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INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

TESE DE DOUTORADO

AVALIAÇÃO DA INSTABILIDADE GENÔMICA E DISFUNÇÕES CELULARES EM FENÓTIPOS DE PREFERÊNCIA PELO ETANOL COM O ZEBRAFISH

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BELO HORIZONTE

2021

UNIVERSIDADE FEDERAL DE MINAS GERAIS

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Izabela Barbosa Moraes

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais.

Orientadora: Dr. Ana Lúcia Brunialti Godard

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ATA DE DEFESA DE DISSERTAÇÃO / TESE

ATA DA DEFESA DE TESE	**140/2021**
IZABELA BARBOSA MORAES	**Entrada 1º/2017** **CPF: 098.382.706-01**

Às nove horas do dia **23 de fevereiro de 2021**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora de Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"AVALIAÇÃO DA INSTABILIDADE GENÔMICA E DISFUNÇÕES CELULARES EM FENÓTIPOS DE PREFERÊNCIA PELO ETANOL COM O ZEBRAFISH"**, requisito para obtenção do grau de Doutora em **Genética.** Abrindo a sessão, a Presidente da Comissão, **Ana Lúcia Brunialti Godard**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos Examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	CPF	Indicação
Ana Lúcia Brunialti Godard	UFMG	107.961.538-50	Aprovada
Larissa Paola Rodrigues Venâncio	UFOB	326.516.068-80	Aprovada
Luciana Karen Calábria	UFU	052.995.806-65	Aprovada
Maria Raquel Santos Carvalho	UFMG	349.651.730-15	Aprovada
Érika Cristina Jorge	UFMG	261.370.228-11	Aprovada

Pelas indicações, a candidata foi considerada: **APROVADA**

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora.

Belo Horizonte, 23 de fevereiro de 2021.

Ana Lúcia Brunialti Godard - Orientadora (UFMG)

Larissa Paola Rodrigues Venâncio (UFOB)

Luciana Karen Calábria (UFU)

Maria Raquel Santos Carvalho (UFMG)

Érika Cristina Jorge (UFMG)

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
Instituto de Ciências Biológicas
Programa de Pós-Graduação em Genética

FOLHA DE APROVAÇÃO

"AVALIAÇÃO DA INSTABILIDADE GENÔMICA E DISFUNÇÕES CELULARES EM FENÓTIPOS DE PREFERÊNCIA PELO ETANOL COM O ZEBRAFISH"

IZABELA BARBOSA MORAES

Tese aprovada pela banca examinadora constituída pelos Professores:

Ana Lúcia Brunialti Godard - Orientadora
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Sumário

Lista de Figuras

Introdução

Capítulo 1

Capítulo 2

Lista de Tabelas

Capítulo 1

Capítulo 2

Discussão e Conclusão

Lista de Abreviaturas

AUD	Alcohol Use Disorder
AW	After Withdrawal preference
B	Basal Preference
CAT	Catalase
CPP	Conditioned Place Preference
CYPE21	Citocromo P450 2E1
DNA	Ácido desoxirribonucleico
EROs	Espécies Reativas de Oxigênio
FRAP	Ferric Reducing Antioxidant Power
GPX	Glutationa peroxidase
GSH	Glutationa Reduzida
GST	Glutathione S-Transferase
MtDNA	DNA mitocondrial
OMS	Organização Mundial de Saúde
PBS	Phosphate Buffered Saline
PC	Post-Conditioning preference
Qpcr	PCR quantitativa (tempo real)
RNA	Ácido ribonucleico
ROS	Reactive Oxygen Species
SAM	S-adenosilmetionina
SEM	Standard Error of the Mean
SOD	Superóxido dismutase
TERC/TR	RNA da telomerase
TERT	Telomerase reverse transcriptase
TL	Telomere Length
WHO	World Health Organization

Resumo

O transtorno do uso do álcool é uma condição patológica que afeta milhares de pessoas em todo o mundo. Há uma relação entre alterações no padrão de encurtamento dos telômeros, estresse oxidativo, apoptose e o uso crônico de etanol. No entanto, muitos desses parâmetros ainda não foram elucidados em conjunto em modelo de exposição aguda e/ou considerando os aspectos individuais de preferência pelo etanol. Nesse contexto, o objetivo deste trabalho foi avaliar a instabilidade genômica e disfunções celulares nos fenótipos de preferência pelo etanol com o *zebrafish* adulto. Os animais foram submetidos ao paradigma comportamental de Preferência Condicionado a Lugar (*Conditioned Place Preference*), e distinguidos em 4 fenótipos em relação à preferência pelo etanol após uma única exposição (20 min): Leve, Pesado, Inflexível e Reforço Negativo. Após a eutanásia, o cérebro, o fígado e as brânquias foram dissecados e imediatamente congelados e mantidos a -80ºC. Para as análises moleculares foi feita a extração de DNA e RNA dos respectivos tecidos, o tamanho dos telômeros, o número de cópias de mtDNA e a quantificação dos transcritos dos genes *bax* e *bcl2* foram realizados por qPCR. Para as avaliações das enzimas antioxidantes, os tecidos foram homogeneizados em tampão fosfato, e posteriormente determinado o potencial antioxidante total e a atividade das enzimas catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GPx). O encurtamento do telômero foi observado nos fenótipos Pesado, Inflexível e Reforço Negativo. O aumento dos transcritos do gene *bax* foi evidenciado no fenótipo Reforço Negativo. O aumento da atividade das enzimas antioxidantes foi evidenciado principalmente nos fenótipos Inflexível e Reforço Negativo. Os fenótipos Inflexível e Pesado apresentaram maior número de cópias de mtDNA. O conjunto de resultados demonstra que uma única exposição ao etanol altera significativamente os parâmetros analisados, sobretudo há o encurtamento de telômeros e aumento da atividade das enzimas antioxidantes nos fenótipos de preferência pelo etanol, principalmente no fenótipo Inflexível. No entanto, nenhum desses parâmetros se mostrou alterado no fenótipo Leve (exposto ao etanol, mas sem preferência), sugerindo que as bases moleculares relacionadas ao comportamento de preferência pelo etanol vão além dos seus efeitos nocivos, apresentando uma forte relação com o perfil do fenótipo.

Palavras-chave: álcool, telômeros, enzimas antioxidantes, mtDNA, apoptose, *Danio rerio.*

Abstract

Alcohol use disorder (AUD) is a pathological condition that affects people around the world. There is a relationship between telomere shortening, oxidative stress, apoptosis, and chronic ethanol abuse. However, many of these parameters have not yet been elucidated together in an acute exposure model and/or considering individual aspects of ethanol preference. In this context, the aim of this work was to evaluate the genomic instability and cellular dysfunctions in adult zebrafish ethanol preference phenotypes. The animals were submitted to behavioral paradigm of Conditioned Place Preference and distinguished in 4 phenotypes after an acute ethanol exposure (20 min): Light, Heavy, Inflexible and Negative Reinforcement. After euthanasia, the brain, liver, and gills were dissected and immediately frozen and stored at -80ºC. For molecular analyzes, DNA and RNA were extracted from specific tissues, the telomeres length, the mtDNA copy number, a transcription of the *bax* and *bcl2* genes were performed by qPCR. For the evaluation of antioxidant enzymes, the tissues were homogenized in phosphate buffer, the total antioxidant potential, and the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were subsequently determined. Telomere shortening was observed in the Heavy, Inflexible and Negative Reinforcement phenotypes. An upregulation of the *bax* gene was evidenced in Negative Reinforcement phenotype. The increased activity of antioxidant enzymes was evidenced mainly in the Inflexible and Negative Reinforcement phenotypes. The Inflexible and Heavy phenotypes had the highest mtDNA copy number. These results demonstrate that a single ethanol exposure significantly alters the parameters analyzed, especially considering the telomere shortening and increased antioxidant enzymes activities in the ethanol preference phenotypes, especially in the Inflexible. However, none of these parameters was altered in the Light phenotype (exposed to ethanol, but without preference). Suggesting the molecular basis related to the ethanol preference behavior go beyond its harmful effects, presenting a strong relationship with the phenotype.

Keywords: alcohol, telomeres, antioxidant enzymes, mtDNA, apoptosis, *Danio rerio*.

1. INTRODUÇÃO

1.1 Transtorno do uso do álcool

O uso de bebidas alcóolicas é provavelmente o hábito social mais antigo do mundo, sendo prevalente em muitas culturas. No entanto, o consumo excessivo de álcool apresenta consequências prejudiciais, incluindo problemas físicos, psicológicos e sociais (EDENBERG; FOROUD, 2014). Segundo o último relatório da Organização Mundial de Saúde (OMS), mais de 3 milhões de pessoas morreram como resultado do uso nocivo de álcool em 2016. No geral, o uso nocivo do álcool causa mais de 5% da incidência global de doenças (WHO, 2018). No Brasil, há um grande consumo de álcool em diversas faixas etárias, desde a adolescência (WILLHELM *et al.*, 2018), até a idade adulta (GUIMARÃES *et al.*, 2010), sendo predominante em adultos com idade entre 18 e 29 anos (GARCIA; FREITAS, 2015).

O transtorno do uso do álcool é uma condição crônica, com aspectos comportamentais e socioeconômicos, caracterizado pelo consumo compulsivo de álcool (ZOU *et al.*, 2017). Esse processo envolve tolerância, sintomas de abstinência após a retirada da bebida, perda de controle, preocupação constante com a aquisição e/ou uso da substância, desejo persistente ou esforços malsucedidos para parar de beber, perda de habilidades sociais, ocupacionais ou recreativas (BATRA *et al.*, 2016).

No que se refere ao uso abusivo do etanol, existem diferentes padrões de comportamento relacionados à quantidade de bebida consumida, frequência do consumo, fatores genéticos, epigenéticos, psicológicos e sociais (KUNTSCHE *et al.*, 2017; PANDEY *et al.,* 2017). Por exemplo, há a definição do “beber pesado” (também chamado de beber prejudicial ou abuso de álcool) relacionado aos indivíduos que apresentam evidências de danos à saúde (físicos ou mentais) pelo consumo excessivo do álcool, porém sem evidências de dependência (JASTRZABSKA *et al.*, 2016). Também temos a definição de Dependente Alcóolico (ou adicto), sendo este considerado o estágio mais grave, de modo que os indivíduos apresentam dependência física e psicológica à droga (FLUYAU; CHARLTON, 2020). De acordo com a Associação Americana de Psiquiatria, o termo adicção se refere a uma condição complexa, uma doença cerebral que se manifesta pelo uso compulsivo de substâncias, apesar da consequência nociva

(AMERICAN PSYCHIATRIC ASSOCIATION, 2013). Nesse contexto, a adicção foi definida como um ciclo de três estágios: (1) compulsão/intoxicação, (2) abstinência/efeito negativo e (3) estágios de preocupação/antecipação, os quais envolvem circuitos neuronais e domínios funcionais distintos (REILLY *et al.*, 2017).

As consequências do abuso crônico do álcool envolvem distúrbios de uma grande variedade de funções no organismo, como hiperlactacidemia, elevação dos níveis de gorduras, inibição da síntese proteica, desestruturações no sistema de canais de membrana, disfunção mitocondrial, ativação acelerada de metabólitos tóxicos, hipoxemia severa, aumento do estresse oxidativo, instabilidade genômica e apoptose (ALBANO, 2006; MARIANO; GUZMÁN, 2000; MARTINS DE CARVALHO *et al.*, 2019; NOWOSLAWSKI *et al.,* 2005). Como consequência surgem diversos problemas clínicos, incluindo cirrose alcoólica, pancreatite alcoólica, câncer do trato gastrointestinal superior e do fígado, doenças cardiovasculares, câncer de mama, diabetes e síndrome alcoólica fetal (EDENBERG; FOROUD, 2013).

A exposição aguda ao etanol tem efeitos marcantes no metabolismo cerebral e promove alterações comportamentais, funcionais e morfológicas (KONG *et al.*, 2019; PAIVA *et al.*, 2020; TIPPS *et al.*, 2015). Os efeitos agudos da exposição ao etanol variam de efeitos ansiolíticos e desinibitórios leves, sedação e perda da coordenação motora, amnésia, emese, hipnose e eventualmente inconsciência (HARRISON *et al.*, 2017).

Considerando o contexto do uso abusivo do álcool, nem todos os indivíduos estão igualmente propensos a se tornar dependentes (STICKEL *et al.*, 2017). Para que o indivíduo se torne dependente do álcool, é fundamental que haja vulnerabilidade e suscetibilidade à dependência, fomentadas por condições biológicas, psicológicas, sociais e ambientais (HECKMANN; SILVEIRA, 2009). No que se refere às condições biológicas relacionadas à adicção, o componente genético destaca-se como um dos principais (KATSAROU *et al.*, 2017; NEWMAN *et al.*, 2016). Além disso, evidências recentes sugerem que a instabilidade genômica e alterações celulares, sobretudo relacionadas ao encurtamento de telômeros (KANG *et al.*, 2017; WANG *et al.,* 2017), alteração das enzimas do sistema antioxidante, disfunção mitocondrial e aumento do índice de apoptose são

parâmetros associados ao consumo excessivo do etanol (KANG *et al.*, 2017; LU *et al.*, 2015; WANG *et al.*, 2017).

1.2 Instabilidade genômica

A instabilidade genômica é definida como sendo o aumento da frequência de alterações genéticas variando de mutações em genes específicos, amplificações, inserções, deleções ou rearranjos de segmentos cromossômicos, ganho ou perda de um cromossomo inteiro e outras alterações que afetam a arquitetura genômica das células (KALIMUTHO *et al.*, 2019). A instabilidade genômica surge de muitas vias diferentes, tais como danos nos telômeros, amplificação do centrossomo, modificações epigenéticas e danos ao DNA de fontes endógenas e exógenas (FERGUSON *et al.*, 2015).

Considerando o processo evolutivo dos seres vivos, o genoma procariótico circular desenvolveu uma complexa maquinaria biológica relacionada com a manutenção da integridade do DNA, garantindo a homeostase celular e a fidelidade da transmissão de informações genéticas (BRILLI *et al.*, 2013; SHCHERBAKOVA; FIJALKOWSKA, 2006). Em eucariotos, o genoma circular ancestral evoluiu para estruturas cromossômicas lineares (KROUSTALLAKI; GAGOS, 2015). No entanto, a estrutura linear dos cromossomos apresenta uma importante restrição biológica, relacionada ao “problema da replicação do final dos cromossomos” (LEVY *et al.*, 1992), de modo que os telômeros não são totalmente replicados durante a divisão celular, e portanto, apresentam um processo natural de desgaste ao longo do tempo (SHAY, 2018).

Os telômeros correspondem a estrutura final dos cromossomos cuja função é proteger o genoma (BLACKBURN, 2001) e têm um papel essencial na garantia de que as extremidades naturais dos cromossomos não sejam confundidas com locais de danos no DNA (KEEFE, 2016). Os telômeros são constituídos por um longo arranjo de fita dupla de repetições de TTAGGG ligados pelo complexo de proteínas *Shelterin* que forma interações em *t-loop* **(Figura 1**) (MACIEJOWSKI; DE LANGE, 2017). A estrutura em *loop* é formada através da atividade nucleolítica nas extremidades do DNA telomérico para produzir uma região rica em guanina (G) de cadeia simples. Isso faz um *loop* para trás e invade

o trato de telômeros de fita dupla, garantindo que as extremidades de DNA soltas sejam alojadas internamente dentro da estrutura de nucleoproteínas *(*TURNER *et al.,* 2019). O DNA telomérico em associação com o complexo *Shelterin* tem um papel essencial na estabilização das extremidades dos cromossomos, formando uma estrutura de cobertura que os protege da degradação e fusão terminal (NEUMANN; REDDEL, 2002).

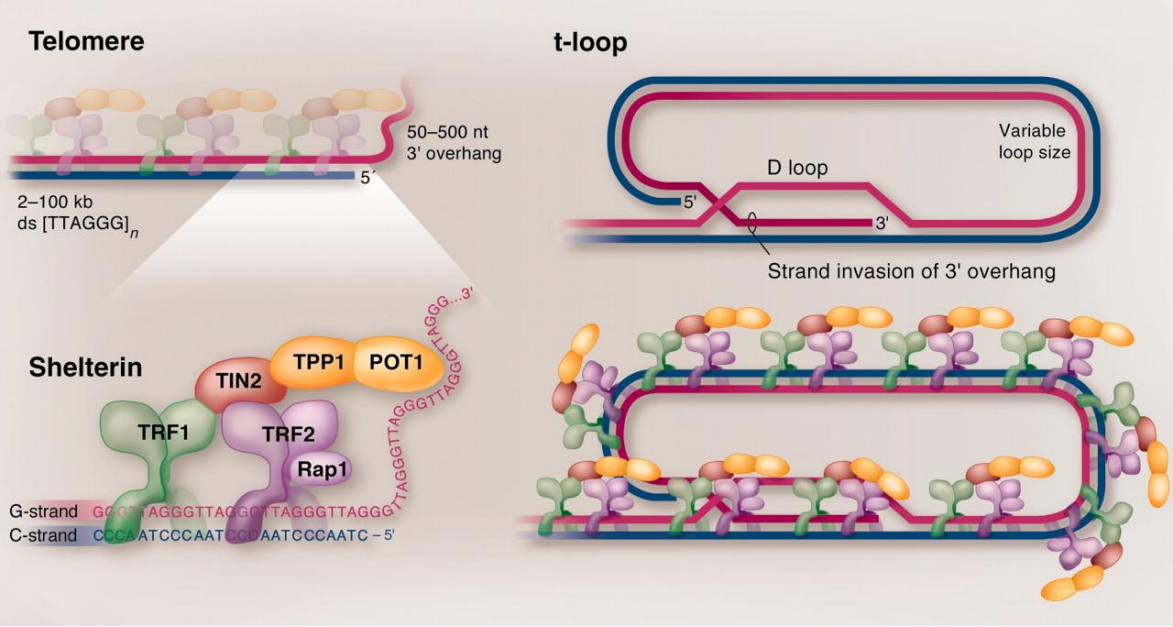

Figura 1. Estrutura dos telômeros. Os telômeros apresentam conformação em alça t (t-loop), que resulta da invasão da projeção da extremidade 3 'da fita simples no DNA telomérico de fita dupla. O complexo *Shelterin* é constituído de seis proteínas diferentes e três delas se ligam especificamente às sequências repetidas do telômero. TRF1 e TRF2 ligam-se às sequências repetidas bifilamentares e POT1 (do inglês, *Protection Of Telomeres* 1) liga-se às sequências repetidas unifilamentares. As subunidades TIN2 e TPP1 fixam POT1 a TRF1 e TRF2 ligadas ao DNA, enquanto a proteína Rap1 associada a TRF2 ajuda a regular o comprimento dos telômeros. O complexo *Shelterin* é suficientemente abundante para cobrir a maior parte do DNA telomérico de fita dupla. Fontes: (DE LANGE, 2009; SNUSTAD; SIMMONS, 2013). Modificada a partir de SHAY; WRIGHT, 2019.

A maioria dos eucariotos evoluiu para manter seus telômeros com maquinários específicos, incluindo a enzima telomerase (LIU, JUN *et al.*, 2019), que consiste de dois componentes essenciais: o RNA da telomerase (TERC/TR) e a

transcriptase reversa da telomerase (TERT), associados a proteínas (OKAMOTO; SEIMIYA, 2019). A telomerase adiciona sequências de repetição telomérica às extremidades dos cromossomos, alongando-as para compensar a perda dessas regiões após o processo de replicação celular (BLACKBURN *et al.,* 2006), de modo que ela possa neutralizar a senescência replicativa, mantendo a estabilidade genômica (SARETZKI, 2009). A telomerase está presente em células-tronco embrionárias, células germinativas e algumas células somáticas proliferativas, tais como células da medula óssea e células que revestem o intestino (HIYAMA, E.; HIYAMA, 2007; HIYAMA *et al.*, 1996; LANSDORP, 2005), todas as quais devem sofrer divisão celular contínua. No entanto, a maioria das células somáticas tem pouca ou nenhuma atividade de telomerase, de modo que os cromossomos nessas células encurtam progressivamente a cada divisão celular (FORSYTH *et al.*, 2002). Quando os telômeros se encurtam além de um ponto crítico, o cromossomo torna-se instável, tem uma tendência a sofrer rearranjos e é degradado (PRESTON, 1997).

