Mestrando: Bruno Camargo dos Santos Orientadora: Dra. Percília Cardoso Giaquinto

UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CENTRO DE AQUICULTURA DA UNESP

CANABIDIOL AUMENTA O BEM-ESTAR DE PEIXES

Bruno Camargo dos Santos

Jaboticabal, SP
2022

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

unesp
Centro de Aquicultura

UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CENTRO DE AQUICULTURA DA UNESP

CANABIDIOL AUMENTA O BEM-ESTAR DE PEIXES

Bruno Camargo dos Santos

Orientadora: Dra. Profa. Adj. Percília Cardoso Giaquinto

Dissertação apresentada ao Programa de Pós-graduação em Aquicultura do Centro de Aquicultura da UNESP - CAUNESP, como parte dos requisitos para obtenção do título de Mestre.

Jaboticabal, SP
2022

S237c

Santos, Bruno Camargo dos
Canabidiol aumenta o bem-estar de peixes / Bruno Camargo dos Santos. –
– Jaboticabal, 2022
vi, 81 p. : il. ; 29 cm

Dissertação (mestrado) - Universidade Estadual Paulista, Centro de Aquicultura, 2022
Orientadora: Percília Cardoso Giaquinto
Banca examinadora: Ana Carolina Luchiari, Renato Filev
Bibliografia

1. Comportamento social dos animais. 2. Cannabis. 3. Stress (Fisiologia). 4. Reprodução. 5. Tilápia-do-Nilo. I. Título. II. Jaboticabal-Centro de Aquicultura.

CDU 639.3.05

Ficha Catalográfica elaborada pela STATI - Biblioteca da UNESP
Campus de Jaboticabal/SP - Karina Gimenes Fernandes - CRB 8/7418

CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: Canabidiol aumenta o bem-estar de peixes

AUTOR: BRUNO CAMARGO DOS SANTOS
ORIENTADORA: PERCILIA CARDOSO GIAQUINTO

Aprovado como parte das exigências para obtenção do Título de Mestre em AQUICULTURA, pela Comissão Examinadora:

Cardoso

Profa. Dra. PERCILIA CARDOSO GIAQUINTO (Participaçao Virtual)
Departamento de Biologia Funcional e Estrutural / UNESP, Campus de Botucatu

Ana C.

Profa. Dra. ANA CAROLINA LUCHIARI (Participaçao Virtual)
Departamento de Fisiologia e Comportamento / Universidade do Estado do Rio Grande do Norte

Dr. RENATO FILEV (Participaçao Virtual) Departamento de Psiquiatria e Psicologia Médica / Universidade Federal de São Paulo (UNIFESP)

Jaboticabal, 28 de janeiro de 2022

SUMÁRIO

AGRADECIMENTOS

Gostaria de agradecer primeiramente minha família. Durante toda minha vida sempre ouvi que a família é a nossa maior base, mas acho que nunca de fato me dei conta do sentido real e mais profundo dessa frase. Ao longo de minha vida venho a ressignificando e hoje posso dizer que tenho muita sorte em ter uma família como a minha, na qual me sinto muito amado e acolhido por todos sem exceção, muito obrigado de verdade por todo carinho.

Gostaria de fazer um agradecimento especial às mulheres mais incríveis desse mundo, minha mãe Selma Maria Leite de Camargo e minha avó Marta Guidolino. Não tenho nem palavras pra descrever tudo o que vocês já fizeram em minha vida. Obrigado por nunca deixarem faltar amor, por sempre me apoiarem de todas as formas possíveis, por todos os ensinamentos e por todos os valores transmitidos. A cada dia que passa, vejo cada vez mais que sou uma versão menor de dona Selma, com lampejos de dona Marta. E tenho muito orgulho disso! Mãe você é a pessoa mais batalhadora que eu conheço, determinada, honesta, muito “humana” em um mundo cada vez menos humano, consegue enfrentar qualquer parada, carregar o peso do mundo nas costas, e sempre com uma alegria inabalável e com um olhar carinhoso pra quem está ao seu lado. Obrigado por sempre apoiar meu sonho de talvez um dia vir a me tornar um pesquisador. Num mundo onde a profissão incrível de “biólogo” ainda é muito desvalorizada e mal remunerada, você nunca me questionou sobre tal escolha, sempre me deixou livre pra trilhar meus caminhos e correr atrás de meu sonho. Até mesmo em cenários muito adversos como o que vivemos hoje na ciência, você nunca deixou de me apoiar e acreditar em mim, você não tem ideia da força que me dá pra seguir em frente. Escrever essa dissertação hoje é o mínimo de retribuição que eu poderia dar por todo esse apoio, espero poder lhe retribuir muito ainda com a pessoa e profissional que venho me tornando durante essa caminhada, obrigado por me permitir sonhar. E vó, não posso deixar de reconhecer o quão batalhadora a senhora também é, além de uma excelente mãe, pois criou 7 filhos sozinha (todos excelentes pessoas) e também contribuiu para minha criação e formação como uma segunda mãe. Obrigado por ser uma grande ouvinte e conselheira, por toda paciência, por todos ensinamentos e por sempre me nutrir com muito amor. Como eu disse acima sou uma pequena Selma, mas as vezes tenho que resgatar minha

Dona Marta interior pra contrabalancear essa vida louca. Meus mais sinceros agradecimentos às duas, amo vocês demais!

Agradeço também a todo meu laboratório. Estava meio reflexivo durante a escrita da dissertação e me dei conta que fazem 6 anos que faço parte deste laboratório. Nestes 6 anos conheci pessoas incríveis, que me fizeram crescer como profissional e também como pessoa. Obrigado à: Vanessa, Rafaela, Adriana, Clarissa, Marciano, Juliane Campos, Isabela Mello, Nina, João, Juliana Lunardi, Raúl, Fábio, Assaf, Prof. Rodrigo, Prof. Helton, Renata, e outros que tiveram passagens mais rápidas no laboratório, pela convivência, ajuda neste trabalho, crescimento e também por me aturarem, que as vezes não é fácil. Gostaria de agradecer ao Bruno Goiano em especial, por todo o acolhimento logo que entrei no laboratório, foi muito bom e importante ter alguém tão curioso, empolgado e pronto pra discutir ciência em qualquer momento como você ao meu lado, você não tem ideia do quanto contribuiu para minha formação! Não poderia deixar de agradecer minha orientadora Dra. Percília Cardoso Giaquinto, que durante todos estes 6 anos esteve presente em minha caminhada. Lembro de quando fui pedir estágio para senhora, numa aula de fisiologia comparada. Perguntei como estava minha situação de faltas na disciplina, e logo em seguida já emendei um estágio. Nunca imaginei que continuaria 6 anos neste lugar que pedi esse estágio tão despretensioso. E hoje agradeço por ter me acolhido, pela convivência e todo aprendizado durante todos esses anos. Gosto muito da relação orientador-orientado que construímos, pautada sempre em muita honestidade e debate. Obrigado por toda paciência, por sempre me deixar livre para trabalhar com o que tenho interesse, por sempre buscar transmitir ensinamentos sobre escrita e metodologia científica, os quais hoje vejo que fazem total diferença na formação de pesquisadores de qualquer área, essa escola Volpato é forte mesmo. Obrigado também por me acolher no mestrado, onde passava por um momento difícil em minha vida, e por confiar no meu trabalho. Acho que meu perfil profissional encontrou um ótimo "fit" com sua orientação, o que contribuiu demais para meu crescimento científico. Além da relação orientador-orientado também agradeço pela amizade que desenvolvemos durante todo esse período. Ainda temos mais um ano juntos pela frente no laboratório, e depois, independente dos caminhos espero levar essa relação sempre junto de mim.

Agradeço também a CNPq pelo financiamento e ao CAUNESP por todo suporte. Agradeço ao Dr. Renato Filev, à Prof. Dra. Eliane Gonçalves-de-Freitas, ao Prof. Dr. Rafael Henrique Nóbrega, à Maira da Silva Rodrigues e ao Daniel Fernandes da Costa pelo apoio de cada um neste trabalho, que foi fundamental para a construção desta dissertação. Agradeço também ao meu psicólogo Edson Laino, que não me ajudou com o conteúdo específico dessa dissertação, mas sua ajuda foi fundamental para que eu completasse esse ciclo do mestrado.

Gostaria de agradecer a todos os amigos que Botucatu me proporcionou, com certeza vocês contribuíram e contribuem muito também para a pessoa e profissional que venho me tornando. Obrigado a todo pessoal da não mais existente república Só-Kanela que me acolheu ao chegar em Botucatu. A convivência naquela sala tão marcante em minha memória, ajudou a contribuir e muito pelo meu interesse pela ciência. Conviver com alunos de graduação, mestres e doutores das mais distintas áreas, tomando uma Bavária e discutindo ciência, da aplicação dos nano sensores com o Bogomiu até o silenciamento genético das larvas do Lacrimoso, tentando juntar tudo isso com comportamento e fisiologia de peixes, gerando ideias que nunca saíram do plano dos pensamentos (mas que um dia sairão), foi um diferencial em minha trajetória, obrigado! Agradecer também em especial ao meu querido amigo Tonelada, por todo apoio emocional, companheirismo, e aumento de bem-estar que você me proporcionou durante todo mestrado. O bar do Concha não é mais o mesmo sem você, obrigado por me ajudar a me manter bem e lúcido durante esse período tão desgastante psicologicamente.

