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INSTITUTO DE ESTUDOS EM SAÚDE E BIOLÓGICAS
CURSO DE CIÊNCIAS BIOLÓGICAS**

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**EFEITOS COMPORTAMENTAIS E BIOQUÍMICOS DA ABSTINÊNCIA
ALCOÓLICA NO ZEBRAFISH (*Danio rerio* Hamilton, 1822)**

**MARABÁ-PA
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Trabalho de Conclusão de Curso apresentado à Universidade Federal do Sul e Sudeste do Pará como requisito parcial para a obtenção do grau de Bacharela em Ciências Biológicas, sob orientação do prof. Dr. Caio Maximino de Oliveira.

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*À minha mãe, Antonia e ao meu irmão,
Sandinny, por todo amor e carinho que faz dos
meus dias mais especiais.*

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RESUMO

O uso crônico de álcool induz adaptações e toxicidade que podem induzir sintomas de ansiedade, hiperexcitabilidade autonômica e convulsões quando o álcool é removido (síndrome de abstinência). O zebrafish recentemente ganhou ampla atenção como modelo comportamental para estudar os efeitos neurocomportamentais do uso crônico e agudo de álcool, incluindo a abstinência. A literatura, no entanto, é muito contraditória em relação aos resultados de retirada, com alguns estudos relatando aumento da ansiedade, enquanto outros relatam nenhum efeito. Uma meta-análise foi realizada para encontrar as fontes dessa heterogeneidade. A concentração de etanol durante a exposição e a duração da exposição foram as principais fontes de variação. Uma replicação conceitual também foi feita usando exposição contínua por 16 dias em etanol a base de água (0,5%) e avaliando o comportamento tipo ansiedade no teste claro / escuro após retirada de 60 min. Os animais demonstraram diminuição da ansiedade, que pode ser comprovado pela redução da preferência pela escuridão. Em contrapartida, houve um aumento na avaliação de risco, demonstrando um aumento da ansiedade. Os animais também foram submetidos ao protocolo de retirada e injetados com pilocarpina em uma dose subconvulsiva para avaliar a suscetibilidade ao comportamento convulsivo. O protocolo foi suficiente para aumentar a suscetibilidade ao comportamento convulsivo em animais expostos ao etanol. Finalmente, a retirada também diminuiu a atividade da catalase no cérebro, mas não no rim cefálico, sugerindo mecanismos associados aos efeitos comportamentais da retirada do etanol.

Palavras-chave: Ansiedade, *Danio rerio*, Síndroma de abstinência, Atividade da Catalase, Convulsões.

ABSTRACT

Chronic alcohol use induces adaptations and toxicity that can induce symptoms of anxiety, autonomic hyperarousal, and epileptic seizures when alcohol is removed (withdrawal syndrome). Zebrafish has recently gained wide attention as a behavioral model to study the neurobehavioral effects of acute and chronic alcohol use, including withdrawal. The literature, however, is very contradictory on findings regarding withdrawal effects, with some studies reporting increased anxiety, while others report no effect. A meta-analytic approach was taken to find the sources of this heterogeneity, and ethanol concentration during exposure and exposure duration were found to be the main sources of variation. A conceptual replication was also made using continuous exposure for 16 days in waterborne ethanol (0.5%) and assessing anxiety-like behavior in the light/dark test after 60 min withdrawal. Withdrawal was shown to reduce preference for darkness, consistent with decreased anxiety, but to increase risk assessment, consistent with increased anxiety. Animals were also subjected to the withdrawal protocol and injected with pilocarpine in a sub-convulsive dose to assess susceptibility to epileptic seizure-like behavior. The protocol was sufficient to increase susceptibility to epileptic seizure-like behavior in animals exposed to ethanol. Finally, withdrawal also decreased catalase activity in the brain, but not in the head kidney, suggesting mechanisms associated with the behavioral effects of ethanol withdrawal.

Keywords: Anxiety, *Danio rerio*, Ethanol withdrawal, Catalase activity, Epileptic seizures.

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1 INTRODUÇÃO

O uso crônico de álcool (etanol, EtOH) produz adaptações e toxicidade que podem levar à tolerância e dependência, manifestando-se como sofrimento físico e mental quando o EtOH é removido (abstinência); Os sintomas da abstinência de etanol incluem ansiedade, insônia e hiperexcitabilidade autonômica (KRYSTAL; TABAKOFF, 2002). Em níveis mais severos de dependência, com episódios repetidos de abstinência, a síndrome pode incluir hiperexcitabilidade autonômica bastante significativa, agitação, mudanças perceptuais, confusão mental, e convulsões (GATCH; LAI, 2001; KRYSTAL; TABAKOFF, 2002). Esses sintomas podem emergir em um contexto de *delirium tremens*, uma complicação possivelmente fatal que normalmente desenvolve-se na primeira semana de sobriedade, hipertermia, arritmia cardíaca e complicações de convulsões epilépticas induzidas por abstinência (LONGO; SCHUCKIT, 2014). Esses sintomas também se apresentam com um curso típico no tempo, com sinais marcantes de ansiedade aparecendo tão cedo quanto 6 horas após a cessação do consumo de álcool, e convulsões epilépticas aparecendo de 12 a 48 horas após a retirada (TREVISAN et al., 1998). Uma vez que os sintomas de abstinência são geralmente reduzidos após o consumo de EtOH, a dependência de EtOH pode ser mantida por reforço negativo (KOOB; LE MOAL, 2008), e, portanto, investigar esses mecanismos motivacionais pode abrir novos caminhos para o tratamento de transtornos relacionados ao consumo de EtOH.

Em modelos animais, a abstinência de EtOH altera a excitabilidade de neurônios localizados em regiões do cérebro associadas a comportamento defensivo, ansiedade e medo (BONASSOLI; MILANI; DE OLIVEIRA, 2011; CHAKRAVARTY; FAINGOLD, 1998; TSIKAS, 2007; YANG; LONG; FAINGOLD, 2001). Além disso, a abstinência também sobre-regula a atividade do eixo hipotálamo-pituitária-adrenal, responsável pela organização endócrina das respostas de estresse (RASMUSSEN et al., 2002). Faz sentido, então, que a maioria dos modelos animais de abstinência de EtOH sejam focados no comportamento de ansiedade, com efeitos consistentes observados em modelos de roedores como o labirinto em cruz elevado, caixa clara / escura, teste de interação social e discriminação de drogas, utilizando ensaio com pentilenotetrazol (GATCH; LAI, 2001).

O zebrafish (*Danio rerio*) é um modelo útil para o estudo da genética comportamental e neurociência comportamental, neuropsicofarmacologia e neurotoxicologia (BONAN; NORTON, 2015; CACHAT et al., 2010; SHAMS et al., 2017). A sua utilização possibilita uma série de vantagens, tais como o baixo custo de aquisição e manutenção, facilidade de manuseio e acomodação, curto tempo de desenvolvimento e facilidade de reprodução em laboratório (GERLAI, 2014; KALUEFF; ECHEVARRIA; MICHAEL, 2014). O grau relativamente alto de homologia genética, neural e endócrina com roedores e seres humanos também é citado como uma vantagem(KOKEL; RANDALL, 2008). O zebrafish também é um bom modelo para o estudo da ansiedade e do estresse, com testes bem validados para ansiedade e interações induzidas por novidade e conflito, interação social e comportamento anti predatório(GERLAI, 2010; MAXIMINO et al., 2010; OLIVEIRA, 2013). É importante ressaltar que, os comportamentos tipo convulsões também foram caracterizados na espécie (HORTOPAN; DINDAY; BARABAN, 2010), embora ainda não em um contexto de abstinência de EtOH.

O comportamento tipo ansiedade do zebrafish tem sido usado para demonstrar os efeitos da retirada da droga, incluindo cocaína (LÓPEZ-PATIÑO et al., 2008; LÓPEZ et al., 2008), morfina (CACHAT et al., 2010) e EtOH (TRAN; FACCIO; GERLAI, 2016). No último caso, a literatura é inconsistente, com alguns estudos relatando efeitos significativos de abstinência no comportamento semelhante à ansiedade (CACHAT et al., 2010), enquanto outros foram incapazes de detectar efeitos de abstinência de EtOH. Diferenças processuais, como tipo de ensaio, concentração durante a exposição ou duração da retirada, podem ser responsáveis por essa diferença.

Uma consistência importante encontrada na literatura diz respeito aos resultados utilizando o teste claro / escuro. Por exemplo, Benneh et al. (2017) e Mathur e Guo. (2011) não encontraram nenhum efeito de abstinência na preferência pelo escuro, enquanto Holcombe et al. (2013) descobriu que o zebrafish submetido à retirada do EtOH inverteu sua preferência, passando mais tempo no compartimento branco em vez do compartimento preto. O teste claro / escuro é conceitualmente diferente do teste de distribuição vertical eliciada pela novidade, em que um o conflito aproximação-fuga parece sublinhar o comportamento no teste claro / escuro, enquanto a fuga do topo parece motivar o comportamento no teste de distribuição vertical eliciada pela novidade (MAXIMINO et al., 2012). Uma meta-análise recente (KYSIL et al., 2017) também sugeriu que o teste claro / escuro é mais sensível a tratamentos farmacológicos do que o

teste de distribuição vertical eliciada pela novidade. As discrepâncias na literatura sobre os efeitos da retirada do EtOH em ambos os testes podem representar diferentes bases neurobiológicas subjacentes.