O tamanho adequado dos telômeros é necessário para garantir a sua função e evitar a ativação de vias de danos ao DNA (SARETZKI, 2009). No entanto, diversos mecanismos resultam no desgaste dos telômeros, como consequência, a célula perde sua capacidade proliferativa, atingindo o seu limite de replicação (TURNER *et al.*, 2019). Dessa forma, a divisão e proliferação celular são limitadas pelo encurtamento contínuo dos telômeros em cada ciclo, levando à senescência e apoptose (SHAY, 2018; STÖGBAUER *et al.*, 2020). O encurtamento dos telômeros se deve à síntese incompleta de DNA na região telomérica, dano oxidativo e eventos de processamentos exonucleolíticos (SHAY; WRIGHT, 2019).

O comprimento dos telômeros não é determinado apenas geneticamente, podendo ser moldado também por fatores ambientais (ZHU *et al.,* 2011). De modo que alterações no comprimento dos telômeros estão relacionadas a diversos mecanismos de doenças (BLACKBURN *et al.,* 2015; KAPURIA *et al.*, 2019). Por exemplo, o alongamento dos telômeros é considerado uma das características marcantes dos tecidos com alta renovação celular, como os tumores malignos (STÖGBAUER *et al.*, 2020; VENTURA *et al.*, 2019). Enquanto o encurtamento dos telômeros abaixo de um certo comprimento crítico limita a proliferação celular; e, consequentemente, tem implicações para a oncogênese, doenças

cardiovasculares, diabetes, cirrose hepática, distúrbios mentais/cognitivos e depressão (CARULLI, 2015; EVANS; LUNDBLAD, 2000; HAYCOCK *et al.*, 2014; MONROY-JARAMILLO *et al.*, 2018; SCHUTTE; MALOUFF, 2015).

O estresse físico e psicológico está associado a menor atividade da telomerase e ao encurtamento dos telômeros (DE PUNDER *et al.*, 2019). Maus-tratos na infância, eventos adversos na vida, doenças crônicas e graves também estão associados a telômeros mais curtos (BOKS *et al.*, 2015; TYRKA *et al.*, 2016). Além disso, foram evidenciados processos de encurtamento telomérico em indivíduos adictos, relacionando esse fenômeno ao uso de drogas como a cocaína (LEVANDOWSKI *et al.*, 2016), e ao álcool (HARPAZ *et al.*, 2018; YAMAKI *et al.*, 2019).

Além do processo de senescência e morte celular programada, a instabilidade genômica mediada pelo encurtamento telomérico apresenta relação com diversos distúrbios funcionais da célula, como estresse oxidativo e disfunção mitocondrial (BERNABEU-WITTEL *et al.*, 2020; PASSOS *et al.*, 2007). Os telômeros são altamente sensíveis a danos por estresse oxidativo, devido ao alto teor de resíduos de guanina (ZHU *et al.*, 2011). O dano oxidativo prolongado também diminui a atividade da telomerase e acelera o encurtamento dos telômeros (SMITH, 2018). As disfunções mitocondriais causam desgaste dos telômeros, enquanto os danos dos telômeros levam à reprogramação da biossíntese e disfunções mitocondriais, o que tem implicações tanto no processo de envelhecimento quanto em doenças (ZHENG *et al.*, 2019).

1.3 Enzimas antioxidantes, disfunção mitocondrial e apoptose

Organismos aeróbios possuem um sistema de defesa antioxidante que lida com as espécies reativas de oxigênio (EROs) produzidas como consequência da respiração aeróbia e oxidação de substratos (FUKAI; USHIO-FUKAI, 2011). As EROs correspondem a moléculas instáveis como o ânion superóxido (O_2^-), o radical hidroxila (OH•) e o peróxido de hidrogênio (H_2O_2) que podem causar danos a qualquer macromolécula (proteínas, DNA e lipídios) (LI, ROBERT *et al.*, 2016). No entanto, a presença de um sistema de defesa antioxidante, em condições normais, mantém a concentração intracelular dessas espécies reativas em um nível seguro

(OKTYABRSKY; SMIRNOVA, 2007). Existem várias enzimas associadas aos mecanismos de defesa e reparo antioxidantes contra o estresse oxidativo, como a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx). A SOD acelera a conversão de superóxido em peróxido de hidrogênio, enquanto a CAT e a GPx convertem o peróxido de hidrogênio em água (FUKAI; USHIO-FUKAI, 2011; GLORIEUX; CALDERON, 2017; MARGIS *et al.*, 2008). Além dos componentes enzimáticos, o sistema antioxidante envolve moléculas com capacidade de inativar rapidamente os radicais e oxidantes, como a glutationa reduzida (GSH), proteínas de ligação a metais, ácido úrico, melatonina, bilirrubina, poliaminas, vitaminas e coenzima Q (MIROŃCZUK-CHODAKOWSKA *et al.*, 2018).

O equilíbrio entre a produção de EROs e as defesas antioxidantes determina o grau de estresse oxidativo celular (FINKEL; HOLBROOK, 2000). De modo que alterações nos padrões de atividade dessas enzimas acontece em resposta ao aumento da produção de EROs, o que pode levar as células a um estresse prolongado, associado a doenças como diabetes (MORAES *et al.*, 2015), doenças hepáticas (LI, SHA *et al.*, 2015), Alzheimer (TÖNNIES; TRUSHINA, 2017), depressão (GORLOVA *et al.*, 2019), e abuso de drogas e álcool (RIEZZO *et al.*, 2012; XU *et al.*, 2019).

As EROs causam deterioração das células neuronais por meio da modulação da função de biomoléculas, como o DNA, RNA, lipídios e proteínas (SINGH *et al.*, 2019). O cérebro consome uma grande quantidade de oxigênio para manter o seu funcionamento adequado, e por isso, produz um alto índice de espécies reativas (HALLIWELL, 2006). O tecido nervoso é mais susceptível ao dano oxidativo por apresentar menos defesas antioxidantes, comparado a outros tecidos, como o fígado (COBLEY *et al.,* 2018). No fígado, o estresse oxidativo apresenta um risco para doença hepática alcoólica, incluindo patologias como esteatose hepática, hepatite e cirrose, que podem levar ao desenvolvimento de carcinoma hepatocelular (CICHOZ-LACH; MICHALAK, 2014).

As mitocôndrias ocupam uma posição central no controle da vida, pois essa organela é indispensável na produção de energia necessária para o funcionamento do organismo, por meio da respiração celular (FERNÁNDEZ-CHECA *et al.*, 1998). Na realização dessa tarefa, as mitocôndrias consomem grandes quantidades de

oxigênio molecular e contribuem significativamente com a produção de EROs, e, ao mesmo tempo, são os principais alvos do dano oxidativo (SARETZKI, 2009). A alta taxa de respiração e fosforilação oxidativa no cérebro, fígado, coração e músculo esquelético está relacionada à alta demanda de energia nesses tecidos. Nesse contexto, o número de mitocôndrias pode variar de acordo com a demanda de energia das células, ou como resposta a condições estressantes (LEE; WEI, 2001; TYRKA *et al.*, 2016). Os níveis de antioxidantes e pró-oxidantes podem desempenhar um papel nesse mecanismo de ajuste da massa mitocondrial ou número de cópias do DNA mitocondrial (mtDNA) nas células do tecido (LIU, CHIN SAN *et al.*, 2003). O aumento da biogênese mitocondrial também pode acontecer devido à instabilidade genômica mediada pelo encurtamento dos telômeros (ZHENG *et al.*, 2019).

Evidências apontam que a telomerase desempenha uma função de proteção nas mitocôndrias, de modo que o componente proteico da telomerase (TERT) se move entre o núcleo e as mitocôndrias sob condições de estresse oxidativo (AHMED *et al.*, 2008; HAENDELER *et al.*, 2009). Por sua vez, o aumento da biogênese mitocondrial associada à disfunção dessa organela, produz quantidades indiscriminadas de EROs, o que pode causar danos oxidativos aos constituintes celulares incluindo o DNA telomérico, resultando no encurtamento dos telômeros (PASSOS; ZGLINICKI, 2005), ocasionando um efeito cíclico que envolve o desgaste telomérico, estresse oxidativo e disfunção mitocondrial, conforme representado na **Figura 2**.

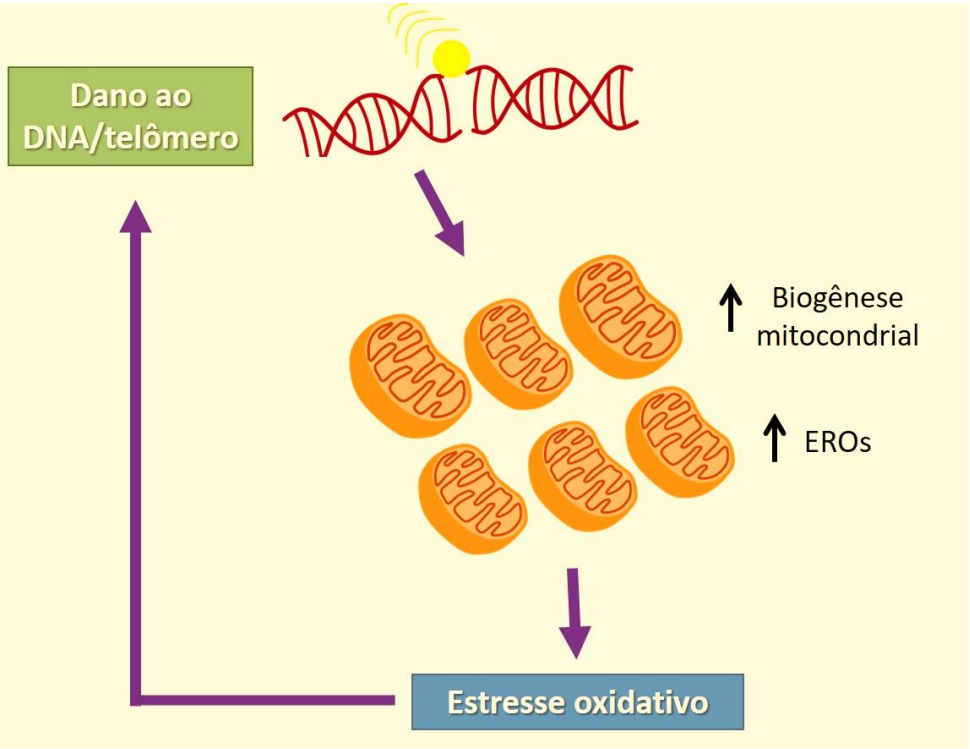

Figura 2. Relação entre a instabilidade genômica e disfunções celulares. O encurtamento dos telômeros está associado ao aumento da biogênese mitocondrial, que por sua vez contribui para produzir espécies reativas de oxigênio (EROs), promovendo o estresse oxidativo. O estresse oxidativo aumenta os danos ao DNA, ocasionando o encurtamento dos telômeros. Todo esse ciclo contribui para o envelhecimento celular e apoptose. Fonte: Próprio autor.

A instabilidade genômica associada às disfunções celulares no contexto do estresse oxidativo e envolvendo as mitocôndrias pode levar à apoptose (BERNABEU-WITTEL *et al.*, 2020; SHAO *et al.*, 2005). A morte celular programada ou apoptose é um mecanismo de autodestruição celular envolvido em uma série de eventos biológicos, como desenvolvimento embrionário e homeostase dos tecidos (MEIER *et al.*, 2000; VOSS; STRASSER, 2020). No entanto, a apoptose excessiva contribui para várias doenças neurodegenerativas, autoimunes, e está envolvida no crescimento e regressão de tumores (KACZANOWSKI, 2016; NAGATA, 2010; RADI *et al.*, 2014).

Duas vias apoptóticas são bem descritas: a via intrínseca, na qual a mitocôndria desempenha um papel central, envolvendo membros da família Bcl-2 que apresenta componentes pró- e anti-apoptóticos; e a via extrínseca, consistindo na família de receptores relacionados ao TNF (Fator de Necrose Tumoral) na

membrana celular (JEONG; SEOL, 2008). A família Bcl-2 (*B Cell Lymphoma 2*) inclui as proteínas anti-apoptóticas, como Bcl2-2 e Bcl-xL (*Bcl-2 like survival factors*), e as proteínas pró-apoptóticas Bax (proteína X associada a Bcl-2; codificada por *Bax*) ou Bak (antagonista/assassino de Bcl-2; codificada por *Bak1*) (SINGH *et al.*, 2019). As interações mútuas entre os membros pró- e anti-apoptóticos estabelecem o limiar que determina se a célula deve ou não entrar em apoptose (BORNER, 2003). Em estudo com linhagem de células tumorais foi demonstrado um efeito positivo entre a redução da atividade da enzima telomerase, encurtamento de telômeros e o aumento da razão Bax/Bcl-2, como um mecanismo de indução da apoptose (KHAW *et al.*, 2013; VAFAIYAN *et al.,* 2015).

1.4 Instabilidade genômica, disfunções celulares e etanol

O encurtamento dos telômeros está associado às alterações de comportamento (BATESON; NETTLE, 2018) e fatores de risco para doenças mentais, incluindo exposição ao estresse e adversidades na infância (EPEL *et al.*, 2004; SHALEV *et al.*, 2013a; SHALEV *et al.*, 2013b; ZAHRAN *et al.*, 2015). Além disso, o encurtamento dos telômeros também está associado com depressão (SCHUTTE; MALOUFF, 2015), ansiedade (HOEN *et al.*, 2013), esquizofrenia (GALLETLY *et al.*, 2017) e abuso de drogas (MONROY-JARAMILLO *et al.*, 2018). O uso abusivo crônico do etanol também apresenta relação com o encurtamento de telômeros em leucócitos (DIXIT *et al.*, 2019; MARTINS DE CARVALHO *et al.*, 2019; PAVANELLO *et al.*, 2011), esôfago (AIDA *et al.*, 2011) e epitélio oral (AIDA *et al.*, 2020). Há indicativos de que o acetaldeído, o primeiro metabólito resultante da oxidação do etanol, desempenhe um papel no processo de encurtamento dos telômeros (YAMAKI *et al.*, 2019). De fato, o processo de oxidação do etanol em acetaldeído parece ter um efeito importante nos parâmetros comportamentais, fisiológicos e moleculares associados ao abuso do etanol (MCBRIDE *et al.*, 2002).

No fígado e no cérebro a oxidação do etanol através da citocromo P450 2E1 (CYP2E1) pode causar aumento significativo de EROs, o que pode contribuir para o estresse oxidativo (LI, SHA *et al.*, 2015). No cérebro, além do CYP2E1, a enzima catalase também desempenha um papel essencial para a oxidação do etanol em acetaldeído (HERNÁNDEZ *et al.,* 2016). Normalmente, a formação das

EROs é balanceada pelo sistema antioxidante, que depende da disponibilidade de GSH, cuja manutenção está interligada com a atividade do ciclo da metionina. O ciclo da metionina é também a fonte de S-adenosilmetionina (SAM), o doador universal do grupo metil para todas as reações de metilação (VADIGEPALLI; HOEK, 2018). Há evidência de que a atividade do ciclo da metionina é suprimida pela ingestão crônica de etanol (KHARBANDA, 2009).

No cérebro, a exposição aguda ao etanol ocasiona modulações na atividade das enzimas antioxidantes, de modo a compensar o efeito tóxico dessa molécula (BALIÑO *et al.*, 2019; ENACHE *et al.*, 2008). Evidências apontam que o aumento da atividade da CAT no tecido nervoso está relacionado ao comportamento de uso abusivo do etanol (ARAGON *et al.*, 1985; MATTALLONI *et al.*, 2019). O consumo de etanol também produz modificações no estado antioxidante do fígado (KOCH *et al.*, 2004). Nesse órgão, o estresse oxidativo pode ser explicado pela metabolização do álcool: a álcool desidrogenase (ADH) oxida o etanol em acetaldeído, o qual é posteriormente oxidado em acetato pela acetaldeído desidrogenase (ALDH) (CENI *et al.*, 2014). Este produto é instável e facilmente se decompõe em água e dióxido de carbono. No entanto, a formação de acetaldeído é destrutiva para as células do fígado por ser um agente reativo que pode causar danos ao DNA que resultam em lesão do tecido (CICHOZ-LACH; MICHALAK, 2014). No fígado, padrões diversos da resposta enzimática do sistema antioxidante foram evidenciados em estudos de exposição aguda e crônica ao etanol (DEY; CEDERBAUM, 2006; IGNATOWICZ *et al.*, 2013).

Nos últimos anos, vários estudos mostraram que o álcool afeta a homeostase celular em órgãos como o fígado e o cérebro alterando a função mitocondrial por meio do comprometimento da cadeia de transporte de elétrons, gerando estresse oxidativo, reduzindo a produção de energia e induzindo processos de apoptose (ALMANSA *et al.*, 2009; BAILEY; CUNNINGHAM, 2002; HOEK *et al.*, 2002). A exposição crônica a EROs pode levar ao declínio da função mitocondrial, bem como o estresse oxidativo pode induzir a proliferação mitocondrial, aumentando o número de cópias de mtDNA (MALIK; CZAJKA, 2013). O aumento no número de mitocôndrias foi evidenciado em tecido cardíaco de ratos expostos ao etanol (MARIN-GARCIA *et al.*, 1995), e no sangue de pessoas com carcinoma de células escamosas de cabeça e pescoço em associação com o uso

de tabaco e abuso de álcool (KUMAR *et al.*, 2017). Além disso, a neurotoxicidade pelo uso de substâncias ilícitas, como cocaína, anfetamina e metanfetamina está relacionada ao aumento do estresse oxidativo intracelular, seguido de alterações no número de mtDNA e disfunção mitocondrial (SADAKIERSKA-CHUDY, 2014).

O etanol ativa a via intrínseca da apoptose em neurônios, contribuindo para os efeitos neuropatológicos da exposição ao álcool (NOWOSLAWSKI *et al.*, 2005), bem como a exposição aguda ao etanol induz a via mitocondrial de apoptose em hepatócitos (HIGUCHI *et al.*, 2001). Considerando a via intrínseca da apoptose, a exposição ao etanol promove alterações na expressão de Bax e Bcl-2 no cérebro (LEE, HAE YOUNG *et al.*, 2008), e em hepatócitos, a translocação de Bax do citosol para a mitocôndria desempenha um papel crucial na apoptose induzida por exposição aguda ao etanol (ADACHI *et al.*, 2004).

1.5 *Danio rerio* (*zebrafish*)

O *Danio rerio* (*zebrafish*) é um teleósteo tropical de água doce, que nos últimos anos vem atraindo a atenção da comunidade científica como um modelo experimental. As vantagens na utilização desses peixes se devem ao seu pequeno porte (3 a 4 cm), fácil manejo, manutenção e alta taxa reprodutiva (SILVEIRA *et al.*, 2012). Dessa forma, o *zebrafish* constitui um excelente modelo para estudos em biologia do desenvolvimento (VELDMAN; LIN, 2008), avaliação de novos agentes terapêuticos (DEVEAU *et al*, 2017), estudos comportamentais (BARTOLINI *et al.*, 2015; LIU, XIUYUN *et al.*, 2016), genéticos (KLEE *et al.*, 2012; PRIETO; ZOLESSI, 2017), toxicológicos (GÓMEZ-CANELA *et al.*, 2017; JAGADEESHAN *et al.*, 2017) e para análise de mecanismos de diversas doenças humanas (BERENS *et al.*, 2016; LULLA *et al.*, 2016), incluindo avaliações sobre o efeito da exposição ao álcool (MATHUR *et al.*, 2011; PAIVA *et al.*, 2020; TRAN *et al.*, 2015).

