igualdade em relação ao padrão de consumo masculino. As mulheres vem apresentando um aumento, não somente na quantidade, mas também na frequência de consumo (PEREIRA, 2012). Tal aumento é atribuído às mudanças no contexto de vida, como seu papel na sociedade e a ampliação de atividades e responsabilidades (PEREIRA, 2012). A **Figura 2** mostra o consumo regular de bebida alcólica nos anos de 2013 e 2019 entre homens e mulhes.

Figura 2 – Consumo de álcool da população brasileira em 2013 e 2019. **(A)** Consumo regular de bebida alcoólica e **(B)** consumo regular de bebida alcoólica uma vez ou mais por semana




**(A)** Percentual de indivíduos de 18 anos ou mais que costumam consumir bebida alcoólica em 5 dias ou mais por semana. **(B)** Percentual de indivíduos de 18 anos ou mais que costumam consumir alguma bebida alcoólica pelo menos 1 dia na semana. Fonte: https://www.pns.icict.fiocruz.br/painel-de-indicadores-mobile-desktop/ (Adaptado) (Acesso em: 06 de Outubro de 2021).

Fatores individuais e ambientais, que vão desde a herança genética até o contexto social, influenciam os hábitos de consumo (CISA, 2020). Enquanto as mulheres bebem para regular o afeto negativo (como angústia e insatisfação) e a reatividade ao estresse, os homens tendem a consumir EtOH devido ao reforço positivo (como a busca de prazer, busca por estímulos para obter satisfação) (PELTIER et al., 2019). Além disso, a ansiedade e a depressão também são fatores chave para o consumo de EtOH nas mulheres, mas não em homens (CISA, 2015; SCHEFFER; ALMEIDA, 2010; ZILBERMAN et al., 2007). Apesar dos mecanismos de ação do EtOH ainda não serem completamente esclarecidos (CISA, 2020), consta-se que nas mulheres, as estruturas cerebrais mais afetadas são as envolvidas com as emoções e com a memória (sistema límbico, hipocampo e amígdala) (ALMEIDA; PASA; SCHEFFER, 2009; DEVAUD; ALELE; RITU, 2003), refletindo um maior índice de depressão e risco de suicídio (ALMEIDA; PASA; SCHEFFER, 2009; GRAHAM et al., 2007). Nos homens, as áreas mais afetadas são as envolvidas no raciocínio, no julgamento de valor e na resolução de problemas

(áreas corticais, especialmente as áreas pré-frontais) (ALMEIDA; PASA; SCHEFFER, 2009; ROSENBLOOM et al., 2005), resultando em alterações comportamentais como aumento da agressividade e impulsividade (ALMEIDA; PASA; SCHEFFER, 2009). Assim, as diferenças farmacológicas do EtOH refletem efeitos comportamentais distintos, com uma ação mais estimulante em homens e mais sedativa em mulheres (FILLMORE; WEAFER, 2004). Evidências sugerem uma influência do sexo no grau em que o EtOH prejudica os mecanismos de controle inibitório, já que, deficiências nesses mecanismos são relacionadas a transtornos caracterizados por comportamentos agressivos ou impulsivos (BARKLEY, 1997; FILLMORE; WEAFER, 2004). Homens que relatam maior estimulação pelo EtOH também exibiram maior agressividade em comparação às mulheres (ADEODATO et al., 2005; FILLMORE; WEAFER, 2004; GIANCOLA; ZEICHNER, 1995; HOAKEN; PIHL, 2000) reforçando uma ação distinta do EtOH no organismo (ALMEIDA; PASA; SCHEFFER, 2009; DEVAUD; ALELE; RITU, 2003). No entanto, mais estudos são necessários para compreender a influência do sexo no efeito do EtOH no controle inibitório, uma vez que os estudos na área ou examinam apenas homens ou envolvem parâmetros limitados, dificultando comparações mais adequadas entre os sexos (DE WIT; CREAN; RICHARDS, 2000; MARCZINSKI; FILLMORE, 2003).

A tendência à equiparação do consumo do EtOH entre os sexos, levanta uma série de preocupações (CISA, 2020), pois, do ponto de vista biológico, as mulheres são metabolicamente menos tolerantes ao EtOH. Isso se deve às características biológicas intrínsecas, como: o peso, uma menor quantidade de água corporal e enzimas metabolizadoras de EtOH, além de uma maior quantidade de gordura (CISA, 2020; MARSHALL et al., 1983; MUMENTHALER et al., 1999; NÓBREGA; OLIVEIRA, 2005; RAMCHANDANI; BOSRON; LI, 2001). Consequentemente, mulheres metabolizam o EtOH de forma mais lenta (NÓBREGA; OLIVEIRA, 2005; OLIVEIRA et al., 2012), levando a uma concentração de EtOH no sangue mais alta em comparação com os homens (FILLMORE; WEAFER, 2004; WHITFIELD; MARTIN, 1994), possibilitando que a intoxicação ocorra com o uso de metade da quantidade de EtOH ingerida (NÓBREGA; OLIVEIRA, 2005). A vulnerabilidade para o desenvolvimento de complicações clínicas é maior entre as mulheres, e as mesmas sofrem mais risco de mortalidade que o sexo oposto (OLIVEIRA et al., 2012). Ademais, o consumo excessivo aumenta as chances para o desenvolvimento de câncer de mama, doenças cardíacas, doenças hepáticas, doenças sexualmente transmissíveis e gravidez indesejada (CISA, 2019; NÓBREGA; OLIVEIRA, 2005). Em suma, compreender as diferentes respostas entre os sexos no consumo de EtOH, torna-se relevante (DEVAUD; ALELE; RITU, 2003) e a utilização de

modelos animais, torna-se viável, visto que, pesquisa em animais auxiliam na compreensão das bases neurobiológicas de inúmeras enfermidades humanas (FESTING; WILKINSON, 2007).

## 2.3 PEIXE-ZEBRA COMO ORGANISMO MODELO

O uso de organismos modelos na pesquisa científica pode ajudar na busca da compreensão dos mecanismos neurobiológicos fundamentais que modulam o comportamento (KUMAR et al., 2009; LIESCHKE; CURRIE, 2007). Assim, o peixe-zebra (*Danio rerio*), um pequeno modelo de vertebrado, tem sido considerado uma ferramenta promissora para pesquisas neurocientíficas e comportamentais (KALUEFF et al., 2013). O genoma do peixe-zebra já é completamente sequenciado e seus genes possuem um alto grau de similaridade genética e fisiológica, o que possibilita modelar doenças humanas (HOWE et al., 2013; SEGNER, 2009). Ele ainda apresenta tamanho reduzido, fácil manutenção, custo baixo, fácil reprodução, dimorfismo sexual em fisiologia e anatomia, e embriões translúcidos que contribuem para seu uso na pesquisa biomédica (FONTANA et al., 2018a; STEWART et al., 2014, 2015; VON HOFSTEN; OLSSON, 2005).

A conservação do genoma do peixe-zebra, quando comparado aos genes de humanos (cerca de 70% de homologia genética), (HOWE et al., 2013) acrescido do comportamento bem caracterizado (KALUEFF et al., 2013), favorecem o uso da espécie como um organismo modelo atraente para estudos de doenças do SNC (LIESCHKE; CURRIE, 2007), como epilepsia, doença de Parkinson, doença de Alzheimer, esquizofrenia, transtornos afetivos e transtornos relacionados a drogas (CANZIAN et al., 2019; DUARTE et al., 2019; FONTANA et al., 2018a; FRANSCESCON et al., 2020; MÜLLER et al., 2020a). Comportamentos complexos, como agressão (ZABEGALOV et al., 2019), ansiedade (FONTANA et al., 2016; MAXIMINO et al., 2010), estresse (CANZIAN et al., 2021; CHAMPAGNE et al., 2010), ousadia (CONRAD et al., 2011; DAHLBOM et al., 2011; ROY; SHUKLA; BHAT, 2017a), medo (MILLER; GERLAI, 2007; SPEEDIE; GERLAI, 2008), dor (COSTA et al., 2019), memória de longo e curto prazo (BLANK et al., 2009; JIA; FERNANDES; GERLAI, 2014), discriminação de objetos (MAY et al., 2016; STEFANELLO et al., 2019) e preferência de cor (BAULT; PETERSON; FREEMAN, 2015) já foram caracterizados através de inúmeros testes comportamentais adaptados para o peixe-zebra (CHAMPAGNE et al., 2010; KALUEFF et al., 2013).