Além desses desfechos comportamentais, análises neuroquímicas e ensaios de estresse oxidativo também foram relatados em modelos de zebrafish de diferenças de retirada de EtOH (MÜLLER et al., 2017). Após exposição ao EtOH a 0,5% durante 22 dias, observaram-se aumentos nos níveis cerebrais de dopamina, serotonina e aspartato com 60 min de retirada (PAN; CHATTERJEE; GERLAI, 2012). Após exposição por 8 dias a 1% de EtOH (por 20 minutos por dia), diminuições na atividade da superóxido dismutase e catalase e consequente aumento no estresse oxidativo foram observados (MÜLLER et al., 2017). Embora ainda exista uma explicação mecanicista, esses resultados são congruentes com a hiperexcitabilidade e a neurotoxicidade induzida pelo EtOH observada em outros modelos (KRYSTAL; TABAKOFF, 2002; ZENKI et al., 2014).

O objetivo do presente trabalho é estudar a heterogeneidade dos efeitos da abstinência de EtOH no comportamento tipo ansiedade do zebrafish na literatura, aplicando técnicas de meta-análise. Além disso, o presente trabalho tenta uma replicação conceitual dos achados sobre a retirada do EtOH, avaliando os efeitos de um protocolo de abstinência no comportamento no teste claro / escuro. Ele também tenta expandir os parâmetros usados na pesquisa de abstinência usando um modelo de comportamento convulsivo quimicamente induzido, com doses subconvulsivas. Finalmente, o presente trabalho também tentou replicar pesquisas anteriores sobre os efeitos da retirada do EtOH sobre a atividade da enzima catalase no cérebro e no rim cefálico. Este trabalho é um relatório completo de todos os estudos realizados para testar o efeito da abstinência do etanol sobre o comportamento ansioso e do tipo convulsivo no zebrafish. Relatamos como determinamos nosso tamanho de amostra, todas as exclusões de dados, todas as transformações de dados e todas as medidas do estudo(SIMMONS et al., 2012).

2 MATERIAL E MÉTODOS

2.1 REVISÃO SISTEMÁTICA E META-ANÁLISE

O protocolo para metanálise foi pré-registrado na base de dados CAMARADES-NC3Rs Preclinical Systematic Review & Meta-analysis Facility (SyRF) (<https://drive.google.com/file/d/0B7Z0eAxKc8ApUjcyQjhwVnFjRFE/view?usp=sharing>)

g). Nenhuma modificação do protocolo pré-registrado foi realizada. Foram procurados artigos com os descritores ‘ethanol withdrawal’ e ‘zebrafish’ na base de dados PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>); foram estabelecidos como critérios de inclusão a apresentação de dados experimentais originais; a apresentação de dados comportamentais; e experimentos que utilizassem a retirada da droga como variável independente (HOOIJMANS et al., 2010). Dados bibliográficos (incluindo DOI, data de publicação, título e resumo) dos estudos identificados na revisão sistemática foram exportados para uma planilha eletrônica. Cada artigo da lista foi revisado em quatro níveis de detalhes (título, resumo, texto completo e uma revisão detalhada do desenho experimental) para determinar sua elegibilidade à meta-análise. Segundo Mohammad et al. (2016), os estudos devem incluir: (1) dados comportamentais primários obtidos em testes para o comportamento tipo ansiedade em zebrafish (teste claro/escuro, teste de distribuição vertical eliciada pela novidade , respostas anti predatórias, respostas de cardumes); (2) relato de controles apropriados; e (3) relato de, pelo menos, tamanhos amostrais e estatísticas (medidas de tendência central e dispersão) resumidas para grupos de controle e abstinência. Embora os testes claro/escuro e distribuição vertical eliciada pela novidade, assim como as respostas anti predatórias representem diretamente o comportamento tipo ansiedade, o cardumeamento foi incluído porque é sensível a manipulações que aumentam a ansiedade e/ou o medo no zebrafish (GREEN et al., 2012), sugerindo um componente defensivo. Quando um experimento avaliou os efeitos de drogas ou outras intervenções sobre os efeitos similares aos da síndrome de abstinência, apenas os grupos controle e de abstinência de EtOH foram considerados, e os dados sobre os efeitos da intervenção não foram analisados; por exemplo, Pittman e Hylton (2015) avaliaram os efeitos ansiogênicos da fluoxetina e da cetamina induzidos pela abstinência, e esses efeitos não foram avaliados na meta-análise. Possíveis confusões em relação ao papel do desenvolvimento foram reduzidos, excluindo estudos que não foram realizados em peixes adultos.

Os seguintes dados foram extraídos de cada estudo incluído: identificação (DOI, autores, ano de publicação); estirpe / fenótipo; concentração de etanol durante a exposição; duração do tratamento com EtOH; duração da retirada; teste comportamental que foi usado; médias e desvios-padrão, bem como estatísticas de teste e graus de liberdade; e tamanhos de amostra (N) para cada grupo. Os dados, que foram representados graficamente, foram extraídos de figuras usando PlotDigitizer (<http://plotdigitizer.sourceforge.net/>). Quando várias variáveis dependentes foram

relatadas, apenas o ponto final primário foi usado (tempo em branco, tempo no fundo, distância entre os peixes, e distância do estímulo). Embora haja considerável variação nos efeitos das intervenções nessas tarefas - e, de fato, variáveis como natação errática ou congelamento podem ser mais sensíveis a certos tratamentos -, a literatura geralmente se refere ao tempo em branco e ao tempo no fundo como desfechos primários. A distância de estímulo foi utilizada como desfecho primário para os experimentos de comportamento anti predador feitos pelo grupo de Robert Gerlai, dado que indica aumento do cardume (distância reduzida ao estímulo do cardume) ou evitação de predadores (aumento da distância ao estímulo predador). Todas as estimativas foram transformadas em diferenças médias padronizadas (SMD), corrigidas pelo seu viés positivo (HEDGES, 1984), com estimativas não-viesadas de variâncias de amostragem e intervalos de nível de confiança de 95%, valores de heterogeneidade I^2 e τ^2 , e p-valor usando um modelo de efeitos mistos, com concentração, duração de exposição e duração de retirada usados como moderadores. Diferentemente dos outros desfechos, as distâncias diminuídas para o estímulo de cardume ou a diminuição das distâncias entre peixes indicam menor ansiedade, e os SMDs para esses casos foram transformados pela multiplicação por -1 (VESTERINEN et al., 2014). Diagnósticos de caso influentes foram feitos inspecionando gráficos para resíduos externamente padronizados, valores de DFFITS, razões de covariância, estimativas de τ^2 e estatísticas de teste para heterogeneidade residual quando cada estudo é removido, por sua vez, com os valores e pesos para cada estudo incluído na análise. O viés de publicação foi avaliado pela inspeção de um gráfico de funil de contorno aprimorado, com contornos nos intervalos de confiança de 90%, 95% e 99%. Além disso, a assimetria do gráfico de funil foi analisada usando um teste de meta-regressão, com o tamanho total das amostras como preditor (EGGER et al., 1997). A potência observada para cada estudo foi calculada com base nos tamanhos de efeito, tamanhos de amostra e desvios padrão, e ajustada contra SMDs por um modelo aditivo generalizado com a família de curvas de Gaussian e função de link de identidade. Finalmente, uma análise de sensibilidade foi realizada adicionando a qualidade do estudo, avaliada usando a ferramenta Risk of Bias (RoB) da SYRCLE (HOOIJMANS et al., 2014), no modelo de meta-regressão. A meta análise foi feita com o pacote metafor do R (VIECHTBAUER, 2010).

2.2. EFEITOS DA ABSTINÊNCIA DE ETANOL NO COMPORTAMENTO TIPO ANSIEDADE E SUSCETIBILIDADE AO COMPORTAMENTO CONVULSIVO NO ZEBRAFISH

2.2.1. ANIMAIS E ALOJAMENTO

Foram utilizadas diferentes populações endógenas devido ao aumento de sua variabilidade, diminuindo os efeitos da deriva genética aleatória que poderia direcionar para características diferentes (PARRA; ADRIAN; GERLAI, 2009; SPEEDIE; GERLAI, 2008). Assim, espera-se que os animais utilizados nos experimentos representem melhor as populações naturais da natureza. Foram utilizados neste experimento zebrafish adultos (fenótipo longfin). Os animais foram comprados de um vendedor comercial e chegaram ao laboratório com aproximadamente 3 meses de idade (comprimento padrão = $13,2 \pm 1,4$ mm) e foram colocados em quarentena por duas semanas; o experimento começou quando os animais tinham uma idade aproximada de 4 meses (comprimento padrão = $23,0 \pm 3,2$ mm). Os animais foram mantidos em tanques mistos durante a aclimatação, com uma proporção aproximada de 50 machos e 50 fêmeas. O criador foi licenciado para a aquicultura sob a Resolução 95/1993 do Ibama (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis). Os animais foram alojados em tanques de 40 L, com uma densidade máxima de 25 peixes por tanque, por pelo menos 2 semanas antes do início dos experimentos. Foram preenchidos os tanques com água descolorada à temperatura ambiente (28°C) e um pH de 7,0-8,0. Foi fornecida iluminação por lâmpadas fluorescentes em um ciclo de 14-10 h, de acordo com os padrões de cuidados para o zebrafish (LAWRENCE, 2007). Os parâmetros de qualidade da água foram os seguintes: pH 7,0-8,0; dureza 100 a 150 mg / 1 CaCO₃; oxigênio dissolvido 7,5 a 8,0 mg / L; amônia e nitrito <0,001 ppm. Todas as manipulações minimizaram o sofrimento dos animais, e seguiu a legislação brasileira (Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017). Os animais foram utilizados para apenas um experimento e em um único teste comportamental, para reduzir a interferência da exposição do aparato.