O *Danio rerio* pode ser considerado um bom modelo de pesquisa para doenças genéticas humanas, pois 71% dos genes que codificam proteínas no genoma humano são relacionados a genes encontrados no *zebrafish*, e destes, 84% dos genes relacionados a doenças humanas possuem um gene ortólogo em *zebrafish* (HOWE *et al.*, 2013). Estudos de aprendizagem, memória, agressividade, ansiedade e sono sugerem fortemente que os processos regulatórios conservados

fundamentam o comportamento tanto em *zebrafish* quanto em mamíferos (NORTON; BALLY-CUIF, 2010), reforçando o uso dessa espécie como modelo de pesquisa para esses estudos.

Especificamente em relação a avaliação dos efeitos do álcool, diversos trabalhos evidenciaram que o *zebrafish* desenvolve padrões de comportamento (DA SILVA CHAVES *et al.*, 2018; GERLAI *et al.*, 2000), modulações sinápticas (KLEE *et al.*, 2012; TRAN *et al.,* 2017) e bioquímicas (MOCELIN *et al.*, 2018; MÜLLER *et al.*, 2017) que servem como base para o entendimento de alterações moleculares e fisiológicas no contexto do uso abusivo do etanol. O que demonstra que o *Danio rerio* é um bom modelo para estudar os efeitos do álcool tanto em nível de transcrição, alterações bioquímicas e moleculares, quanto no comportamento, sobretudo em relação à preferência pelo etanol (PAIVA *et al.*, 2020).

2. JUSTIFICATIVA

Muitos modelos de pesquisa têm sido utilizados para estudar os efeitos do álcool, com o intuito de identificar padrões genéticos relacionados ao comportamento de preferência e perda de controle em relação ao etanol. Os modelos de pesquisa baseados no estudo de comportamento e que definem a relação de preferência pelo etanol, sugerem respostas diferenciais dos indivíduos, sendo possível a distinção fenotípica quanto à preferência. Em camundongos foram identificados dois padrões de preferência em modelo de livre escolha, de modo que o grupo caracterizado como compulsivo por esses autores corresponde aos indivíduos que continuaram tendo preferência pelo etanol, mesmo após a adição de quinino, demonstrando uma perda de controle em relação ao consumo do álcool (SPOELDER *et al.*, 2015).

No nosso grupo de pesquisa, um padrão similar de comportamento foi identificado em um estudo também com camundongos, cuja definição em relação à preferência pelo etanol se deu em dois fenótipos distintos: os animais de fenótipo pesado e fenótipo inflexível, sendo que esse último manteve o alto consumo de etanol, mesmo após a adição de quinino, enquanto o fenótipo pesado interrompeu o consumo quando o quinino foi adicionado (DA SILVA E SILVA *et al.*, 2016).

Em estudos comportamentais relacionados ao etanol utilizando o *zebrafish* como modelo de pesquisa, também foi possível distinguir a variabilidade em relação à preferência pelo etanol. Mathur e colegas (2011) identificaram uma preferência significativamente maior pelo compartimento onde os peixes receberam etanol durante uma única exposição de 20 minutos, principalmente em indivíduos expostos a 1,5% de etanol, sendo que a avaliação da preferência final foi feita 24h após a exposição (MATHUR et al., 2011).

Com o intuito de aprofundar o entendimento da relação dos fenótipos com as modulações moleculares relacionadas à preferência pelo etanol, o nosso grupo de pesquisa estabeleceu um protocolo com o *zebrafish* utilizando como base o teste de Preferência Condicionado a Lugar (do inglês *Conditioned Place Preference* - CPP). O teste CPP é um método validado para análise de comportamento de preferência baseado em um estímulo (COLLIER *et al.*, 2014; COLLIER; ECHEVARRIA, 2013). O protocolo consiste na definição do lado basal de

preferência do animal, seguido de exposição ao etanol e avaliação quanto à mudança em relação ao lado de preferência no aquário. Como resultado, foram distinguidos quatro fenótipos: Leve (não apresenta preferência pelo lado exposto ao etanol); Pesado (apresenta preferência ao lado exposto ao etanol após 24h de exposição); Inflexível (apresenta preferência ao lado exposto ao etanol, após 24h e após 15 dias da exposição); e Reforço Negativo (apresenta preferência pelo lado exposto ao etanol após 15 dias de exposição) (PAIVA *et al.*, 2020). Nesse trabalho foram identificadas alterações no padrão de transcrição dos principais receptores de neurotransmissores que são alvos do etanol (*drd1, drd2, grin1a, gria2a* e *gabbr1b*).

Tendo em vista que o nosso grupo vem caracterizando e desenvolvendo esses modelos de pesquisa, relacionando os fenótipos de preferência pelo etanol às variações nos níveis de transcritos de genes envolvidos no sistema recompensa, e considerando que mecanismos como encurtamento de telômeros, disfunção mitocondrial e estresse oxidativo podem estar relacionados com o comportamento de uso abusivo do etanol, decidimos investigar a ocorrência de instabilidade genômica e disfunções celulares nos fenótipos de preferência pelo etanol com o *zebrafish*. Portanto, este trabalho teve como objetivo avaliar o padrão de encurtamento de telômeros; analisar a ocorrência de biogênese mitocondrial (pela quantificação de mtDNA por qPCR); avaliar a atividade das enzimas antioxidantes (SOD, GPx, CAT) e do potencial antioxidante total dos tecidos; e quantificar os transcritos dos genes *bax* e *bcl2* (relacionados ao controle da apoptose) em brânquias, fígado e cérebro dos fenótipos de preferência pelo etanol.

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CAPÍTULO 1 – Avaliação do tamanho dos telômeros e quantificação dos marcadores de apoptose *bax* e *bcl2* nos fenótipos de preferência pelo etanol.

ARTIGO

Telomere shortening and apoptosis analysis of the ethanol preference phenotypes in zebrafish

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Abstract

Telomeric shortening is related to genomic instability and increased apoptosis in chronic alcohol abuse. However, there is no evidence of telomere shortening in association with apoptosis an acute ethanol exposure. We aimed to evaluate the telomeres length and the transcriptional regulation of the *bax* and *bcl2* genes in the gills, liver, and brain of ethanol preference phenotypes in adult zebrafish. The ethanol preference of the animals was define using the behavioral Conditioned Place Preference (CPP) paradigm, after a single ethanol exposure. Four phenotypes were identified: Light, Heavy, Inflexible and Negative Reinforcement. The telomere shortening was observed in gills and brain of Heavy, Inflexible and Negative Reinforcement phenotypes, as well in liver of Heavy and Negative Reinforcement. An upregulation of the *bax* gene was evidenced in the brain of Negative Reinforcement. Our results suggest an association of ethanol preference phenotype and telomere shortening in adult zebrafish submitted to CPP test following an acute ethanol exposure.

Keywords: alcohol abuse, *Danio rerio*, telomeres shortening, apoptosis, ethanol preference.

1. Introduction

Telomeres are composed of a long double-ribbon array of TTAGGG repetitions at chromosome's end that protects the genome from degradation and fusion [1,2]. Telomeres extend for 9–15 kb in humans but can be as long as 100 kb in rodents [3] and 5-15 kb in zebrafish [4]. Most eukaryotes have evolved to maintain their telomeres with specific machinery, including telomerase [5].

The telomere sequence is a protection system to prevent the loss of crucial DNA after replication in proliferating cells, however, there are several mechanisms that result in telomere shortening [6]. Thus, progressive telomere shortening occurs as results of cell division and aging due to the incomplete synthesis of DNA in the telomeric region, oxidative damage and exonucleolytic processing events [7,8]. Once the telomeres have shortened to their threshold, they lose the ability to cap the end of the chromosome, which leads to genomic instability, loss of cell viability and apoptosis [9–11].

Alterations in the telomere length (TL) are related to several disease mechanisms [12,13]. For example, stretching of telomeres is considered one of the marks of tissues with high cell proliferation such as malignant tumors [14,15]. Interestingly, telomere shortening may also be considered as an indicator of biological age. Thus, it has been studied as a biomarker of cell aging and therefore associated to the increased risk of pathological conditions such as hypertension, diabetes, Alzheimer and depression [16,17]. In addition, telomere shortening was observed in cocaine addiction and alcohol abuse [18–20].

Alcohol abuse is a worldwide problem of a psychological, physical, and social nature. According to data of World Health Organization (WHO), more than 3 million people died as a result of harmful alcohol use in 2016 [21]. Besides accelerate physiological aging, chronic alcohol abuse has been proposed as one of the factors responsible for telomere shortening which may be a bidirectional relationship [22–24]. In this context, in a behavioral study Kang et al [25] suggested that the impulsive choice for alcohol consumption is associated with shorter telomere length.

Exposure to ethanol may also lead to increased apoptosis rate in hepatocytes, cardiomyocytes, lung, and neurons [26–30]. A growing body of evidence shows that apoptosis may occur as a consequence of telomere shortening [31]. Thus, the relationship between alcohol, shortening of telomeres and apoptosis should not be disregarded. The Bcl-2 protein family are central regulators of apoptosis being subdivided into three groups: group I with anti-apoptotic activity (as Bcl-2), groups II and III that promote cell death (as Bax) [32].

Our research group developed a model of ethanol preference in juvenile zebrafish describing four phenotypes regarding the ethanol preference – Light, Heavy, Inflexible and Negative Reinforcement – and identified patterns of differential gene regulation in these groups associated to ethanol abuse [33]. In the present study, we aimed to evaluate the shortening of telomeres and the transcriptional regulation of the *bax* and *bcl2* genes in the gills, liver, and brain of the ethanol preference phenotypes in adult zebrafish. Considering the behavioral Conditioned Place Preference (CPP) test paradigm, we identified the same four phenotypes of ethanol preference and evidenced the telomere shortening in the gills and the brain of Heavy, Inflexible and Negative Reinforcement phenotypes, as well in liver of Heavy and Negative Reinforcement. An upregulation of the *bax* gene was evidenced in the brain of Negative Reinforcement phenotype.

2. Results

2.1 Determination of the phenotypes based on ethanol preference behavior

The animals subjected to the CPP test were classified into four phenotypes according to their preference for the ethanol: 1) Light (n = 9), animals that did not prefer the conditioning side at any time (Basal Preference - B, Post-Conditioned Preference - PC, and After Withdrawal Preference - AW); 2) Heavy (n = 12), animals that preferred the side where the conditioning to the ethanol occurred in PC but lost preference in AW; 3) Inflexible (n = 15), animals that preferred the conditioning side in both PC and AW; and 4) Negative Reinforcement (n = 14), animals that did not alter their PC preference in relation to B, but began to seek the conditioning side in AW (Table 1 and Figure 1).

In the Control group (with no exposure to the ethanol), no statistical differences were observed compared to the threshold preference in PC or AW (Figure 1A). The Light phenotype presented a significantly lower mean than the preference threshold (50.1%) in B, PC and AW ($p < 0.0001$ for all) (Figure 1B). For the Heavy phenotype, we observed a mean value statistically different and higher than 50.1% in PC ($p < 0.0001$) and statistically different and lower than the same threshold in AW ($p < 0.0001$) (Figure 1C). The Inflexible phenotype showed a mean higher from 50.1% in PC ($p = 0.0029$) and in AW ($p < 0.0001$) (Figure 1D). Lastly, in Negative Reinforcement the mean was statistically lower and different from 50.1% in PC ($p = 0.0005$) and was higher from the same threshold in AW ($p = 0.0011$) (Figure 2E).

Table 1. Description and number of samples (n) of each phenotypic group distinguished by the CPP test.

Phenotype	Description	n
Light	Animals that did not prefer the ethanol conditioning side in any of the observations.	9
Heavy	Animals that preferred the ethanol conditioning side at PC and quit preferring at AW.	12
Inflexible	Animals that preferred the ethanol conditioning side at both PC and AW.	15
Negative Reinforcement	Animals that began to prefer the ethanol conditioning side only at AW.	14

PC – Post-Conditioned Preference. AW – After-Withdrawal Preference.

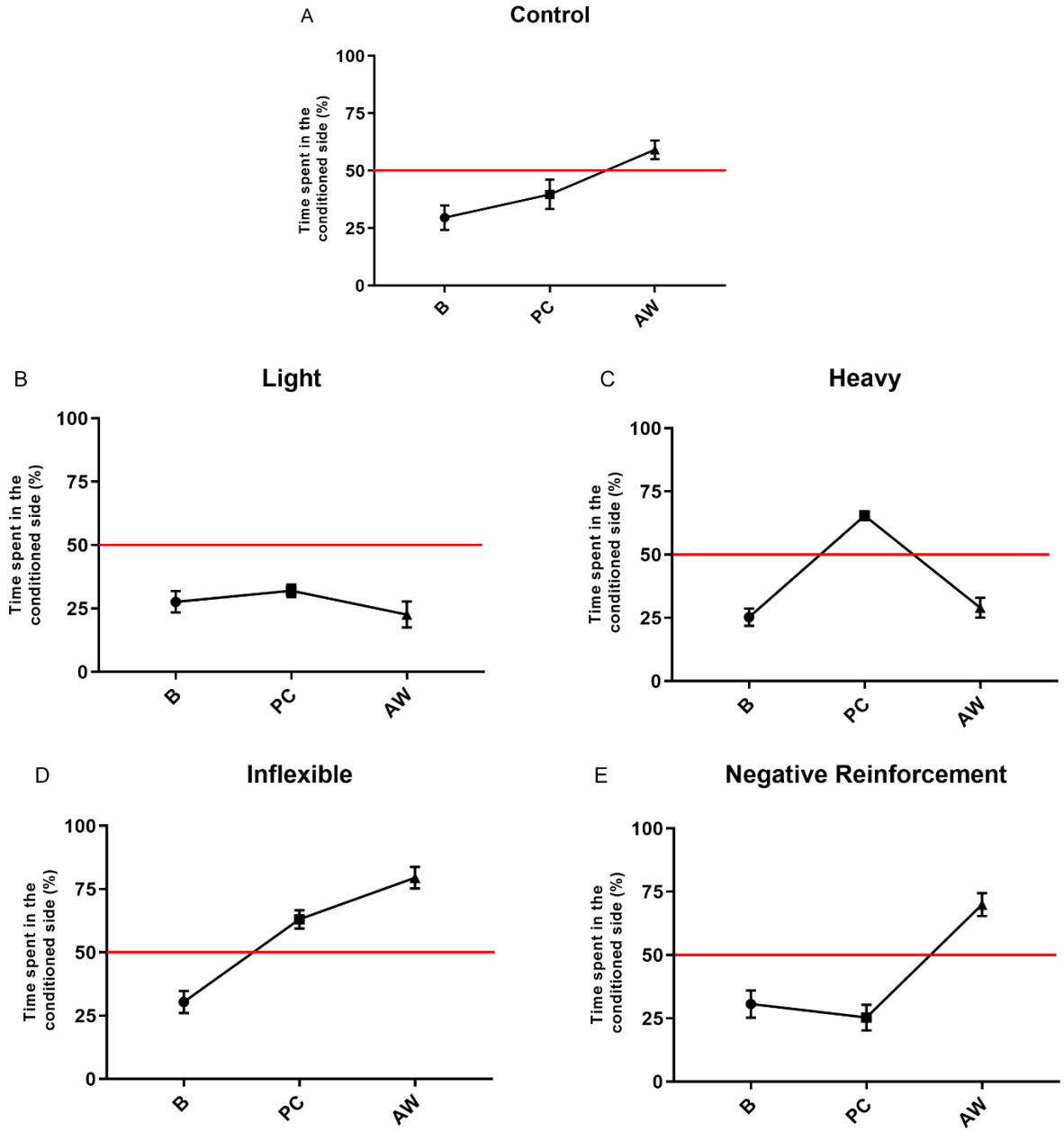

Figure 1. Ethanol preference determined by the CPP test for each phenotypic group. Data are represented as the percentage of time spent on the ethanol conditioning side during the determination of the preferences B, PC, and AW for each phenotype distinguished after a single ethanol exposure of 20 min. Preference moments: B = Basal, before conditioning; PC = Post-Conditioning; AW = After Withdrawal. The preferences were calculated in relation to a hypothetical preference threshold of 50.1% (red line). The graphs represent the preference changes that characterize each phenotype: (A) In the Control group (n = 10). (B) Light phenotype (n = 9). (C) Heavy phenotype (n = 12). (D) Inflexible phenotype (n = 15). (E) Negative Reinforcement phenotype (n = 14). Differences between the means of the groups and the hypothetical value of 50.1% were considered statistically significant when $p < 0.05$. Data are expressed as the mean and standard error of the mean (±SEM).

2.2 Telomere length in gills, liver, and brain

For telomere length analysis we used a protocol for qPCR [34]. As shown in Figure 2, telomere shortening was evidenced in gills (Figure 2A) (F (4,30) = 5.276) of Heavy, Inflexible and Negative Reinforcement phenotypes compared to Control ($p < 0.05$). The same result was found in liver (Figure 2B) (F (4,26) = 3.344) of Heavy and Negative Reinforcement phenotypes compared to Control ($p < 0.05$). In brain (Figure 2C) (F (4,28) = 5.601) the telomere shortening was also observed in Heavy, Inflexible and Negative Reinforcement, compared to Light group ($p < 0.05$).

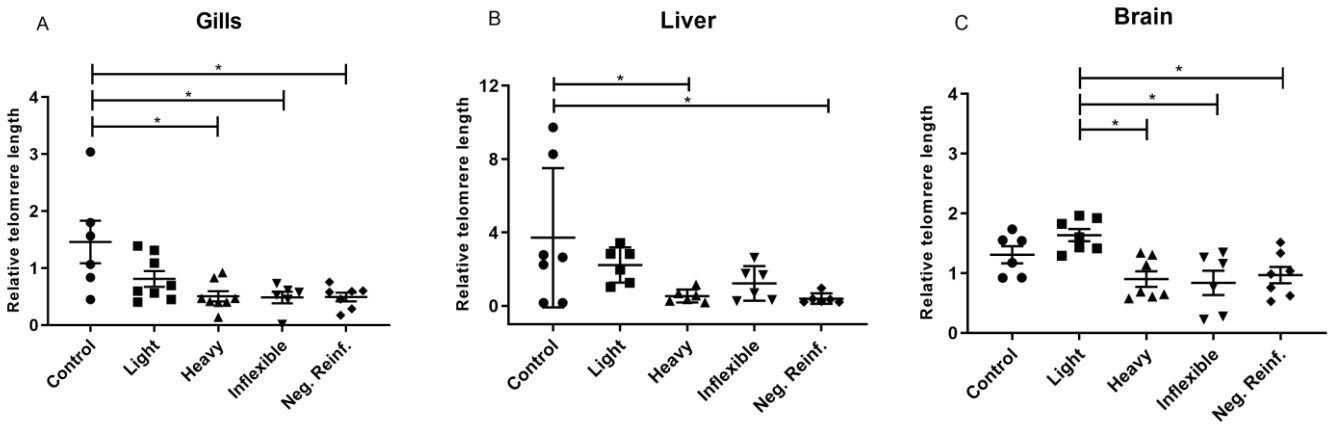

Figure 2. Relative telomeres length of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference. (A) Gills. (B) Liver. (C) Brain. Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$.

2.3 Transcriptional regulation of *bax* and *bcl2* in gills, liver, and brain

Figure 3 shows the transcript levels of the apoptosis molecular markers *bax* and *bcl2* in the zebrafish gills, liver, and brain for each phenotype. We observed a significant difference in the transcript levels of *bax* in brain (F (4,27) = 1.994) of Negative Reinforcement phenotype compared to Light ($p < 0.05$).