O teste de campo aberto (*open field*), originalmente descrito em roedeores (HALL, 1934), tem por finalidade estudar a emocionalidade em modelos de laborarório, e é conhecido

como um dos procedimentos mais populares em psicologia animal (SEIBENHENER; WOOTEN, 2015; WALSH; CUMMINS, 1976). Em peixes, o teste consiste em um tanque novo, geralmente circular (BLASER; GERLAI, 2006; EGAN et al., 2009; GERLAI et al., 2000), onde a exploração avaliada é comumente associada ao comportamento semelhante à ansiedade, através dos parâmetros de timotaxia, congelamento e natação errática (CACHAT et al., 2010; DAHLBOM et al., 2011; MAXIMINO et al., 2010). A tigmotaxia consiste em um comportamento "centrofóbico", no qual os animais evitam a região central do tanque e passam a maior parte do tempo próximo às paredes do aparato (CHAMPAGNE et al., 2010; DAHLBOM et al., 2011; LÓPEZ-PATIÑO et al., 2008; MAXIMINO et al., 2010). Os episódios de congelamento (*freezing*) são contabilizados quando os animais permanecem imóveis, realizando apenas batimentos operculares, movimentos necessários para manter a postura e possíveis respostas oculomotoras (DAHLBOM et al., 2011; MAXIMINO et al., 2010; MÜLLER et al., 2020b). Por outro lado, os movimentos erráticos consistem em um padrão imprevisível de nado (*zig-zagging*) no qual o peixe-zebra continua a se mover, mas a locomoção é interrompida por mudanças abruptas da direção de nado (DAHLBOM et al., 2011; EGAN et al., 2009; MAXIMINO et al., 2010). O teste do campo aberto tem se tornado um procedimento conveniente para medir não apenas comportamentos semelhante à da ansiedade, mas também comportamento semelhante a ousadia (BURNS, 2008) através de parâmetros como tempo gasto explorando o novo ambiente (WRIGHT et al., 2006). A ousadia pode ser quantificada a partir de um maior comportamento exploratório e uma alta atividade na área central do aparato (ARIYOMO; CARTER; WATT, 2013; ARIYOMO; WATT, 2012; COLLIER; KALUEFF; ECHEVARRIA, 2017; WHITE et al., 2013), enquanto indivíduos tímidos são caracterizados por uma resposta passiva combinada com baixa exploração (SIH et al., 2004).

A versatilidade do teste de campo aberto permite a adaptação para a realização de outros ensaios, como o teste de interação com um novo objeto (PRUT; BELZUNG, 2003). Esse teste consiste na inserção de um objeto não familiar na arena (DAHLBOM et al., 2011; WRIGHT et al., 2003) e avaliação da exploração do mesmo. Em peixes-zebra, a interação com o objeto não familiar é frequentemente relacionada ao comportamento semelhante a ousadia. A quantificação de um maior tempo gasto e alta atividade de natação próximo ao objeto são padrões comportametais manifestados por animais ousados (DAHLBOM et al., 2011; NORTON et al., 2011; WHITE et al., 2013; WRIGHT et al., 2003, 2006). No entanto, pouco é explorado sobre a influência do sexo nos fenótipos comportamentais em peixes-zebra (GENARIO et al., 2020b).

## 2.4 INFLUÊNCIA DO SEXO BIOLÓGICO NOS EFEITOS COMPORTAMENTAIS EM PEIXE-ZEBRA

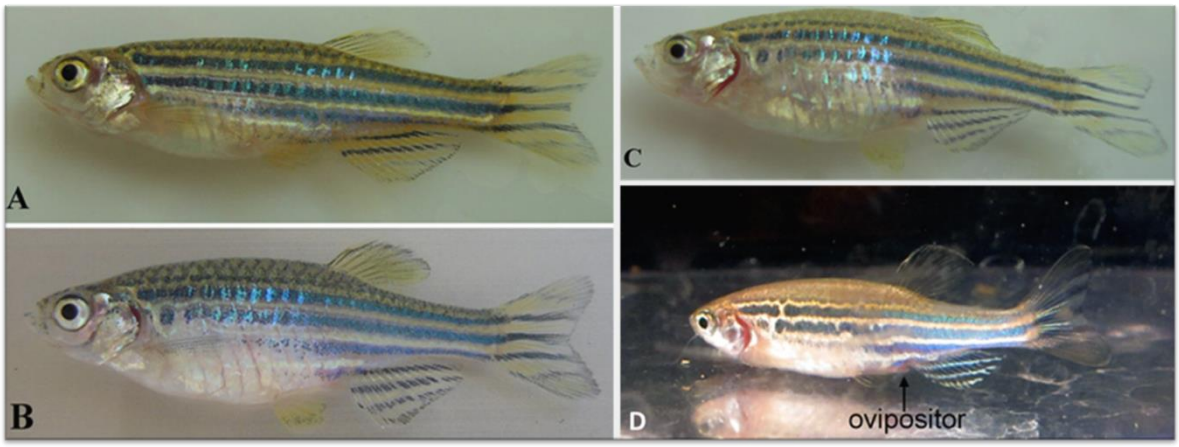
O sexo biológico influência no comportamento animal, levando a respostas comportamentais distintas quando confrontados com estímulos que variam desde comida até co-específicos (FONTANA; CLEAL; PARKER, 2020; MOWREY; PORTMAN, 2012). O peixe-zebra atinge a maturidade sexual por volta de três meses de vida, porém a sexagem ocorre após 21-23 dias após a fertilização (dpf) (VON HOFSTEN; OLSSON, 2005). Antes desde período, os animais apresentam gônadas semelhantes a ovários, sendo entre o 21-30 dpf o período em que ocorre o desenvolvimento dos testículos e a apoptose dos ovários nos machos (VON HOFSTEN; OLSSON, 2005).

Apesar de haver poucos estudos sobre os mecanismos genéticos e a determinação do sexo em peixes, sabe-se que o genoma do peixe-zebra contém 50 cromossomos e nenhum cromossomo sexual específico identificado (VON HOFSTEN; OLSSON, 2005). Assim, acredita-se que variantes alélicas e variações de expressão de genes autossômicos, como gene relacionado com SRY HMG 9 (*Sox9*), gene do hormônio anti-Mulleriano (*amh*), os genes *Fushi Tarazu* fator-1 (*FTZ-F1*), gene relacionado *doublesex-mab 3 (dmrt1)*, WT-1 e o gene *Cyp19* (Aromatase), possam estar envolvidos com a determinação e o desenvolvimento das gônadas (RODRÍGUEZ-MARÍ et al., 2005; VON HOFSTEN; OLSSON, 2005). O Sox9 é um fator de transcrição necessário para a determinação do testículo em mamíferos (CHIANG et al., 2001) e em peixe-zebra, são encontrados dois genes com expressão sexualmente dimórficos (VON HOFSTEN; OLSSON, 2005). O *Sox9a* apresenta um padrão de expressão amplo e é encontrado nos testículos, enquanto o *Sox9b* é encontrado nos ovários (VON HOFSTEN; OLSSON, 2005). O mesmo ocorre com o gene *amh*, o qual é expresso nos testículos, mas não nos ovários (RODRÍGUEZ-MARÍ et al., 2005). Apesar dos teleósteos apresentarem múltiplas variantes dos genes *FTZ-F1*, acredita-se que o gene *ff1a* tenha envolvimento na diferenciação e desenvolvimento das gônadas (VON HOFSTEN; KARLSSON; OLSSON, 2003; VON HOFSTEN; OLSSON, 2005), assim como o gene *dmrt1*, que pode ter um papel importante na determinação do testículo em teleósteos (GUAN; KOBAYASHI; NAGAHAMA, 2000; MARCHAND et al., 2000; VON HOFSTEN; OLSSON, 2005). Além disso, *ff1a*, *ff1b* e o gene *WT1* influenciam na esteroidogênese (CHAI; LIU; CHAN, 2003; VON HOFSTEN; KARLSSON; OLSSON, 2003; VON HOFSTEN; OLSSON, 2005), refletindo um envolvimento com o desenvolvimento primário das gônadas (VON HOFSTEN; OLSSON, 2005). A maioria dos vertebrados tem um único gene *cyp19*, enquanto o peixe-zebra possui

dois genes (*cyp19a* e *cyp19b*) e que apesar de apenas um deles (*cyp19a*) ser expresso no ovário, ambos os genes contribuem para a regulação das respostas estrogênicas e podem, portanto, influenciar a diferenciação sexual (VON HOFSTEN; OLSSON, 2005).

Apesar dos mecanismos genéticos e bioquímicos que influenciam na determinação e diferenciação sexual no peixe-zebra ainda estarem pouco esclarecidos (VON HOFSTEN; OLSSON, 2005), o modelo apresenta dimorfismo sexual na fisiologia e na anatomia (GENARIO et al., 2020b; VON HOFSTEN; OLSSON, 2005), sendo facilmente visualizado (VON HOFSTEN; OLSSON, 2005), uma vez que as fêmeas têm uma forma corporal ventral mais arredondada (AVDESH et al., 2012; SPENCE et al., 2008) e com a presença de papilas genitais identificáveis (YOSSA et al., 2013), os machos possuem nadadeiras anais maiores e mais amareladas (AVDESH et al., 2012; SPENCE et al., 2008) e raios da nadadeira peitoral mais grossos (GENARIO et al., 2020b; MCMILLAN et al., 2013; MCMILLAN; GÉRAUDIE; AKIMENKO, 2015). A distinção entre os sexos pode ser observada na **Figura 3**.

Figura 3 **–** Exemplares de peixe-zebra (*Danio rerio*) adulto **(A)** macho e **(B, C e D)** fêmea.




Fonte: Avdesh et al. (2012) (Adaptado).