2.2.2. EXPOSIÇÃO AO ETANOL E ABSTINÊNCIA

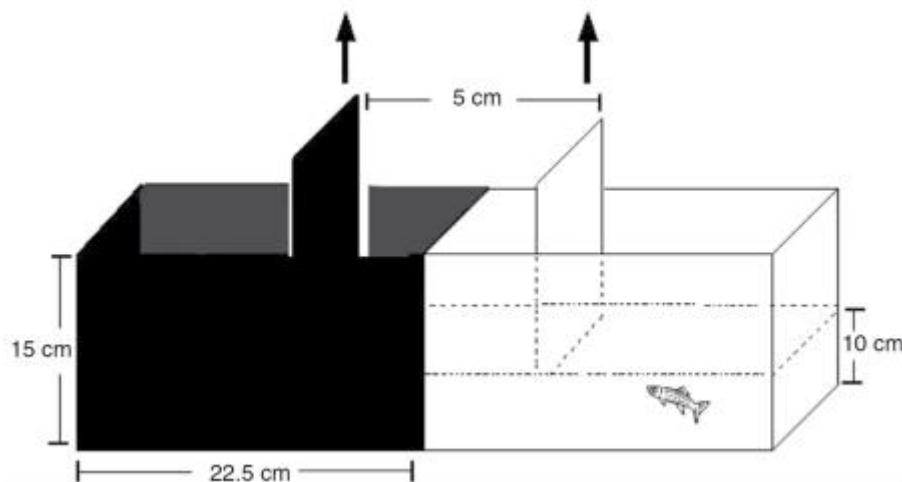
A administração de exposição e retirada de EtOH foi adaptado de Gerlai et al. (2008). Os animais foram expostos as concentrações crescentes de EtOH (0,125% -0,5%), dispersas na água do aquário, por 16 dias, diminuindo a mortalidade associada à exposição prolongada ao EtOH em zebrafish. Concentrações duplicaram a cada quatro dias,

atingindo uma concentração final de 0,5% (v/v). Os animais foram mantidos nesta concentração final durante 4 dias. Outro grupo foi exposto à mesma manipulação, sem exposição a EtOH; os animais foram alocados aleatoriamente para tratamentos através da geração de números aleatórios usando a ferramenta de randomização em <http://www.randomization.com/>. Após o tratamento, os animais foram transferidos para um tanque com água do sistema por 60 min (estágio de retirada). Todos os animais foram incluídos nos experimentos, e nenhuma anormalidade física foi observada durante o período de exposição. Para controlar os efeitos da exposição crônica ao EtOH que não são atribuíveis à retirada, dois grupos adicionais de 8 animais foram expostos como acima e transferidos, sem um estágio de retirada, para o aquário do teste claro/escuro.

2.2.3. TESTE CLARO/ESCURO

Os animais foram testados individualmente em aquário (15 cm altura X 10 cm diâmetro X 45 cm largura), dividido na metade em um compartimento preto e um compartimento branco. O aparato foi iluminado pelo topo por duas lâmpadas fluorescentes de 25 W, produzindo uma média de 270 lumens sobre o tanque. O aparato contém portas deslizáveis que delimitam um compartimento central, no qual o animal foi colocado por 3 minutos para aclimatação. Após esse período, as portas foram retiradas e o comportamento do animal foi registrado por 15 minutos (Figura 1). A ordem com que os animais foram expostos ao tanque foi randomizada e equilibrada entre os tratamentos. Os experimentadores foram cegados para o tratamento. Os vídeos foram transcritos manualmente por dois observadores, cegos para o tratamento, usando o X-Plo-Rat (<https://github.com/lanecunifesspa/x-plo-rat>). Foram analisadas as seguintes variáveis: tempo no compartimento branco; latência para entrada no branco; número de entradas no branco; tempo em congelamento; número de eventos de nado errático; tempo em tigmotaxia; e número de eventos de avaliação de risco. Os dados foram analisados por meio de testes aproximativode permutação de Fisher de Pitman com duas amostras e 10.000 re-amostragens de Monte-Carlo. A análise de dados foi cegado para o tratamento; após análise, os dados foram revelados. Os dados são apresentados usando gráficos de pontos individuais combinados com de intervalos médios em nível de 95% de confiança. Diferenças médias padronizadas com estimativas de variância não enviesadas foram calculadas usando o pacote R "metafor". *Post-hoc* (observado) foi calculado com base em uma aproximação do teste t, com duas hipóteses. Todas as análises e gráficos foram feitos usando a versão R 3.3.0 e pacotes "ggplot2" e "coin".

Figura 1: Modelo de preferência Claro/escuro.



Fonte: Maximino et al., (2010). Esquema do aquário de acrílico fosco, com a metade clara e outra escura, com portas deslizáveis no centro.

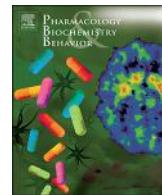
2.2.4. TESTE DE CONVULSÃO INDUZIDO POR PILOCARPINA

Imediatamente após o teste de preferência clara/escuro, os animais foram tratados com pilocarpina em dose de 150 mg/kg, insuficiente para induzir clonia e convulsões tônico-clônicas em animais normais (PINTO, 2015). Em seguida, os animais foram transferidos individualmente para aquários de 1,5 L e filmados por 15 minutos para analisar o perfil das crises convulsivas. Os escores das crises epilépticas foram classificados de acordo com Mussulini et al. (2013): Escore I - Imobilidade e aumento da frequência de abertura opercular; Escore II - Comportamento natatório em forma de redemoinho; Escore III - Movimentos rápidos da direita para esquerda; Escore IV – Clonia e movimentos tônicos e anormais; Escore V – Clônias rápidas de corpo inteiro; Escore VI – Crises tônico-clônicas; Escore VII – Morte. A latência para atingir o escore 4 foi considerada como o principal desfecho para o comportamento convulsivo (MUSSULINI et al., 2013); As latências foram analisadas ajustando-se um modelo de Kaplan-Meier às curvas de sobrevida, usando o pacote R “survival”.

2.3 EFEITOS DA ABSTINÊNCIA ALCÓOLICA NA ATIVIDADE DA CATALASE

Foi usado neste experimento um grupo separado de 12 animais. Os animais foram submetidos ao regime de exposição ao etanol e abstinência descrito em 2.2.2. Após 60 minutos de retirada, os animais foram eutanasiados em água fria seguida de secção espinhal e os seus cérebros e cabeça foram dissecados. A atividade da catalase foi medida utilizando a taxa de dissipação de H₂O₂ espectrofotometricamente, seguindo o método descrito por Aebi (1984). A validação dentro do laboratório teve uma linearidade de $r^2 = 0,9849$ e repetibilidade intermediária de 0,2364 (IC95% [0,0042, 0,4686]; razão de Horwitz). A atividade enzimática foi corrigida pelos níveis de proteína, quantificados pelo método de Bradford. As diferenças entre os grupos foram analisadas usando testes de permutação de duas amostras aproximadas de Fisher-Pitman.

**3 BEHAVIORAL AND BIOCHEMICAL EFFECTS OF ETHANOL
WITHDRAWAL IN ZEBRAFISH (DOI: 10.1016/J.PBB.2018.04.006)**



Behavioral and biochemical effects of ethanol withdrawal in zebrafish

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ABSTRACT

Chronic alcohol use induces adaptations and toxicity that can induce symptoms of anxiety, autonomic hyperarousal, and epileptic seizures when alcohol is removed (withdrawal syndrome). Zebrafish has recently gained wide attention as a behavioral model to study the neurobehavioral effects of acute and chronic alcohol use, including withdrawal. The literature, however, is very contradictory on findings regarding withdrawal effects, with some studies reporting increased anxiety, while others report no effect. A meta-analytic approach was taken to find the sources of this heterogeneity, and ethanol concentration during exposure and exposure duration were found to be the main sources of variation. A conceptual replication was also made using continuous exposure for 16 days in waterborne ethanol (0.5%) and assessing anxiety-like behavior in the light/dark test after 60 min withdrawal. Withdrawal was shown to reduce preference for darkness, consistent with decreased anxiety, but to increase risk assessment, consistent with increased anxiety. Animals were also subjected to the withdrawal protocol and injected with pilocarpine in a sub-convulsive dose to assess susceptibility to epileptic seizure-like behavior. The protocol was sufficient to increase susceptibility to epileptic seizure-like behavior in animals exposed to ethanol. Finally, withdrawal also decreased catalase activity in the brain, but not in the head kidney, suggesting mechanisms associated with the behavioral effects of ethanol withdrawal.

1. Introduction

Chronic alcohol (ethanol, EtOH) use produces adaptations and toxicity that can lead to tolerance and dependence, manifested as physical and mental distress when EtOH is removed (withdrawal); symptoms of ethanol withdrawal include anxiety, insomnia, and autonomic hyperarousal (Krystal and Tabakoff, 2002). In more serious conditions, patients presenting this EtOH withdrawal syndrome can present perceptive changes, agitation, mental confusion, significant increases in autonomic arousal, and epileptic seizures (Gatch and Lal, 2001; Krystal and Tabakoff, 2002). The most serious condition involves *delirium tremens* and death by hyperthermia, cardiac arrhythmia, and complications from withdrawal-induced epileptic seizures (Longo and Schuckit, 2014). These symptoms also present with a typical time course, with marked signs of anxiety appearing as early as 6 h after cessation of alcohol consumption, and epileptic seizures appearing from

12 to 48 h after withdrawal (Trevisan et al., 1998). Since withdrawal symptoms are usually reduced after EtOH consumption, EtOH dependence can be maintained by negative reinforcement (Koob and Le Moal, 2008), and therefore investigating these motivational mechanisms could open new avenues for the treatment of EtOH consumption-related disorders.

In animal models, EtOH withdrawal changes the excitability of neurons located in brain regions associated with defensive behavior, anxiety, and fear (Bonassoli et al., 2011; Chakravarty and Faingold, 1998; Long et al., 2007; Yang et al., 2003, 2002, 2001). Moreover, EtOH withdrawal also dysregulates the activity of the hypothalamus-pituitary-adrenal axis that modulates behavioral and endocrine responses to stress (Rasmussen et al., 2002). It makes sense, then, that the majority of animal models of EtOH withdrawal are focused on anxiety-like behavior, with consistent effects observed in rodent models such as the elevated plus-maze, light/dark box, social interaction test, and a

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drug discrimination assay using pentylenetetrazole (Gatch and Lal, 2001).