Gostaria de agradecer em especial também a minha casa de veraneio em Botucatu, composta por: Isabela Guermandi, Marina Bellot e Stephanie Grimaldi. Essa casa também ganhou um lugar especial em minha memória, seja na realização dos Happy hours depois do laboratório, que de duas cervejinhas acabavam muitas vezes se transformando em muito mais, ou mesmo nas trocas de ideias cotidianas sobre problemas da vida, cada um sempre apoiando o outro e nunca deixando ninguém cair, ou mesmo em momentos que só nos reuníamos pra falar um milhão de groselhas e fofocar. Obrigado de verdade, o meu período de mestrado foi muito mais feliz com vocês ao meu lado. Abro parênteses para a Marina e Isabela, que além de grandes amigas durante longa data, foram também companheiras de laboratório durante todo mestrado e iniciação científica. O crescimento que vocês me proporcionaram profissionalmente e pessoalmente durante o mestrado com certeza me mudou significativamente como pessoa, e pra melhor ($p < 0.001$)! Carrego hoje na forma de pensar, de escrever, de me relacionar com os outros um mix de nós três, e tenho muito orgulho disso, pois vocês são pessoas e profissionais incríveis! Marina e Isabela já defenderam seus trabalhos de mestrado e estão trilhando outros caminhos, e eu também trilharei caminhos diferentes, então independente de onde a vida nos levar saibam que sempre carregarei um pouco de vocês em quem eu sou e sempre serei muito grato por tudo que já fizeram e fazem por mim, contem sempre comigo, amo vocês!

Por último e não menos importante gostaria de agradecer a minha namorada Ana Clara, que ao menor sinal de perturbação de minha homeostase emocional sempre se fez presente e pronta pra me ajudar, seja ouvindo longos desabafos e dando ótimos conselhos, seja com demonstrações de carinho, seja com sua simples presença. Sou muito sortudo em ter uma pessoa com tamanha sensibilidade ao meu lado como parceira. Obrigado muitas vezes por segurar o rojão que explode quando chego em meu limite de estresse e por me ensinar a ser mais paciente, você não tem ideia do quanto nosso convívio e relação me ajudam manter emocionalmente estável num mundo, e caminho profissional tão instáveis. Tenho muito orgulho da relação que estamos construindo, da pessoa que você é e vem se tornando, e por ter o privilégio de poder caminhar e crescer junto com você, muito obrigado por tudo, te amo!

Acho que como conclusão desses longos agradecimentos, tenho que os laços afetivos que construí com familiares, amigos, colegas de trabalho e minha namorada são o que fazem valer a pena toda essa trajetória científica, muito mais que qualquer $p < 0.05$. O carinho de vocês me dá muita força para continuar seguindo atrás do meu sonho de me tornar um pesquisador na área de comportamento animal. Obrigado de coração a todos por estarem presentes e me ajudarem durante toda essa caminhada, a presença de vocês em minha vida aumenta o meu bem-estar muito mais que o CBD ou qualquer fármaco!

APOIO FINANCEIRO

O presente trabalho foi realizado com apoio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Bolsa de Mestrado, Nº do processo: 130881/2019-8.

RESUMO

Na aquicultura, investigar estratégias para melhorar o bem-estar dos peixes, mitigando os efeitos adversos do ambiente de criação, é de grande importância. Uma substância que tem potencial para aumentar o bem-estar dos animais de criação, é o canabidiol (CBD). Em alguns vertebrados, o CBD apresenta propriedades ansiolíticas, diminui a agressividade e o estresse, regula a reprodução, entre outros efeitos. Portanto, nosso objetivo é verificar o efeito de diferentes doses de CBD (0,1,10 e 20 mg/kg) em variáveis comportamentais e morfofisiológicas relacionadas ao bem-estar da tilápia-do-Nilo (*Oreochromis niloticus*), como agressividade, estresse e reprodução. A tilápia-do-Nilo é uma espécie agressiva e um dos peixes mais cultivados em todo o mundo, constituindo um bom modelo de estudo. O CBD foi administrado aos peixes por 5 semanas através da dieta. A dose de 10 mg/kg diminuiu a agressividade dos animais ao longo do tempo, enquanto a dose de 20 mg/kg atenuou o estresse não-social, e ambas as doses diminuíram o nível basal de cortisol e aumentaram o índice gonadossomático. Porém, o CBD diminuiu o número de espermatozoides dos peixes. Nenhuma dose de CBD afetou qualquer variável de alimentação ou crescimento, consequentemente, demonstrando não alterar a produção de carne. Assim, a suplementação com CBD pode trazer benefícios para a vida dos animais e produtores. Nosso estudo foi o primeiro a utilizar o CBD como ferramenta para aumentar o bem-estar dos animais de produção, sendo promissor em peixes e apresentando grande potencial a ser explorado em outros animais de produção e cativeiro.

Palavras-chave: Comportamento social dos animais, *Cannabis*, Stress (Fisiologia), Reprodução, Tilápia-do-Nilo.

ABSTRACT

In aquaculture, investigating strategies to improve fish welfare mitigating adverse effects of the rearing environment is of great importance. A substance that has the potential to increase farming animals' welfare, is cannabidiol (CBD). In some vertebrates, CBD presents anxiolytic proprieties, decreases aggressiveness and stress, regulates reproduction, among other effects. Therefore, we aimed to verify the effect of different CBD doses (0,1,10, and 20 mg/kg) on behavioral and morpho-physiological variables related to the welfare of Nile tilapia (*Oreochromis niloticus*), such as aggressiveness, stress, and reproduction. Nile tilapia is an aggressive species and one of the most farmed fish worldwide. CBD was administered through diet to fish for 5 weeks. The 10 mg/kg dose decreased fish's aggressiveness over time, while the 20 mg/kg dose attenuated non-social stress, and both doses decreased the non-stressed cortisol level and increased the gonadosomatic index. However, CBD decreased the spermatozoa number. CBD did not affect any feeding or growth variable, and consequently the meat production. Thus, CBD supplementation can bring benefits to animals' life and producers. Our study was the first to use CBD as a tool to increase farming animals' welfare, being promising in fish, and presenting a great potential to be explored in other husbandry and captivity animals.

Keywords: Social behavior of animals, *Cannabis*, Stress (Physiology), Reproduction, Nile Tilapia.

CONSIDERAÇÕES INICIAIS

A tese de mestrado intitulada “Canabidiol aumenta o bem-estar de peixes” será apresentada a seguir escrita inteiramente em Inglês. Entretanto, foi incluída uma seção inicial “Introdução” em português, para situar o leitor que não tem domínio da língua inglesa sobre o assunto que foi pesquisado neste trabalho. Gostaria de ressaltar que o conteúdo apresentado nas seções “Introdução” e “Introduction” são iguais, mudando apenas a língua em qual este é apresentado.

1. INTRODUÇÃO

O bem-estar animal (BEA) vem crescendo como ciência, e diversos estudos mostraram a importância de atender às necessidades dos animais para garantir-lhes uma melhor qualidade de vida (BROOM, 2011; SARAIVA; ARECHAVALA-LOPEZ, 2019; VON KEYSERLINGK; WEARY, 2017). Existem três principais concepções científicas do que é AW. A primeira é baseada em uma abordagem “natural-living”, e para estar em um bom nível de bem-estar, os animais precisam ser capazes de expressar em cativeiro comportamentos que eles teriam em ambientes naturais (FRASER et al., 1997; VON KEYSERLINGK ; WEARY, 2017). A segunda concepção de AW leva em conta as preocupações com o “estado afetivo” dos animais. Assim, para estar em boas condições de vida, os animais precisam estar livres de sofrimentos intensos e prolongados de dor, medo, fome e outros estados afetivos negativos (DUNCAN, 2004; FRASER et al., 1997). A terceira e última concepção de AW é a mais adotada pelos pecuaristas e se baseia em uma “abordagem funcional”, na qual, para estar em um bom nível de bem-estar, os animais devem ter uma boa funcionalidade dos sistemas biológicos e boa saúde quando são lidar com seus ambientes (FRASER et al., 1997; MARTINS et al., 2012). No entanto, Fraser et al. (1997) sugeriram que essas três concepções de AW estão intrinsecamente conectadas e que as melhores práticas de AW irão abordar as preocupações dessas três abordagens. Por exemplo, permitir que o gado leiteiro procure sombras em dias quentes (comportamento natural), evita que as vacas se sintam desconfortáveis com o calor (estado afetivo) e também reduz os problemas de saúde associados ao estresse térmico (funcionalidade biológica)

(VON KEYSERLINGK et al., 2009; VON KEYSERLINGK; WEARY, 2017). Os preceitos dessa abordagem integrativa de AW estão sendo incluídos gradativamente na pecuária, como bovinos e aviários, porém, na aquicultura, essa abordagem ainda é incipiente (SARAIVA; ARECHAVALA-LOPEZ, 2019).

Na aquicultura, os animais são geralmente expostos a diversos estressores que afetam seu bem-estar, como, por exemplo, alterações na qualidade da água (MARTINS et al., 2009), manejos (por exemplo, classificação, captura e transporte (CONTE, 2004)) e altas densidades de estoque (ELLIS et al., 2002; MARTINS et al., 2012). Em geral, todos esses estressores induzem alterações fisiológicas, como aumento dos níveis de cortisol por exemplo (BARRETO; VOLPATO, 2006; MARTINS et al., 2012; WINBERG; OVERLI; LEPAGE, 2001). Essas respostas fisiológicas ao estresse podem levar a efeitos comportamentais e morfológicos nos peixes, como diminuição do desempenho reprodutivo (BILLARD; BRY; GILLET, 1981; CONTE, 2004), redução da ingestão alimentar e, consequentemente, ganho de peso (MARTINS et al., 2012; WENDELAAR BONGA, 1997). Em particular, a alta densidade de estoque na aquicultura pode aumentar o número de confrontos agressivos em espécies hierárquicas e territoriais (CONTE, 2004; KEELEY, 2000; MARTINS et al., 2012). Embora as interações agressivas sejam um componente natural do comportamento de muitas espécies de peixes, altas densidades de criação podem aumentar a ocorrência desse tipo de comportamento a níveis acima dos observados em ambientes naturais (abordagem natural-vida) (GONÇALVES-DE-FREITAS et al., 2019). A intensificação da agressividade dos peixes a um nível não natural pode ter diversas consequências em cativeiro, como maior gasto energético dos animais, aumento do estresse social decorrente da hierarquia de dominância, e também aumento de lesões corporais (GONÇALVES-DE-FREITAS et al., 2019). Como consequência do aumento das lesões corporais, os peixes também sentirão mais dor (estado afetivo). Assim, as condições de criação da aquicultura exacerbam o comportamento agressivo dos peixes e diminuem seu valor adaptativo (GONÇALVES-DE-FREITAS et al., 2019).