Apesar de fatores ambientais e hormonais influenciarem nos fenótipos comportamentais dos sexos (LEE et al., 2018) essas diferenças permanecem pouco compreendidas (GENARIO et al., 2020b) e inúmeros estudos mostram-se contraditórios. Enquanto estudos relatam fêmeas de peixe-zebra com um comportamento basal mais agressivo em comparação aos machos (DAHLBOM et al., 2012; RAMBO et al., 2017), outros mostram o oposto (PAULL et al., 2010). O mesmo ocorre com o comportamento do tipo ousadia, onde há estudos que demostram machos mais ousados (DAHLBOM et al., 2011; ROY; SHUKLA; BHAT, 2017b), enquanto

outros (OSWALD et al., 2012; DANIEL; BHAT, 2020) relatam o contrário. Quando avaliado o comportamento semelhante a ansiedade, fêmeas passam mais tempo no nível superior do tanque (teste do mergulho), refletindo uma baixa taxa de ansiedade (AMPATZIS; DERMON, 2016). Contudo, evidências recentes suportam um maior comportamento do tipo ansiedade basal em fêmeas (FONTANA; CLEAL; PARKER, 2020; GENARIO et al., 2020a).

As diferenças comportamentais entre os sexos também se refletem nas respostas farmacológicas (FRANCONI et al., 2007; GENARIO et al., 2020a; MEIBOHM; BEIERLE; DERENDORF, 2002). Em ensaios utilizando tratamento agudo com diazepam, pode-se observar um efeito ansiolítico apenas em machos de peixe-zebra (GENARIO et al., 2020a), enquanto que em tratamento crônico, o diazepam modula negativamente o comportamento social de fêmeas, mas não de machos (CHEN et al., 2021). Além disso, uma maior sensibilidade à exposição crônica ao EtOH é verificada em fêmeas de peixe-zebra da linhagem selvagem (WT) no comportamento de agrupamento, porém, na exposição aguda mais estudos são necessários (DLUGOS; BROWN; RABIN, 2011).

Ensaios baseados nos fenótipos comportamentais do peixe-zebra são úteis para testar os efeitos de fármacos (CHAMPAGNE et al., 2010), dado que a espécie é cada vez mais utilizada em ensaios farmacológicos devido à facilidade de exposição dos animais a diferentes substâncias por imersão, incluindo a exposição ao EtOH (TSANG; ANSARI; GERLAI, 2019).

## 2.5 PEIXE-ZEBRA: MODELO PARA ESTUDO DOS EFEITOS COMPORTAMENTAIS DO ETANOL

O peixe-zebra apresenta os principais sistemas de neurotransmissores que modulam as respostas ao EtOH (CHATTERJEE; SHAMS; GERLAI, 2014; MÜLLER et al., 2017). Tanto em exposições agudas quanto crônicas, animais adultos mostram respostas comportamentais semelhantes às observadas em mamíferos (GERLAI et al., 2000; MÜLLER et al., 2017). O EtOH modula as funções cerebrais envolvidas na impulsividade e avaliação de risco (PARKER et al., 2014). Também produz um efeito bifásico no comportamento do peixe-zebra, onde em baixas concentrações (até 0,5% v/v) gera efeitos estimulantes, como a intensificação do nado vertical (efeito ansiolítico) e aumento da atividade exploratória e agressividade (FONTANA et al., 2016; GERLAI et al., 2000; ROSEMBERG et al., 2012). Em concentrações mais altas (1,0% v/v), o EtOH diminui a locomoção e a preferência social (FONTANA et al., 2016; GERLAI et al., 2000; ROSEMBERG et al., 2012). A redução da atividade de peixes tratados com uma concentração alta de EtOH está relacionada com os efeitos sedativos, causando

prejuízo na coordenação e na atividade natatória (GERLAI et al., 2000). Recentemente, em peixes-zebra adultos, verificou-se um envolvimento direto do sistema serotoninérgico nos comportamentos de agressividade e em respostas ansiolíticas após exposição de 0,25% e 0,5% de EtOH, respectivamente (MÜLLER et al., 2020b). Larvas de peixes-zebra (7 ± 1 dpf) submetidas à exposição a 0,5% de EtOH apresentam um efeito estimulante (frequência reduzida de permanência imóvel, aumento da velocidade de nado, variabilidade temporal da velocidade e virada de nado); enquanto 1% de EtOH induz um efeito sedativo, similar ao observado em peixes-zebra adultos (TSANG; ANSARI; GERLAI, 2019).

Dessa forma, pesquisas envolvendo análises comportamentais, suportam o uso do peixe-zebra como um organismo modelo atraente para o estudo dos mecanismos biológicos relacionados aos efeitos do EtOH (GERLAI et al., 2000), mesmo durante as fases iniciais de seu desenvolvimento (TSANG; ANSARI; GERLAI, 2019). Embora estudos com o objetivo de avaliar os efeitos do EtOH no comportamento do peixe-zebra tenham aumentado rapidamente (MATHUR; GUO, 2011; MÜLLER et al., 2017, 2020b), pouco se sabe em relação aos efeitos do EtOH no comportamento de machos e fêmeas de peixes-zebra, e se, esses efeitos são dependentes da concentração testada.

# 3 JUSTIFICATIVA

O consumo de EtOH tem, entre seus inúmeros efeitos, a capacidade de modular o comportamento social, podendo facilitar ou dificultar as interações interpessoais (CISA, 2004; OSCAR-BERMAN et al., 1997) dependendo, não apenas, da quantidade e frequência ingerida, mas também de acordo com fatores individuais e genéticos, como o sexo biológico (CISA, 2012). Essa modulação no comportamento social varia desde desinibição à sedação até hipnose, com concentrações crescentes de EtOH (SPANAGEL, 2009). Sabe-se que, em humanos, mulheres tendem a ser menos tolerantes ao EtOH (NÓBREGA; OLIVEIRA, 2005), devido a suas características biológicas (MARSHALL et al., 1983; MUMENTHALER et al., 1999; NÓBREGA; OLIVEIRA, 2005; RAMCHANDANI; BOSRON; LI, 2001), apresentando uma maior probabilidade para o desenvolvimento de complicações clínicas (CISA, 2019; NÓBREGA; OLIVEIRA, 2005). O uso de organismos modelos na pesquisa científica pode auxiliar na compreensão dos mecanismos fundamentais que afetam respostas neurocomportamentais (KUMAR et al., 2009; LIESCHKE; CURRIE, 2007). O peixe-zebra (*Danio rerio*), por apresentar inúmeras características vantajosas para a pesquisa translacional, surge como um organismo modelo atrativo para estudos dos efeitos neuroquímicos e comportamentais do EtOH (TRAN; FACCIOL; GERLAI, 2016), pois exibe os principais sistemas de neurotransmissores que interferem as respostas mediadas pelo EtOH (CHATTERJEE; SHAMS; GERLAI, 2014). Assim como em humanos, o EtOH produz um efeito bifásico no comportamento do peixe-zebra: em baixas quantidades, intensifica a atividade vertical e a agressividade, enquanto em concentrações mais altas, diminui a locomoção e a preferência social (FONTANA et al., 2016; ROSEMBERG et al., 2012). Entretanto, poucos estudos realizam a sexagem dos animais para a realização dos experimentos, desconsiderando um fator biológico importante.

# 4 OBJETIVOS

## 4.1 OBJETIVO GERAL

Avaliar se machos e fêmeas de peixe-zebra apresentam diferenças comportamentais quando expostos ao EtOH.

## 4.2 OBJETIVOS ESPECÍFICOS

- Avaliar se machos e fêmeas de peixe-zebra apresentam diferenças nos comportamentos tipo ansiedade, ousadia e atividade exploratória;
- Avaliar se machos e fêmeas de peixe-zebra apresentam diferenças no padrão de interação com um objeto não familiar quando expostos agudamente a baixas (0,25% e 0,50% v/v) e alta (1,00% v/v) concentrações de EtOH;
- Identificar se fatores como ansiedade, padrão locomotor e atividade exploratória exercem uma maior contribuição com as principais respostas neurocomportamentais mensuradas.

# 5 DESENVOLVIMENTO

## 5.1 ARTIGO CIÊNTIFICO

*Acute effects of ethanol on behavioral responses of male and female zebrafish in the open field test with the influence of a non-familiar object*

Thiele P. Souza, Francini Franscescon, Flavia V. Stefanello, Talise E. Müller, Laura W. Santos and Denis B. Rosemberg

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# Acute effects of ethanol on behavioral responses of male and female zebrafish in the open field test with the influence of a non-familiar object

Thiele P. Souza [a,b], Francini Franscescon [a,b], Flavia V. Stefanello [a,b], Talise E. Müller [a,b], Laura W. Santos [a], Denis B. Rosemberg [a,b,c,*]

[a] Laboratory of Experimental Neuropsychobiology, Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria, 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil
[b] Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria, 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil
[c] The International Zebrafish Neuroscience Research Consortium (ZNRC), 309 Palmer Court, Slidell, LA, 70458, USA

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ABSTRACT

In this report, we investigate whether the acute effects of different ethanol (EtOH) concentrations are sex-dependent in zebrafish subjected to the open field test (OFT) with the influence of a non-familiar object. Male and female zebrafish were separated into four groups and exposed to EtOH (0%, 0.25%, 0.5%, or 1.0% v/v) for 1 h. Fish were tested individually in the OFT and the tank was divided into three areas: periphery, intermediate, and center area. An object (black sphere; diameter: 1 cm) was placed in the center of the tank and behaviors were recorded for 5 min. At the baseline, females had a distinct exploratory activity and interaction pattern with the object, reflecting a more anxious and shyer behavior in relation to males. Females exposed to 0.5% EtOH performed more rapid investigation to the object than males, while 1.0% EtOH reduced locomotion in both sexes and increased immobility only in males. Principal component analyses revealed that anxiety-like behaviors, exploratory activity, and locomotion were the components that most accounted for total variances. Collectively, our novel findings show the existence of a sex-dependent effect in the zebrafish models acutely exposed to EtOH tested in the OFT with a non-familiar object.