Zebrafish (*Danio rerio*) is increasingly being considered as useful model organisms for studying both behavioral genetics and behavioral neuroscience, neuropsychopharmacology, and neurotoxicology (Bonan and Norton, 2015; Kalueff et al., 2012; Norton and Bally-Cuif, 2010; Shams et al., 2018; Stewart et al., 2015). The main advantages associated with this species are its low cost of acquisition and upkeep, ease handling, short lifespan, and readiness of reproduction in laboratory environments (Gerlai, 2014; Kalueff et al., 2014). The relatively high degree of genetic, neural, and endocrine homology with rodents and human beings is also cited as an advantage (Kokel and Peterson, 2008). Zebrafish is also a good model for studying anxiety and stress, with well-validated assays for novelty- and conflict-induced anxiety, social interaction, and antipredatory behavior (Gerlai, 2010; Maximino et al., 2010; Oliveira, 2013). Importantly for EtOH withdrawal, epileptic seizure-like behaviors were also characterized in the species (Hortopan et al., 2010), although not yet in a context of EtOH withdrawal.

Zebrafish anxiety-like behavior has been used to demonstrate the effects of drug withdrawal, including cocaine (López-Patiño et al., 2008a, 2008b), morphine (Cachat et al., 2010; Khor et al., 2011; Wong et al., 2010), and EtOH (Tran et al., 2016). In the last case, the literature is inconsistent, with some studies (e.g., Tran et al., 2015a) reporting significant effects of withdrawal on anxiety-like behavior, while others (e. g., Cachat et al., 2010) were unable to detect effects of EtOH withdrawal. Procedural differences, such as assay type, strain, concentration during exposure, or withdrawal duration, could be responsible for this difference.

One important consistency that is found in the literature regards results using the light/dark test. For example, Benneh et al. (2017) and Mathur and Guo (2011) found no effect of withdrawal on dark preference, while Holcombe et al. (2013) found that zebrafish subjected to EtOH withdrawal reversed their preference, spending more time in the white compartment instead of the black compartment. The light/dark test is conceptually different from the novel tank test (one of the most commonly used assays in zebrafish withdrawal research) in that an approach-avoidance conflict appears to underline behavior in the light/dark test, while escape from the top appears to motivate behavior in the novel tank test (Maximino et al., 2012); a recent meta-analysis (Kysil et al., 2017) also suggested that the light/dark test is more sensitive to pharmacological treatments than the novel tank test. The discrepancies in the literature regarding the effects of EtOH withdrawal on both tests could represent different underlining neurobiological bases, or methodological differences.

In addition to these behavioral endpoints, neurochemical analyses and oxidative stress assays were also reported in zebrafish models of EtOH withdrawal (Müller et al., 2017; Tran et al., 2015b). After exposure to 0.5% EtOH for 22 days, increases in brain levels of dopamine, serotonin, and aspartate were observed with 60 min withdrawal (Pan et al., 2012). After exposure for 8 days to 1% EtOH (for 20 min per day), decreases in superoxide dismutase and catalase activity and consequent increases in oxidative stress were observed (Müller et al., 2017). While a mechanistic explanation is still lacking, these results are congruent with the hyperexcitability and EtOH-induced neurotoxicity observed in other models (Krystal and Tabakoff, 2002; Zenki et al., 2014).

The aim of the present work is to study the heterogeneity of effects of EtOH withdrawal on zebrafish anxiety-like behavior in the literature, by applying meta-analytical techniques. Moreover, the present work attempts a conceptual replication of findings on EtOH withdrawal by assessing the effects of a withdrawal protocol on behavior in the light/dark test. It also attempts to expand the range of endpoints used in withdrawal research by using a chemically-induced epileptic seizure-like behavior model with sub-convulsive doses. Finally, the present work also attempted to replicate previous research on the effects of EtOH withdrawal on the activity of the enzyme catalase in the brain and head kidney. This manuscript is a complete report of all the studies

performed to test the effect of ethanol withdrawal on anxiety-like and convulsive-like behavior in zebrafish. We report how we determined our sample size, all data exclusions (if any), all data transformations, and all measures in the study (Simmons et al., 2012).

2. Methods

2.1. Systematic review and metaanalysis

The protocol for the meta-analysis was pre-registered in the CAMARADES-NC3Rs Preclinical Systematic Review & Meta-analysis Facility (SyRF) database (<https://drive.google.com/file/d/0B7Z0eAxKc8ApUjcyQjhwVnFjRFE/view?usp=sharing>). No modification from the pre-registered protocol was made. Article with the descriptors 'ethanol withdrawal' and 'zebrafish' were searched for in PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>), using a search filter optimized to finding studies on animal experimentation on PubMed (Hooijmans et al., 2010). Bibliographic data (including DOI, publication date, title, and abstract) from the studies identified in the systematic review were exported to a spreadsheet. Each article from the list was reviewed in four levels of detail (title, abstract, full text, and a detailed revision of the experimental design) in order to determine its eligibility to meta-analysis. Following Mohammad et al. (2016), studies should include (1) primary behavioral data obtained in tests for anxiety-like behavior in zebrafish (light/dark test, novel tank test, antipredator responses, shoaling responses); (2) reporting of appropriate controls; and (3) reporting of at least sample sizes and summary statistics (central tendency and dispersion measures) for control and withdrawal groups. While the light/dark and novel tank tests, as well as antipredator responses, straightforwardly represent anxiety-like behavior, shoaling has been included because it is sensitive to manipulations which increase anxiety and/or fear in zebrafish (Green et al., 2012), suggesting a defensive component. When an experiment evaluated the effects of drugs or other interventions on withdrawal syndrome-like effects, only control and EtOH withdrawal groups were considered, and data on intervention effects was not analyzed; e.g., while Pittman and Hylton (2015) assessed the effects of fluoxetine and ketamine on withdrawal-induced anxiogenesis, these effects were not assessed in the meta-analysis. Possible confounds in relation to the role of development were reduced by excluding studies which were not performed on adult fish.

The following data were extracted from each included study: identification (DOI, authors, publication year); strain/phenotype; concentration of ethanol during exposure; duration of EtOH treatment; duration of withdrawal; behavioral test that was used; means and standard deviations, as well as a test statistics and degrees of freedom; and sample sizes (N) for each group. Data, which were represented graphically, were extracted from figures using PlotDigitizer (<http://plotdigitizer.sourceforge.net/>). When multiple dependent variables were reported, only the primary endpoint was used (time on white, time on bottom, inter-fish distance, and distance from stimulus). While there is considerable variation in the effects of interventions on these tasks – and indeed variables such as erratic swimming or freezing can be more sensitive to certain treatments –, the literature usually refers to time on white and time on bottom as the primary endpoints. Distance from stimulus was used as a primary endpoint for the shoaling and antipredator behavior experiments made by Robert Gerlai's group, given that it indicates increased shoaling (decreased distance to shoal stimulus) or predator avoidance (increased distance to predator stimulus). All estimates were transformed to standardized mean differences (SMD), corrected for its positive bias (Hedges and Olkin, 1985), with unbiased estimates of sampling variances and confidence intervals at the 95% level, I^2 and τ^2 heterogeneity values, and p -values using a mixed-effects model, with concentration, exposure duration, and withdrawal duration used as moderators. Differently from the other endpoints, decreased distances to the shoal stimulus or decreased inter-

fish distances indicate less anxiety, and the SMDs for these cases were transformed by multiplying by -1 (Vesterinen et al., 2014). Influential case diagnostics was made by inspecting plots for externally standardized residues, DFFITS values, Cook's distances, covariance ratios, estimates of τ^2 and test statistics for residual heterogeneity when each study is removed in turn, hat values, and weights for each study included in the analysis. Publication bias was assessed by inspection of a contour-enhanced funnel plot, with contours at the 90%, 95% and 99% confidence intervals. Moreover, funnel plot asymmetry was analyzed using a meta-regression test, with total samples size as predictor (Egger et al., 1997). Observed power for each study was calculated based on effect sizes, sample sizes and standard deviations, and fitted against SMDs by a generalized additive model with Gaussian curve family and identity link function. Finally, a sensitivity analysis was performed by adding study quality, assessed using SYRCLE's Risk of Bias (RoB) tool (Hooijmans et al., 2014), in the meta-regression model. The meta-analysis was made with the metafor package from R (Viechtbauer, 2010).

2.2. Effects of EtOH withdrawal on anxiety-like behavior and epileptic seizure-like behavior susceptibility in zebrafish

2.2.1. Animals, housing, and baseline characteristics

Outbred populations were used due to their increased genetic variability, decreasing the effects of random genetic drift which could lead to the development of uniquely heritable traits (Parra et al., 2009; Speedie and Gerlai, 2008). Thus, the animals used in the experiments are expected to better represent the natural populations in the wild. Twenty-four-adult zebrafish from the wildtype strain (longfin phenotype) were used in this experiment. Animals were bought from a commercial seller, and arrived in the laboratory with 3 months of age, approximately (standard length = 13.2 ± 1.4 mm), and were quarantined for two weeks; the experiment begun when animals had an approximate age of 4 months (standard length = 23.0 ± 3.2 mm). Animals were kept in mixed-sex tanks during acclimation, with an approximate ratio of 50 male:50 female. The breeder was licensed for aquaculture under Ibama's (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) Resolution 95/1993. Animals were group-housed in 40 L tanks, with a maximum density of 25 fish per tank, for at least 2 weeks before experiments began. Tanks were filled with non-chlorinated water at room temperature (28°C) and a pH of 7.0–8.0. Lighting was provided by fluorescent lamps in a cycle of 14–10 h (LD), according to standards of care for zebrafish (Lawrence, 2007). Water quality parameters were as follow: pH 7.0–8.0; hardness 100–150 mg/L CaCO₃; dissolved oxygen 7.5–8.0 mg/L; ammonia and nitrite < 0.001 ppm. All manipulations minimized their potential suffering of animals, and followed Brazilian legislation (Conselho Nacional de Controle de Experimentação Animal - CONCEA, 2017). Animals were used for only one experiment and in a single behavioral test, to reduce interference from apparatus exposure.