Todas essas alterações comportamentais e fisiológicas desencadeadas pelos ambientes de aquicultura comprometem o bem-estar dos peixes do ponto de vista de uma abordagem integrativa de AW, levando em conta as preocupações das 3 principais concepções de AW: vida natural, estado afetivo e funcional.

Portanto, investigar estratégias que melhorem o bem-estar dos peixes em cativeiro, mitigando ao máximo os efeitos adversos gerados pelo ambiente de criação da aquicultura, com baixo custo e fácil implantação, mesclando benefícios à vida dos animais e do agricultor, é de grande importância.

Uma substância que atua em diversos sistemas do organismo e, portanto, pode ter um grande potencial para aumentar o bem-estar dos animais de criação, é o canabidiol (CBD) (IFFLAND; GROTENHERMEN, 2017). O CBD apresenta muitas propriedades farmacológicas e grande potencial medicinal, auxiliando no tratamento de diversas doenças e transtornos psiquiátricos (CUNHA et al., 1980; DEVINSKY et al., 2014; HARTMANN et al., 2019). O CBD é um dos principais canabinoide da planta Cannabis sativa, e em mamíferos apresenta efeitos ansiolíticos (ALMEIDA et al., 2013; MOREIRA; AGUIAR; GUIMARÃES, 2006) e antidepressivos (SARTIM; GUIMARÃES; JOCA, 2016; ZANELATI et al., 2010), diminui a agressividade (CORSETTI et al., 2021; HARTMANN et al., 2019; HUSSAIN et al., 2015) e o estresse (CAMPOS; FERREIRA; GUIMARÃES, 2012; RESSTEL et al., 2009), tem efeitos anti-inflamatórios (BURSTEIN, 2015), auxilia a ingestão alimentar e ganho de peso (GÁLL et al., 2020), e regula a fertilidade (AMOAKO et al., 2013; PANDELIDES et al., 2020; STEGER et al. , 1990).

Os mecanismos de ação do CBD responsáveis pela maioria de seus efeitos ainda não estão totalmente elucidados. No entanto, sabe-se que a droga atua indiretamente na ativação dos receptores endocanabinoides CB1 e CB2 (HARTMANN et al., 2019), e atua diretamente na ativação dos receptores serotoninérgicos 5-HT1A (HARTMANN et al., 2019; RUSSO et al. al., 2005), entre outros receptores (BISOGNO et al., 2001; O'SULLIVAN, 2016; RYBERG et al., 2007). O CBD diminui a agressividade em mamíferos por meio de um mecanismo associado à ativação de ambos os receptores, 5-HT1A e CB1 (HARTMANN et al., 2019). A ativação dos receptores 5-HT1A pela droga também está relacionada à diminuição do estresse e da ansiedade (XIN et al., 2020). Os receptores endocanabinoides também exercem efeito regulatório sobre o eixo hipotálamo-hipófise-adrenal (HPA), responsável por mediar as respostas ao estresse em mamíferos (HILL; GORZALKA, 2006), além de ter papel fundamental na ingestão alimentar e no ganho de peso corporal (IGNATOWSKA -JANKOWSKA; JANKOWSKI; SWIERGIEL, 2011). Além disso, o sistema endocanabinoide está envolvido na regulação da fertilidade masculina (AMOAKO et al., 2013; BATTISTA et al., 2007; PANDELIDES et al., 2020) e feminina (CECCONI et al., 2019).

Embora muitos desses efeitos do CBD e seus mecanismos subjacentes descritos em mamíferos não sejam comprovados em peixes, ambos os táxons apresentam receptores 5-HT1A muito semelhantes (KHAN; DESCHAUX, 1997; WINBERG; NILSSON, 1996), e o sistema endocanabinóide é altamente conservado entre os táxons (ELPHICK, 2012), em que os receptores CB1 de peixes apresentam cerca de 70% de similaridade com os receptores CB1 de roedores e humanos (OLTRABELLA et al., 2017). Assim, espera-se que os efeitos do CBD em mamíferos envolvam a ativação desses dois receptores, sendo semelhantes em peixes. De fato, alguns estudos já mostraram que em zebrafish (Danio rerio) o CBD apresenta um efeito ansiolítico (NAZARIO et al., 2015), diminui o ritmo natatório, auxilia na diferenciação e regulação de genes de imunidade (JENSEN et al., 2018), e também regula a fecundidade masculina e feminina, apresentando toxicidade reprodutiva para machos e melhorando alguns parâmetros reprodutivos em fêmeas (PANDELIDES et al., 2020). Na tilápia do Nilo (Oreochromis niloticus), o CBD diminui ligeiramente alguns biomarcadores secundários de estresse em peixes estressados e não estressados, porém, essas diferenças não são significativas (MORTUZA; MASON; MUSTAFA, 2021). Embora essas reduções não tenham sido significativas, isso pode ser um indicador de que o CBD também pode modular as respostas ao estresse dos peixes, se administrado em uma dosagem e tempo apropriados. Além disso, outros efeitos do CBD demonstrados em mamíferos, como diminuição da agressividade, aumento da ingestão de alimentos e ganho de peso corporal ainda não foram estudados em peixes.

Assim, espera-se que os efeitos do CBD em mamíferos envolvendo a ativação desses dois receptores sejam semelhantes em peixes. De fato, alguns estudos já demonstram que em zebrafishs (*Danio rerio*) o CBD apresenta efeito ansiolítico [47], diminui o ritmo natatório, auxilia na diferenciação e regulação de genes de imunidade [48] e também regula a fecundidade de machos e fêmeas, apresentando toxicidade reprodutiva para machos e melhorando alguns parâmetros reprodutivos em fêmeas [32]. Na tilápia-do-Nilo (*Oreochromis niloticus*), o CBD diminui ligeiramente alguns biomarcadores secundários de estresse em indivíduos estressados e não estressados, no entanto, essas diferenças não são significativas [49]. Mesmo que essas diminuições não tenham sido significativas em estudos anteriores, sua ocorrência pode ser um indicador de que o CBD também pode modular as respostas ao estresse dos peixes, se administrado em uma dosagem e tempo adequados. Além disso, outros efeitos do CBD demonstrados em mamíferos, como diminuição da agressividade, aumento da ingestão alimentar e ganho de peso corporal, ainda não foram estudados em peixes.

Portanto, conhecendo os efeitos do CBD em mamíferos e os potenciais efeitos da droga em peixes, o CBD pode auxiliar na melhoria do bem-estar dos peixes em ambientes de cativeiro, principalmente em espécies agressivas de alta importância na aquicultura, como a tilápia do Nilo. A tilápia do Nilo é uma espécie territorialista e hierárquica, altamente agressiva (GIAQUINTO; VOLPATO, 1997), e um dos peixes mais cultivados no mundo (FAO, 2020). Um estudo recente testou os efeitos da suplementação da dieta da tilápia do Nilo com óleo de cannabis no crescimento, hematologia e metabolismo de peixes (SAOUD et al., 2018). O óleo de cannabis apresentou efeito negativo na taxa de conversão alimentar e também não melhorou a resposta imune em peixes (SAOUD et al., 2018). No entanto, o óleo de cannabis apresenta todos os compostos vegetais, incluindo outros canabinóides, como o tetrahidrocanabinol (THC). Juntos, todos os canabinóides vegetais podem causar efeitos diferentes no organismo em comparação ao CBD sozinho (FREEMAN et al., 2019). Além disso, mesmo sem significância estatística, o CBD diminui levemente alguns biomarcadores de estresse secundário da espécie (MORTUZA; MASON; MUSTAFA, 2021). Provavelmente, se administrado em dose e tempo adequados, pode apresentar alguns resultados positivos quanto à atenuação do estresse. Neste estudo, objetivamos verificar o efeito de diferentes doses de CBD sobre variáveis comportamentais e morfofisiológicas relacionadas ao bem-estar da tilápia do Nilo, como agressividade, estresse e reprodução. O CBD foi administrado por meio da dieta, por ser um método de administração simples e não invasivo, e de fácil replicação em pisciculturas. Devido às propriedades farmacológicas e ampla gama de efeitos do CBD no organismo, levantamos a hipótese de que o medicamento terá um grande potencial de melhorar muitas variáveis relacionadas ao bem-estar dos animais de criação.

2. INTRODUCTION

Animal welfare (AW) has been growing as a science, and several studies showed the importance of meeting animal needs to ensure them a better quality of life (BROOM, 2011; SARAIVA; ARECHAVALA-LOPEZ, 2019; VON KEYSERLINGK; WEARY, 2017). There are three main scientific conceptions of what is AW. The first one is based on a “natural-living” approach, and to be at a good welfare level, animals need to be able to express in captivity behaviors that they would perform in natural environments (FRASER *et al.*, 1997; VON KEYSERLINGK; WEARY, 2017). The second conception of AW takes into account concerns about the “affective state” of animals. Thus, to be in good living conditions, animals need to be free of suffering intense and prolonged pain, fear, hunger, and other negative affective states (DUNCAN, 2004; FRASER *et al.*, 1997). The third and last conception of AW is the most adopted by animal farmers and is based on a “functional approach”, in which to be at a good welfare level, animals must have a good functionality of the biological systems and good health when they are coping with their environments (FRASER *et al.*, 1997; MARTINS *et al.*, 2012). However, Fraser et al.(FRASER *et al.*, 1997) suggested that these three conceptions of AW are intrinsically connected and that the best practices of AW will address the concerns of these three approaches. For example, allowing dairy cattle to search for shades on hot days (natural behavior), prevents cows from feeling uncomfortable with the heat (affective state) and also reduces health problems associated with thermal stress (biological functionality) (VON KEYSERLINGK *et al.*, 2009; VON KEYSERLINGK; WEARY, 2017). The precepts of this integrative approach of AW are gradually be included in animal farming, such as cattle and poultry, however, in aquaculture, this approach is still incipient (SARAIVA; ARECHAVALA-LOPEZ, 2019).