## 1. Introduction

Ethanol (EtOH) is one of the most consumed drugs worldwide (WHO, 2014) mainly due to its capacity of reducing anxiety and stress, providing well-being sensation (Rozin and Zagonel, 2012). The effects of EtOH can vary, not only based on the quantity and frequency of consumption, but also on individual and genetic factors (Fillmore and Weafer, 2004; Gerlai et al., 2009; WHO, 2018). Because EtOH elicits deleterious effects on the central nervous system, excessive alcohol consumption has been associated with high morbidity and mortality rates (WHO, 2018). In general, EtOH triggers depressant effects and impairs memory and coordination due to various changes in neurotransmitter systems (Chastain, 2006). Moreover, EtOH modulates the social behavior, ranging from disinhibition to sedation and even hypnosis, in a dose-dependent manner (Komáreková and Janík, 2012; Spanagel, 2009).

In humans, the motivation for ingesting EtOH can also be sex-dependent. For example, while women drink to regulate negative affect (*e.g.*, anguish and dissatisfaction) and stress reactivity, men tend to consume EtOH due to the positive reinforcement (*e.g.*, pursuit of pleasure, when seeks stimuli to obtain satisfaction) (Peltier et al., 2019). Because women have lower amounts of body water, a greater amount of fat, and a reduced expression of EtOH-metabolizing enzymes (*e.g.*, alcohol dehydrogenase and cytochrome P450 2E1), they tend to be less tolerant to EtOH than men (Nóbrega and Oliveira, 2005; Szabo, 2018; Ward and Coutelle, 2003). Thus, women usually become intoxicated more easily than men when the same dose of EtOH is given (Haut et al., 1989), presenting higher blood alcohol levels (Ward and Coutelle, 2003). In women, the occurrence of impulsivity (Reed et al., 2012) and alcoholic hepatitis is higher, which can be caused by ingesting half of the cumulative amount of alcohol when compared to that ingested by men (Szabo, 2018). Moreover, excessive EtOH consumption in women increases the risk for breast cancer, heart disease, sexually transmitted infections, and unwanted pregnancy (Nolen-Hoeksema, 2004; White

* Corresponding author at: Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria, 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil.
*E-mail address:* dbrosemberg@gmail.com (D.B. Rosemberg).

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et al., 2015).

In experimental research, animal models represent valuable tools to assess behavioral and physiological responses of distinct drugs (Conn, 2008; Van der Staay, 2006; Van der Staay et al., 2009). The zebrafish (*Danio rerio*) is a well consolidated vertebrate system to investigate the neural bases involved in EtOH consumption and associated behavioral phenotypes (Fontana et al., 2018b, 2020b; Gerlai et al., 2000; Müller et al., 2017, 2020; Rosemberg et al., 2010, 2012). The high degree of genetic similarity when compared to the human genes (Fontana et al., 2018a; Howe et al., 2013), sexual dimorphism, and the well characterized behaviors represent key aspects to assess how EtOH affects brain functions in neurobehavioral research (Von Hofsten and Olsson, 2005). Similar to humans, zebrafish show a biphasic effect following EtOH exposure. Lower EtOH concentrations elicit anxiolytic-like responses (*e.g.*, increase vertical activity and aggressiveness) and higher concentrations decrease locomotion and social preference, thereby supporting depressant/sedative-like effects (Fontana et al., 2016; Gerlai et al., 2000; Rosemberg et al., 2012).

Studies aiming to evaluate the effects of EtOH on zebrafish behaviors do not usually analyze the influence of sex on the parameters measured (Fontana et al., 2020a, 2016; Müller et al., 2017). Such approach could represent a relevant factor in translational research, since female zebrafish may be more sensitive to the effects of alcohol than males (Dlugos et al., 2011). The influence of sex on boldness behavior of zebrafish is still controversial and a context-dependent effect has been suggested (Conrad et al., 2011). Notably, while studies describe male zebrafish bolder than female (Dahlbom et al., 2011; Roy et al., 2017), other reports show the opposite (Conrad et al., 2011; Norton and Bally-Cuif, 2012). However, it is conceivable that anxiety-like behavior in zebrafish is sex-dependent (*e.g.*, females are more anxious than males) (Fontana et al., 2020a) and this fact could reflect possible differences in the exploratory pattern when fish are challenged to a non-familiar object following EtOH exposure in the open field test (OFT). In humans, for example, behaviors like anxiety (Comeau et al., 2001; Pihl and Peterson, 1995; Varlinskaya et al., 2015) and impulsivity (de Wit et al., 2000; Reed et al., 2012) are affected by EtOH consumption. Here, we aimed to investigate the acute effects of different EtOH concentrations on the behavioral responses of male and female zebrafish subjected to the OFT with the presence of a non-familiar object. The results of this study can help elucidate whether the exploratory activity to novel objects following acute EtOH exposure are sex- and/or-concentration-dependent in this aquatic species.

## 2. Materials and methods

### 2.1. Animals

Subjects were adult (4–6 months-old) male and female zebrafish (*Danio rerio*) of the wild-type *short-fin* (WT-SF) phenotype. Fish were obtained from a local supplier (Hobby Aquários, RS, Brazil) and acclimated in 40-L tanks for two weeks in a maximum density of four fish per liter. Animals were fed with a commercial flake fish food (Alcon BASIC$^{TM}$, Alcon, Brazil) two times per day. Tanks were filled with non-chlorinated water under constant mechanical, biological, and chemical filtration. Water temperature, pH, and conductivity were set at 26 ± 2 °C, 7.2 ± 0.5, and 400 ± 50 μS, respectively. Animals were kept on a 14/10 light/dark photoperiod cycle (lights on at 7:00 a.m.). This study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and all protocols were approved by the Institutional Animal Care and Use Committee (process number 6894010616).

### 2.2. Experimental design

#### 2.2.1. Separation of male and female zebrafish

Male and female zebrafish were separated and kept in 4 L tanks (20 cm length ×15 cm height ×20 cm width) before the experiments. The identification of both sexes was carried out as described previously (Avdesh et al., 2012; Gupta and Mullins, 2010; Nusslein-Volhard and Dahm, 2002; Reed and Jennings, 2011; Westerfield, 2007). Basically, females have a larger belly and ovipositor, while males are slenderer, with a darker shade and a more yellowish anal fin. Sex was further confirmed by gonadal extraction after euthanasia (Gupta and Mullins, 2010).

#### 2.2.2. EtOH exposure protocol

Subjects were 38 males and 39 females randomly separated by sex into 4 subgroups: control, 0.25% EtOH; 0.5% EtOH, or 1.0% EtOH ($n$ = 8–10 males or females per experimental group), EtOH% represents the corresponding EtOH concentration (percentage volume) in which fish were exposed individually in a 500 mL beaker for 1 h (Gerlai et al., 2000; Rosemberg et al., 2012). Control fish were handled in a similar manner, but kept in non-chlorinated water for the same period in the absence of EtOH. Importantly, the EtOH concentrations used here were prepared from a 95% stock solution (Merck, Darmstadt, Germany). To ensure appropriate alcohol concentration, as well as to exclude a potential interference of stress-related hormones, the water was changed after each exposure for the individuals tested.

#### 2.2.3. Open field test (OFT) with the presence of a non-familiar object

Behavioral analyses in the OFT with the presence of a non-familiar object was used to investigate whether the exploratory pattern of zebrafish exposed to EtOH in such context is sex- and/or concentration-dependent. Importantly, we chose the OFT with the presence of a non-familiar object since this test allows measuring numerous behaviors, such as overall locomotor activity, anxiety (Krook et al., 2019), and boldness (Norton et al., 2011; Wright et al., 2003). Interaction-like behaviors with the non-familiar object, such as increased time spent close to the object (Norton et al., 2011; White et al., 2013; Wright et al., 2003), and more entries to the stimulus area (Wright et al., 2006) can be suggestive of boldness. Other behaviors, such as decreased time spent near the object associated with increased thigmotaxis and immobility may reflect anxiety-like responses (Gerlai et al., 2000; Krook et al., 2019; Maximino et al., 2010; Speedie and Gerlai, 2008). Importantly, a higher swimming activity in the center of the arena (a more risky area of the apparatus) may also be indicative of reduced anxiety in bolder animals (Winberg and Thörnqvist, 2016).