2.2.2. Ethanol exposure and withdrawal

The EtOH exposure and withdrawal regimen was adapted from Gerlai et al. (2009). In brief, animals were exposed to increasing EtOH concentrations (0.125%–0.5%), dispersed on the tank water, for

16 days, therefore decreasing mortality that is associated with prolonged exposure to EtOH in zebrafish. Concentrations doubled every four days, reaching a final concentration of 0.5% (v/v). Animals were kept in this final concentration for 4 days. Another group was exposed to the same manipulation, without EtOH exposure; animals were randomly allocated to treatments via generation of random numbers using the randomization tool in <http://www.randomization.com/>. Caretakers were blinded for treatment. After treatment, animals were transferred to a tank with system water for 60 min (withdrawal stage). All animals were included in the experiments, and no gross physical abnormalities were observed during the exposure period. To control for effects of chronic EtOH exposure that are not attributable to withdrawal, two additional groups of 8 animals were exposed as above and transferred, without an withdrawal stage, to the light/dark tank.

2.2.3. Light/dark test

After EtOH withdrawal, animals (10 animals in the control group, 14 in the withdrawal group) were individually tested in a tank (15 cm height \times 10 cm width \times 45 cm length) that was divided in half into a black compartment and a white compartment. The tank was made of matte acrylic. The apparatus was illuminated from above by two fluorescent 25 W lamps, which produced an average of 270 lm above the tank (light levels were measured using a hand photometer). The tank contained sliding doors that defined a central compartment in which the animal was positioned for a 3-min acclimation. After this stage, the sliding doors were removed, allowing the animal to freely explore the apparatus for 15 min.

The order with which animals were exposed to the tank was randomized and balanced across treatments. Experimenters were blinded to treatment. Videos were manually transcribed by two observers, blinded to treatment, using X-Plo-Rat (<https://github.com/lanec-unifesspa/x-plo-rat>). The following variables were analyzed: time on the white compartment (s); transitions to white; total locomotion on white (number of virtual 4.5 cm² squares crossed by the animal in the compartment); mean duration of entries in the white compartment (total duration divided by the number of transitions); time freezing (s); number of erratic swimming events; time in thigmotaxis (s); number of risk assessment events. Operational definitions of these endpoints can be found in Table 1.

Data were analyzed via approximative two-sample Fisher-Pitman permutation tests with 10.000 Monte-Carlo re-samplings. The data analyst was blinded to treatment by cell scrambling; after analysis, data was unblinded. Data are presented using individual dot plots combined with summaries of mean and bootstrapped confidence intervals at 95% level. Standardized mean differences with unbiased variance estimates were calculated using the R package 'metafor'. Post-hoc (observed) power was calculated based on an approximation of the t-test, with two-tailed hypotheses. All analyses and graphs were made using R version 3.3.0 and packages 'ggplot2' and 'coin'.

2.2.4. Pilocarpine-induced epileptic seizure-like behaviors

Immediately after the light/dark test, animals were injected with a dose of 150 mg/kg of pilocarpine. This dose has been shown to be insufficient to produce clonic and tonic-clonic epileptic seizure-like behavior in adult animals (Pinto, 2015). Fifteen minutes after injection,

Table 1

Operational definitions for behavioral endpoints assessed in the light/dark test. Whenever available, codes used in the Zebrafish Behavioral Catalog (Kaluff et al., 2013) are provided.

Behavioral endpoint	Definition
Freezing	Time spent immobile, with the exception of opercular and eye movements, in seconds, observed in the white compartment (ZBC 1.68)
Erratic swimming	Sharp changes in direction or velocity of swimming, associated with repeated fast acceleration bouts, observed in the white compartment (ZBC 1.51)
Thigmotaxis	Percentage of time swimming in a distance of 2 cm or less from the white compartment's walls (ZBC 1.173)
Risk assessment	Fast (< 1 s) entries in the white compartment, followed by re-entry in the black compartment, or partial entries in the white compartment (i.e., the pectoral fin does not cross the midline)

Table 2

Behavioral phenotypes scored in the pilocarpine-induced seizure model. Scores were based on Mussolini et al. (2013).

Score	Behavioral phenotype
0	Short swim mainly in the bottom of the tank.
1	Increased swimming activity and high frequency of opercular movement.
2	Burst swimming, left and right movements, and erratic movements.
3	Circular movements.
4	Clonic seizure-like behavior (abnormal whole-body rhythmic muscular contraction).
5	Fall to the bottom of the tank, tonic seizure-like behavior (sinking to the bottom of the tank, loss of body posture, and principally by rigid extension of the body).
6	Death

animals were individually transferred to 1.5 L tanks, and filmed for 15 min to analyze the profile of epileptic seizure-like behavior. Epileptic seizure-like behaviors were scored according to Mussolini et al. (2013), as in Table 2.

Score 4 is the minimum behavioral phenotype that can be considered epileptiform (Mussolini et al., 2013); therefore, the latency to reach Score 4 was considered as the main endpoint for epileptic seizure-like behavior. Latencies were analyzed by fitting a Kaplan-Meier model to survival curves, using the R package “survival”.

2.3. Effects of EtOH withdrawal on catalase activity

A separate group of 12 animals were used in this experiment. Animals were subjected to the exposure and withdrawal regimen described in 2.2.1. Sixty minutes after withdrawal, animals were euthanized in cold water followed by spinal section, and their brains and head kidneys were dissected. Catalase activity in those organs was measured using the rate of disappearance of H₂O₂ spectrophotometrically, following the method described by Aebi (1984). Within-laboratory validation yielded a linearity of r² = 0.9849, and intermediary repeatability of 0.2364 (IC95% [0.0042, 0.4686]; Horwitz ratio). Enzyme activity was corrected by protein levels, quantified by the Bradford method. Differences between groups were analyzed using Approximative Two-Sample Fisher-Pitman Permutation Tests.

Table 3

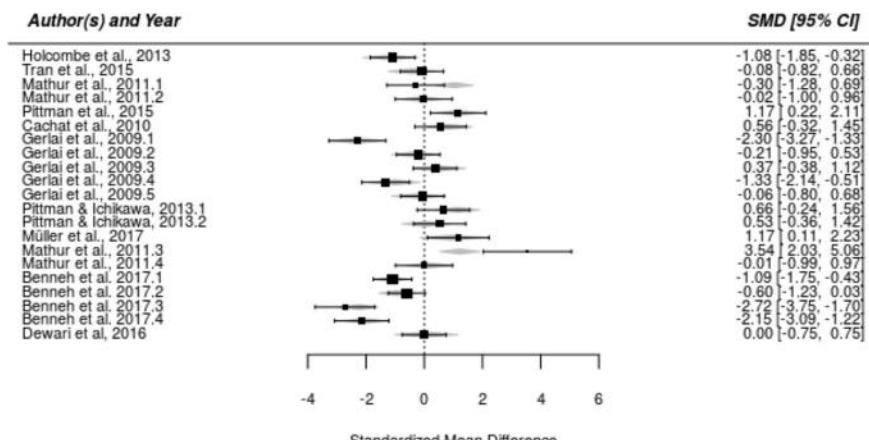
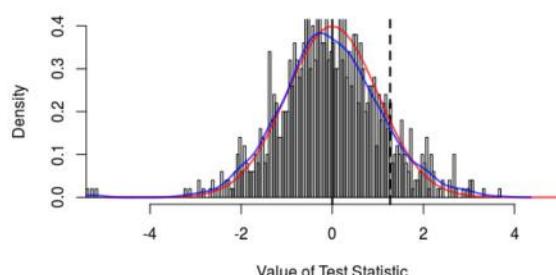
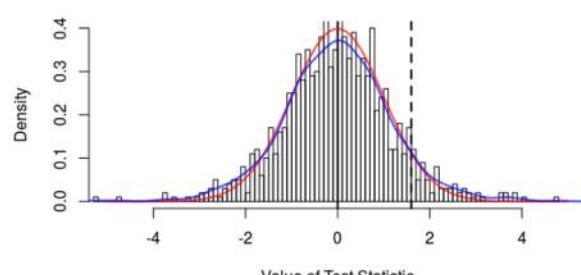
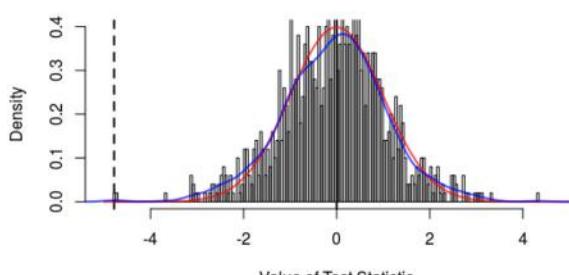
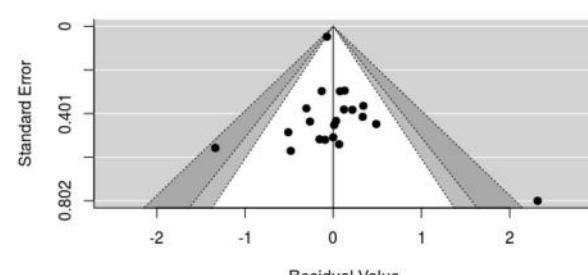
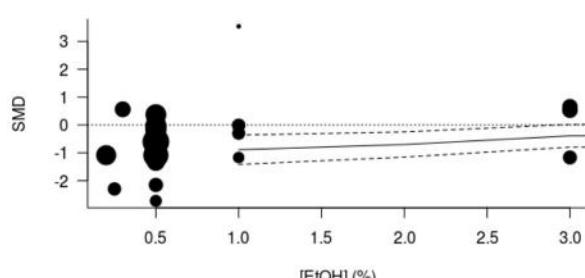
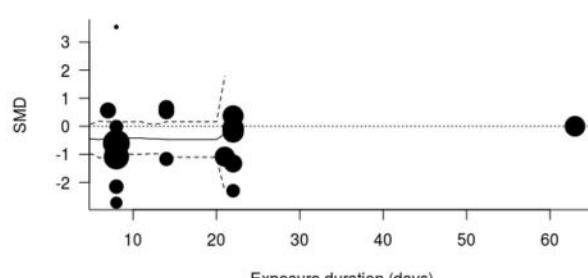
Studies included in the metanalysis. Studies were ordered by strain used (BSF = blue shortfin; WT = non-specified wild-type); behavioral assay (LDT = light/dark test; NTT = novel tank test); ethanol concentration during exposure ([EtOH]); exposure duration; and withdrawal duration.