In aquaculture, animals are usually exposed to diverse stressors that affect their welfare, for example, changes in water quality (MARTINS *et al.*, 2009), handlings, (e.g., grading, capturing, and transporting (CONTE, 2004)), and high stock densities (ELLIS *et al.*, 2002; MARTINS *et al.*, 2012). In general, all these stressors induce physiological alterations, such as increasing in cortisol levels for example (BARRETO; VOLPATO, 2006; MARTINS *et al.*, 2012; WINBERG; OVERLI; LEPAGE, 2001). These physiological stress responses can lead to behavioral and morphological effects in fish, such as decreasing in reproductive performance (BILLARD; BRY; GILLET, 1981; CONTE, 2004), a reduction in food intake, and consequently weight gain (MARTINS *et al.*, 2012; WENDELAAR BONGA, 1997). In particular, the high stock density in aquaculture can increase the number of aggressive confronts in hierarchical and territorial species (CONTE, 2004; KEELEY, 2000; MARTINS *et al.*, 2012). Although aggressive interactions are a natural component of the behavior of many fish species, high rearing densities can increase the occurrence of this type of behavior to levels above the observed in natural environments (natural-living approach) (GONÇALVES-DE-FREITAS *et al.*, 2019). The intensification of fish aggressiveness to an unnatural level can have several consequences in captivity, such as a higher energy expenditure of animals, increasing social stress arising from the dominance hierarchy, and also an increase in body injuries (GONÇALVES-DE-FREITAS *et al.*, 2019). As a consequence of the body injuries increase, fish will also experience more pain (affective state). Thus, the aquaculture rearing conditions exacerbate fish’s aggressive behavior and shrink its adaptive value (GONÇALVES-DE-FREITAS *et al.*, 2019).

Collectively, all these behavioral and physiological alterations triggered by the aquaculture environments compromise fish welfare from the point of view of an integrative AW approach, taking accounting the concerns of the 3 main AW conceptions: natural living, affective state, and functional. Therefore, investigating strategies that improve fish welfare in captivity, mitigating as many as possible the adverse effects generated by the aquaculture rearing environment, with a low cost and easy implantation, merging benefits to animals’ life and farmers, is of great importance.

A substance that acts in diverse systems of the organism, and thus, may have a great potential to increase the welfare of farm animals, is cannabidiol (CBD) (IFFLAND; GROTENHERMEN, 2017). The CBD presents many pharmacological properties and great medicinal potential, assisting in the treatment of many diseases and psychiatric disorders (CUNHA *et al.*, 1980; DEVINSKY *et al.*, 2014; HARTMANN *et al.*, 2019). The CBD is one of the major cannabinoids from the *Cannabis sativa* plant, and in mammals presents anxiolytic (ALMEIDA *et al.*, 2013; MOREIRA; AGUIAR; GUIMARÃES, 2006) and antidepressant effects (SARTIM; GUIMARÃES; JOCA, 2016; ZANELATI *et al.*, 2010), decreases the aggressivity

(CORSETTI *et al.*, 2021; HARTMANN *et al.*, 2019; HUSSAIN *et al.*, 2015) and stress (CAMPOS; FERREIRA; GUIMARÃES, 2012; RESSTEL *et al.*, 2009), has anti-inflammatory effects (BURSTEIN, 2015), assists the food intake and weight gain (GÁLL *et al.*, 2020), and regulates the fertility (AMOAKO *et al.*, 2013; PANDELIDES *et al.*, 2020; STEGER *et al.*, 1990).

The action mechanisms of CBD responsible for most of its effects are still not totally elucidated. However, it is known that the drug indirectly acts in the activation of CB1 and CB2 endocannabinoids receptors (HARTMANN *et al.*, 2019), and directly acts in the activation of 5-HT1A serotoninergic receptors (HARTMANN *et al.*, 2019; RUSSO *et al.*, 2005), among other receptors (BISOGNO *et al.*, 2001; O'SULLIVAN, 2016; RYBERG *et al.*, 2007). The CBD decreases aggressivity in mammals through a mechanism associated with the activation of both receptors, 5-HT1A and CB1 (HARTMANN *et al.*, 2019). The activation of 5-HT1A receptors by the drug is also related to the decrease in stress and anxiety (XIN *et al.*, 2020). The endocannabinoid receptors also have a regulatory effect on the hypothalamic-pituitary-adrenal axis (HPA), responsible for mediating stress responses in mammals (HILL; GORZALKA, 2006), besides having a key role in the food intake and body weight gain (IGNATOWSKA-JANKOWSKA; JANKOWSKI; SWIERGIEL, 2011). Moreover, the endocannabinoid system is involved in the regulation of male (AMOAKO *et al.*, 2013; BATTISTA *et al.*, 2007; PANDELIDES *et al.*, 2020) and female fertility (CECCONI *et al.*, 2019).

Although many of these CBD effects and their underlying mechanisms described in mammals are not proven in fish, both taxa present 5-HT1A receptors very similar (KHAN; DESCHAUX, 1997; WINBERG; NILSSON, 1996), and the endocannabinoid system is highly conserved between the taxons (ELPHICK, 2012), in which fish CB1 receptors have about 70% similarity with CB1 receptors of rodents and humans (OLTRABELLA *et al.*, 2017). Thus, it is expected that the CBD effects in mammals involve the activation of these two receptors, being similar in fish. Indeed, some studies already showed that in zebrafish (*Danio rerio*) CBD presents an anxiolytic effect (NAZARIO *et al.*, 2015), decreases the natatory rhythm, assists in the differentiation and regulation of immunity genes (JENSEN *et al.*, 2018), and also regulates the male and female fecundity, presenting reproductive toxicity for males and improving some reproductive parameters in females

(PANDELIDES *et al.*, 2020). In the Nile tilapia (*Oreochromis niloticus*), CBD slightly decreases some secondary stress biomarkers in stressed and non-stressed fish, however, these differences are not significant (MORTUZA; MASON; MUSTAFA, 2021). Even though these decreases were not significant, this could be an indicator that CBD can also modulate fish stress responses whether administrated in an appropriate dosage and time. Moreover, other CBD effects demonstrated in mammals, such as aggressivity decrease, increase in food intake, and body weight gain was not studied in fish yet.

Therefore, knowing the CBD effects has on mammals and the potential effects of the drug in fish, CBD can assist to improve fish welfare in captivity environments, mainly in aggressive species of high importance in aquaculture, such as the Nile tilapia. The Nile tilapia is a territorialist and hierarchical species, highly aggressive (GIAQUINTO; VOLPATO, 1997), and one of the most farmed fish in the world (FAO, 2020). A recent study tested the effects of the supplementation of the Nile tilapia diet with cannabis oil in fish growth, hematology, and metabolism (SAOUD *et al.*, 2018). The cannabis oil presented a negative effect on food conversion rate and also did not improve the immune response in fish (SAOUD *et al.*, 2018). However, cannabis oil presents all the plant compounds, including other cannabinoids, such as tetrahydrocannabinol (THC). Together, all the plant cannabinoids can cause different effects in the organism compared to the CBD alone (FREEMAN *et al.*, 2019). In addition, even no statistical significance was found, CBD slightly decreases some secondary stress biomarkers of the species (MORTUZA; MASON; MUSTAFA, 2021). Probably, whether administrated in a suitable dose and time it can present some positive outcomes regarding stress attenuation. In this study, we aim to verify the effect of different CBD doses on behavioral and morpho-physiological variables related to the welfare of Nile tilapia, such as aggressiveness, stress, and reproduction. The CBD was administered through diet, since it is a simple and non-invasive method of administration, and it is easy replication in piscicultures. Due to the pharmacological proprieties and wide range of CBD effects in the organism, we hypothesized that the drug will have a great potential of improving many variables related to the welfare of farmed animals.

3. OBJECTIVES

Here, we tested the effect of different CBD doses (0,1,10 and 20 mg/kg) on the (i) aggressive behavior, (ii) stress and (iii) reproductive development of the Nile tilapia.

4. MATERIAL AND METHODS

4.1. ANIMALS AND EXPERIMENTAL CONDITIONS

Juveniles of Nile tilapia (Supreme strain and sexually reversed – all males) were obtained from fish farming (at Botucatu – SP, Brazil) and used to constitute a stock population. These animals were maintained in a 500L tank (100 fish) for a month and were fed three times a day with a commercial diet (Presence Nutripiscis – extruded ration, pellets of 3-4mm) at 1% of their body weight (3% per day). The water temperature was maintained between 25-27ºC, and the stock tank was supplied with constant aeration and a recirculating water system with a continuous flow of dechlorinated water. After one month at the stock tank, 60 fish were selected and individualized in 23L aquaria (40 x 23 x 25 cm) without visual contact at the beginning of the experiment. The experimental aquaria were supplied with constant aeration, and 40% of water was partially changed every two days, to maintain acceptable and good water quality parameters, that were checked twice a week. The pH was maintained between 6.8-7.2 and ammonia and nitrite were below 0.5 and 0.05 ppm, respectively. The temperature of the experimental aquaria was maintained between 25-27ºC. Fish received daily a commercial diet corresponding to 3% of their body weight fractionated at three meals (9:00h, 13:00h, and 17:00h; 1% per meal). The average fish weight at the beginning of the experiment was 29.5 ± 2.8g (mean ± SD).

4.2. CANNABIDIOL AND TREATED FEED PREPARATION

The CBD dose of each treatment (0 mg/kg, 1 mg/kg, 10 mg/kg, and 20 mg/kg) was calculated based on the initial fish weight (approximately 30g; Table 1), without adjustments through all experimental period, since such adjustments may induce additional stress to animals. Fish were fed three times a day, with 4h intervals between each feeding period (9:00h, 13:00h, and 17:0hh), a time that CBD maintains its peak action through oral administration (MILLAR *et al.*, 2018). The CBD remains bioavailable in the organism after an oral and chronic administration for 5 days (MILLAR *et al.*, 2018).

The CBD used in this study was isolated in salt (99% concentration) and was obtained from the Brazilian Center of Information about Psychotropic Drugs – CEBRID (Psychobiology department – UNIFESP). CBD is a drug highly liposoluble (MECHOULAM; PARKER; GALLILY, 2002), therefore, to add it to the fish diet, CBD was diluted in soybean oil and a top coating with this CBD oil was applied on commercial pellets (Presence Nutripiscis – extruded ration, pellets of 3-4mm).