The OFT with the presence of a non-familiar object was based on the protocol described elsewhere (Dahlbom et al., 2011). Immediately after the EtOH exposure, each fish was individually placed in the open field tank (diameter of 30 cm with a 4 cm non-chlorinated water column). Water conditions in the apparatus were similar to those described for the holding tanks. The respective water level was adopted to allow fish to swim freely horizontally, reducing their vertical activity. This strategy was chosen to ensure a more precise identification of the exploratory activity with the use of a single camera located above the apparatus (Stefanello et al., 2019). The tank was divided into three areas: periphery, intermediate, and center. A black sphere of 1 cm in diameter was placed in the center of the tank, characterizing a non-familiar object. Here, we standardized the size and color of the object aiming to facilitate reproducibility among different laboratories (Stefanello et al., 2019). A representative scheme of the apparatus is shown in Fig. 1.

Initially, fish were individually placed in the tank and acclimated for a brief period. After 30 s, their behavioral activities were recorded for 5 min using a webcam (Multilaser WC045) located 60 cm above the apparatus. Male and female zebrafish were tested in different tanks to avoid a possible interference of sex hormones on the behaviors measured. Moreover, the apparatus was cleaned after the trial and the water was changed for each animal tested. The following parameters were measured: distance traveled, number of immobile episodes, number of transitions to the center and periphery areas, time in the center and periphery areas, average speed moving towards the object, as well as the minimum, maximum, and mean distances from the object. All

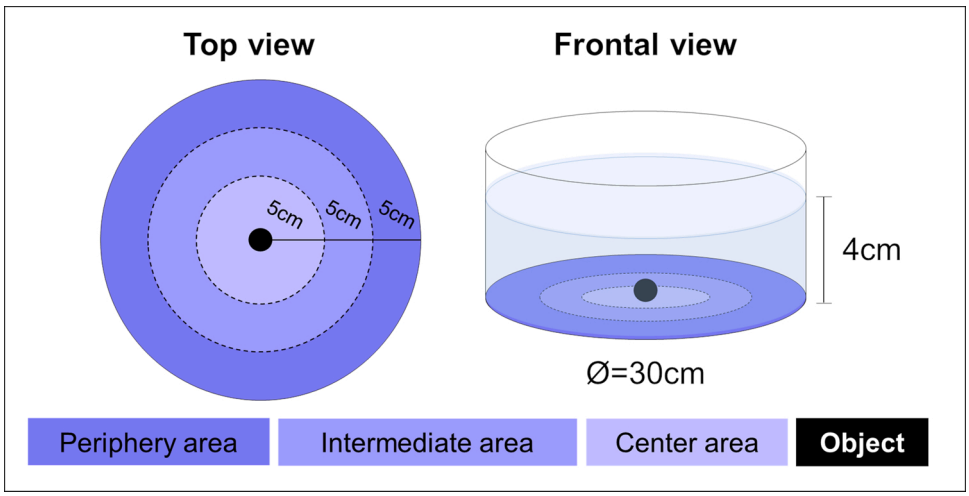


**Fig. 1.** Schematic representation of the OFT apparatus with the non-familiar object. The figure shows the top view of the tank with the respective areas and the frontal view of the apparatus depicting the water column level.

data were obtained from automated analysis using the ANY-maze$^{TM}$ software and further extracted offline. Since the behavioral endpoints were fully assessed in an automated fashion, which excludes potential observer bias, no blind analysts were used here. Importantly, all data extracted from the software were checked by two independent experimenters in Excel spreadsheets before performing statistical analysis.

### 2.3. Statistical analysis

Normality of data and homogeneity of variances were analyzed by Kolmogorov-Smirnov and Bartlett's tests, respectively. Because all data were normally distributed and homoscedastic, results were expressed as means ± standard error of the mean (S.E.M.) and analyzed by two-way analysis of variance (ANOVA) (sex and treatment as factors), followed by the Student-Newman-Keuls post-test whenever necessary. Effects sizes to all reported $F$ values were expressed as partial eta squared ($\eta_p^2$).

We also performed a principal component analysis (PCA) to investigate putative association between the behavioral endpoints measured, as well as the contribution of each component on data variance. The component matrix was further subjected to Varimax rotation with Kaiser normalization. Components (or factors) with eigenvalue lower than 1 were disregarded and measures with loadings greater than 0.3 were retained. Data were run in SPSS 19 (IBM SPSS Statistics, version 19) and values for each component were further analyzed by two-way ANOVA followed by the Student-Newman-Keuls multiple comparison test whenever necessary. Results were considered significant when $p \leq 0.05$.

## 3. Results

The effects of EtOH on locomotion-related parameters of male and female zebrafish are shown in Fig. 2. Two-way ANOVA revealed a significant effect of EtOH on the distance traveled ($F_{(3,69)} = 8.546$, $p = 0.0001$, $\eta_p^2 = 0.271$) and on the number of immobile episodes ($F_{(3,69)} = 3.315$, $p = 0.0249$, $\eta_p^2 = 0.126$). Post-hoc analyses revealed a decreased distance traveled in both males and females treated with 1.0% EtOH compared to their respective controls. Moreover, females exposed to 1.0% EtOH swam less than those of 0.25% and 0.5% EtOH groups (Fig. 2A). At the baseline, females were more immobile than males, while males exposed to 1.0% EtOH increased the number of immobile episodes when compared to untreated males (Fig. 2B).

Regarding the exploration in the periphery and center areas (Fig. 3), two-way ANOVA revealed significant effects of EtOH on transitions to the center area ($F_{(3.69)} = 6.4$, $p = 0.0007$, $\eta_p^2 = 0.218$), time spent in the center area ($F_{(3.69)} = 9.362$, $p = 0.0001$, $\eta_p^2 = 0.289$), and transitions to the periphery area ($F_{(3.69)} = 5.046$, $p = 0.0032$, $\eta_p^2 = 0.180$). We also verified a significant effect of sex on time spent in the periphery area ($F_{(1,\ 69)} = 12.05$, $p = 0.0009$, $\eta_p^2 = 0.149$). Males treated with 1.0% EtOH showed less transitions to the center area there when compared with their respective control group. Females exposed to 1.0% EtOH displayed a significant decrease in the number of transitions to the center area compared to females treated with 0.25% EtOH (Fig. 3A). Control males spent more time in the center area than control females and males exposed to 1.0% EtOH. Females treated with 0.25% EtOH spent more time in the center area than females exposed to 0.5% and

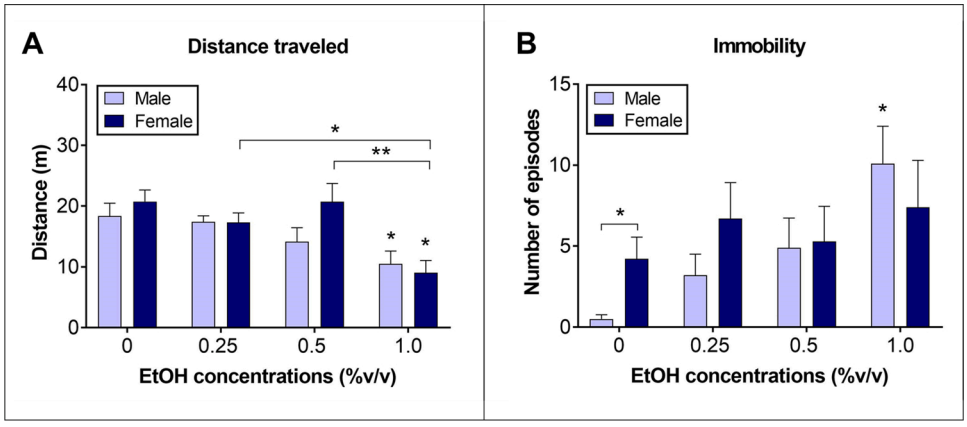


**Fig. 2.** Effects of EtOH (0%, 0.25%, 0.5%, and 1.0% v/v) on locomotor-related endpoints of male and female zebrafish submitted to the OFT with the presence of a non-familiar object. **(A)** Distance traveled. **(B)** Number of immobile episodes. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA (sex and treatment as factors), following by Newman–Keuls multiple comparison test whenever appropriate. The asterisks above bars express significant differences compared to the respective control group, while asterisks above brackets indicate statistical differences between other groups ($n = 8$–10 animals per group; * $p <$ 0.05; ** $p < 0.01$; 0% EtOH concentration denotes the control groups).