Study	Strain	Assay	[EtOH]	Exposure duration	Withdrawal duration
Holcombe et al., 2013	BSF	LDT	0.2%	21 d	48 h
Tran et al., 2015a	AB	NTT	0.5%	22 d	1 h
Mathur and Guo, 2011, study 1	AB	NTT	1%	8 d	48 h
Mathur and Guo, 2011, study 2	AB	LDT	1%	8 d	24 h
Mathur and Guo, 2011, study 3	AB	NTT	1%	8 d	144 h
Mathur and Guo, 2011, study 4	AB	LDT	1%	8 d	168 h
Pittman and Hylton, 2015	WT	NTT	3%	14 d	48 h
Cachat et al., 2010	BSF	NTT	0.3%	7 d	12 h
Gerlai et al., 2009, study 1	WT	Predator avoidance	0.25%	22 d	1 h
Gerlai et al., 2009, study 2	AB	Predator avoidance	0.25%	22 d	1 h
Gerlai et al., 2009, study 3	SF	Predator avoidance	0.25%	22 d	1 h
Gerlai et al., 2009, study 4	WT	Shoaling	0.25%	22 d	1 h
Gerlai et al., 2009, study 5	AB	Shoaling	0.25%	22 d	1 h
Gerlai et al., 2009, study 6	SF	Shoaling	0.25%	22 d	1 h
Pittman and Ichikawa, 2013, study 1	WT	NTT	3%	14 d	48 h
Pittman and Ichikawa, 2013, study 2	WT	LDT	3%	14 d	48 h
Müller et al., 2017	BSF	Shoaling	1%	8 d	24 h
Benneh et al., 2017, study 1	WT	NTT	0.5%	8 d	96 h
Benneh et al., 2017, study 2	WT	NTT	0.5%	8 d	192 h
Benneh et al., 2017, study 3	WT	LDT	0.5%	8 d	96 h
Benneh et al., 2017, study 4	WT	LDT	0.5%	8 d	192 h
Dewari et al., 2016	WT	NTT	0.5%	63 d	1512 h

3. Results

3.1. Metanalysis

To evaluate the effect of EtOH withdrawal on zebrafish anxiety-like behavior, we applied a mixed-effects meta-regression model on the results from the systematic review. Characteristics from the articles found in the systematic review can be found in Table 3; raw data and analysis scripts for this metanalysis can be found in our GitHub repository (<https://github.com/lanec-unifesspa/etoh-withdrawal/tree/master/metanalysis>). EtOH concentrations ranged from 0.25% to 3% v/v (median 1%); exposure durations ranged from 7 to 63 days (median 14 days), and withdrawal durations ranged from 1 to 1.512 h (median 48 h). In general, a high risk of bias was observed, since most studies did not report blinding or random allocation (<https://github.com/lanec-unifesspa/etoh-withdrawal/blob/master/metanalysis/etoh-withdrawal-metanalysis-rob.csv>). Behavioral test, strain/phenotype, EtOH concentration, exposure duration, and withdrawal duration were used as moderators. Results from this analysis are presented in the forest plot found in Fig. 1A. Residual heterogeneity was estimated as $\tau^2 = 0.1667$, significantly high ($QE_{[df=11]} = 22.6338$, $p = 0.0199$), suggesting that although about 88% of the total heterogeneity can be explained by including the five moderators in the model, other factors might influence the effect. After applying a permutation test with 1.000 replications, a significant effect of exposure duration ($p = 0.029$) and EtOH concentration ($p = 0.001$) were found, but the other mediators did not affect withdrawal-like behavior (Fig. 1B–D). The contour-enhanced funnel plot (Fig. 1E) suggest that most studies failed to reach statistical significance, with only one study falling in the 95% CI range, and one in the 99% CI range. Egger's test on this funnel plot did not suggest publication bias ($t_{[df=10]} = 0.439$, $p = 0.67$). An analysis of influential observations suggests that the Mathur and Guo (2011) study on the effects on the NTT with the longer withdrawal duration and the Dewari et al. (2016) study produced most residual heterogeneity (Fig. S1). Absolute SMDs were significantly explained by observed power (slope = 1.2819, $p = 0.0174$; Fig. S2). Finally, sensitivity analysis suggested that study quality did not influence results, as including RoB scores in the model did not improve fit (model without RoB: AIC = 46.4816, BIC = 50.88 = 585; model with RoB: AIC = 47.2908, BIC = 50.9218).

A**B****C****D****E****F****G**

(caption on next page)

Fig. 1. Metanalysis of EtOH withdrawal experiments in zebrafish reveal a significant increase in anxiety-like behavior, but high heterogeneity driven by methodological differences. (A) Forest plot showing the results of 16 studies examining the effect of ethanol withdrawal on zebrafish anxiety-like behavior. The figure shows the standardized mean difference (SMD) between control and withdrawal-exposed groups with corresponding 95% confidence intervals in the individual studies, based on a mixed-effects model. A negative standardized mean difference (SMD) corresponds to decreased anxiety-like behavior, while a positive SMD corresponds to increased anxiety-like behavior after ethanol withdrawal. Studies are ordered by total degrees of freedom. (B–D) Permutation distribution of the test statistic for the mediators: behavioral test (B), ethanol concentration during exposure (C), exposure duration, in days (D), withdrawal duration, in hours (E), and strain/phenotype (F). Distributions were based on a permutation test with 1000 replications. The blue contour represents kernel density estimates of the permutation distributions; the red curve represents the standard normal density; the full line represents the null hypothesis of no difference; and the dashed line represents the observed values of test statistics. (G) Contour-enhanced funnel plot of meta-analysis. Estimated standardized mean differences were plotted against precision (1/standard error) were a negative estimate corresponds to decreased anxiety-like behavior, while a positive estimate corresponds to increased anxiety-like behavior after ethanol withdrawal. The unshaded region corresponds to p -values > 0.1, the gray-shaded region to p -values between 0.1 and 0.05, the dark gray-shaded region corresponds to p -values between 0.05 and 0.01, and the region outside of the funnel corresponds to p -values below 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Effects of EtOH withdrawal on anxiety-like behavior in zebrafish

Withdrawal increased time on white ($Z = 2.1207$, $p = 0.0261$; $SMD_{UB} = -0.995$; observed power = 0.6414; Fig. 2A), without affecting entry duration ($Z = 0.92305$, $p = 0.4301$; $SMD_{UB} = -0.392$; observed power = 0.147; Fig. 2B). Withdrawal did not increase erratic swimming ($Z = 1.9389$, $p = 0.0564$, $SMD_{UB} = 0.890$; observed power = 0.5467; Fig. 2C), but it increased risk assessment ($Z = 1.9895$, $p = 0.0405$, $SMD_{UB} = 0.918$; observed power = 0.5724; Fig. 2D). Freezing ($Z = 0.8082$, $p = 0.7003$, $SMD_{UB} = 0.286$; observed power = 0.098; Fig. 2E) and thigmotaxis ($Z = 0.41291$, $p = 0.7024$, $SMD_{UB} = -0.2133$; observed power = 0.0718; Fig. 2F) were unaffected. As for motor effects, transitions to white were not affected by withdrawal ($Z = 0.91765$, $p = 0.3763$, $SMD_{UB} = 0.39$; observed power = 0.1469; Fig. 2G), but total locomotion was higher in animals exposed to withdrawal ($Z = 2.5965$, $p = 0.0034$, $SMD_{UB} = 1.31$; observed power = 0.863; Fig. 2H).

To control for effects of chronic exposure, independent groups were chronically exposed to EtOH (0.5%), and tested in the light/dark test immediately after the last exposure (i.e., without withdrawal). Animals exposed to EtOH spent more time in the white compartment ($Z = -1.9535$, $p = 0.0467$; $SMD_{UB} = -1.033$; observed power = 0.482; Fig. S3A), but did not show changes in entry duration ($Z = -1.264$, $p = 0.2632$; $SMD_{UB} = 0.611$; observed power = 0.204; Fig. S3B). Erratic swimming was decreased by chronic EtOH treatment ($Z = 2.521$, $p = 0.01$; $SMD_{UB} = 1.516$; observed power = 0.803; Fig. S3C), but no changes were observed on risk assessment ($Z = 0.478$, $p = 0.793$; $SMD_{UB} = 0.212$; observed power = 0.058; Fig. S3D) or freezing ($Z = 0.393$, $p = 0.736$; $SMD_{UB} = 0.180$; observed power = 0.052; Fig. S3E). Thigmotaxis was decreased by chronic EtOH ($Z = 2.2879$, $p = 0.012$; $SMD_{UB} = 1.295$; observed power = 0.671; Fig. S3F). Neither transitions to white ($Z = -1.790$, $p = 0.074$; $SMD_{UB} = -0.922$; observed power = 0.402; Fig. S3G) nor total locomotion ($Z = -1.643$, $p = 0.097$; $SMD_{UB} = -0.829$; observed power = 0.336; Fig. S3H) were changed by chronic treatment with EtOH.