The treated feed preparation was conducted in a room without any light due to the CBD photosensitive property (MECHOULAM; HANUŠ, 2002). For each treatment 2Kg of pellets were used. The CBD amount applied to the feed of each treatment was calculated based on the following formula:

$$\begin{aligned} &CBD\ amount\ applied\ to\ the\ feed\ of\ each\ treatment \\ &= \ Total\ CBD\ amount\ ingested\ daily\ by\ a\ fish\ of\ each\ treatement\ (mg) \\ &\times 15\ fish\ (N\ by\ treatment)\ \times 35\ days (experimental\ time) \end{aligned}$$

The total CBD amount of each treatment feed (Table 1) was dissolved in a quantity of soybean oil corresponding to 2% of the total ration weight (2 Kg). To dissolve CBD in the soybean oil, a Heat Magnetic Stirrer (Biomixer AM-10), at 60ºC, rotating at 3000 rpm was utilized. After dissolving CBD in the oil, the feed pellets were placed homogeneously on a smooth and straight surface, and the oil with CBD was applied to it with a hand sprayer. The pellets were turned four times during the oil application, to ensure that the CBD was applied in all superficies. After this top coating was described, the pellets were kept in a plastic bag and manually shaken for 3 minutes, to ensure CBD homogenization in all feed. Posteriorly, the pellets were placed again over a surface and left out at room temperature to dry for one day. After this procedure, the treatment feeds were stored in opaque pots that did not allow the entrance of light. The same procedure was done to the preparation of the control group feed, however, without the CBD addition.

Table 1 – General information about the CBD amount applied in each treatment feed (2 Kg of feed per treatment) and ingested daily by fish of each treatment. The CBD doses of the treatments (0, 1, 10 e 20 mg/kg) were calculated based on the fish's initial weight.

Treatments (mg of CBD[1]/ Kg of fish)	Initial fish weight (kg)	Total CBD amount ingested daily by a fish (mg)	Total CBD amount applied to diet (mg)	CBD percentage on the feed (%)
Control	0.03 ± 0.003	0	0	0
CBD1 (1 mg/kg)	0.02967 ± 0.003	0.03	16.5	0.0033
CBD10 (10 mg/kg)	0.02867 ± 0.002	0.3	166.5	0.0333
CBD 20 (20 mg/kg)	0.02833 ± 0.003	0.6	333	0.0666

[1] CBD – Cannabidiol.

In "Initial fish weight", values are mean ± SD.

4.3. EXPERIMENTAL DESIGN AND PROCEDURES

To verify the CBD effect on behavioral and morpho-physiological variables related to Nile tilapia welfare, fish were distributed in four treatments (N = 15). Each treatment was submitted to diets supplemented with different CBD doses, as follow: (1) CBD 1 treatment, received 1 mg/kg body weight of CBD per day; (2) CBD 10 treatment, received 10 mg/kg body weight of CBD per day; (3) CBD 20 treatment, received 20 mg/kg body weight of CBD per day; and (4) control treatment, received a diet with the same formulation but without the CBD addition. The animals were allowed to acclimatize to the experimental aquaria for 1 week. During this period all fish received a diet without CBD. The experimental period in which fish received the treated diet, started after the acclimatization week and lasted 5 weeks (35 days). All tests were performed during this period were conducted at 8:00-12:00h, with fasted animals.

During the first four experimental weeks, the aggressive behavior and the responses to a social stressor were evaluated in fish (Figure 1A). The aggressive behavior ("Mirror Test"; more details below) and the responses to a social stressor ("Ventilation Rate -VR"; more details below) were measured in five sampling time points: basal (the first day of the experimental period, before fish received the treated diets for the first time) and once a week (week 1, week 2, week 3 and week 4) (Figure 1A). Firstly, the VR pre-stress was measured, followed by the Mirror Test (aggressive behavior test and also a social stressor), and lastly, the VR post-stress was collected (Figure 1A). Fish were measured and weighted right before being placed at experimental aquaria and after the end of the fourth experimental week.

In the fifth experimental week, some stress responses induced by a non-social stressor (confinement – Figure 1B) were measured. On the first day of this week, a blood sample was collected for further analysis of fish baseline cortisol. On the last day, confinement stress was applied to the fish. The VR was collected pre-stress and post-stress. Right after the VR post-stress measurement, fish were anesthetized and another blood sample was collected for further analysis of the stress-induced cortisol level (post-stress). Posteriorly, the animals were euthanized after an overdose of clove oil (1500µl/l (STRYKOWSKI; SCHECH, 2015), measured, weighed and their gonads were collected for further morphological and histological analysis (Figure 1B).

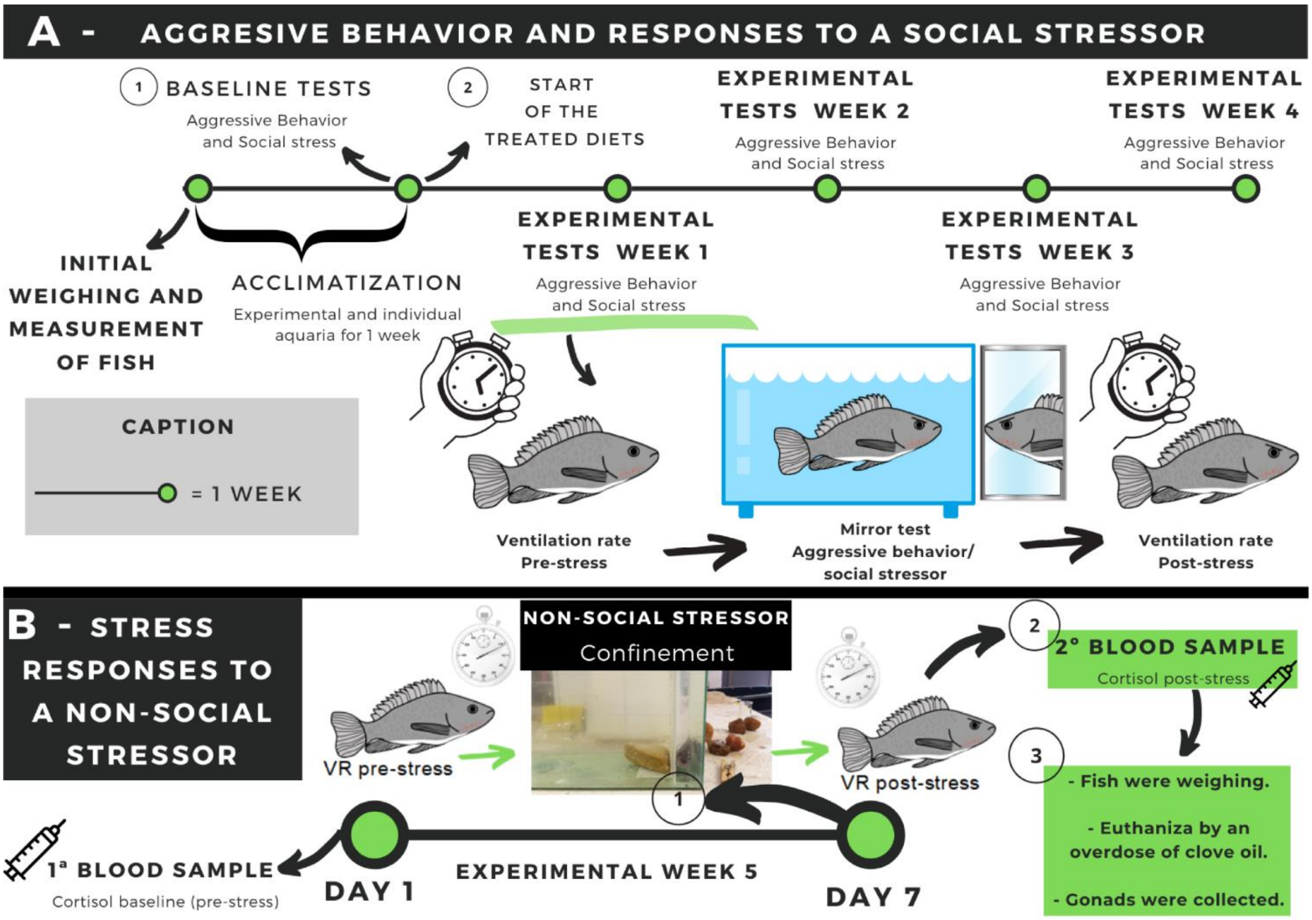

Figure 1 – Experimental design. The sequence of the experimental procedures is divided into two phases: **(A)**The first phase, in which fish were acclimatized for 1 week to individual aquaria followed by the start of the CBD administration through diet and the beginning of

the experimental weeks. In the first four experimental weeks the aggressive behavior and responses to a social stressor were accessed; and **(B)** the second phase, composed of the fifth and last experimental week, in which some stress responses to a non-social stressor were evaluated.

4.4. SOCIAL STIMULI – MIRROR TEST

4.4.1. Aggressive behavior

The aggressive behavior was analyzed through the mirror test in the first four experimental weeks, and was measured over five sampling time points (basal, week 1, week 2, week 3, and week4). The mirror test is a reliable method to evaluate fish aggressive behavior in several species, including the Nile tilapia (ARCHARD; BRAITHWAITE, 2011; BARRETO *et al.*, 2009; CAMARGO-DOS-SANTOS *et al.*, 2021; DESJARDINS; FERNALD, 2010; REDDON; BALSHINE, 2010; ROS; BECKER; OLIVEIRA, 2006). A recent study showed that a cleaner fish species (*Labroides dimidiatus*) can recognize its own reflected image on the mirror (KOHDA *et al.*, 2019). However, the cichlid fishes (for example, the Nile tilapia) are not able to recognize their image on the mirror, perceiving it as another conspecific entering their territory, and thus, exhibiting aggressive interactions against the mirror (HOTTA; KOMIYAMA; KOHDA, 2018). The mirror test was chosen as a stimulus to evaluate fish aggressivity instead of another conspecific to avoid any possible bias created by the use of a conspecific in the aggressive behavior of focal fish. Size, aggressiveness, color, and sex are examples of characteristics of conspecifics that could influence the aggressive behavior of the focal fish. Thus, the advantage of the mirror test is the control of the “virtual opponent”, and the results are assigned only to the focal individual behavior (REDDON *et al.*, 2012). Besides that, there is no difference in the aggressivity motivation when the Nile tilapia encounters a conspecific or their image in the mirror (REDDON *et al.*, 2012).