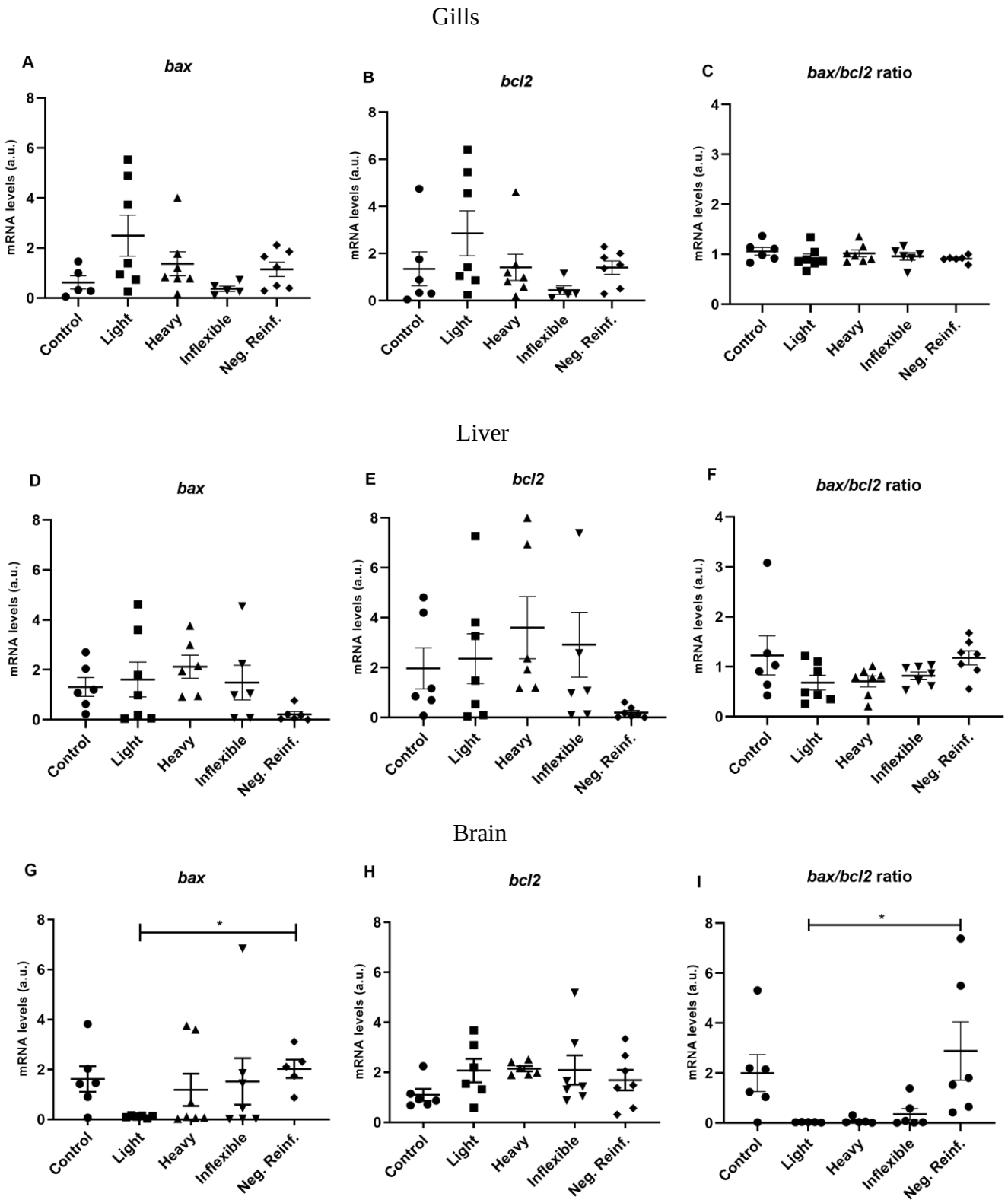

Figure 3. Relative amount of mRNA of apoptosis genes in the zebrafish submitted to CPP test and distinguished in phenotypes of ethanol preference. (A) *bax* in gills. (B) *bcl2* in gills. (C) *bax/bcl2* ratio in gills (D) *bax* in liver. (E) *bcl2* in liver. (F) *bax/bcl2* ratio in liver. (G) *bax* in brain: upregulated in Negative Reinforcement in comparison to the Light $p < 0.05$. (H) *bcl2* in brain. (I) *bax/bcl2* ratio in brain. Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$. (n = 6). a.u.= arbitrary units.

2.4 Correlation between telomere length and *bax/bcl2* ratio

Pearson's correlation was performed to evaluate the association between the results of the telomeres length and the relative quantification of the *bax* and *bcl2* transcripts, considering the tissues analyzed, as well the ethanol preference phenotypes. As shown in Table 2, there was no statistical evidence of a correlation between shortening of telomeres and indicative of increased apoptosis in the ethanol preference phenotypes.

Table 2. Correlation between telomere length and *bax*/*bcl2* ratio in gills, liver and brain of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference.

Phenotypes	r	p-value
Gills		
Control	-0.214	0.684
Light	0.360	0.427
Heavy	-0.089	0.849
Inflexible	-0,357	0.555
Negative Reinforcement	-0.314	0.493
Liver		
Control	0.090	0.866
Light	0.431	0.394
Heavy	-0.585	0.222
Inflexible	0.490	0.402
Negative Reinforcement	-0.231	0.618
Brain		
Control	-0.511	0.301
Light	-0.556	0.195
Heavy	-0.289	0.578
Inflexible	-0.510	0.302
Negative Reinforcement	-0.188	0.687

Coefficients (r) and p-values were calculated by the Pearson's correlation model.

3. Discussion

Paiva et al [33] characterized the ethanol preference phenotypes using juvenile zebrafish (20 days post fertilization (dpf)) submitted to CPP test paradigm, by describing the behavior and molecular alterations related to alcohol abuse. In the present work we evidenced the same phenotypes in heterogenetic adult zebrafish reinforcing the use of this model to study ethanol preference. As shown in Table 1 there was found four different phenotypes: Light, Heavy, Inflexible and Negative Reinforcement.

In the context of alcohol abuse, changes in telomeres length present an important association with risk factors, such as cancer, liver cirrhosis [35,36] and premature aging [37]. There are many reports about shortening telomeres associated with chronic alcohol abuse in human

leukocyte [20,22], blood [24], esophagus [38] and oral epithelium [39], although, there are a missing information related to acute ethanol exposure and telomere shortening. Harpaz and colleagues [40] evidence telomere shortening in human cells cultures exposure to moderate concentrations of ethanol, equivalent to social drinking, and suggests that may contribute to the carcinogenic potential of acute alcohol consumption. The animals used in our experiment were exposure to ethanol once and for 20 min, and present relevant alterations related to genomic and cellular instability by the telomere shortening. These finds contribute to reinforce the harmful effects of ethanol consumption even in low doses, specially associated to seeking behavior.

Considering the ethanol preference in adult zebrafish, telomere shortening was evidenced in the gills and brain of Inflexible, Negative Reinforcement and Heavy phenotypes. In Heavy and Negative Reinforcement phenotypes the telomere shortening was also evidenced in the liver. These phenotypes shown ethanol preference in CPP test, especially the Inflexible, whose prefers the ethanol side in both PC and AW tests. However, no evidence of telomere shortening was found in Light phenotype, also exposure to ethanol, but without ethanol preference, so that the results found in this group are similar to control.

In a study of early stress associated with anxiety and substance use disorders in human, Tyrka and colleagues [41] evidenced shorter telomeres. Our results present the same pattern of telomere shortening in the gills, liver, and brain of ethanol preference phenotypes. In the liver, this characteristic may represent a genetic risk factor for the development of cirrhosis [35,42]. In brain telomere shortening has been associated to neurodegenerative disorders [43] and neuroinflammation [44]. Considering our results, we present the occurrence of shortening telomeres in the brain, liver, and gills in individuals with ethanol preference for the first time.

Telomere shortening is also related to cellular loss by triggering apoptosis [31,45]. To better understand the context of apoptosis considering the ethanol preference phenotypes, we performed the analysis of transcriptional regulation of the *bax* and *bcl2* genes. We identified an upregulation of *bax* gene in the brain of Negative Reinforcement phenotype, suggesting a possible increased apoptosis index in this group. *Bax* gene codify a pro-apoptotic protein and presents an important role in the context of ethanol-induced apoptosis [46]. Britton and Miller [47] also demonstrated this in a study with *Bax* knockout mice which no present neuronal loss after ethanol exposure. There was no evidence of alterations in transcriptional regulation of the *bax* and *bcl2* genes for the other tissues of the ethanol preference phenotypes, thus, other studies can be performed to evidence the occurrence of apoptosis in these phenotypes by another pathways. In order to evaluate the association

between telomere shortening and the *bax*/*bcl2* ratio, we performed Pearson's correlation analysis. However, there was no evidence of a correlation between these parameters considering the ethanol preference phenotypes in our study.

Nevertheless, our results demonstrated a differential cellular response in telomere shortening regarding ethanol preference. One consideration about our experiment is the phenotypic definition related to the behavior using heterogenetic animals. Bateson and Nettle [48] discuss how individual differences in telomere length are associated with individual differences in behavior, and by their finds, suggests a possible relation of telomere length and higher impulsivity in choices between delayed rewards, higher propensity to take risky decisions, higher probability of smoking, higher alcohol consumption, higher stress reactivity, and more neurotic and pessimistic personality types. Furthermore, there is an association between telomere shortening and biobehavioral research [49], such as depression [16], stress [50] and violence [51]. In our model of study, the Light group presented similar results compared to control, i.e., despite have been exposed to ethanol, this phenotype does not present the alterations observed in the other phenotypes which ethanol preference. These data reinforce the association between the telomere shortening and the behavior changes associated to ethanol preference, seeking and consumption, even considering an acute ethanol exposure.

In conclusion, we present genomic alterations related to telomere shortening and evaluation of transcriptional regulation of *bax* and *bcl2* genes in the gills, liver and brain of adult zebrafish submitted to CPP test and distinguished in phenotypes according to their ethanol preference. All the animals with ethanol preference: Heavy, Inflexible and Negative Reinforcement, showed telomere shortening in gills and brain, demonstrating the susceptibility of these tissues to ethanol damage. Our results also present an association of genomic vulnerability related to ethanol preference phenotypes, mainly considering our finds for the brain, elucidating the individual's response associated to genomic instability and behavior. We also evidenced an upregulation of *bax,* a pro-apoptotic gene, in the brain of Negative Reinforcement phenotype. Furthermore, our protocol using zebrafish demonstrates the relevance of this model for studies of behavior and ethanol preference. In addition, the patterns of molecular changes that we present are consistent with the data found in rodents and humans, with the differential to establishing a relationship between the behavior and the telomere shortening considering the acute ethanol exposure.

4. Methods

4.1 Animals and experimental conditions

Were used seventy wild type adult zebrafish (*Danio rerio*), *short-fin* of heterogeneous background and of both sexes obtained from the Aquatic Animal Housing of Universidade Federal de Lavras (Minas Gerais, Brazil). The animals presented an average weight of 0.2709 ± 0.078 grams. The fish were housed in an automated system (Rack Hydrus, model ZEB-40-Alesco), in polycarbonate aquariums with 2.5L capacity (11.5 x 34.5 x 15.5 cm), containing a recirculation system, under a 14:10 hour photoperiod (light/dark). The temperature maintained was 28°C ± 1ºC and pH 7.0. All animals were fed three times a day, twice (once at 8:00 and 16:00) with commercial alcon BASIC® as flake fish food (Alcon, Brazil), and once at 11:00 with live food (*Artemia sp*.). Rearing and welfare conditions were in accordance with the standards for the species. The protocol was approved by the Ethics Committee of CEUA-UFMG (Protocol number: 64/2016).

4.2 Experimental design

The experiment was conducted according to the protocol described by Paiva and colleagues [33], as shown in Figure 4. Sixty animals were used to define the ethanol preference phenotypes and ten animals were designated as a Control group, without drug exposure. The animals were individualized and acclimated for seven days before the beginning of the experiment and were kept in the rack (ALESCO® Indústria e Comércio Ltda, Brazil).

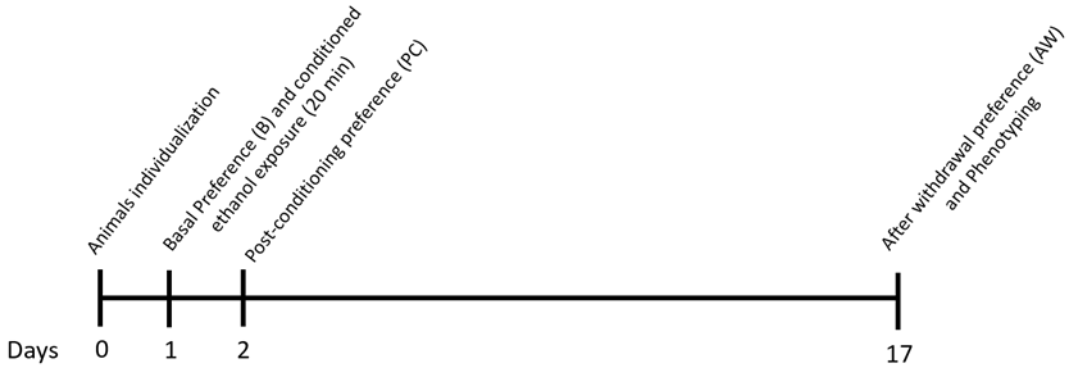

Figure 4. Experimental design. Acclimation: animals were randomly divided for ethanol preference phenotyping and Control group. The fish were maintained in the experimental environment for seven days for adaptation. After this period, on the experimental day 1, we began the CPP test, which lasted 16 days. For this test, the animals of both groups were individualized. Preferences were determined by analyses of filming records (10 min each) from three moments: Basal (B), Post-Conditioning (PC), and After Withdrawal (AW). Preference in B was measured to establish the intrinsic preference of each animal for the different compartments of the tank (white and dotted) in the absence of the drug. Following determination of the preference B, each animal of the Acute group was exposed to ethanol conditioning (1% v/v) on the least preferred side for 20 min, followed by another period of 20 min on the preferred side containing only water. During this phase, the animals of the Control group were similarly conditioned on both sides of the tank, but in the absence of ethanol. Twenty-four hours after the conditioned exposure, the PC preference was determined and, after 16 days (on day 17), we determined the AW preference. Later in the same day, the animals were classified into Light, Heavy, Inflexible, and Negative Reinforcement, based on data from the three preference determination time points (B, PC, and AW). The animals were, then, euthanized and their gills, liver and brain collected for analysis. (Modified from Paiva et al [33]).

4.2.1. Conditioned Place Preference (CPP)

To determine the ethanol preference phenotypes, we used the protocol described before [33]. The preference was evaluated in three moments: basal (B), post-conditioning (PC), and after withdrawal (AW). We used 5L (30 x 15 x 12 cm) experimental tanks with two patterns of texture in the bottom, i. e., half of the tank had a plain white bottom and the other half had a white bottom with evenly distributed black circles (environmental clue) [52]. The experimental tanks were separated from each other by isolators to prevent the animals from seeing the neighboring tank.

Determining the basal (B) preference - The previously individualized animals were carefully transported to the behavioral evaluation room (with controlled lighting, temperature, and noise conditions) and transferred to the experimental tanks being careful to

minimize the stress due to the transference and hypoxia. Then, in the absence of ethanol, the animals were filmed for ten minutes to determine the preference. The basal (B) preference was defined as the side of the tank that the animal explored for longer (%).

Conditioned exposure to the ethanol – Following determination of the B preference, the animals were placed in the conditioning tank, on the side opposite to the preference. These tanks were identical to those used to determine the preference, except for the presence of a sealed central divider to prevent the passage of water or drug, thus avoiding cross-contamination effects. On the least-preferred side, each animal from the Acute group was exposed to ethanol (Merck, Damstadt, Alemanha) at a concentration of 1% (v/v) for 20 minutes. Then the fish were removed from that compartment, left in a beaker with water for 5 minutes to remove ethanol excess. Next, the animals were placed in the compartment corresponding to the preferred side in B, with only water, and were held there for another 20 minutes. After conditioning on both sides, the animals were taken back to the maintenance tanks. Both the water and the ethanol solution were exchanged for each animal.

Determining the post-conditioning (PC) preference - The PC preference was determined following the same procedures described for establishing the B preference. The evaluation was performed 24 hours after the conditioning period to ensure the elimination of any residual effects of the drug administered on the previous day.

Determining the preference after withdrawal (AW) - Following determination of the PC preference, the animals were kept in the maintenance tanks without ethanol for 16 days. At the end of this period, the post-withdrawal (AW) preference was established following the procedures previously described.

4.2.2. Behavioral Assessment and Phenotyping

At the end of all phases of the CPP test, the animals were classified according to the individual preference for ethanol based on the video recording. The initial two minutes of filming were disregarded, and the next five minutes were used for analysis in the behavioral software EthoVision XT 12 (Noldus - Wageningen, Netherlands) [53]. This software generated data of average speed and total time always spent by each animal on the white and dotted sides of the tank (in B, PC, and AW tests). The preference results were expressed as the percentage of time spent on the side opposite to that of preference B (the side on which they were exposed to the ethanol). Percentages of time on the conditioning side that were higher than and statistically different from the hypothetical value of 50.1% were considered as a

preference for ethanol. Values below and different from the threshold were considered as an aversion for the compartment. Animals with freezing behavior were excluded from all analyses.

4.3 DNA and RNA extraction

After the AW test animals were euthanized with an overdose of the anesthetic benzocaine (ethyl p-aminobenzoate, 250 mg/L) [54]. The gills, liver, and brain were dissected, washed in phosphate buffered saline (PBS) and stored at -80°C. DNA was extracted from the tissues using the Direct-Zol DNA / RNA Kit (Zymo Research São Paulo, Brazil), according to the manufacturer's instructions. This kit allows the extraction of DNA and RNA from the same sample and free of contaminants. The quantification was performed on the DeNovix DS-11 (DeNovix, Delaware, USA).

4.4 Telomere length

64ng of DNA were used to perform the qPCR, using CFX 96TM Real Time system (BioRad) and QuantiNova SYBR Green RT-PCR Kit (Qiagen, São Paulo, Brazil). PCR amplification was performed using the following protocol: 95 ℃ for 15 minutes, followed by 40 cycles at 95 ℃ for 15 seconds and 54 ℃ for 2 minutes. The fluorescence quantification was done during the last stage of the cycle (54 ℃). The negative control, without the template DNA, was tested in all reactions. The analysis of real-time PCR data was calculated by the Ct delta-delta method using a reference gene (*rps11* - ribosomal protein S11) for normalization. For telomeres length we used the primers sequences: 5'GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT3' (Forward primer), and 5'TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA3' (Reverse primer) [39]. For *rps11* we used the sequences: 5'CTCTGACGACACTGCCTTATG3' (Forward primer) and 5'GAAGATGGTGGGCTGTTTCT3' (Reverse primer) [This study].

4.5 Primer design, Reverse transcription, and qPCR

Primers were designed as described in Martins e collaborators [55]. The sequences used are available in Table 3. For each sample, 400 ng of total mRNA were used for reverse transcription using oligo (dT20), primers (Prodimol Biotecnologia, Belo Horizonte, Brazil)

and Revertaid® (Thermo, São Paulo, Brazil) according to the manufacturer's instructions. Target gene transcripts were quantified by qPCR using the CFX 96TM Real Time system (BioRad) and QuantiNova SYBR Green RT-PCR Kit (Qiagen, São Paulo, Brazil). PCR amplification was performed without the extension step (95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds and 60 °C for 10 seconds). The fluorescence quantification was done during the last stage of the cycle (60 °C). A negative control without a sample (NTC) was tested in all reactions. The qPCR data were analyzed by the Ct delta-delta method using the reference gene *eef1a1a* (eukaryotic translation elongation factor 1 alpha 1a) for normalization. The relative amount of mRNA of the genes of interest was calculated as described by Vandesompele [56].

Table 3. Sequence of the qPCR primers and respective amplicon sizes.