3.3. Effects of EtOH withdrawal on epileptic seizure-like behavior susceptibility

Only one animal from the control group exhibited Score IV epileptic seizure-like behaviors after 150 mg/kg pilocarpine, replicating findings from Pinto (2015); therefore, this dose is indeed sub-convulsive in zebrafish. All animals from the withdrawal group exhibited Score IV epileptic seizure-like behaviors after 150 mg/kg pilocarpine; after applying a log-rank model for the differences in latencies to Score IV, a significant difference was seen in the withdrawal group ($\chi^2_{[df=1]} = 8.8$,

$p = 0.00308$; Fig. 3). This data can be found in our GitHub repository (<https://github.com/lanec-unifesspa/etoh-withdrawal/tree/master/seizure>).

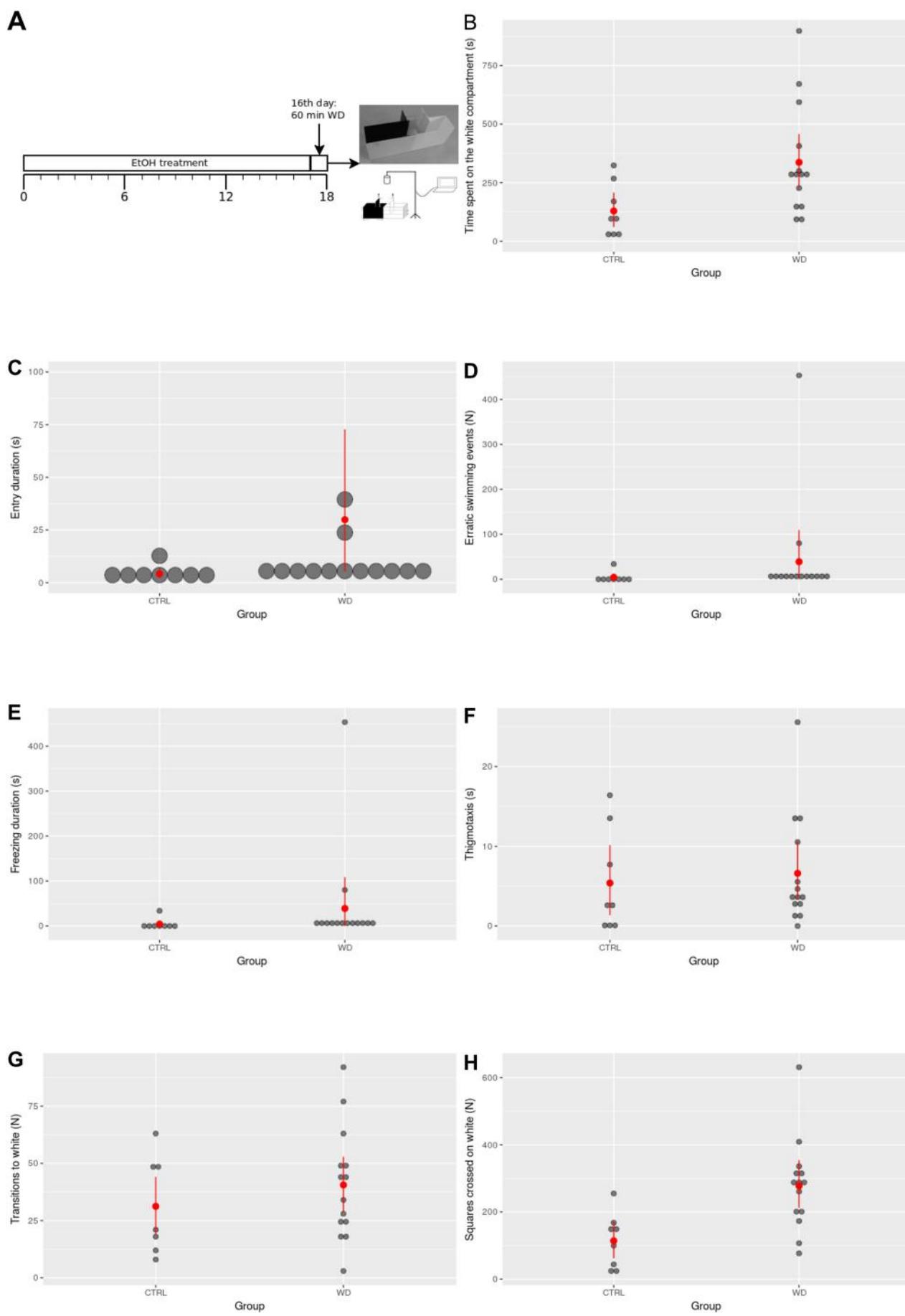
3.4. Effects of EtOH withdrawal on catalase activity

Catalase activity was reduced in the brain of animals exposed to the withdrawal regime ($Z = 2.0885$, $p = 0.0156$; Fig. 4A), while no significant differences were found in the head kidney ($Z = 1.2547$, $p = 0.1863$; Fig. 4B). Müller et al. (2017) also observed decreased catalase activity in the brains of zebrafish after 24 h EtOH withdrawal. Data and scripts for this experiment are available at <https://github.com/lanec-unifesspa/etoh-withdrawal/tree/master/catalase>.

4. Discussion

The present work reinforced the utility of using zebrafish as a model organism in studying ethanol withdrawal by searching for broader patterns in the literature, providing a conceptual replication of some findings regarding anxiety-like behavior and catalase activity, as well as expanding the range of behavioral domains for study. We found that the literature is inconsistent in what regards the effects of ethanol withdrawal. This heterogeneity is associated with the great procedure differences which are reported; the results of the metanalysis suggest that the main driving factors are ethanol concentration during exposure and exposure duration, with lower concentrations and longer durations more likely to induce anxiety-like behavior. Moreover, we found that ethanol withdrawal (after exposing animals for 16 days to 0.5% ethanol and 1 h withdrawal) decreased scototaxis, but increased risk assessment, in the light/dark test, and decreased the threshold for chemically-induced epileptic seizure-like behavior.

Zebrafish is increasingly being considered as a model organism in behavioral research (Bonan and Norton, 2015; Kalueff et al., 2012; Norton and Bally-Cuif, 2010; Stewart et al., 2015), with a great deal of studies on alcohol (Tran et al., 2016). Behavioral effects of drug withdrawal have been demonstrated with different drugs, including cocaine (López-Patiño et al., 2008a; López-Patiño et al., 2008b) and morphine (Cachat et al., 2010; Khor et al., 2011; Wong et al., 2010); in all cases, anxiety-like behavior was assessed. In the same direction, ethanol withdrawal has been studied mainly with models for anxiety-like behavior (Table 3). Our metanalysis revealed a significant effect of EtOH withdrawal on anxiety-like or defensive behavior in zebrafish, but a high degree of heterogeneity. Most of the heterogeneity was explained by procedural aspects; significant effects of exposure duration and EtOH concentration were found, suggesting that longer exposure and higher concentrations are critical to induce withdrawal. Other factors, including statistical inference and lack of control factors (Gerlai, 2018),



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Fig. 2. Effects of EtOH withdrawal (increasing concentration of up to 0.5% for 16 days, followed by 1 h withdrawal) on behavior in the light/dark test. (A) Scototaxis; (B) Erratic swimming; (C) Risk assessment; (D) Freezing; (E) Thigmotaxis; (F) Transitions to white; (G) Locomotion on white. Red dots represent mean, and red error bars represent nonparametric bootstrapped confidence intervals for the mean at the 95% level. To facilitate visualization, data points were jittered; therefore, their absolute position does not reflect the actual value, and values can appear to be below 0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

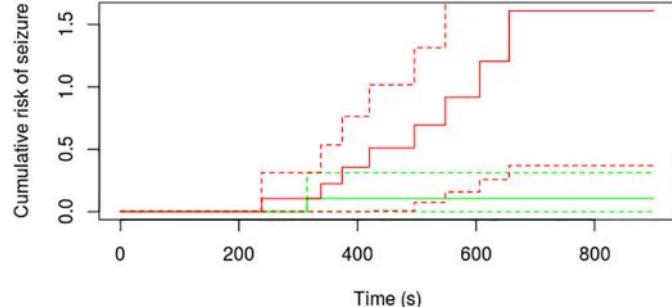


Fig. 3. Effects of EtOH withdrawal (increasing concentration of up to 0.5% for 16 days, followed by 1 h withdrawal) on Score IV seizure latencies after sub-convulsive pilocarpine injection. Animals were observed after injection of 150 mg/kg pilocarpine in controls (green lines) and withdrawal (red lines). The dashed lines represent 95% confidence intervals around the Kaplan-Meier estimates of seizure probability at each time interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

could influence the heterogeneity as well. Most of the studies were underpowered, and there was an association between observed power and effect size. Risk of bias (RoB) was very high for all studies, which usually did not report blinding and random allocation; nonetheless, study quality (i.e., RoB) did not influence the results of the metaanalysis, evidencing the robustness of the method. While publication bias is a relevant issue in the reproducibility and replicability of zebrafish research (Gerlai, 2018), no evidence for it was found in the systematic review.