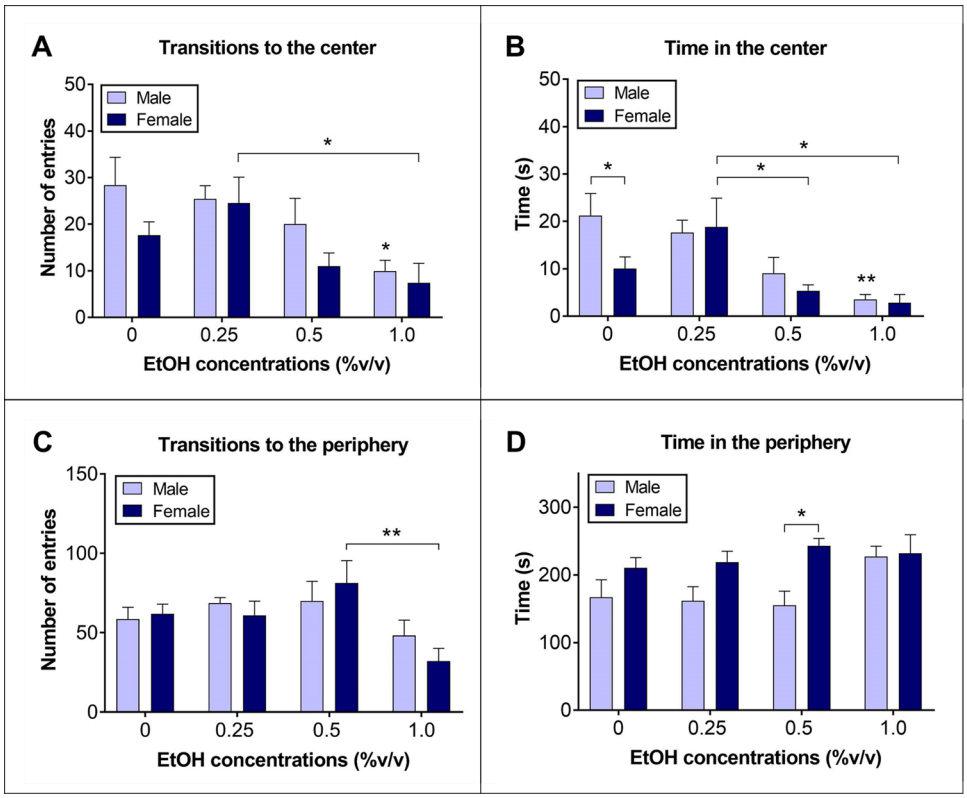


**Fig. 3.** Effects of EtOH (0%, 0.25%, 0.5%, and 1.0% v/v) on exploration-related parameters in male and female zebrafish submitted to the OFT with the presence of a non-familiar object. **(A)** Number of transitions in the center area. **(B)** Time in the center area. **(C)** Number of transitions to the periphery area. **(D)** Time in the periphery area. Data are represented as means ± S.E.M. and analyzed by two-way ANOVA (sex and treatment as factors), following by Newman–Keuls multiple comparison test whenever appropriate. The asterisks above bars express significant differences compared to the respective control group, while asterisks above brackets indicate statistical differences between other groups ($n = 8$–10 animals per goup; * $p < 0.05$; ** $p < 0.01$; 0% EtOH concentration denotes the control groups).

1.0% EtOH (Fig. 3B). The transitions to the periphery area also increased in females exposed to 0.5% EtOH compared to females treated with 1.0% EtOH (Fig. 3C). Additionally, the time in the periphery area was higher in females treated with 0.5% EtOH compared to males with same treatment (Fig. 3D).

Behavioral parameters reflecting interaction with the non-familiar object are shown in Fig. 4. Two-way ANOVA revealed a significant effects of EtOH x sex interaction ($F_{(3,69)} = 3.552$, $p = 0.0187$, $\eta_p^2 = 0.134$)

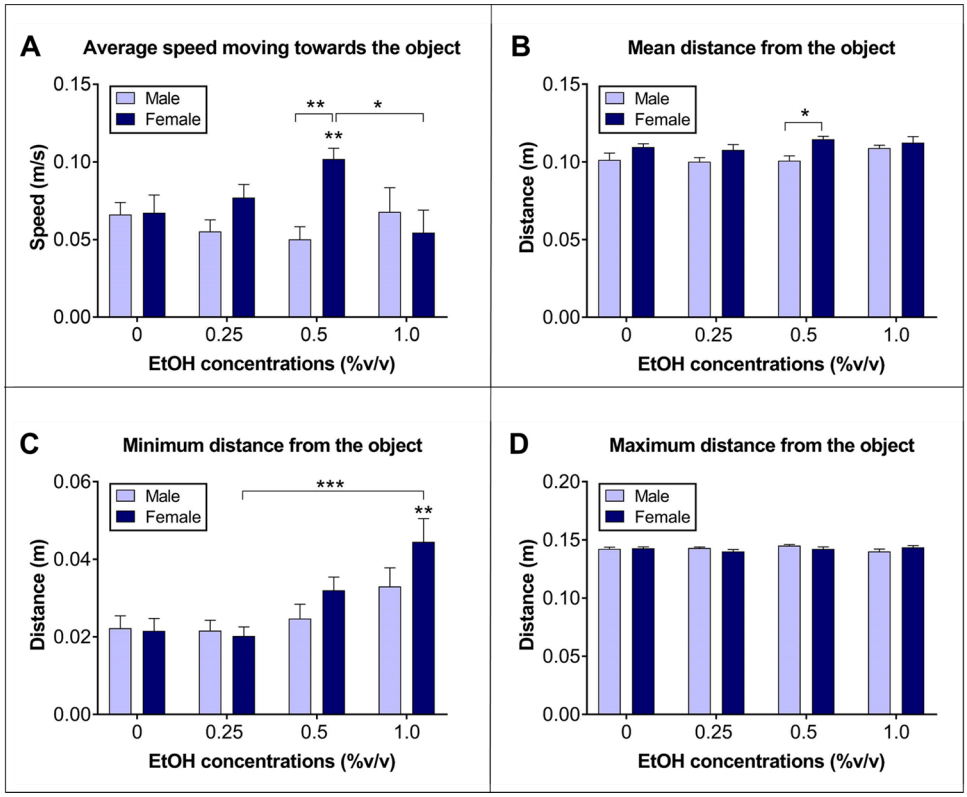


**Fig. 4.** Interaction-like behaviors of male and female zebrafish exposed to EtOH (0%, 0.25%, 0.5%, and 1.0% v/v). **(A)** Average speed moving towards the object. **(B)** Mean distance from the object. **(C)** Minimum distance from the object. **(D)** Maximum distance from the object. Data were represented as means ± S.E.M. and analyzed by two-way ANOVA (sex and treatment as factors), following by Newman–Keuls multiple comparison test whenever appropriate. The asterisks above bars express significant differences compared to the respective control group, while asterisks above brackets indicate statistical differences between groups ($n = 8$–10 animals per group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; 0% EtOH concentration denotes the control groups).

and sex ($F_{(1,69)} = 4.047$, $p = 0.0481$, $\eta_p^2 = 0.06$) on the average speed moving towards the object. We also found a significant effect of sex on the mean distance from the object ($F_{(1,69)} = 15{,}17$, $p = 0.002$, $\eta_p^2 = 0.180$), as well a significant effect of EtOH on the minimum distance from the object ($F_{(3,69)} = 3.552$, $p = 0.0187$, $\eta_p^2 = 0.134$). The average speed moving towards the object increased in female treated with 0.5% EtOH compared to group control females, males exposed to 0.5% EtOH and females exposed to 1.0% EtOH (Fig. 4A). The mean distance from the object increased in the females treated with 0.5% EtOH compared to males exposed to 0.5% EtOH (Fig. 4B). The minimum distance from the object increased in females exposed to 1.0% EtOH compared to control females and females exposed to 0.25% EtOH (Fig. 4C). No differences in the maximum distance from the object were observed between groups (Fig. 4D).

Using the PCA to simplify the structure of complex data set, the Kaiser-Meyer-Olkin measure of sampling adequacy obtained was 0.682 and Bartlett's tests of sphericity was significant ($\chi^2 = 498.566$, df = 45, $p < 0.0005$). PCA extracted three principal components (PC1, PC2, and PC3), which accounted for more than 73% of the total variance (35.287% for PC1, 22.836% for PC2, and 15.063% for PC3, respectively). PC1 was associated with anxiety-like behavior, showing positive correlations with the mean distance from the object, time in the periphery, and minimum distance from the object. Negative loadings were found for transitions to the center and time in the center. Since PC2 showed positive loadings for transitions to the center, distance traveled, transitions to the periphery, and average speed moving towards the object with negative correlations with minimum distance from the object and immobility, this component was associated with exploratory activity. PC3 was associated with the locomotor pattern, displaying positive correlations for distance traveled and maximum distance from the object, while a negative loading was found for immobility. Data from the principal component analysis rotated component matrix are shown in Table 1.

The influence of different EtOH concentrations on the principal components detected were further investigated (Fig. 5). For PC1 values, two-way ANOVA showed significant effects of EtOH ($F_{(3,69)} = 6.860$, $p = 0.0004$, $\eta_p^2 = 0.230$) and sex ($F_{(1,69)} = 15.02$, $p = 0.0002$, $\eta_p^2 = 0.179$). At the baseline, females showed higher PC1 values than males, while 1.0% EtOH increased PC1 values in males compared to their respective baseline. Moreover, females showed higher PC1 values than males exposed to 0.5% EtOH. Increased PC1 values were also observed in females at 0.5% EtOH when compared to those exposed to 0.25% EtOH. Concerning PC2 values, two-way ANOVA showed significant effects of EtOH x sex interaction ($F_{(3,69)} = 4.573$, $p = 0.0056$, $\eta_p^2 = 0.166$), EtOH ($F_{(3,69)} = 4.078$, $p = 0.0100$, $\eta_p^2 = 0.151$) and sex ($F_{(1,69)} = 4.829$, $p = 0.0313$, $\eta_p^2 = 0.07$). Post hoc analyses yielded significant increased PC2 values in females exposed to 0.5% EtOH compared to males, while 1.0% EtOH reduced PC2 in females compared to those exposed to 0.25% and 0.5% EtOH. The analysis of PC3 values revealed significant effects of EtOH ($F_{(3,69)} = 5.738$, $p = 0.0015$, $\eta_p^2 = 0.200$), where 1.0% EtOH decreased PC3 in both male and female compared to their respective controls.