Gene symbol	Gene description	Primer Forward (5'-3')	Primer Reverse (5'-3')	Amplicon (pb)
eef1a1a	*Eukaryotic translation elongation factor 1 alpha 1a*	CTACTCTTCTTGATGCCCTTGAT	TGTCTCCAGCCACATTACCAC	309
bax	*BCL2 associated X, apoptosis regulator a*	GGAGATGAGCTGGATGGAAATG	GGCGACAGGCAAAGTAGAAA	160
bcl2	*BCL2 apoptosis regulator a*	CTGGATGACTGACTACCTGAAC	CAGGCAGAGAAAGTGGAATAGA	242

4.6 Statistical Analysis

All data were analyzed for normality by the Shapiro-Wilk test. To determine the existence of preference i.e. if the mean time spent on the conditioning side was different from the hypothetical threshold of 50.1%, the one-sample t-test was performed (GraphPad Prism version 7). The one-way ANOVA test (GraphPad Prism version 7) was used to analyze the molecular data, Pearson's correlation coefficients were calculated to evaluate the association between telomere length and *bax*/*bcl2* ratio. The results were expressed as the mean and standard error of the mean (± SEM). A p value < 0.05 was considered significant for all tests.

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

All fish were kept following the welfare parameters for the species and the protocols were conducted according to the rules of the Ethics Committee on the Use of Animals (Comitê de Ética no Uso de Animais) of the Federal University of Minas Gerais, Minas Gerais, Brazil (Protocol number 64/2016).

Author contributions

All authors contributed to the study conception and design. The CPP test was performed by IBM and IMP. Material preparation and data collection were performed by IBM. The molecular analysis was performed by IBM, BMS and REMJ. The first draft of the manuscript was written by IBM and all authors commented on previous versions of the manuscript. LDSM commented on previous versions of the manuscript. ALBG Conceptualization, Project administration, Resources, Funding acquisition, Supervision, Writing - review and editing.

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CAPÍTULO 2 – Avaliação da atividade das enzimas antioxidantes e do número de cópias de DNA mitocondrial (mtDNA) nos fenótipos de preferência pelo etanol.

Artigo Submetido

Acute ethanol exposure increases antioxidant enzymes activity and mtDNA copy number in zebrafish preference phenotypes

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Abstract

Ethanol can cause a significant increase in reactive oxygen species and an imbalance in antioxidants enzymes, which contributes to oxidative stress and behavioral changes. Mitochondria is susceptible to oxidative stress, to compensate there is an increase in mitochondrial biogenesis. We aimed to validate the protocol of ethanol preference phenotypes in adult zebrafish; evaluate the antioxidant enzymes activity and mtDNA copy number. The ethanol preference was define using the behavioral Conditioned Place Preference paradigm. To validate the protocol, the transcriptional regulation of ethanol targets receptors in brain was evaluated (*drd1, drd2, grin1a, gria2a, gabbr1b* and *lrrk2).* The total antioxidant potential was measured by FRAP, SOD, CAT and GPx activities were performed in gills, liver and brain. mtDNA copy number was measured by qPCR. Four phenotypes were observed: Light, Heavy, Inflexible and Negative Reinforcement. The transcripts of ethanol target receptors in brain showed the same regulation described before for juveniles animais. A decrease in total antioxidant potential was showed in the gills and brain of ethanol preference phenotypes, as in the liver of Negative Reinforcement. Gills showed increased SOD and CAT activities while GPx was reduced for the Negative Reinforcement. SOD activity also increased in gills of the Heavy. In the liver of Inflexible SOD and CAT increased and the Negative Reinforcement showed increased GPx. In brain there was increased SOD, CAT and GPx activities in the Negative Reinforcement while in the Heavy and Inflexible SOD/GPx and SOD/CAT increased, respectively. Was also evidenced an increased mtDNA copy number in gills and liver of Inflexible, as in brain of Heavy. Considering these results, we can suggest an association between the increased antioxidant enzymes activity and ethanol preference phenotypes in adult zebrafish. Our results also indicate that ethanol exposure may be related to the increase in mitochondrial biogenesis in Inflexible and Heavy.

Keywords: alcohol abuse, CPP test, *Danio rerio*, behavior, antioxidants, mtDNA copy number

1. Introduction

Alcohol drinking is probably the oldest social habit in the world, being present in many cultures (Jastrzabska et al., 2016). Excessive alcohol consumption has adverse consequences, including physical, psychological, and social problems (Edenberg and Foroud, 2014). In addition to the harmful effects, ethanol and their first metabolite acetaldehyde (Smith et al., 1997) can cause a significant increase in reactive oxygen species (ROS), which contributes to oxidative stress (Albano, 2006) in the liver (Ceni et al., 2014), brain (Cui et al., 2019) and other tissues (Scott et al., 2000; Tamura et al., 2014).

Oxidative stress is described as an imbalance between the levels of ROS and the inadequate availability of the antioxidants (Sies and Jones, 2007). The antioxidants substances are divided into non-enzymatic, like reduced glutathione (GSH), metal-binding proteins, uric acid, melatonin, bilirubin, polyamines, and coenzyme Q, (Mirończuk-Chodakowska et al., 2018) and enzymatic. There are several enzymes associated with antioxidant defense and repair mechanisms against oxidative stress, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Superoxide dismutases (SODs) are the major antioxidant defense systems against superoxide anion, which consist of three isoforms of SOD in mammals: the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3) (Fukai and Ushio-Fukai, 2011). CAT is a key enzyme in the metabolism of hydrogen peroxide and reactive nitrogen species, and is mainly located in peroxisomes (Glorieux and Calderon, 2017). In the brain, CAT oxidizes ethanol in acetaldehyde and has been associate with ethanol consumption (Gill et al., 1996). GPx is a family of multiple isozymes that catalyze the reduction of hydrogen peroxide to water using GSH as an electron donor (Margis et al., 2008), and is the major antioxidant system of the brain (Teixeira et al., 2017).

Mitochondria dysfunction are intimately involved in the generation of oxidative stress (Hoek et al., 2002), producing indiscriminate amounts of ROS that may lead to oxidative damage to cellular constituents (Gonzalez-Ebsen et al., 2017). Measurement of mitochondrial DNA (mtDNA) content has been proposed as a biomarker for mitochondrial dysfunctions studies, using real time quantitative PCR (qPCR) (Malik and Czajka, 2013). There is evidences of changes in mtDNA copy number in human leukocytes and lymphocytes under oxidative stress conditions (Chan et al., 2013; Liu et al., 2003), and higher mtDNA copy numbers were seen in individuals with major depression, depressive disorders, and anxiety disorders (Tyrka et al., 2016). Further, high mtDNA copy number was

evidenced in blood of people with head and neck squamous cell carcinoma in association with smoke and smokeless tobacco, betel quid chewing, and alcohol (Kumar et al., 2017).

Ethanol abuse promotes alterations in antioxidants enzymes activities contributing to oxidative stress, cellular disturbances and damage (Koch et al., 2004; Oyenihi et al., 2016). Oxidative stress and mitochondrial dysfunctions are associated with alcoholic liver disease (Bailey and Cunningham, 2002; Li et al., 2015) that includes hepatic steatosis, hepatitis and cirrhosis, which may lead to the development of hepatocellular carcinoma (Cichoz-Lach and Michalak, 2014). In brain, ROS accumulation triggering alterations in transduction signaling pathways (Halliwell, 2006), a variety of molecular cascades that increase blood-brain barrier permeability, causing neuroinflammation (Sajja et al., 2016), cell disturbance and death (Flora et al., 2012). In addition to cellular and molecular damage, oxidative stress is associated to ethanol-induced aggressive and suicidal behavior (Tobore, 2019), drug abuse (Riezzo et al., 2012; Yamamoto and Raudensky, 2008), food addiction (Tobore, 2020), and neuropsychiatric disorders such as Alzheimer's (Tönnies and Trushina, 2017), schizophrenia (Barron et al., 2017), depression (Gorlova et al., 2019), and anxiety (Smaga et al., 2015). In brain, oxidative stress induced by alcohol abuse is related to behavioral changes associated to addiction (Yedy Israel et al., 2019). In zebrafish, ethanol altered social behavior, fear response, and exploration (Gerlai et al., 2006; Heloysa et al., 2018), also promotes oxidative stress in the brain (Agostini et al., 2018; Rosemberg et al., 2010) and liver (Howarth et al., 2011; Tsedensodnom et al., 2013). The gills are responsible for gas exchange and plays a dominant role in osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous wastes (Evans et al., 2005), also are important for the absorption of ethanol (Tran et al., 2016) and is an essential target for toxicity and pathology studies in fish (Evans, 1987). However, information about the effects of alcohol in this structure is scarce.

Studies of reward, learning, memory, aggression, anxiety, and sleep behavior strongly suggest that conserved regulatory processes underlie behavior in zebrafish and mammals (Norton and Bally-Cuif, 2010). Furthermore, zebrafish develops behavior patterns (da Silva Chaves et al., 2018; Mathur et al., 2011; Paiva et al., 2020b), synaptic modulations (Klee et al., 2012) and biochemical changes (Müller et al., 2017) that provide a basis for understanding cellular alterations in the context of addiction. In zebrafish, Mocelin et al (Mocelin et al., 2018) showed a protective association of N-acetylcysteine (a GSH precursor with potent antioxidant properties) in both the behavioral alterations and the oxidative stress observed in animals exposed to ethanol. Another study demonstrated an association of

anxiety-like behavior in zebrafish populations and changes in oxidative stress-related parameters in brain, such as increased CAT and glutathione S-transferase (GST) activities (Quadros et al., 2019). Considering the evidences of a relationship between the imbalance in anitoxidant status and behavior modulations, we proposed this study to evaluate the antioxidant enzymes activity using a model of ethanol preference.

Our research group developed a model of ethanol preference in juvenile zebrafish describing four phenotypes regarding the ethanol preference – Light, Heavy, Inflexible and Negative Reinforcement – and identified patterns of differential gene regulation in these groups associated to ethanol abuse (Paiva et al., 2020a). In the present work, we aimed to: validate this same protocol for adult zebrafish; and characterize the phenotypes of ethanol preference in adult zebrafish in a biochemical and molecular context of antioxidant enzymes activity and mitochondrial biogenesis. Considering the behavioral Conditioned Place Preference (CPP) test paradigm, we identified the same four phenotypes of ethanol preference and the same transcriptional regulation of ethanol targets receptors in brain (*drd1, drd2, grin1a, gria2a, gabbr1b* and *lrrk2)* of adult zebrafish. Besides that, we evidenced an increased antioxidant enzymes activities in the gills, liver, and brain of Heavy, Inflexible and Negative Reinforcement phenotypes. We also evidenced an increased mtDNA copy number in the gills and liver of Inflexible phenotype, as in the brain of Heavy animals. Considering these results, we can suggest a relationship between the increased antioxidant enzymes activities and increased mtDNA copy number, induced by acute ethanol exposure, and the ethanol preference behavioral in adult zebrafish.

2. Methods

2.1 Animals and experimental conditions

Two experiments were carried out. The first was used to validate the protocol described by Paiva for juveniles (Paiva et al., 2020a), in adult animals. The second experiment was conducted to perform the analysis of the antioxidant enzymes activities and evaluation of mtDNA copy number in the ethanol preference phenotypes. For the Experiment 1, were used eighty-five wild type zebrafish (*Danio rerio*) *short-fin* of heterogeneous background and of both sexes obtained from Ecofish (Minas Gerais, Brazil). Animals were 4-5 months old and presented an average weight of 0.39 grams. For the Experiment 2, were used one hundred and forty wild type adult zebrafish obtained from the

Aquatic Animal Housing of Universidade Federal de Lavras (Minas Gerais, Brazil), presented an average weight of 0.387 grams. For the both experiments, the animals were housed in an automated system (Rack Hydrus, model ZEB-40-Alesco), in polycarbonate aquariums with 2.5L capacity (11.5 x 34.5 x 15.5 cm), containing a recirculation system, under a 14:10 hour photoperiod (light: dark), the temperature maintained was 28°C ± 1ºC and pH 7.0. All animals were fed three times a day, twice (once at 8:00 and 16:00) with commercial alcon BASIC® as flake fish food (Alcon, Brazil), and once at 11:00 with live food (artemia). Rearing and welfare conditions were in accordance with the standards for the species. The protocol was approved by the Ethics Committee of CEUA-UFMG (Protocol n°64/2016, UFMG, Minas Gerais, Brazil).

2.2 Experimental design

The two experiments was conducted according to the protocol described by Paiva and colleagues (Paiva et al., 2020a), as shown in Fig. 1. On Experiment 1, seventy-five animals were used to define the ethanol preference phenotypes and ten as Control group, without drug exposure. For the Experiment 2, one hundred and twenty animals were used to define the ethanol preference phenotypes and twenty animals were designated as a Control group. The animals were individualized and acclimated for seven days before the beginning of the experiment and were kept in the rack (ALESCO® Indústria e Comércio Ltda, Brazil).

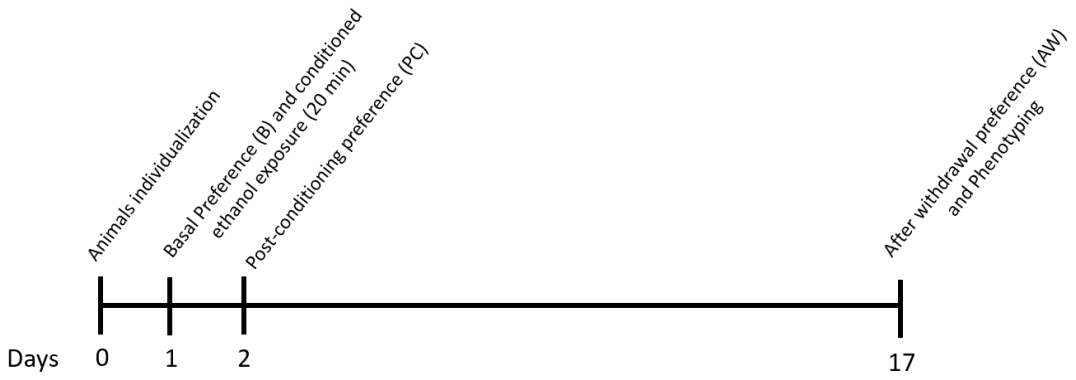

Fig. 1 Experimental design. Acclimation: animals were randomly divided for ethanol preference phenotyping and Control group. The fish were maintained in the experimental environment for seven days for adaptation. After this period, on the experimental day 1, we began the CPP test, which lasted 16 days. For this test, the animals of both groups were individualized. Preferences were determined

by analyses of filming records (10 min each) from three moments: Basal (B), Post-Conditioning (PC), and After Withdrawal (AW). Preference in B was measured to establish the intrinsic preference of each animal for the different compartments of the tank (white and dotted) in the absence of the drug. Following determination of the preference B, each animal of the Acute group was exposed to ethanol conditioning (1% v/v) on the least preferred side for 20 min, followed by another period of 20 min on the preferred side containing only water. During this phase, the animals of the Control group were similarly conditioned on both sides of the tank, but in the absence of ethanol. Twenty-four hours after the conditioned exposure, the PC preference was determined and, after 16 days (on day 17), we determined the AW preference. Later on the same day, the animals were classified into Light, Heavy, Inflexible, and Negative Reinforcement, based on data from the three preference determination time points (B, PC, and AW). The animals were, then, euthanized and their tissues were collected for analysis (Modified from Paiva et al (2020a)).

2.2.1 Conditioned Place Preference (CPP)

To determine the ethanol preference phenotypes, we used the protocol described (Paiva et al., 2020a). The preference was evaluated in three moments: basal (B), post-conditioning (PC), and after withdrawal (AW). We used 5L (30 x 15 x 12 cm) experimental tanks with two patterns of texture in the bottom, i. e., half of the tank had a plain white bottom and the other half had a white bottom with evenly distributed black circles (environmental clue) (Mathur et al., 2011). The experimental tanks were separated from each other by isolators to prevent the animals from seeing the neighboring tank.

Determining the basal (B) preference - The previously individualized animals were carefully transported to the behavioral evaluation room (with controlled lighting, temperature, and noise conditions) and transferred to the experimental tanks being careful to minimize the stress due to the transference and hypoxia. Then, in the absence of ethanol, the animals were filmed for ten minutes to determine the preference. The basal (B) preference was defined as the side of the tank that the animal explored for longer (%).

Conditioned exposure to the ethanol – Following determination of the B preference, the animals were placed in the conditioning tank, on the side opposite to the preference. These tanks were identical to those used to determine the preference, except for the presence of a sealed central divider to prevent the passage of water or drug, thus avoiding cross-contamination effects. On the least-preferred side, each was exposed to ethanol (Merck, Damstadt, Alemanha) at a concentration of 1% (v/v) for 20 minutes. Then they were removed from that compartment, left in a beaker with water for 5 minutes to remove ethanol

excess. Next, they were placed in the compartment corresponding to the preferred side in B, with only water, and were held there for another 20 minutes. After conditioning on both sides, the animals were taken back to the maintenance tanks. Both the water and the ethanol solution were exchanged for each animal.

Determining the post-conditioning (PC) preference - The PC preference was determined following the same procedures described for establishing the B preference. The evaluation was performed 24 hours after the conditioning period to ensure the elimination of any residual effects of the drug administered on the previous day.

Determining the preference after withdrawal (AW) - Following determination of the PC preference, the animals were kept in the maintenance tanks without ethanol for 16 days. At the end of this period, the post-withdrawal (AW) preference was established following the procedures previously described.

2.2.2 Behavioral Assessment and Phenotyping

At the end of all phases of the CPP test, the animals were classified according to the individual preference for ethanol based on the video recording. The initial two minutes of filming were disregarded, and the next five minutes were used for analysis in the behavioral software EthoVision XT 12 (Noldus - Wageningen, Netherlands). This software generated data of average speed and total time spent by each animal on the white and dotted sides of the tank at all times (B, PC, and AW). The preference results were expressed as the percentage of time spent on the side opposite to that of preference B (the side on which they were exposed to the ethanol). Percentages of time on the conditioning side that were higher than and statistically different from the hypothetical value of 50.1% were considered as a preference for ethanol. Values below and different from the threshold were considered as an aversion for the compartment. Animals with freezing behavior were excluded from all analyses.

2.3 Total RNA extraction

After the AW test, the animals of Experiment 1 were euthanized with an overdose of the anesthetic benzocaine (ethyl p-aminobenzoate, 250 mg/L) (Ross et al., 2009). The brains were dissected, immersed on a phosphate buffered saline solution (PBS), frozen in liquid nitrogen, and stored at −80 °C. Total mRNA was extracted using ReliaPrep™ RNA Miniprep Systems (Promega, Fitchburg, USA) according to the manufacturer's instructions. Samples were quantified using the DeNovix DS-11 (DeNovix, Delaware, EUA). All samples showed purity ratios of 260/280 and 230/ 260 between 1.8 and 2.2.

2.4 Primer design, Reverse transcription, and qPCR

Primers were designed as described in Martins de Carvalho et al. (2019). The sequences used are available in Table 1. For each sample of Experiment 1, 800 ng of total mRNA were used for reverse transcription using oligo (dT20), primers (Prodimol Biotecnologia, Belo Horizonte, Brazil), dNTP mix (10 mM), Reaction Buffer 5X (Thermo Fisher Scientific, São Paulo, Brazil), Ribolock RNase Inhibitor (Thermo Fisher Scientific), and Revertaid® Reverse Transcriptase (Thermo Fisher Scientific, São Paulo, Brazil) according to the manufacturer's instructions. Target gene transcripts were quantified by qPCR using the CFX 96TM Real Time system (BioRad) and Kapa SYBR Fast qPCR Kit Master Mix (Kapa Biosystems, São Paulo, Brazil). Amplification was conducted without the extension step (95 °C for 3 min, followed by 40 cycles at 95 °C for 3 s and 60 °C for 20 s). Fluorescence quantification was performed during the last step of the cycle (60 °C). A negative control without a sample (NTC) was tested in all reactions. The qPCR data were analyzed by the Ct delta-delta method using the geometric mean of the reference genes *eef1a1a* (eukaryotic translation elongation factor 1 alpha 1a) and *rpl13* (ribosomal protein L13) for normalization. Stability of the reference genes was confirmed using the algorithms BestKeeper and Genorm (Pfaffl et al., 2004; Wan et al., 2010) . The relative amount of mRNA of the genes of interest was calculated as described by Vandesompele et al. (2002).