In the mirror test, each fish was recorded for ten minutes. On one side of the aquarium, a mirror of the same size as the lateral wall is placed parallel to this, covered by an opaque divisor. The recording started when the opaque divisor is removed. The latency for the animal to perform the first aggressive behavior against the mirror was analyzed, besides the frequency of the following aggressive behaviors: number of bites, number of touches, and lateral fights (ALVARENGA;

VOLPATO, 1995). Moreover, the total number of aggressive behaviors was also accounted for.

4.4.2. Stress response – Ventilation rate

The mirror test used to measure the aggressive behavior is a social stimulus that triggers some stress responses in fish REF 60. The mirror image can induce increase the EGR-1 activity in the hippocampus, a transcription factor for later acting genes that code for stress responses (DESJARDINS; FERNALD, 2010). Also, the mirror image can cause fear in fish, because it is a completely new and unpredictable stimulus (DESJARDINS; FERNALD, 2010). The VR in response to the mirror test was measured. The VR is a physiological stress response of fish and a reliable measure of stress level since it increases in response to a stressor and is related to the metabolic rate (BARRETO; VOLPATO, 2011). The stress can induce catabolic responses and thus, modulates the metabolism and consequently causes alterations in VR (BARRETO; VOLPATO, 2011; MOMMSEN; VIJAYAN; MOON, 1999). To measure the VR, the time that fish took to realize 20 successive opercular or buccal movements was accounted for (ALVARENGA; VOLPATO, 1995; BARRETO; VOLPATO, 2011). Through an estimate, it was calculated how many opercular beats per minute each fish executed. The VR before the stress applying (VR pre-stress) and after the stress applying (VR post-stress) were measured. Both VRs (pre-and post-stress) were measured three times per fish in three consecutive minutes. The mean for each fish was calculated and used for further analysis (BARRETO; VOLPATO, 2011). In addition, the variation of VR in response to a stressor (ΔVR = VR post-stress – VR pre-stress) was calculated for each fish.

4.5. NON-SOCIAL STRESS – CONFINEMENT

In the fifth week of the experiment, the fish stress responses to non-social stress were measured. On the last day of this week, a confinement acute stressor was applied as a non-social stressor. For this, an opaque partition was used to confine fish to a small side of the aquarium, restricting the animal to only 10% of the aquarium volume for 30 minutes. Two physiological stress responses to confinement were measured: the VR and the plasmatic cortisol level.

4.5.1. Ventilation rate

The same procedure described above to measure the VR in response to a social stimulus was done to measure the VR in response to a non-social stressor. The VRs before confinement (VR pre-stress) and after the confinement (VR post-stress) were collected, and afterward, the ΔVR was calculated for each fish.

4.5.2. Plasmatic cortisol level

Two blood samples were collected during a week for further cortisol analysis. The first blood collection was done on the first day of the week (baseline cortisol level) and the second one was done on the last day of the week, post confinement. The isolation period of fish for five weeks before the first blood sample collection was necessary to ensure that animals had similar environments before the sampling, and to guarantee there was no other factor modulating fish baseline cortisol level that was not the CBD. For example, fish social hierarchy position, or the number of aggressive confronts between individuals in the rearing tanks could have influenced the baseline cortisol level of fish if it was measured in the first experimental weeks (ANTUNES *et al.*, 2021; GONÇALVES-DE-FREITAS *et al.*, 2019).

For the blood sample procedure, fish were taken out of their aquaria with an aquarium dip net, anesthetized by immersion in an aquarium with clove oil (280 mg/l), and afterward, a blood sample was collected (0.4ml) through cardiac punction, with hypodermic needles and heparinized syringes. Posteriorly to this procedure, fish were returned to their experimental aquaria. The handling time between removing and returning fish to the aquarium, lasted less than 5 minutes for all individuals, to avoid any bias of handling time in the cortisol levels. The blood was centrifuged at 3000 rpm for 10 minutes, to the separation of approximately 0.1 ml of plasma, which was frozen at -20ºC for further cortisol assays. The cortisol was measured by ELISA – Enzyme-Linked Immunosorbent Assay, using commercial cortisol kits (DRG).

4.6. FEED INGESTION AND GROWTH VARIABLES

During the first four experimental weeks, the fish feed ingestion and some growth variables were measured. The fifth experimental week was not included in the analysis due to the handling of the animals to the blood sample, stressful even which can alter the motivation to feed (LEAL *et al.*, 2011). However, to ensure that was no effect of possible different feed ingestion between CBD treatments on the stress responses to confinement, the feed ingestion was also measured and analyzed separated in the fifth week. The feed ingestion was measured every day, accounting for the number of pellets eaten daily by each fish. Each day, 39 pellets were offered (approximately 0.9g) to each fish, which is equivalent to about 3% of the animals' initial weight. This daily amount of pellets was divided into three meals (9:00h, 13:00h, and 17:00h). After 4h of a feeding period, the number of pellets left in each aquarium was accounted for, and afterward, these pellets were removed from the aquaria. The feed ingestion was measured by taking into account the number of pellets eaten per day per fish, and the consumed mass of feed was inferred from this value. The mean mass of feed consumed by each week was calculated for further analysis. Regarding fish growth, the following response variables were analyzed: Final standard length, final weight, average weight gain (AWG), feed conversion (FC), specific growth rate (SGR), and condition factor (K). The AWG, FC, SGR, and K were calculated through the following formulas respectively:

$$AWG = Final\ weight\ (g) - Initial\ weight\ (g)$$

$$FC = \frac{Offered\ feed\ (g)}{AWG}$$

$$SGR = \frac{\ln Final\ weight\ (g) - \ln Initial\ weight\ (g)}{Experimental\ period\ (days)} \times 100$$

$$K = \frac{Final\ Weight\ (g)}{Final\ standard\ lengt\ (cm)^3} \times 100$$

4.7. MORPHOLOGICAL AND HISTOLOGICAL VARIABLES OF REPRODUCTION

At the end of the fifth experimental week, testes were collected for morphological and histological analyzes (N = 10 for each treatment). Right after being collected, the gonads were weighed and the gonadosomatic index (GI) were calculation flowing the formula:

$$GI = \frac{Gonad\ weight\ (g)}{Fish\ weight\ (g)} \times 100$$

Afterward, the testicular explants were fixed in 4% Karnovsky at 4°C for at least 24h, dehydrated, and embedded in a Technovit (7100) historesin (Heraeus Kulzer, Germany). Subsequently, the samples were sectioned at 3µm of thickness and stained with 0.1% toluidine blue to further quantify spermatozoa number. The relative number of spermatozoa in fish was estimated per field as previously described by Fallah et al., Tovo Neto et al., and Rodrigues et al.(FALLAH *et al.*, 2019, 2021; RODRIGUES *et al.*, 2021; TOVO-NETO *et al.*, 2020). Thus, 20 non-overlapping fields were chosen randomly and photographed using a Leica DMI6000 microscope (100x objective lens total magnification). The ImageJ software was used to analyze the number of spermatozoa in each field. For this purpose, photos were transformed from “RGB-color” type to “32-bits” type and the threshold adjustment tool was applied, resulting in a black and white image, in which cells were highlighted in black color. The photos were treated with the “filling holes” tool and with the “watershed tool”, which splits merged cells. Areas containing spermatozoa in the image were identified by the cell’s classic characteristics (the small area compared to other spermatogenesis cells and the circle format (SCHULZ *et al.*, 2010) and selected using the “freehand selection” tool. Afterward, the spermatozoa number by field was counted through the "analyze particle" tool. The areas with spermatozoa were selected in the image to avoid any interference from other small and circle black particles in the cell counting.

4.8. STATISTICAL ANALYSIS

All the statistical analyses were performed in the R environment software (v3.6.0.). Shapiro-Wilk and Levene tests were used to test respectively the normality and homoscedasticity of data. For the non-social stress responses (VR and cortisol level pre-stress, VR and cortisol level post-stress, ΔFV and Δcortisol level), growth response variables (final standard length, final weight, AWG, FC, SGR, and K) and morphological variables of reproduction (GI and spermatozoa number by field) an ANOVA one-way were performed when the data met the parametric assumptions and a Kruskal-Wallis test was done when data did not meet any parametric assumption. All the response variables mentioned above were predicted by "treatment", a 4-level (levels: control, CBD 1, CBD 10, CBD 20) categorical independent variable. Post-hoc comparisons were performed using the Tukey HSD test.

Regarding the following responses variables: aggressive behavior (number of attacks, latency for the first aggressive behavior, number of bites, number of touches, and lateral fights), social stress responses (VR pre-stress, VR post-stress, and ΔFV) and feed ingestion, Linear Mixed Models were performed. The independent categorical variable "treatment" (levels: control, CBD 1, CBD 10, CBD 20) and sampling time points (e.g., levels: basal, week 1, week 2, week 3, and week 4) were set as fixed factors in the models, and "fish ID" was included as a nested random factor. The normality of residuals assumption was checked through visual analysis of Normal quantile-quantile plots (QQ plots) of residuals (using the "qqnorm" function in R), and also Shapiro-Wilk tests, and the assumption was valid for all mixed models built on this study. Post-hoc comparisons were performed using the Tukey HSD test. The significance level for all statistical tests performed in this work was set at $\alpha = 0.05$.

5. RESULTS

5.1. AGGRESSIVE BEHAVIOR

In the total number of attacks, we did not observe a significant treatment effect ($F_{(3,\ 55.94)} = 0.362$, $p = 0.78$). However, we observed a significant effect of the sampling time points ($F_{(4,\ 222.1)} = 2.516$, $p = 0.042$) and the interaction between treatments and sampling time points ($F_{(12,\ 222.11)} = 2.424$, $p = 0.006$; Figure 2A). Fish from the CBD 10 treatment decreased the number of attacks significantly over the sampling time points, comparing their basal mean with the attack numbers of weeks 3 and 4 ($p < 0.001$). This decrease in the number of attacks was not observed in the other treatments (Figure 2A).