**Table 1**
Principal component analysis rotated component matrix for zebrafish submitted to the OFT with the presence of a non-familiar object. Loadings < |0.3| are not shown. The percentage of total variance explained by each component is depicted below the loading matrices. The analysis is based upon bivariate Pearson correlation coefficients calculated for all behavioral endpoints measured.

| Rotated component matrix | | | |
|---|---|---|---|
| | Component | | |
| | 1 | 2 | 3 |
| Mean distance from the object | 0.922 | | |
| Time in the periphery | 0.853 | | |
| Transitions to the center | −0.837 | 0.313 | |
| Time in the center | −0.827 | | |
| Minimum distance from the object | 0.615 | −0.31 | |
| Distance traveled | | 0.848 | 0.359 |
| Transitions to the periphery | | 0.786 | |
| Average speed moving towards the object | | 0.78 | |
| Maximum distance from the object | | | 0.861 |
| Immobility | | −0.312 | −0.732 |
| Extraction method: principal component analysis. | | | |
| Rotation method: varimax with Kaiser normalization. | | | |
| Percentage of total variance | 35.287 | 22.836 | 15.063 |

## 4. Discussion

Overall, the present report assessed some behavioral parameters that may reflect both anxiety-related phenotypes and boldness in male and female zebrafish acutely exposed to different EtOH concentrations. At the baseline, both sexes showed a distinct exploratory activity and interaction pattern with the non-familiar object, in which females tend to display a more anxious and shyer behavior than males. Different than males, females exposed to 0.5% EtOH showed a prominent rapid investigation to the non-familiar object. Exposure to 1.0% EtOH, increased immobility in males but not in females, suggesting an influence of sex on sedative/depressant effects of EtOH.

The OFT is a suitable task to analyze the exploratory and anxiety-like behaviors of zebrafish (Champagne et al., 2010; Godwin et al., 2012). When fish are challenged to a non-familiar object, this task allows the simultaneous measurement of distinct behaviors (*e.g.,* anxiety-like responses, boldness) (Norton et al., 2011; Wright et al., 2006). Boldness is known as the reaction of a dangerous situation or the tendency to explore a new or challenging situation. Notably, boldness is correlated with a variety of behaviors, ranging from increased activity, exploration, aggression, dispersion, and even choosing partners (Ariyomo and Watt, 2012; Mustafa et al., 2019; Norton et al., 2011; Roy et al., 2017; Sih et al., 2004; Wilson and Godin, 2009). In experimental animals, such phenotype may also be sex- and strain-dependent (Mustafa et al., 2019; Wright et al., 2003; Zala et al., 2012). Genetic and life experience factors generate behavioral variability in individuals of the same species and habitat (Toms and Echevarria, 2014; Wright et al., 2006, 2003). Notably, the test applied can also become a determining factor to assess boldness-related behaviors (Mustafa et al., 2019; Réale et al., 2007). Considering the lack of standardization of the objects to assess interaction-related behaviors (Dahlbom et al., 2011; Dean et al., 2020; Norton et al., 2011; Toms and Echevarria, 2014), the use of specific objects with a well-defined color and shape allow a more precise replication of data among laboratories. At the baseline, although parameters of interaction with the object did not differ, males spent more time in the center area of the OFT than females, reflecting a bolder phenotype. Conversely, females showed decreased time in the center area and increased freezing episodes in the absence of EtOH, corroborating a shyer behavior and, hence, a prominent anxiety-like phenotype (Fontana et al., 2020a). In relation to boldness, some studies may also show divergent results than those reported here (Oswald et al., 2012). This fact may be linked to the lack of standardization of the protocols to measure specific behaviors (Réale et al., 2007), since boldness can be assessed by measuring different endpoints, leading to conflicting results (Toms et al., 2010). Here, our findings reinforce an effect of sex on anxiety- and boldness-related behaviors in zebrafish subjected to the OFT with the presence of a non-familiar object, which is clearly sensitive to pharmacological manipulations.

Acute EtOH exposure changes various behavioral responses in adult zebrafish (*e.g.*, locomotion, anxiety-like behavior, social interaction, and aggression) (Fontana et al., 2020b, 2018b; Gerlai et al., 2000; Müller et al., 2020; Rosemberg et al., 2012), but the influence of EtOH on sex is poorly explored yet. Here, we observed that 0.5% EtOH elicited a stimulating effect on zebrafish females, since they swam faster towards the object, but also had increased mean distance from the object and spent more time in the periphery area, reflecting a phenotype known as

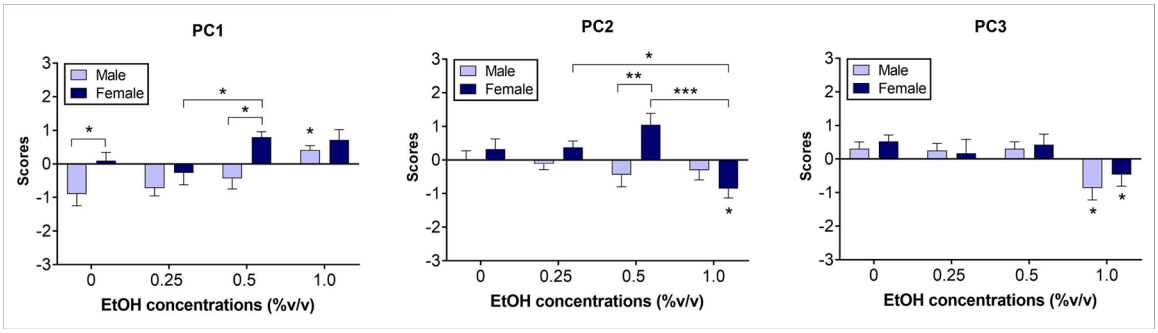


**Fig. 5.** Principal component analysis (PCA) for behavioral endpoints measured in male and female zebrafish exposed to EtOH (0%, 0.25%, 0.5%, and 1.0% v/v) submitted to the OFT with the presence of a non-familiar object. Comparison of the values of three PC with eigenvalues greater than 1. Data were represented as means ± S.E.M. and analyzed by two-way ANOVA (sex and treatment as factors), following by Newman–Keuls multiple comparison test whenever appropriate. The asterisks above bars express significant differences compared to the respective control group, while asterisks above brackets indicate statistical differences between other groups ($n$ = 8–10 animals per group; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; 0% EtOH concentration denotes the control groups).

fast investigation. This exploratory behavior has been characterized by a fast swim towards the object with a rapid return to periphery area, similar to a risk assessment episode (Stefanello et al., 2019). The effects observed on this interaction-related response may result from baseline differences, in which females are shyer than males and, therefore, can be more affected by the stimulatory effect of 0.5% EtOH. Acute exposure to intermediate EtOH concentrations is known to increase dopamine in SF population (Gerlai et al., 2009), causing disinhibition in shy animals (Araujo-Silva et al., 2018) by producing an initial feeling of pleasure followed by addiction (Vengeliene et al., 2008). Moreover, 0.5% EtOH can reduce risk aversion in shy animals, modulating the behavioral activity according to the characteristics/personality of the animal (Araujo-Silva et al., 2018).

The assessment of animal behavior involves a large number of endpoints correlated with distinct phenotypes (Loss et al., 2014). To simplify the structure of the data set, we have run a PCA aiming to verify which behaviors mostly contribute to the responses measured. Three principal components were extracted from the experiment, which were associated to anxiety-like behaviors (PC1), exploratory activity (PC2), and locomotion (PC3), respectively. As expected, PC1 values were lower in males than females at the baseline, corroborating the higher anxiety-like state in female zebrafish. Notably, the higher PC1 and PC2 values in female exposed to 0.5% EtOH than those found in males reinforce the fast investigation as a behavioral phenotype more linked to a defensive approach of fish. Although the risk-taking behavior may be indicative of boldness, in which animals become more susceptible to a potentially dangerous situation (Wilson and Godin, 2009), increased risk assessment can also reflect a protective response. Indeed, defensive behaviors when animals face a non-familiar object are pronounced to ensure appropriate safe conditions (Dahlbom et al., 2011; Toms and Echevarria, 2014).

It is well established that EtOH induces neurophysiological effects in a dose-dependent manner (Araujo-Silva et al., 2018). Acute exposure to 1.0% EtOH elicits sedative effects, associated with lethargy and immobility (Gerlai et al., 2000; Rosemberg et al., 2012). Accordingly, we found a robust sedative response in adult zebrafish. As corroborated by the PC3 values analysis, both sexes showed decreased locomotion when exposed to 1.0% EtOH, but they differed in the number of immobile episodes. Here, 1.0% EtOH did not change immobility in females compared to the respective control, while males showed a concentration-dependent effect on immobility. Because female zebrafish tend to show increased anxiety-like behaviors than males (Fontana et al., 2020a), the effects of EtOH on immobility could be less pronounced due to a possible ceiling effect. Because sex hormones can play a role in EtOH-mediated responses in rodents (Lenz et al., 2012), we cannot rule out hormonal influences on the behavioral parameters measured (Clayman et al., 2017). Assessing the effects of 1.5% or 3.0% EtOH (Lockwood et al., 2004; Mathur et al., 2011; Mathur and Guo, 2011; Peng et al., 2009) can represent an interesting strategy to verify how both sexes respond to higher EtOH concentrations in the future. Although the neurochemical mechanisms underlying the behavioral responses described here still merit further scrutiny, we suggest the existence of a different behavioral profile of male and female zebrafish following 1.0% EtOH exposure. While the reduced locomotion in females may apparently result from a decreased swimming velocity and reduced exploration (as observed by lower PC2 values), the hypolocomotion in males may result from reduced activity associated with increased immobility and anxiety-like behavior (as observed by PC1 analysis). Together, these data support that sedative/depressant effects of EtOH in zebrafish may occur in a sex-dependent manner.