2.5 Tissue preparation for biochemical and molecular analysis

After the AW test, the animals of Experiment 2 were euthanized with an overdose of the anesthetic benzocaine (ethyl p-aminobenzoate, 250 mg/L) (Ross et al., 2009). Animals of each phenotype and control group were randomly divided for antioxidant enzymes

activities or mtDNA copy number analysis. The gills, liver, and brain were dissected, washed in phosphate buffered saline (PBS) and stored at -80°C.

2.6 Antioxidant defenses assays for FRAP, SOD, CAT and GPx activities

Tissue homogenization - Tissue samples were homogenized on ice in 200uL of phosphate homogenization buffer (pH 7.4). The homogenate was centrifuged at 800×g for 10 min at 4°C and proteins concentrations were then measured by Qubit Protein Assay (Invitrogen, Brazil) in Qubit 2.0 Fluorometer, following the manufactory instructions.

Total antioxidant capacity – Total antioxidant capacity was evaluated by the ferric reducing antioxidant power (FRAP) method (Benzie and Strain, 1996). The antioxidants present in the samples reduce Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-tris(2-pyridyl)-striazine (TPTZ) to form a Fe^{2+}−TPTZ complex. The tissue homogenates were incubated with the FRAP reagent (0.3 M sodium acetate buffer - pH 3.6 -, 10 mM TPTZ, and 20 mM ferric chloride) at 37 °C for 6 min. The absorbance was measured at 593 nm. Antioxidant capacity was determined using a standard curve with Trolox.

Superoxide Dismutase activity - SOD activity was assessed by the inhibition of autoxidation of pyrogallol by SOD present in the tissue samples (MARKLUND and MARKLUND, 1974). The homogenates were mixed with 50 mM Tris-HCl buffer (pH 8.2) containing EDTA (1mM), catalase (80 U/mL), and pyrogallol (24 mM), and the kinetic assay was monitored during 10 min at 420 nm.

Catalase – CAT activity was determined by the method described by Aebi (Aebi, 1984). CAT activity evaluation was based upon hydrogen peroxide decomposition by CAT presents in the samples. The homogenates were incubated with 10% Triton X-100 and mixed with 10 mM potassium phosphate buffer, pH 7.0, containing 0.2% hydrogen peroxide. The hydrogen peroxide decomposition was monitored at 240 nm for 10 min.

Glutathione Peroxidase activity - GPx dismutes the t-butyl hydroperoxide (t-BuOOH) of the test, generating a disulfide bridge between two GSH (GS-GS) which returns to the reduced state (2 GSH), by the action of Glutathione Reductase (GR). GR acts by oxidizing NADPH. Thus, the test is a measure that consists of recording the decrease in NADPH (Flohé and Günzler, 1984). The tissue homogenates were mixed with Buffer GPx (100 mM

potassium phosphate buffer and 1mM EDTA, pH 7.7), 40 mM Sodium azide, GSH, GR, NADPH and Tert-butyl. The kinetic assay was monitored for 10 min at 340 nm.

2.7 DNA extraction

DNA was extracted from the tissues samples of Experiment 2 using the Direct-Zol DNA / RNA Kit (Zymo Research São Paulo, Brazil), according to the manufacturer's instructions. This kit allows the extraction of DNA and RNA from the same sample and free of contaminants. The quantification was performed on the DeNovix DS-11 (DeNovix, Delaware, USA).

2.8 mtDNA copy number

For mitochondrial DNA copy number analyses, 10ng of DNA were used to perform the qPCR, using the CFX 96TM Real Time system (BioRad) and QuantiNova SYBR Green RT-PCR Kit (Qiagen, São Paulo, Brazil). PCR amplification was performed without the extension step (95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds and 60 °C for 10 seconds). The fluorescence quantification was done during the last stage of the cycle (60 °C). The negative control, without the template DNA, was tested in all reactions. The analysis of the real-time PCR data was calculated by the Ct delta-delta method using a nuclear reference gene (*rps11* - ribosomal protein S11) for normalization. The primers were designed considering the sequence of mitochondrial *cox1* and *atp6* genes and were synthesized by IDT (Integrated DNA Technologies). The sequences are listed in Table 2.

2.9 Statistical Analysis

All data were analyzed for normality by the Shapiro-Wilk test. Using the RStudio statistical package, we performed the principal component analysis (PCA) to distinguish the phenotypes in relation to the ethanol preference of Experiment 1. To determine the existence of preference i.e. if the mean time spent on the conditioning side was different from the hypothetical threshold of 50.1%, the one-sample t-test was performed (GraphPad Prism version 7). The one-way ANOVA test (GraphPad Prism version 7) was used to analyze the antioxidant enzymes activities and molecular data. The results were expressed as the mean

and standard error of the mean (± SEM). A p value < 0.05 was considered significant for all tests.

3. Results

3.1 Determination of the phenotypes based on ethanol preference behavior

Animals from Experiments 1 and 2 subjected to the CPP test were classified into four phenotypes according to their preference for the ethanol: 1) Light - animals that did not prefer the conditioning side at any time (B, PC, and AW); 2) Heavy - animals that preferred the side where the conditioning to the ethanol occurred in PC but lost preference in AW; 3) Inflexible - animals that preferred the conditioning side in both PC and AW; and 4) Negative Reinforcement - animals that did not alter their PC preference in relation to B, but began to seek the conditioning side in AW (Table 3 and Fig. 2). For the Experiment 2, fourteen animals showed freezing behavior and were excluded from phenotype analysis.

We analyzed the behavior results of the two experiments together. In the Control group (with no exposure to the ethanol), no statistical differences were observed compared to the threshold preference in PC or AW (Fig. 2A). The Light phenotype presented a significantly lower mean than the preference threshold (50.1%) in B, PC and AW ($p < 0.0001$ for all) (Fig. 2B). For the Heavy phenotype, we observed a mean value statistically different and higher than 50.1% in PC ($p < 0.0001$) and statistically different and lower than the same threshold in AW ($p < 0.0001$) (Fig. 2C). The Inflexible phenotype showed a mean higher from 50.1% in PC ($p < 0.0001$) and in AW ($p < 0.0001$) (Fig. 2D). Lastly, in Negative Reinforcement the mean was statistically lower and different from 50.1% in PC ($p < 0.0001$) and was higher from the same threshold in AW ($p < 0.0001$) (Fig. 2E).

3.2 Analysis of clustering phenotypes

PCA analysis considering the variance of the preference data and the effects of the determination moments (B, PC, and AW) corroborated the grouping of the animals of Experiment 1 into the four observed phenotypes (Fig. 3).

3.3 Transcriptional regulation of ethanol target receptors in brain

To validate the protocol described before in juveniles, we performed the analyse of the transcripts of ethanol target receptors in adult zebrafish's brain of Experiment 1. Differential regulation was observed between phenotypes for transcript levels of *drd1* ($F(4.28) = 4.316$, $p = 0.0076$) and *drd2* ($F(4, 26) = 19.10$, $p < 0.0001$), from *grin1a* ($F(4.29) = 5.317$, $p = 0.0025$) and *gria2a* ($F(4, 25) = 17.95$, $p < 0.0001$) and also *gabbr1b* ($F(4, 25) = 19.77$, $p < 0.0001$). Post hoc analyzes revealed that *drd1* is hyper-regulated in the Inflexible phenotype in relation to all others (Figure 4A). For *drd2* and *grin1a*, a hyporegulation of all phenotypes was observed in relation to the Control (Figure 4B and C, respectively). *gria2a* and *gabbr1b* were hyper-regulated in all phenotypes also in relation to the Control (Figure 4D and 4E, respectively). The results showed a group-specific effect ($F(4.29) = 3.777$, $p = 0.0136$) on the *lrrk2* transcription, a hyperregulation of this gene was observed in the Inflexible phenotype (Figure 4F).

3.4 Total antioxidant potential in the gills, liver, and brain among ethanol preference phenotypes

For the analysis of antioxidant status, evaluations of the total antioxidant potential were performed using the FRAP method in the gills, liver, and brain of the animals of Experiment 2. In the gills ($F(4,23) = 11.95$) there was a decrease in the antioxidant potential for all groups exposed to ethanol compared to Control: Light ($p = 0.0021$), Heavy ($p = 0.0001$), Inflexible ($p < 0.0001$) and Negative Reinforcement ($p = 0.0002$) (Fig. 5A). In the liver ($F(4,25) = 2.060$), there was a decrease in total antioxidant potential for Negative Reinforcement ($p = 0.0374$) (Fig. 5B) in relation to Ligth group, and in the brain ($F(4,25) = 7.084$) there was a decrease in the total antioxidant capacity for the Heavy ($p = 0.0016$), Inflexible ($p = 0.0167$) and Negative Reinforcement ($p = 0.0028$) phenotypes compared to the Light group (Fig. 5C).

3.5 SOD, CAT and GPx activities in the gills, liver, and brain of ethanol preference phenotypes

The results for SOD activity are shown in Fig. 6. In the gills (Fig. 6A) ($F(4,25) = 19.78$) there was an increase in SOD activity in Negative Reinforcement group compared to Control ($p < 0.0001$) and Light ($p < 0.0001$). In addition, in the gills of Heavy group there

was also an increase in SOD activity compared to Control ($p = 0.0003$) and Light ($p = 0.0004$). The same was found in liver ($F(4,25) = 4.546$) for Inflexible group (Fig. 6B), which showed an increase in SOD activity compared to Control ($p = 0.0244$) and Light ($p = 0.0127$). There was found a significant increased SOD activity in brain ($F(4,24) = 5.716$) of Heavy, Inflexible and Negative Reinforcement groups (Fig. 6C), compared to Light ($p = 0.0084$; $p = 0.0055$; $p = 0.0269$ respectively) and for Heavy ($p = 0.0266$) and Inflexible ($p = 0.0182$) compared to Control group.

As shown in Fig. 7A, Negative Reinforcement group showed an increased CAT activity in gills ($F(4,22) = 3.808$) compared to Control ($p = 0.0073$) and Light ($p = 0.0194$). There was an increased activity of CAT in liver ($F(4,25) = 4.375$) (Fig. 7B) of Inflexible compared to Control ($p = 0.0018$) and Light ($p = 0.0282$) groups. In brain, CAT activity ($F(4,25) = 5.275$) was higher in Inflexible and Negative Reinforcement groups compared to both Control ($p = 0.0176$; $p = 0.055$) and Light ($p = 0.0298$; $p = 0.0096$) (Fig. 7C).

The results of GPx activity for all tissues analysed are presented in Fig. 8. In the gills ($F(4,21) = 5.021$) there is a decrease in GPx activity in the Inflexible group compared to Control group ($p = 0.0234$) (Fig. 8A). In the liver ($F(4,25) = 2.607$), the Negative Reinforcement showed greater GPx activity compared to Control ($p = 0.0291$) and Light ($p = 0.0390$) (Fig. 8B). Finally, in the brain ($F(4,23) = 5.102$) an increase in GPx activity was evidenced in Heavy group compared to Control ($p = 0.0436$) and Negative Reinforcement compared to both Control ($p = 0.0036$) and Light ($p = 0.0485$) (Fig. 8C).

3.6 mtDNA copy number in the gills, liver and brain

The mtDNA copy number was analysed by qPCR, considering the amplification of two mitochondrial genes: *atp6* and *cox1*. The results are shown in Fig 9. As demonstrated in Fig 9D, we found an increased mtDNA copy number in the gills ($F(4, 29) = 3.490$) of Inflexible phenotype compared to Light ($p < 0.03$) considering the gene *cox1*. In the liver (Fig 9B and 9E), the increased of mtDNA was evidenced in Inflexible compared to Control ($p < 0.01$) and Light ($p < 0.02$), and considering both *atp6* ($F(4, 31) = 4.733$) and *cox1* ($F(4, 30) = 4.669$). Finally, an increased mtDNA copy number (*atp6* and *cox1*) ($F(4, 30) = 5.981$; $F(4, 31) = 3.892$) was observed in the brain (Fig 9C and 9F) of Heavy phenotype compared to Control ($p < 0.05$) and Light ($p < 0.005$).

4. Discussion

Our research group has been working with the phenotypic characterization of ethanol preference in juvenile zebrafish (20 days post fertilization (dpf)) (Paiva et al., 2020a). At the present study we were able to validate the protocol in adult zebrafish by showing the same transcriptional regulation of the genes commonly associated with addiction-like phenotypes (*drd1, drd2, grin1a, gria2a, gabbr1b* and *lrrk2*), and we replicate the same phenotypes: Light phenotype - had no influence of ethanol on the behavior modulation in relation to the preferred side. The Heavy phenotype - shows preference for the side exposed to ethanol in the PC test but is no longer preferred in the AW test. Inflexible phenotype - prefers the side exposed to ethanol in the two tests analyzed (PC and AW). Finally, the Negative Reinforcement phenotype - shows preference for ethanol only in the AW test, in other words, after the alcohol withdrawal period there is a change of preference about the side of the aquarium in which the animal was exposed to ethanol.

We analyzed the antioxidant status and mitochondrial biogenesis in the gills, liver, and brain of ethanol preference phenotypes, considering the total antioxidant potential and activities of SOD, CAT, GPx, and the measurement of mtDNA copy number by qPCR. According to our results, a single ethanol exposure increases the antioxidant enzymes activities in the individuals of Heavy, Inflexible and Negative Reinforcement groups. Using the FRAP method, we observed a decrease in total antioxidant potential in the gills and the brain of all ethanol preference phenotypes, as well in the liver of the Negative Reinforcement. Reinforcing our results, other authors have shown decreased antioxidant status in studies of acute ethanol intake in rats (Basarslan et al., 2017; Ozkol et al., 2017). For ethanol preference phenotypes (Heavy, Inflexible and Negative Reinforcement), there was a significant increase in SOD, CAT and GPx activities in the gills, liver, and brain. We found an increased mtDNA copy number in the gills and liver of Inflexible phenotype, and in brain of Heavy animals. Our results are in accordance with other studies that demonstrate a relationship between the occurrence of oxidative stress and behavioral patterns associated to ethanol abuse (Müller et al., 2017; Nkpaa et al., 2019) and to alcohol preferring in mice (Xu et al., 2019). Additionally, higher mtDNA numbers were evidenced in a study of early stress associated with anxiety and substance use disorders (Tyrka et al., 2016).

The harmful effects of ethanol are related to its metabolism and oxidation (Koch et al., 2004). Evidence from previous studies has shown that acetaldehyde, the first ethanol metabolite, is an oxidative stress inducer (Cui et al., 2019; Tamura et al., 2014; Yan et al.,

2016). The oxidation of ethanol to acetaldehyde can occur through pathways that involve CAT, cytochrome CYP2E1, and alcohol dehydrogenase (ADH) (Hernández et al., 2016). The main pathway to metabolize ethanol in the liver involves ADH and CYP2E1 (Ceni et al., 2014), while CAT is particularly important for this process in the brain (Zimatkin et al., 2006). Moreover, associated with SOD and GPx, CAT acts as key enzyme of the cellular antioxidant system (Li et al., 2015). A single ethanol exposure promotes an increase in SOD (Enache et al., 2008) CAT, (Oyenihi et al., 2016; Rosemberg et al., 2010), and GPx (Baliño et al., 2019) activities.

Studies indicates an increase in antioxidant enzymes activities probably as a compensatory regulatory response to oxidative stress (Caixeta et al., 2018; Justino et al., 2017; Moraes et al., 2015), including acute ethanol exposure (Casañas-Sánchez et al., 2016; Schlorff et al., 1999). We found an increase in SOD activity in the gills of Heavy and Negative Reinforcement phenotypes, and in the liver of Inflexible phenotype, as well in the brain of all these phenotypes. An elevated SOD activity in zebrafish gills has been associated to oxidative stress induced by toxic substances (Chen et al., 2012; Paravani et al., 2019). There is scarce evidence on the effects of alcohol in the gills, therefore, our findings are important because they demonstrate the susceptibility of this structure to the drug, associated to ethanol preference phenotypes. We also evidenced an increase in hepatic SOD activity of Inflexible phenotype. Similar results were found by Ignatowicz et al (Ignatowicz et al., 2013), who demonstrated an elevated SOD activity in the liver of alcohol-addicted rats after a single-dose ethanol exposure. These findings reinforce the relationship between the occurrence of oxidative stress in this organ and the profile of ethanol-seeking behavior. Considering our results for brain, there was an increase in SOD activity for Heavy, Inflexible and Negative Reinforcement phenotypes. Ethanol increases superoxide radical generation in the brain (Ribiere et al., 1994) that can be probably related to the increased SOD activity evidenced in our and other studies. In the central nervous system, the same pattern of high SOD activity in hippocampus was found by Enache et al (Enache et al., 2008) in a study with adolescent male rats acutely exposed to ethanol, as in brain from chronic alcoholics (Marklund et al., 1983), and in serum of alcohol-dependent patients (Thome et al., 1997).

There was an increase in CAT activity in the gills of Negative Reinforcement, the same was evidenced in the liver of Inflexible, and in the brain of both phenotypes. Oyenihi et al (Oyenihi et al., 2016) also showed an increased CAT activity in the liver of rats acutely exposed to ethanol. Similar results for CAT activity were found in brain of zebrafish acutely

exposed to ethanol (Rosemberg et al., 2010). High CAT activity in our phenotypes may be associated with an increase in hydrogen peroxide production, associated to ethanol exposure (Hernández et al., 2016). Beyond that, studies investigate the role of CAT in ethanol intake, since in brain CAT oxidize ethanol in acetaldehyde, which, as has been proposed, mediates some behavioral effects produced by ethanol (McBride et al., 2002). Additionally, our results demonstrate a possible association of high CAT activity, induced by the acute ethanol exposure, and the ethanol preference. As shown before, we found an increased activity of CAT in the liver and especially in the brain of Inflexible phenotype. Aragon (Aragon et al., 1985) suggests that cerebral CAT may be involved in the regulation of the affinity of animals to consume ethanol. To support this, it was evidenced a lower ingestion of ethanol in rats treated with a CAT blocker (Mattalloni et al., 2019), showing the importance of this enzyme in ethanol consumption. Interestingly, in a study of ethanol-induced conditioned place preference (CPP) with mice, the authors suggest that catalase–H_2O_2 system may be an important step in the experiencing of the positive affective effects of ethanol regarding contextual cues during the development of CPP (Font et al., 2008).

In the gills of Inflexible phenotype there was a decrease in GPx activity. The same was found in blood of mice exposed to ethanol (Das et al., 2009), what might be explained by elevations in ethanol-induced ROS (Bailey et al., 2001). On the other hand, GPx activity was higher in the liver and the brain of the Negative Reinforcement and in the brain of Heavy phenotype. The same pattern was evidenced in a study with neuronal cell culture that demonstrated an increase in GPx activity 48h after an acute ethanol exposure (Casañas-Sánchez et al., 2016). The increased GPx activity is probably associated with the oxidative damage caused by the ethanol in these individuals, since GPx plays a critical role in protecting cells from damage by ROS, providing a mechanism for peroxide detoxification in living cells (Baliño et al., 2019). In association with abusive ethanol behavior, Wu et al (Wu et al., 2020) observed an increased GPx activity in alcohol use disorders (AUD) patients and suggest that GPx levels might be AUD state biomarker.

The mitochondria play an important role in the context of oxidative stress related to drug addiction (Sadakierska-chudy, 2014) and induced by ethanol (Bailey and Cunningham, 2002; Hoek et al., 2002). The alterations in mtDNA copy number has been proposed as a biomarker of mitochondrial dysfunction (Wang et al., 2017), in this sense, mitochondrial genome to nuclear genome ratio (Mt/N) values would increase as a result of increased mitochondrial biogenesis (Malik and Czajka, 2013). Considering studies of chronic alcohol

consumption, it seems there is no difference in mtDNA copy number of alcoholics individuals (von Wurmb-Schwark et al., 2008; Vyas et al., 2020). However, we found an increased mtDNA copy number in ethanol preference phenotypes, after an acute ethanol exposure, precisely in Heavy and Inflexible groups. Beyond that, we also demonstrated an increase in antioxidant enzymes activities in these same phenotypes. Evidence suggests that during oxidative stress, ROS generation can be considered as a signal to the nucleus to limit cell proliferation through telomere shortening as sensors to damage in the mitochondria (Passos and Zglinicki, 2005). Furthermore, during oxidative stress, the telomerase reverse transcriptase (TERT) component of telomerase have been shown to translocate to the mitochondria (Ahmed et al., 2008; Haendeler et al., 2009), suggesting that TERT may protect mitochondrial function indirectly by regulation of mitochondrial biogenesis (Gonzalez-Ebsen et al., 2017). In the gills and the liver of Inflexible phenotype and in the brain of Heavy, we evidence both, an increased mtDNA copy number andelevated antioxidant enzymes activities. The mechanism related to TERT translocation from nucleus to mitochondria, considering the occurrence of oxidative stress, presents clues that can explain our results for these phenotypes.