Regarding the latency for the first aggressive behavior, we observed a significant effect of the sampling time points ($F_{(4,\ 222.638)} = 3.03$, $p = 0.018$) and a significant effect of the interaction between treatments and sampling time points ($F_{(12,\ 222.644)} = 1.986$, $p = 0.027$; Figure 2B), while we did not observe a significant effect of the treatments ($F_{(3,\ 56.266)} = 1.253$, $p = 0.299$). Fish from the control treatment became more motivated to attack the mirror image, decreasing their latency to the first aggressive behavior over the sampling time points. In the third week, fish from the control group presented a significant decrease in the latency to attack the mirror compared to their basal mean ($p = 0.042$). This pattern was not observed in any other treatment exposed to CBD (1, 10, and 20 mg/kg). Interestingly, in the 10 mg/kg treatment we observed even the opposite pattern: fish present an increase in the latency for the first aggressive behavior over the sampling time points. However, this increase was not statistically significant ($p = 0.7347$; Figure 2B).

In the number of bites, we did not observe a significant effect of the treatments ($F_{(3,\ 55.905)} = 0.504$, $p = 0.681$), or sampling time points ($F_{(4,\ 222.041)} = 0.433$, $p = 0.785$). However, we observed a significant effect of the interaction treatments and sampling time points ($F_{(12,\ 222.043)} = 2.469$, $P = 0.005$; Figure 2C). Similar to the number of attacks, fish from treatment 10 mg/kg decreased their number of bites over the sampling time points, significantly decreasing the bite number between the basal measure and the fourth week ($p = 0.039$). This decrease over the sampling time points was not observed in the other treatments (Figure 2C).

In the number of lateral confronts we did not observe a significant effect of the treatments ($F_{(3,\ 56.015)} = 0.412$, $p = 0.745$), or interaction between treatments and sampling time points ($F_{(12,\ 222.262)} = 1.305$, $p = 0.217$), however, we observed a significant effect of the sampling time points ($F_{(4,\ 222.258)} = 11.452$, $p < 0.001$; Figure 2D). In general, fish from all treatment decrease their number of touches over the sampling time points. In the weeks 2, 3 and 4 fish significantly decrease their number of touches against the mirror in relation to their basal measure ($p < 0.001$; Figure 2D). Regarding the number of lateral fights, we did not observe a significant effect of the treatments ($F_{(3,\ 56.063)} = 1.126$, $p = 0.346$), or sampling time points ($F_{(4,\ 222.217)} = 1.175$, $p = 0.323$) or the interaction between treatments and sampling time points ($F_{(12,\ 222.22)} = 0.643$, $p = 0.804$; Figure 2E).

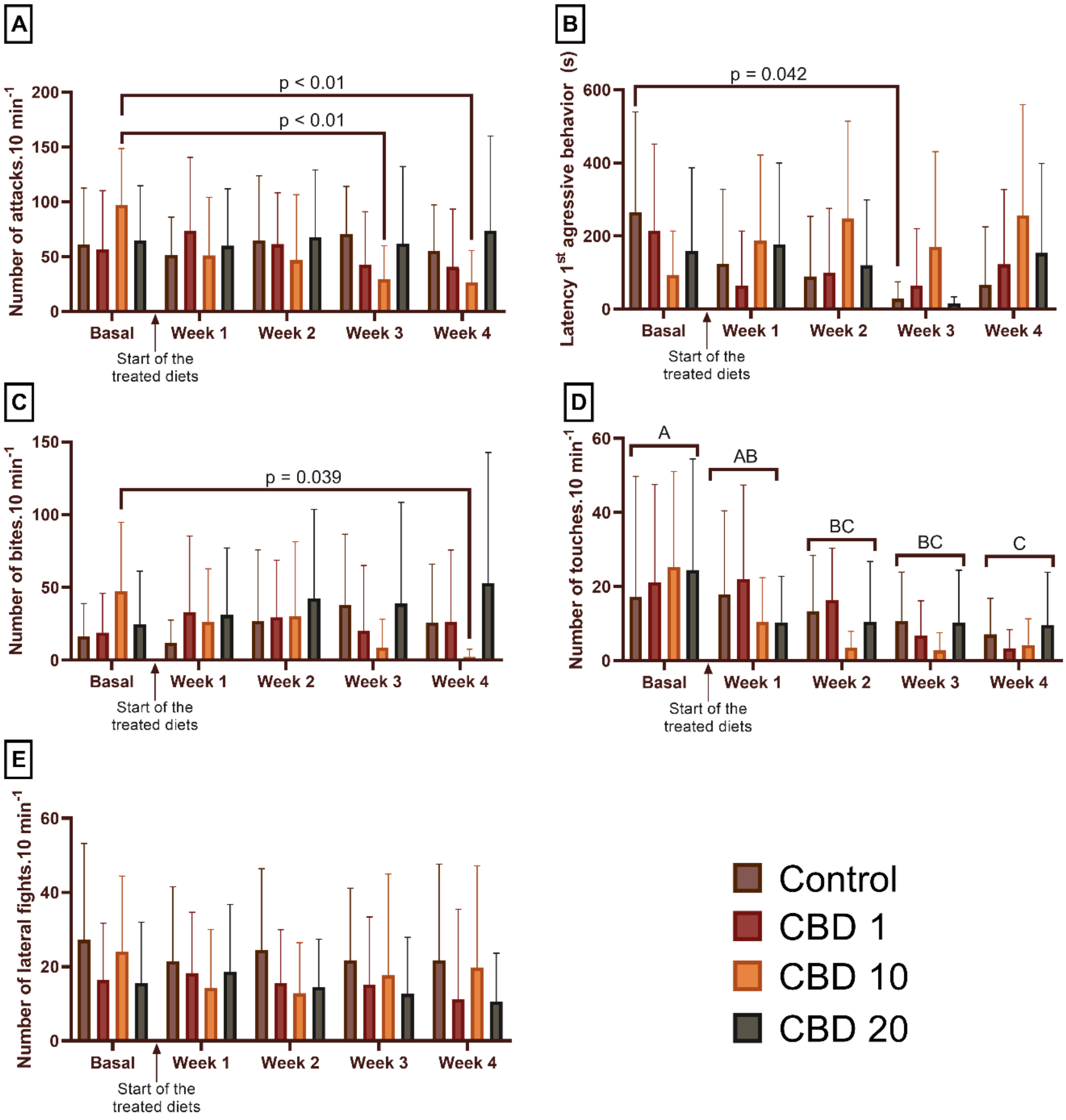

Figure 2 – Effect of diets containing different CBD doses, 0 (control), 1, 10, and 20 mg/kg on **(A)** the number of attacks, **(B)** the latency for the first aggressive behavior, **(C)** the number of bites, **(D)** the number of touches and **(E)** the number of lateral fights of Nile tilapias. These response variables were evaluated over five sampling time points, Basal, Week 1, 2, 3, and 4. The arrow indicates the beginning of the application of the treated diets. In Figures 2A, 2B, and 2C, the highlighted p-values indicate significant differences between the groups under the Tukey Test ($p < 0.05$). In Figure 2C, the uppercase letters indicate a significant difference between the sampling time points, unifying all treatments (Tukey Test; $p < 0.05$). The absence of highlighted p-values or letters indicates there is no significant difference between the treatments or sampling time points (Figure 2E; $p > 0.05$). Values are mean ± SD (N = 15).

5.2. SOCIAL STRESS

In the VR pre-stress, we did not observe a significant treatment effect ($F_{(3, 60)} = 0.589$, $p = 0.624$), or sampling time points ($F_{(4, 240)} = 1.785$, $p = 0.132$), however, we observed a significant effect of the interaction between treatments and sampling time points ($F_{(12, 240)} = 3.742$, $p < 0.001$; Figure 3A). In general, in the week 1, the VR was significantly higher in the CBD 10 treatment compared to the control ($p < 0.01$) and CBD 1 ($p = 0.045$) groups. Moreover, in the week 1, the VR of the CBD 10 treatment was significant higher compared to their basal measure ($p = 0.045$) and to the week 4 ($p < 0.01$). Further, in the week 1, the VR pre-stress of the control treatment was significant lower compared to the basal measure ($p = 0.045$) and the week 3 of the group ($p = 0.037$; Figure 3A).

Regarding the VR post-stress, we did not observe significant treatment effect ($F_{(3, 60)} = 1.346$, $p = 0.268$), however, we observed a significant effect of the sampling time points ($F_{(4, 240)} = 4.529$, $P = 0.001$) and a significant effect of the interaction treatments and sampling time points ($F_{(12, 240)} = 4.503$, $p < 0.001$; Figure 3B). The CBD 1 treatment decreased the VR in week 1 compared to their basal measure ($p < 0.01$). On the other hand, the CBD 10 treatment increased the VR in week 1 compared to their basal ($p = 0.019$), week 3 and 4 ($p < 0.01$) measures. Moreover, in the week 1, fish from the CBD 10 treatment also presented a higher VR compared to the control ($p = 0.027$) and CBD1 ($p < 0.01$) treatments within the same sample time point (Figure 3B).

In relation the variation in the VR, naming ΔVR (VR post – VR pre-stress), we did not observe a significant treatment effect ($F_{(3, 60)} = 1.929$, $p = 0.134$), or sampling time points effect ($F_{(4, 240)} = 0.867$, $p = 0.484$), or effect of the interaction between treatments an time sampling points ($F_{(12, 240)} = 0.62$, $p = 0.824$; Figure 3C).