Our conceptual replication used 60 min withdrawal, translationally relevant to the initial symptoms of EtOH withdrawal in humans (which include anxiety symptoms and epileptic seizures; Trevisan et al., 1998). Using the exposure method described in Gerlai et al. (2009), we showed that withdrawal reduces scototaxis. This is in line with the “daily-moderate” condition in Holcombe et al. (2013), which showed a decrease in scototaxis after using the same exposure profile we presented. As can be appreciated from the meta-analysis, the effect sizes calculated for scototaxis in Holcombe et al.’s (2013) experiment are similar in direction and magnitude. The Mathur and Guo (2011) scototaxis study used a higher concentration of EtOH (1%) but a shorter exposure period (8 days). While Pittman and Ichikawa (2013) used a similar exposure period (14 days), the concentration was much higher (3%); in both studies, EtOH withdrawal did not affect scototaxis.

While following only effects on scototaxis confirms findings reported in Holcombe et al. (2013), these findings contradict the overall results from the meta-analysis, which suggested that EtOH withdrawal increases anxiety-like behavior in zebrafish. If other variables are considered, however, the picture changes, with increases in risk assessment and number of transitions. The increase in risk assessment is consistent with increased anxiety-like behavior (Maximino et al., 2014), but, since scototaxis was decreased, this conclusion is only provisional. These results are difficult to interpret, but could be explained by the increased risk assessment; however, an exploratory analysis suggests negative correlation between risk assessment and time on white in the withdrawal group ($r^2 = -0.443$, vs. -0.122 in the control group). Risk

assessment was defined as fast (< 1 s) entries in the white compartment, followed by re-entry in the black compartment, or partial entries in the white compartment (Table 1); as such, it is unlikely that risk assessment impacted measures of scototaxis or mean entry duration. The increase in transitions without an apparent increase in locomotion in the white compartment could be interpreted as psychomotor agitation, an important symptom of EtOH withdrawal.

A reduction in scototaxis would be expected of animals chronically exposed to EtOH, which could decrease anxiety levels, and therefore not be a direct effect of withdrawal. While initially unplanned, we performed additional experiments to test this hypothesis by exposing a different group of animals to chronic ethanol and analyzing its behavior without withdrawal. We observed decreased scototaxis, but we also observed decreased erratic swimming and thigmotaxis. These results present preliminary evidence that withdrawal itself, and not the chronic exposure to EtOH, produced the reported behavioral effects. A limitation of the present experiments is that biochemical (catalase) effects and seizure-like behavior was not assessed in this negative control groups, and therefore it is not possible to assess whether these reflect the effects of withdrawal or of chronic exposure instead.

In addition to this behavioral profile, EtOH withdrawal was also shown to decrease the threshold for chemically-induced epileptic seizure-like behavior in zebrafish. Pilocarpine is a muscarinic agonist which induces epileptic seizure-like behavior in rodents (Scorza et al., 2009), and has recently been shown to induce a similar profile in zebrafish (Pinto, 2015). The rationale of using a sub-convulsive dose is that, if EtOH withdrawal increases susceptibility to epileptic seizure-like behavior, zebrafish should present epileptic seizure-like behavior with a dose which does not induce this state in control animals. We observed increased probability of entering Stage 4 epileptic seizure-like behaviors in EtOH withdrawal animals, and a shorter latency to this event, suggesting that the protocol presented here is able to model susceptibility to epileptic seizure-like behavior, increasing the range of endpoints for studying EtOH withdrawal in zebrafish. While ethanol was shown to produce anticonvulsant effects in mice (McQuarrie and Fingl, 1958), a limitation of the present experiments is that seizure-like behavior was not assessed in this negative control groups, and therefore it is not possible to assess whether these reflect the effects of withdrawal or of chronic exposure instead.

Finally, we also observed decreased catalase activity in the brain, but not in the head kidney of EtOH withdrawal animals. Catalase is a detoxifying enzyme that catalyzes the transformation of hydrogen peroxide, a free radical, into water and oxygen (Aebi, 1984); in the brain, catalase is poorly expressed, but usually associated with microglial activity (Dringen, 2005). The inhibition of enzymatic activity observed here replicates results by Müller et al. (2017) in the zebrafish brain; in that study, however, the exposure duration was shorter (8 days), the concentration of EtOH was higher (1%), and the pattern of exposure was different (animals were exposed for 20 min per day, instead of continuously, to ethanol) compared to the present investigation. The lack of effect on the head kidney, where interrenal cells (the teleost functional equivalent of the mammalian adrenal cortex) lie, suggesting that possible effects on cortisol (e.g., Cachat et al., 2010) are not due to effects in these cells, but upstream in the hypothalamus-hypophyseal-interrenal axis.

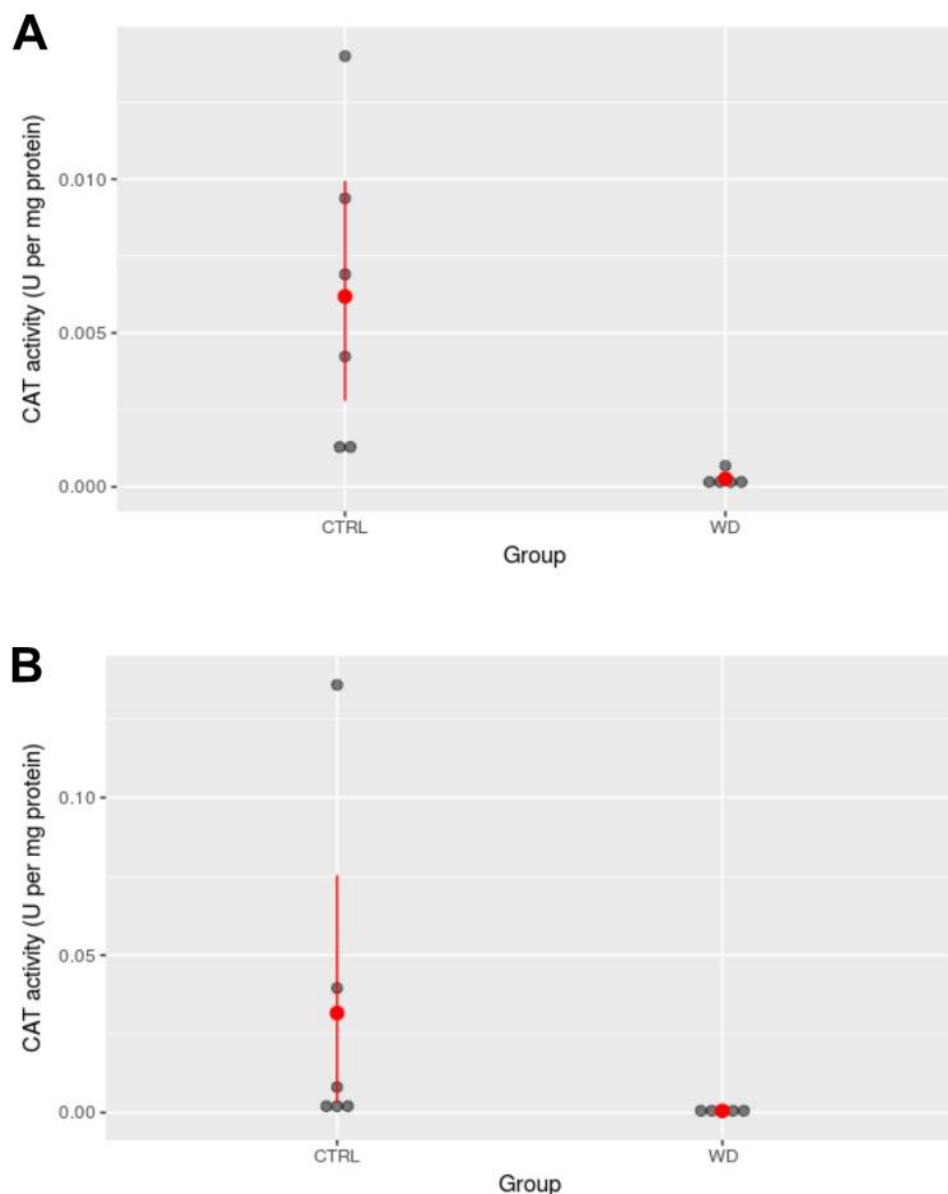


Fig. 4. Effects of EtOH withdrawal (increasing concentration of up to 0.5% for 16 days, followed by 1 h withdrawal) on catalase activity. Enzyme activity was assessed in (A) brain and (B) head kidney of zebrafish from control (CTRL) and withdrawal (WD) groups. Red dots represent mean, and red error bars represent nonparametric bootstrapped confidence intervals for the mean at the 95% level. To facilitate visualization, data points were jittered; therefore, their absolute position does not reflect the actual value, and values can appear to be below 0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The present work contributed to the use of zebrafish as a model in EtOH withdrawal research by: A) identifying sources of heterogeneity in the literature on EtOH withdrawal and anxiety-like behavior in the species; B) presenting a conceptual replication of withdrawal-induced anxiogenesis in the light/dark test; C) extending the behavioral phenotypes to include epileptic seizure-like behavior susceptibility; and D) replicating the effects on catalase activity, suggesting that EtOH withdrawal-elicted oxidative stress could be a mechanism of anxiogenesis in the species. Further work will characterize these mechanisms with care.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pbb.2018.04.006>.

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4. CONSIDERAÇÕES FINAIS

O presente trabalho contribuiu para o uso do zebrafish como modelo na pesquisa de abstinência alcóolica por: A) identificar fontes de heterogeneidade na literatura sobre a abstinência alcóolica e comportamento tipo ansiedade na espécie; B) apresentar uma replicação conceitual da ansiogênese induzida pela abstinência no teste claro/ escuro; C) através da aplicação de dose sub-convulsivante de pilocarpina, evidências sugestivas de diminuição do limiar convulsivo na espécie; e D) replicando os efeitos na atividade da catalase, sugerindo que o estresse oxidativo induzido pela abstinência alcóolica poderia ser um mecanismo ansiogênico na espécie. Trabalhos adicionais caracterizarão esses mecanismos com cuidado.

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