In summary, we demonstrated an increased antioxidant enzymes activity in Heavy, Inflexible and Negative Reinforcement phenotypes, as well an increased mtDNA copy number in Heavy and Inflexible. Heavy phenotype showed a decreased antioxidant potential in the gills and the brain, as an increased activity of SOD in the gills and both SOD and GPx in the brain. Heavy also demonstrated an increased mtDNA copy number in the brain. The Inflexible phenotype present a lower antioxidant potential in the gills and brain, a decreased activity of GPx in gills, a high activity of SOD and CAT in both liver and brain, and increased mtDNA copy number in the gills and liver. A decreased antioxidant potential was evident in the gills, liver and brain of Negative Reinforcement phenotype, additionally, an increased activity of SOD and CAT was found in the gills and brain of this group, as well a high activity of GPx in their liver and brain. The 3 phenotypes cited correspond to animals whose shown ethanol preference at least one moment of the CPP test. On the other hand, the Light group was the one without ethanol preference, still, the animals of this phenotype were exposure to ethanol, but we didn't find any alterations in antioxidant status in gills, liver or brain of them. Considering that and the heterogenetic characteristic of the animals used in our experiment, and the evidences discussed before related to the occurrence of oxidative stress and increased mitochondria biogenesis mainly in liver and brain associated to

voluntary ethanol consumption, we suggest an association between the antioxidant imbalance, mitochondria dysfunction, and the ethanol preference phenotypes described by our group.

In conclusion, these results show the individual differences in relation to the voluntary seeking for alcohol, by establishing a phenotypic distinction in relation to ethanol preference, and also demonstrates variations in antioxidant status and mtDNA copy number in specific tissues, considering a single ethanol exposure. We are still trying to understand the mechanisms involved in the loss of control that promotes the abuse of ethanol, and many studies need to be done to better elucidate the vulnerability that some individuals have in relation to the addiction. In this line, we show in a model that is easy to develop, with low maintenance cost, and that presents a great similarity with the results found in murine models and human studies, that there is a differential response in antioxidant enzymes activity and mitochondrial biogenesis, considering the phenotypes. Additionally, the modulation of behavior in the ethanol preference goes beyond a physiological response, and involves patterns of characteristics previously existing, which ethanol triggers modulating the behavior. Furthermore, other studies are necessary to improve our knowledge about these phenotypes and their implications.

Table 1. Oligonucleotides used to validate the protocol of ethanol preference phenotypes in adult zebrafish. Sequence of the qPCR primers and respective amplicon sizes.

Target	Gene description	Primer Forward (5'-3')	Primer Reverse (5'-3')	Amplicon pb
rpl13a	*Ribosomal protein L13*	TCTGGAGGACTGTAAGAGGT	AGACGCACAATCTTGAGAGCAG	148
eef1a1l1	*Eukaryotic translation elongation factor 1 alpha 1a*	CTACTCTTCTTGATGCCCTTGAT	TGTCTCCAGCCACATTACCAC	309
drd1	*Dopamine receptor D1a*	CTCAAATGTGGACTCGGAAAG	GAGAGAAGAGTTAGCCCATCCA	226
drd2	*Dopamine receptor D2a*	ATACTTCCGCTCTTTGGATGAA	ATCAGGTAGTTGGTGGTGGTCT	275
grin1a	*Glutamate receptor, ionotropic, N-methyl Daspartate 1a*	CACCAGGATGTCCATTTATTCA	CCTTAGGTCCTCTCTTGTTGTCA	269
gria2a	*Glutamate receptor, ionotropic, AMPA 2a*	TCACTGTGGAGAGAATGGTGTC	GATAAGCGTATTTGCCTTTGGA	237
gabbr1b	*Gamma-aminobutyric acid (GABA) B receptor, 1b*	AAGCATCACTGAAGGACCATCT	GGGAGACACTTTCTGACCTTTG	306
lrrk2	*Leucine-rich repeat kinase 2*	GATGCTACTGGAAGACCTGCTC	AAGACCCACCAAACTAGGATGA	346

Table 2. Oligonucleotides used to evaluate the mt-DNA copy number by qPCR in gills, liver and brain of adult zebrafish submitted to CPP test.

Target	Gene description	Primer Forward (5'-3')	Primer Reverse (5'-3')	Amplicon pb
rps11	*Ribosomal protein S11*	CTCTGACGACACTGCCTTATG	GAAGATGGTGGGCTGTTTCT	205
cox1	*Cytochrome c oxidase subunit I*	ACCAGGATTCGGCATTATCTC	CTCGGGTGTCTACATCCATTC	164
atp6	*ATP synthase 6*	CCTTATCCTCGTTGCCATACTT	GTTTGTGAATCGTCCAGTCAATC	115

Table 3. Description and number of samples (n) of each phenotypic group distinguished by the CPP test in the Experiments 1 (Exp1) and 2 (Exp2).

Phenotype	Description	Exp1 n	Exp2 n
Light	Animals that did not prefer the ethanol conditioning side in any of the observations.	20	27
Heavy	Animals that preferred the ethanol conditioning side at PC and quit preferring at AW.	20	32
Inflexible	Animals that preferred the ethanol conditioning side at both PC and AW.	20	24
Negative Reinforcement	Animals that began to prefer the ethanol conditioning side only at AW.	15	23

PC = Post-Conditioned Preference. AW = After Withdrawal Preference.

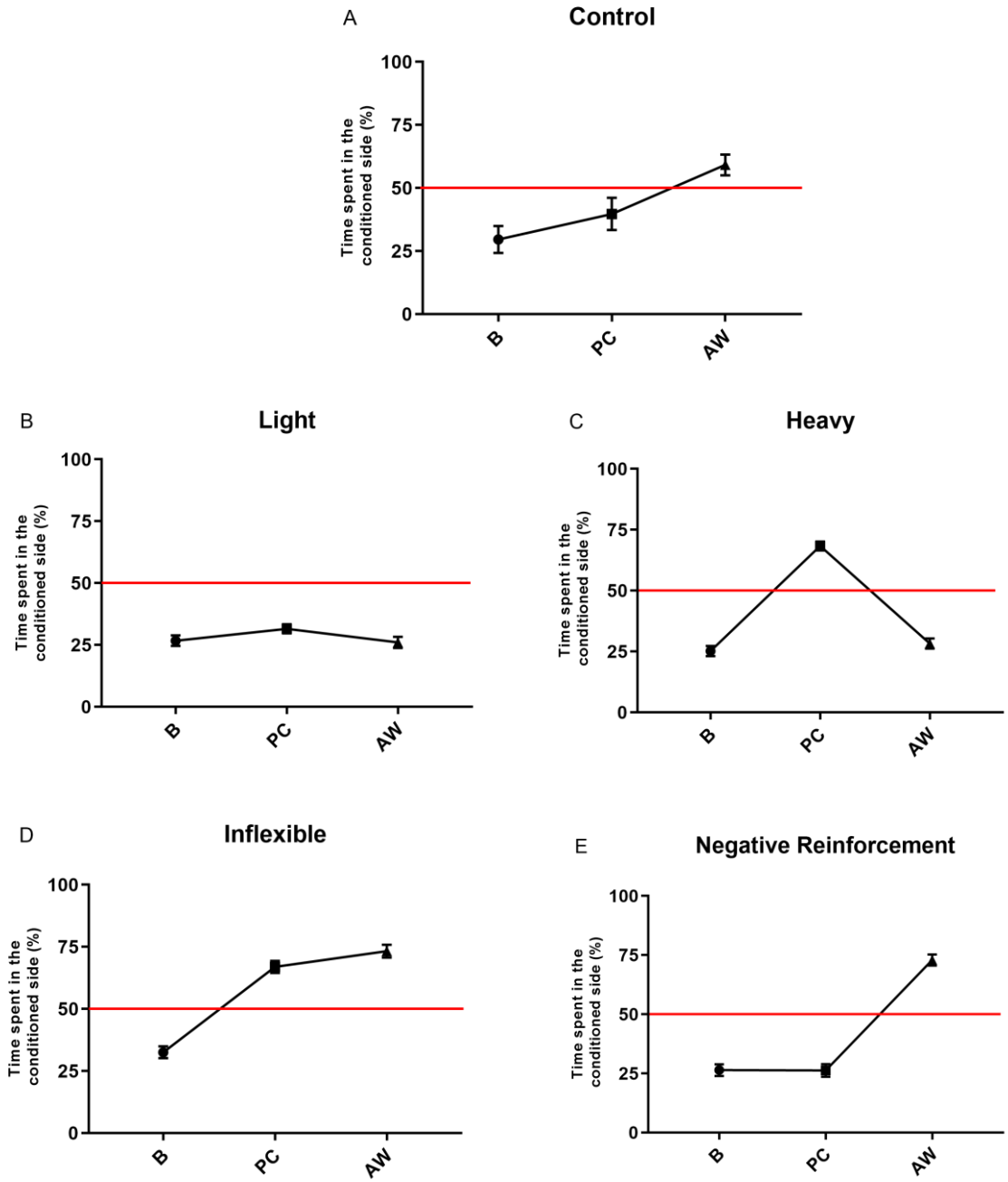

Fig. 2 Ethanol preference determined by the CPP test for each phenotypic group. Data are represented as the percentage of time spent on the ethanol conditioning side during the determination of the preferences B, PC, and AW for each phenotype distinguished after a single ethanol exposure of 20 min. Moments of preference determination: B = Basal, before conditioning; PC = Post-Conditioning, 24h after conditioning; AW = After Withdrawal, 16 days after withdrawal. The preferences were calculated in relation to a hypothetical preference threshold of 50.1% (red line), with mean values higher than and different from 50.1% indicating a preference for the ethanol conditioning side and mean values below this threshold indicating an aversion to the conditioning compartment. The graphs represent the preference changes that characterize each phenotype: (A) In the Control group (n = 20) no statistical differences were observed compared to the threshold

preference in PC or AW. (B) Light phenotype (n = 27) presented a significantly lower mean than the preference threshold (50.1%) in B, PC and AW ($p < 0.0001$ for all). (C) For the Heavy phenotype (n = 32), we observed a mean value statistically different and higher than 50.1% in PC ($p < 0.0001$) and statistically different and lower than the same threshold in AW ($p < 0.0001$). (D) The Inflexible phenotype (n = 24) showed a mean higher from 50.1% in PC ($p < 0.0001$) and in AW ($p < 0.0001$) (Fig. 3D). (E) In Negative Reinforcement (n = 23) the mean was statistically lower and different from 50.1% in PC ($p < 0.0001$) and was higher from the same threshold in AW ($p < 0.0001$). Differences between the means of the groups and the hypothetical value of 50.1% were considered statistically significant when $p < 0.05$. Data are expressed as the mean and standard error of the mean (±SEM).

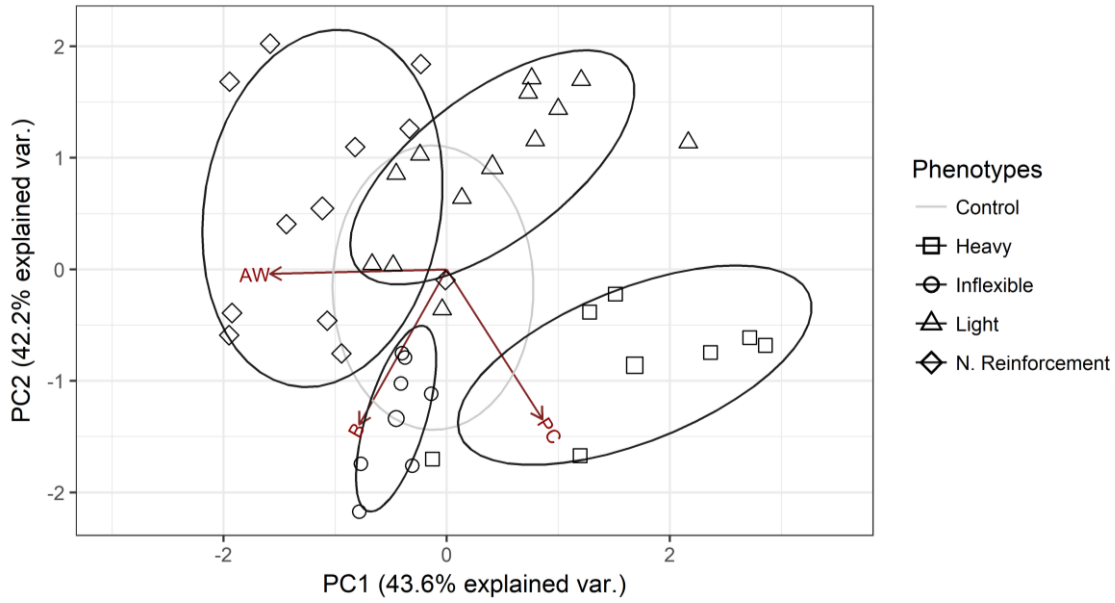

Fig 3. Scree plot showing the distribution of the preference data and its relationship to the moments of preference determination (B, PC and AW). Ellipses indicate grouping of data into four phenotypes (Light, Heavy, Inflexible and Negative Reinforcement) and the arrows indicate the moments of preference determination (B, PC and Aw). The proximity of ellipses (phenotypes) in relation to the arrows indicate which moment best explains the variance of the data that phenotypic category. Width and size of ellipses indicate the variability. The direction of arrows indicates the correlation between the variables. B – Basal Preference. PC – Post-Conditioned Preference. AW – After-Withdrawal Preference.

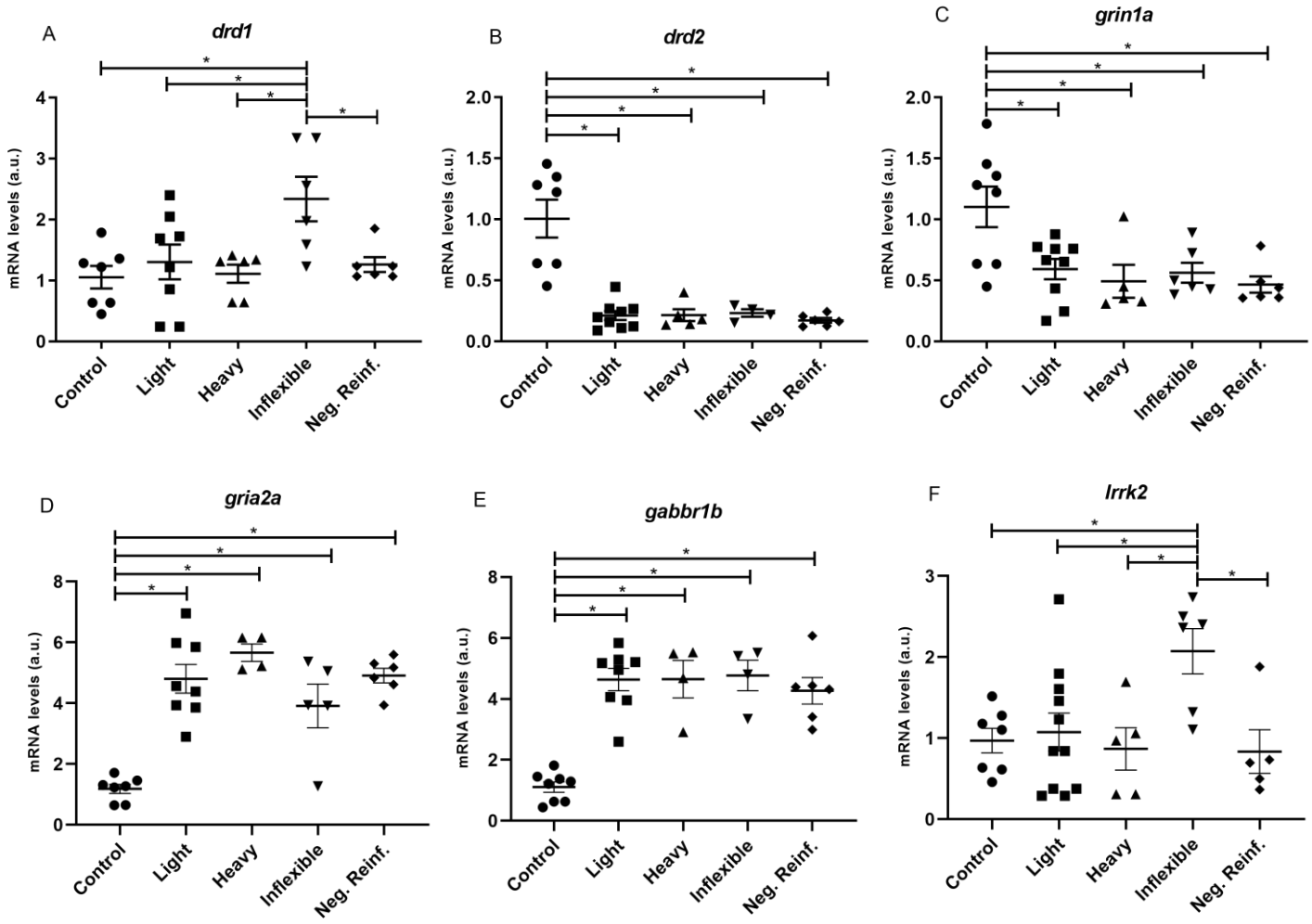

Fig 4. Transcriptional regulation of ethanol targets receptors in brain of the adult zebrafish submitted to CPP test and distinguished in ethanol preference phenotypes. (A) *drd1*. (B) *drd2*. (C) *grin1a.* (D) *gria2a*. (E) *gabbr1b*. (F) *lrrk2.* Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$.

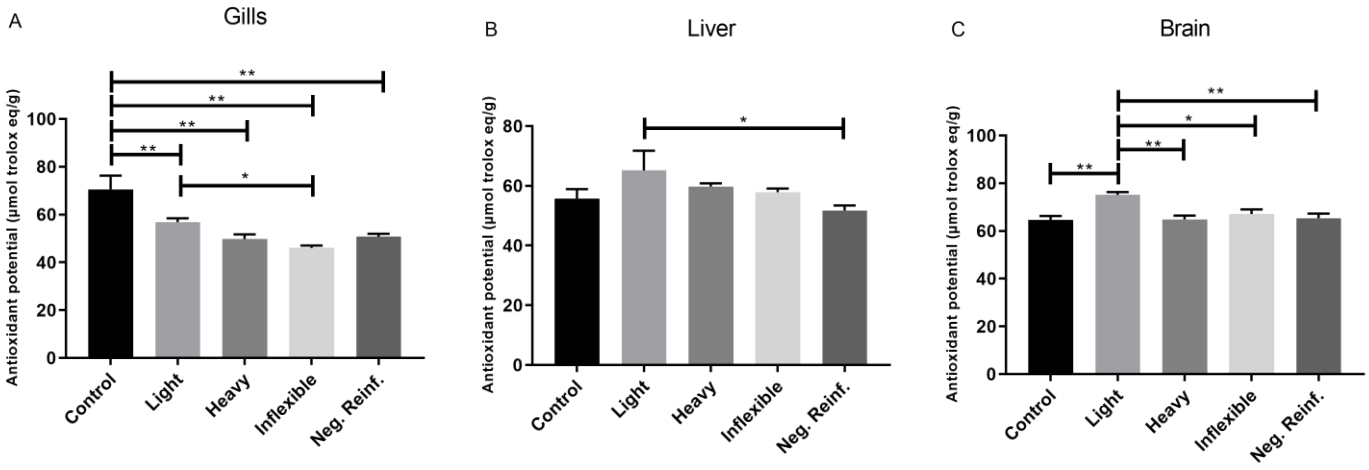

Fig. 5 Total antioxidant potential by the FRAP method in tissues of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference. (A) Gills. (B) Liver. (C) Brain. Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$, ** $p < 0.005$ ($n = 6$).