Finally, we emphasize potential limitations of this study. It is known that the type of objects seems to influence only the immediate or initial approach responses, since zebrafish tend to gradually explore aversive conditions as novelty stress reduces (Dahlbom et al., 2011; Toms and Echevarria, 2014). Because the use of a single object may be considered a limiting factor, the influence of non-familiar objects with distinct sizes, colors, and shapes on the behavioral measures reported here still merits further scrutiny. The assessment of EtOH-mediated responses in zebrafish populations may also provide relevant findings. For example, WT females are more susceptible to the chronic effects of alcohol than those of long-fin (LF) phenotype when shoal-related behaviors are measured, supporting an influence of sex and population on EtOH sensitivity (Dlugos et al., 2011). Pre-exposure to EtOH during the juvenile phase also reflects sex-dependent responses in the conditioned place preference test (Clayman et al., 2017), supporting the use of other behavioral paradigms to investigate how individual differences influence the overall exploratory pattern of zebrafish. In line with this, complementing the behavioral analyses using other tasks (*e.g.*, exposure to the predator, environment with shelter, or to dyadic contest) to elucidate how EtOH affects boldness in male and female fish subjected to different contexts should not be ruled out. Based on the assumption that zebrafish metabolize EtOH similarly to mammals (Tran et al., 2015), further experiments are needed to explore whether sex differences in terms of alcohol metabolism may occur, as well as their potential correlations with a wide range of behavioral responses.

## 5. Conclusion

In conclusion, we report the acute effects of different EtOH concentrations on the exploratory activity of male and female zebrafish subjected to the OFT with the presence of a non-familiar object. At the baseline, we observed higher anxiety-like and shyness behavior in females, corroborating previous findings (Dahlbom et al., 2011; Fontana et al., 2020a; Roy et al., 2017). Our novel data revealed a sex-dependent

effect of EtOH when zebrafish were exposed to intermediate and high concentrations. In general, 0.5% positively modulated interaction-like behaviors in females, while sedative responses of 1.0% EtOH result from different effects on the exploratory pattern of male and female zebrafish. Although more studies to investigate the influence of sex on zebrafish behavioral responses are necessary, our findings reinforce the biological sex as a relevant factor in translational research, which must be taken into account in future pharmacological studies.

## Author statement

Material preparation, data collection, and analysis were performed by T.P.S, F.F, F.V.S, T.E.M, and L.W.S. All other aspects of the study, from conception to manuscript preparation and editing were performed by D.B.R. and T.P.S. D.B.R. was responsible to supervise the research and acquire funding. All authors approved the final version of the manuscript.

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## Declaration of Competing Interest

The authors report no competing interest.

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# 6 CONCLUSÕES

Em suma, nossos novos achados mostram um efeito dependente do sexo em peixes-zebra expostos agudamente a diferentes concentrações de EtOH testados no teste de campo aberto na presença de um objeto não familiar. Essa conclusão é sustenta a partir dos seguintes achados:

- As fêmeas apresentaram um comportamento distinto na atividade exploratória e no padrão de interação com o objeto não familiar, refletindo um comportamento mais ansioso e tímido em relação aos machos;
- A exposição à concentração intermediária de EtOH (0,5% v/v) induziu um comportamento de investigação rápida ao objeto não familiar apenas em fêmeas de peixe-zebra, enquanto a que a exposição a alta concentração de EtOH (1,0% v/v) reduziu a locomoção em ambos os sexos, mas acarretou um aumento na imobilidade apenas em machos;
- A análise de componente principal (PCA) revelou que os efeitos comportamentais são explicados principalmente por mudanças nos comportamentos do tipo ansiedade, exploração e locomoção.

Por fim, os dados da presente Dissertação reforçam o sexo biológico como um fator relevante na pesquisa, o qual deve ser levado em consideração em estudos farmacológicos futuros. Além disso, o estudo também contribui para a investigação dos efeitos neurobiológicos promovidos pelo EtOH em peixes-zebra machos e fêmeas em futuras pesquisas translacionais.

# 7 PESPECTIVAS DO ESTUDO

Os resultados apresentados na presente Dissertação contribuem para a validação do peixe-zebra como um organismo modelo de estudo da influência do sexo nos efeitos comportamentais a exposição a diferentes concentrações de EtOH. No entanto, muitos aspectos do modelo ainda devem ser abordados e explorados, a fim de fornecer um avanço científico na área. A realização de novos protocolos e testes comportamentais, tal como o teste de exposição ao predador (FONTANA et al., 2018b) e teste do tanque novo (FONTANA et al., 2020; MATHUR; GUO, 2011), tornam-se uma perspectiva interessante para expandir os estudos em relação às diferenças entre os padrões comportamentais de machos e fêmeas de peixes-zebra. A realização de protocolos de exposição crônica repetida ao EtOH (MATHUR; GUO, 2011; MÜLLER et al., 2017) também é interessante com o intuito de explorar aspectos de dependência ao EtOH entre os sexos no modelo.

No geral, os estudos sobre o metabolismo do EtOH em modelos de peixe-zebra não fazem distinção entre o sexo dos animais (TRAN et al., 2015, 2016). Contudo, estudos já apontaram a influência do sexo na sensibilidade ao EtOH (DLUGOS; BROWN; RABIN, 2011), assim como a pré-exposição ao EtOH durante a fase juvenil que induz diferenças nas respostas entre machos e fêmeas (CLAYMAN et al., 2017). Considerando que as evidências atuais sugerem que o peixe-zebra metaboliza o EtOH de forma semelhante aos mamíferos (TRAN et al., 2015) e que em humanos há diferenças entre os sexos, explorar se/como o fator sexo influencia o metabolismo do EtOH no peixe-zebra, torna-se um tópico relevante para estudos futuros. Tais achados podem ajudar a elucidar os resultados da presente Dissertação, além de acrescentar o sexo biológico como um fator relevante que deve ser levado em consideração em estudos farmacológicos futuros.

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## ANEXO A - PRODUÇÕES CIENTÍFICAS EM COLABORAÇÃO

Artigos produzidos em parceria durante o período do mestrado:

1. Canzian J, Franscescon F, Müller TE, Stefanello FV, **Souza TP**, Rosa LV, Rosemberg DB. Stress increases susceptibility to pentylenetetrazole-induced seizures in adult zebrafish. Epilepsy Behav. 2021 Jan;114(Pt A):107557. doi: 10.1016/j.yebeh.2020.107557. Epub 2020 Nov 24. PMID: 33243678.

2. Mezzomo NJ, Müller TE, Franscescon F, Michelotti P, **Souza TP**, Rosemberg DB, Barcellos LJG. Taurine-mediated aggression is abolished via 5-HT1A antagonism and serotonin depletion in zebrafish. Pharmacol Biochem Behav. 2020 Dec;199:173067. doi: 10.1016/j.pbb.2020.173067. Epub 2020 Nov 2. PMID: 33144206.

3. Cristina da Costa Araldi I, **Piber de Souza T**, de Souza Vencato M, de Andrade Fortes T, Emanuelli Mello CB, Sorraila de Oliveira J, Dornelles GL, Melazzo de Andrade C, Maciel RM, Danesi CC, Gindri AL, Machado AK, de Freitas Bauermann L. Preclinical safety assessment of the crude extract from Sida rhombifolia L. aerial parts in experimental models of acute and repeated-dose 28 days toxicity in rats. Regul Toxicol Pharmacol. 2021 Aug;124:104974. doi: 10.1016/j.yrtph.2021.104974. Epub 2021 Jun 15. PMID: 34139276.

4. Franscescon F, **Souza TP**, Müller TE, Michelotti P, Canzian J, Stefanello FV, Rosemberg DB. Taurine prevents MK-801-induced shoal dispersion and altered cortisol responses in zebrafish. Prog Neuropsychopharmacol Biol Psychiatry. 2021 Jul 9;111:110399. doi: 10.1016/j.pnpbp.2021.110399. Epub ahead of print. PMID: 34246730.

5. Stefanello FV, Müller TE, Franscescon F, Quadros VA, **Souza TP**, Canzian J, Leitemperger J, Loro VL, Rosemberg DB. Taurine modulates behavioral effects of intermittent ethanol exposure without changing brain monoamine oxidase activity in zebrafish: Attenuation of shoal- and anxiety-like responses, and abolishment of memory acquisition deficit. Pharmacol Biochem Behav. 2021 Oct;209:173256. doi: 10.1016/j.pbb.2021.173256. Epub 2021 Aug 17. PMID: 34416220.