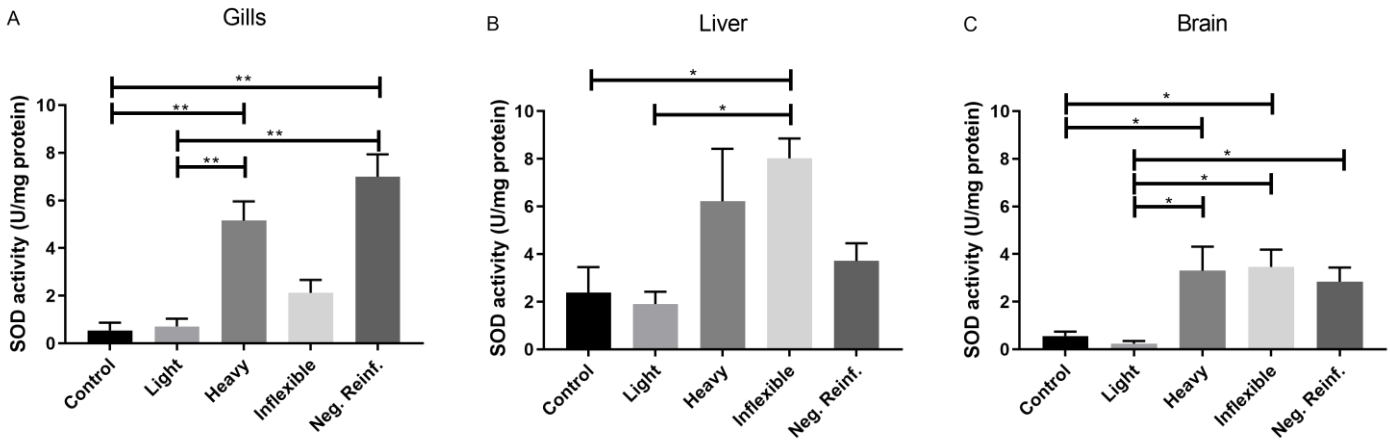

Fig. 6 SOD activity in tissues of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference. (A) Gills. (B) Liver. (C) Brain. Statistical analyses were performed by one-way ANOVA followed by the Tukey's po st-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$. ($n = 7$).

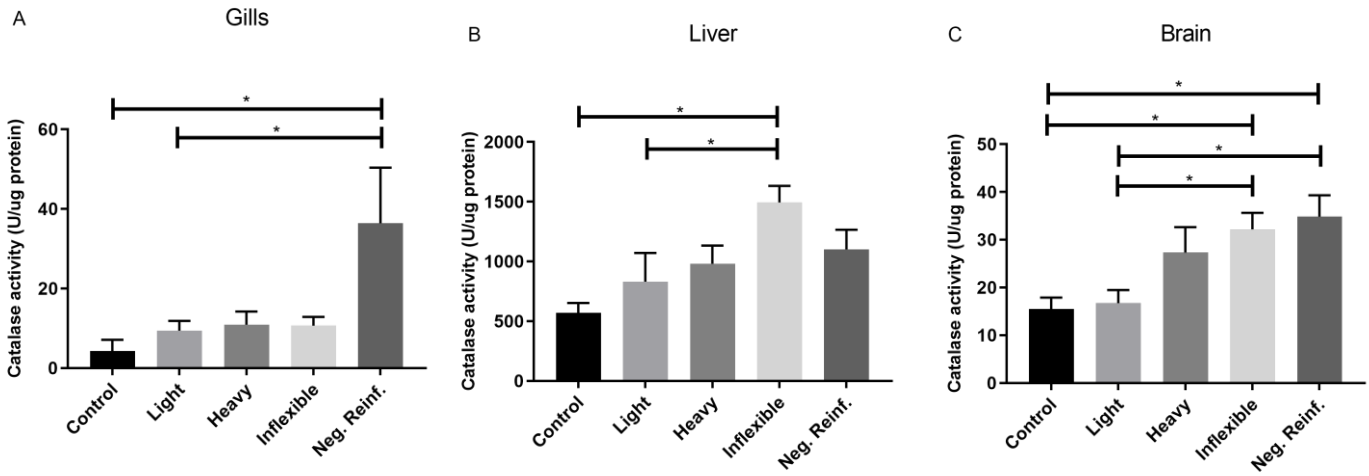

Fig. 7 Results of CAT activity in tissues of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference. (A) Gills. (B) Liver. (C) Brain. Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$.(n = 7).

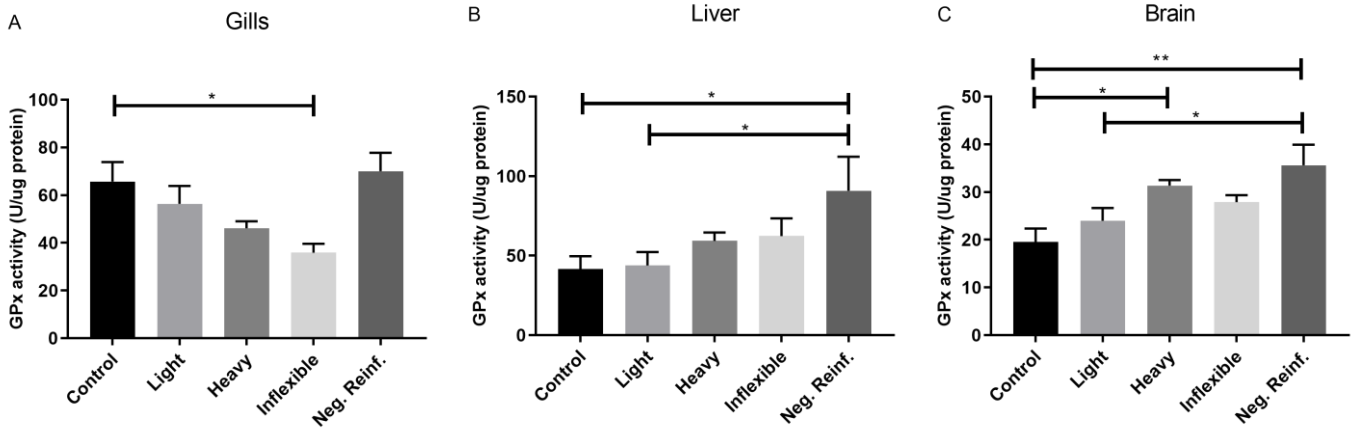

Fig. 8 Results of GPx activity in tissues of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference. (A) Gills. (B) Liver. (C) Brain. Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$, ** $p < 0.005$; (n = 7).

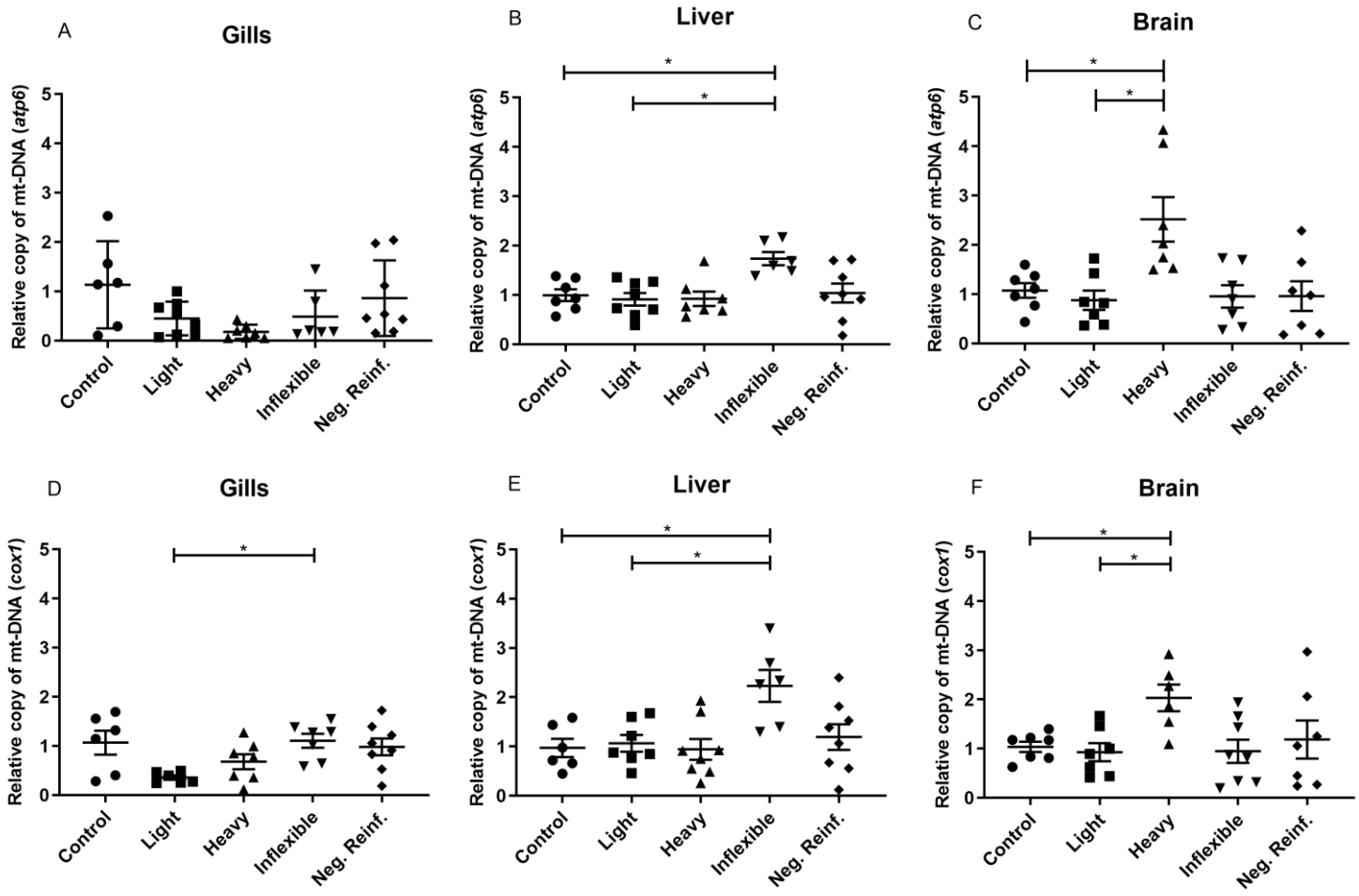

Fig. 9 Relative mtDNA copy number of adult zebrafish submitted to the CPP test and the distinguished in phenotypes of ethanol preference. A-C results for *atp6*. D-F results for *cox1*. Statistical analyses were performed by one-way ANOVA followed by the Tukey's post-hoc test. Data are expressed as the mean and standard error of the mean (± SEM). Statistically significant differences are represented by * $p < 0.05$ ($n = 8$).

Declarations

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

All fish were kept following the welfare parameters for the species and the protocols were conducted according to the rules of the Ethics Committee on the Use of Animals (Comitê de Ética no Uso de Animais) of the Federal University of Minas Gerais, Minas Gerais, Brazil (Protocol number 64/2016).

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4. DISCUSSÃO E CONCLUSÃO

Tabela 1. Síntese dos resultados para os parâmetros avaliados considerando os fenótipos de preferência pelo etanol e o tecido em que as alterações foram observadas.

Alterações/Fenótipos	Leve	Pesado	Inflexível	Reforço Negativo
Encurtamento de telômeros	-	Brânquias Fígado Cérebro	Brânquias Cérebro	Brânquias Fígado Cérebro
bax/bcl2	-	-	-	Cérebro
↓ **FRAP**	-	Brânquias Cérebro	Brânquias Cérebro	Brânquias Fígado Cérebro
↑ **SOD**	-	Brânquias	Fígado Cérebro	Brânquias Cérebro
↑ **CAT**	-	-	Fígado Cérebro	Brânquias Cérebro
↑ **GPx**	-	Cérebro	Brânquias (reduziu)	Fígado Cérebro
↑ **Mt-DNA**	-	Cérebro	Brânquias Fígado	-

O transtorno do uso do álcool é um grave problema de cunho biológico, psicológico e social, que causa uma série de complicações para os indivíduos e a sociedade. Demonstramos que o uso abusivo do etanol promove alterações funcionais e comportamentais a longo prazo. Muitas pesquisas têm sido feitas com o intuito de descrever a relação entre esses mecanismos, no entanto, trata-se de uma condição humana extremamente complexa e que envolve não apenas aspectos biológicos e psicológicos, mas também a história de vida pessoal e o contexto social no qual o indivíduo está inserido. Dessa forma, os modelos de pesquisa conseguem se aproximar das condições humanas, contudo, apresentam limitações.

Embora essas limitações sejam reais, os modelos de pesquisa fornecem pistas para o entendimento de mecanismos de doenças humanas, e, portanto, apresentam grande relevância científica. Conforme descrito anteriormente, o nosso grupo de pesquisa tem trabalhado com a definição de fenótipos em relação à preferência pelo etanol. Considerando o modelo *zebrafish*, foi estabelecido o protocolo de análise de comportamento e avaliação dos transcritos de genes relacionados à adicção, em indivíduos juvenis. Os mesmos parâmetros foram identificados em adultos, contribuindo para a consolidação desse modelo para o estudo de fenótipos de preferência pelo etanol. Dentre os fenótipos, destaca-se o Inflexível, que corresponde aos indivíduos com preferência pelo etanol nos dois momentos analisados, sendo este o nosso fenótipo de maior interesse.

Tendo em vista os resultados apresentados na **Tabela 1**, identificamos um significativo encurtamento dos telômeros nos fenótipos de preferência pelo etanol, dentre eles o Inflexível. Os telômeros apresentam uma importante associação com distúrbios de comportamento incluindo o uso abusivo do etanol, de modo que o estado funcional dos telômeros pode ter efeitos profundos no fenótipo das células e, por extrapolação, nos organismos (RODIER *et al.*, 2005). Evidenciamos nesse mesmo fenótipo um aumento da atividade de enzimas do sistema antioxidante, indicando a possibilidade de ocorrência de estresse oxidativo concomitantemente com a instabilidade genômica. Além disso, os fenótipos Pesado e Reforço Negativo também apresentaram instabilidade genômica e disfunções celulares. Esses dois fenótipos apresentam preferência pelo etanol em um dos momentos do teste (Pesado = no teste Pós-Condicionamento; Reforço Negativo = no teste Após a

Retirada). Entretanto, o fenótipo Leve, surpreendentemente, não apresentou alterações em nenhum dos parâmetros analisados (**Tabela 1**), o que significa que mesmo tendo sido expostos ao etanol, os indivíduos desse grupo não apresentaram instabilidade genômica ou disfunções celulares. Vale ressaltar que os animais foram expostos ao etanol, e após o período de 15 dias foram eutanasiados, ou seja, no momento da coleta dos tecidos o etanol já não estava mais presente no organismo. Dessa forma, estamos avaliando os efeitos a longo prazo da exposição aguda ao etanol. A característica mais importante aqui, é o padrão diferencial de regulação molecular e celular que foram identificados nos fenótipos de preferência pelo etanol, e que não foram evidenciados nos indivíduos do fenótipo Leve, demonstrando que as alterações nesses processos de regulação são desencadeadas pelo etanol, mas também estão relacionados ao fenótipo de preferência.

Considerando os nossos resultados, principalmente no tecido nervoso, os fenótipos de preferência pelo etanol apresentaram redução do tamanho dos telômeros e disfunções celulares, mesmo que tenham sido expostos uma única vez ao etanol e por 20 min. Na maioria das pesquisas realizadas nesse contexto, utilizou-se o sangue periférico (ou seja, leucócitos), de modo que a originalidade do nosso trabalho consiste na apresentação do processo de encurtamento de telômeros em tecidos específicos como o cérebro, fígado e brânquias, e após uma exposição aguda ao etanol. Esses dois parâmetros ainda não tinham sido avaliados dessa forma. Além disso, os nossos resultados referentes às disfunções celulares corroboram com a premissa de que o álcool desencadeia eventos de regulação que se relacionam com o perfil de comportamento individual, sugerindo que as bases moleculares relacionadas ao comportamento de preferência pelo etanol vão além dos seus efeitos nocivos, apresentando uma forte relação com o perfil fenotípico.

CERTIFICADO DO COMITÊ DE ÉTICA

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CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o Protocolo nº. 64 / 2016, relativo ao projeto intitulado "ESTUDO FUNCIONAL DO GENE LRRK2 E DE microRNAs NA REGULAÇÃO E MANUTENÇÃO DO FENÓTIPO DE USO ABUSIVO DE ETANOL NO MODELO DANIO RERIO (ZEBRAFIS", que tem como responsável Ana Lúcia Brunialti Godard, está de acordo com os Princípios Éticos da Experimentação Animal, adotados pela Comissão de Ética no Uso de Animais (CEUA/UFMG), tendo sido aprovado na reunião de 24/05/2016. Este certificado espira-se em 24/05/2021.

CERTIFICATE
We hereby certify that the Protocol nº. 64 / 2016, related to the Project entilted " FUNCTIONAL STUDY OF THE LRRK2 GENE AND microRNAS IN THE REGULATION AND MAINTENANCE OF ETHANOL ABUSE PHENOTYPE OF THE DANIO rerio MODEL (ZEB", under the supervision of Ana Lúcia Brunialti Godard, is in agreement with the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee in Animal Experimentation (CEUA/UFMG), and was approved in 24/05/2016. This certificates expires in 24/05/2021.

Cleuza Maria de Faria Rezende
Coordenador(a) da CEUA/UFMG
Belo Horizonte, 24/05/2016.

Atenciosamente.

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ARTIGOS SUBMETIDOS

Pharmacology, Biochemistry and Behavior

Acute ethanol exposure increases antioxidant enzymes activity and mtDNA copy number in zebrafish preference phenotypes

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Abstract:	Ethanol can cause a significant increase in reactive oxygen species and an imbalance in antioxidants enzymes, which contributes to oxidative stress and behavioral changes. Mitochondria is susceptible to oxidative stress, to compensate there is an increase in mitochondrial biogenesis. We aimed to validate the protocol of ethanol preference phenotypes in adult zebrafish; evaluate the antioxidant enzymes activity and mtDNA copy number . The ethanol preference was define using the behavioral Conditioned Place Preference paradigm. To validate the protocol, the transcriptional regulation of ethanol targets receptors in brain was evaluated (drd1, drd2, grin1a, gria2a, gabbr1b and lrrk2). The total antioxidant potential was measured by FRAP, SOD, CAT and GPx activities were performed in gills, liver and brain. mtDNA copy number was measured by qPCR. Four phenotypes were observed: Light, Heavy, Inflexible and Negative Reinforcement. The transcripts of ethanol target receptors in brain showed the same regulation described before for juveniles animais. A decrease in total antioxidant potential was showed in the gills and brain of ethanol preference phenotypes, as in the liver of Negative Reinforcement. Gills showed increased SOD and CAT activities while GPx was reduced for the Negative Reinforcement. SOD activity also increased in gills of the Heavy. In the liver of Inflexible SOD and CAT increased and the Negative Reinforcement showed increased GPx. In brain there was increased SOD, CAT and GPx activities in the Negative Reinforcement while in the Heavy and Inflexible SOD/GPx and SOD/CAT increased, respectively. Was also evidenced an increased mtDNA copy number in gills and liver of Inflexible, as in brain of Heavy. Considering these results, we can suggest an association between the increased antioxidant enzymes activity and ethanol preference phenotypes in adult zebrafish. Our results also indicate that ethanol exposure may be related to the increase in mitochondrial biogenesis in Inflexible and Heavy phenotypes.

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