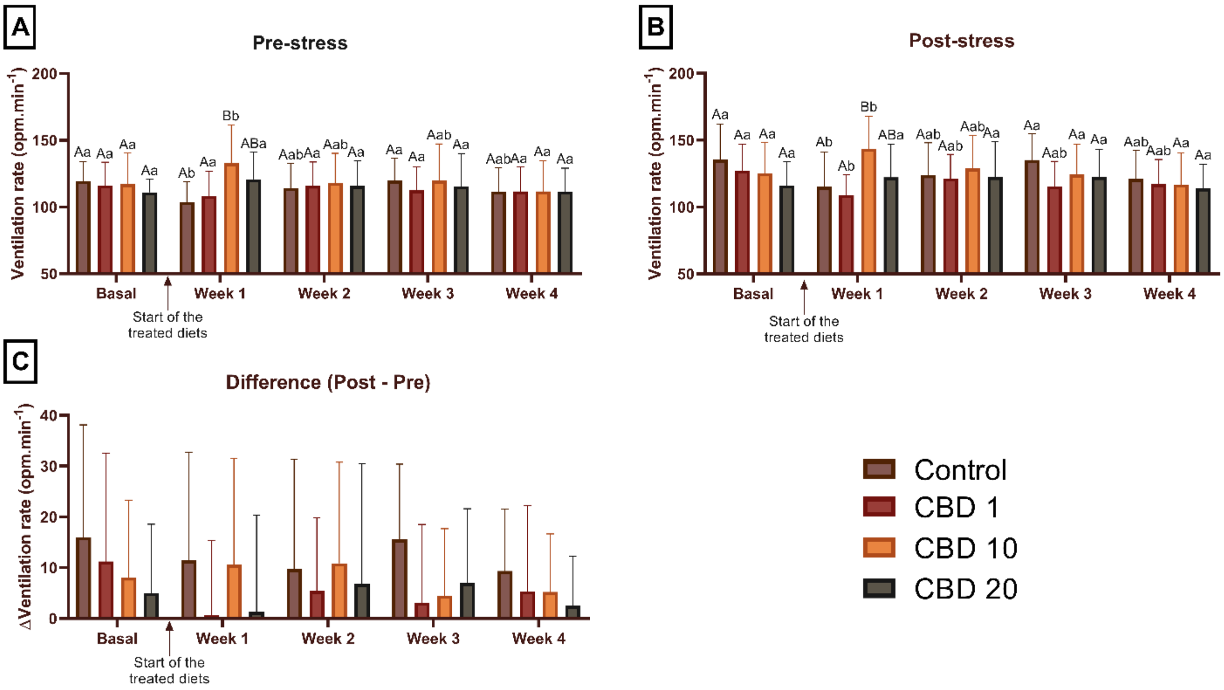

Figure 3 – Effect of diets containing different CBD doses, 0 (control), 1, 10, and 20 mg/kg on **(A)** the ventilation rate (VR) pre-stress, **(B)** the VR post-stress, and **(C)** the ΔVR (VR post – VR pre-stress) of Nile tilapias. The stress applied was social stress, using the fish image reflected on the mirror to induce stress in fish. These response variables were evaluated over five sampling time points, Basal, Week 1, 2, 3, and 4. The arrow indicates the beginning of the application of the treated diets. In Figures 3A and 3B, the uppercase letters indicate significant differences between treatments within a sampling time point (Tukey Test, p <0.05), and lowercase letters indicate significant differences between sampling time points within the same treatment (Tukey Test, p <0.05). The absence of uppercase letters indicates there is no significant difference between the treatments or sampling time points (Figure 3C; p > 0.05). Values are mean ± SD (N = 15).

5.3. NON-SOCIAL-STRESS

We did not observe a significant effect of CBD on the VR pre-stress ($H_{(3)}$= 4.258, p = 0.235; Figure 4A) and post-stress ($F_{(3, 45)}$ = 0.336, p = 0.799; Figure 4B). However, we observed a significant effect of CBD on the ΔVR (VR post – VR pre-stress). Fish from treatment CBD 20 increased less the VR after the confinement stressor, presenting a lower ΔVR compared to the control fish (p = 0.045; Figure 4C).

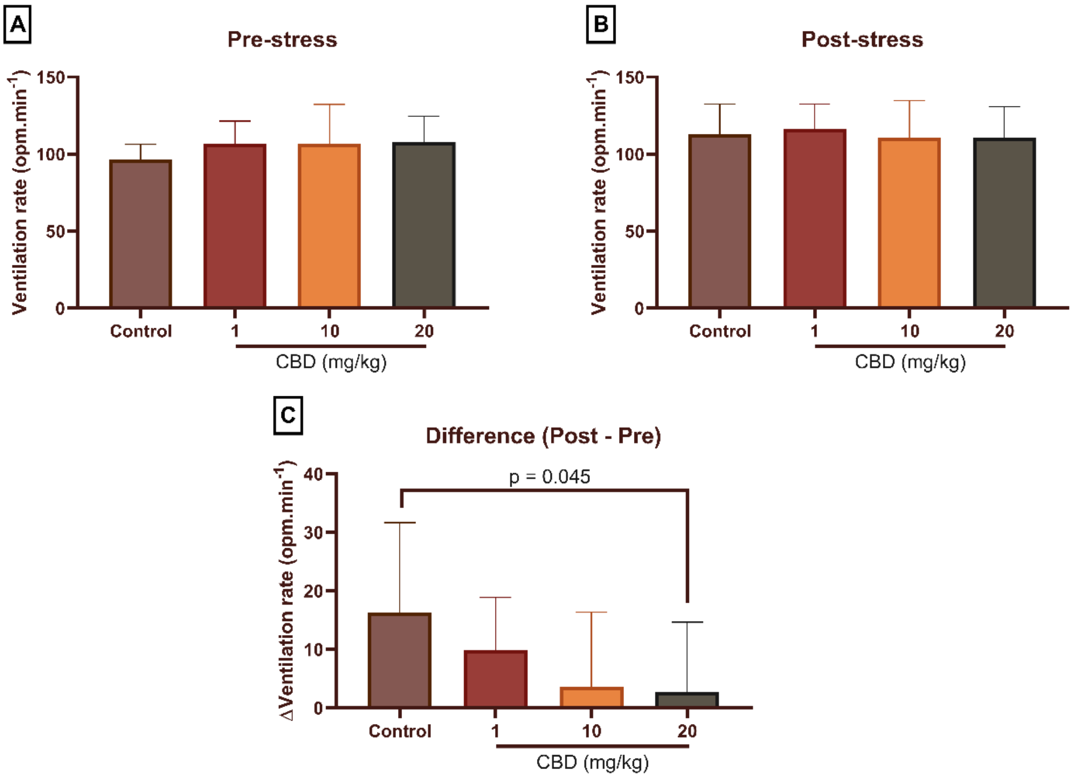

Figure 4 – Effect of diets containing different CBD doses, 0 (control), 1, 10, and 20 mg/kg on **(A)** the ventilation rate (VR) pre-stress, **(B)** the VR post-stress, and **(C)** the ΔVR (VR post – VR pre-stress) of Nile tilapias. The stress applied was non-social stress through a confinement stressor. In Figure 4C, the highlighted p-value indicates a significant difference between the groups under the Tukey Test ($p < 0.05$). The absence of highlighted p-values indicates there is no significant difference between the treatments (Figure 4A and 4B; $p > 0.05$). Values are mean ± SD (N = 15).

Regarding the cortisol level pre-stress, we observed a significant effect of the CBD ($F_{(3, 24)} = 7.621$, $p < 0.001$; Figure 5A). The cortisol level pre-stress of fish from the CBD 10 and CBD 20 groups was lower compared to the control group ($p = 0.023$ and $p < 0.01$, respectively). However, in relation to the cortisol level post-stress and the variation of cortisol level after the confinement stressor (Δcortisol), we did not observe a significant effect of the CBD ($F_{(3, 27)} = 0.99$, $p = 0.412$, Figure 5B; $F_{(3, 24)} = 0.688$, $p = 0.568$, Figure 5C, respectively).

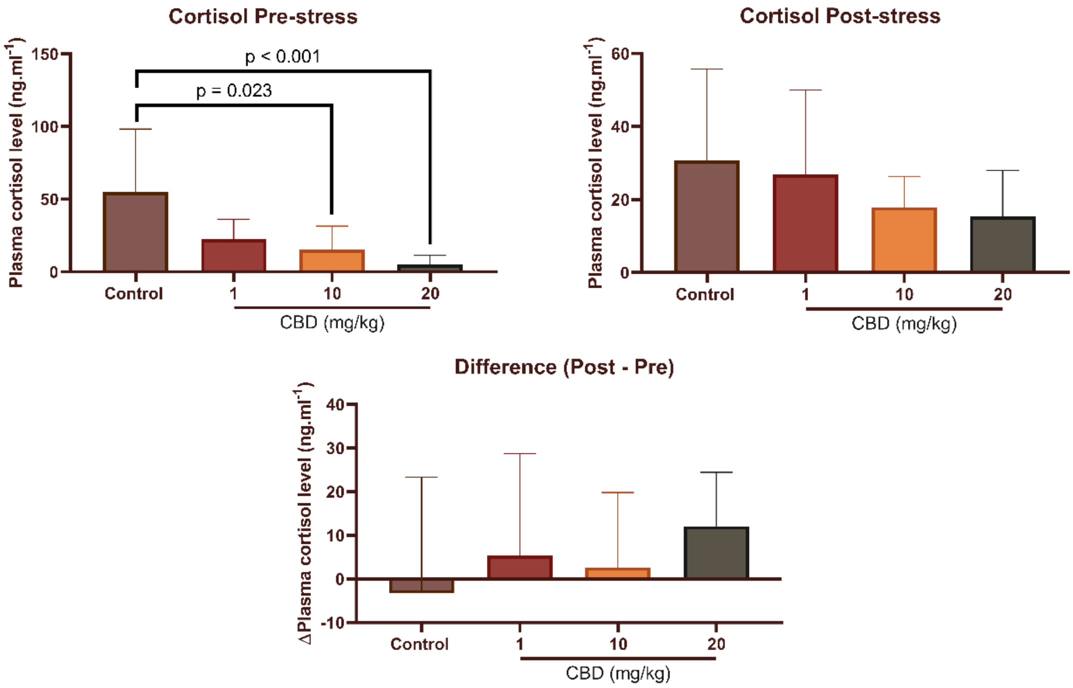

Figure 5 – Effect of diets containing different CBD doses, 0 (control), 1, 10, and 20 mg/kg on **(A)** the plasma cortisol level pre-stress, **(B)** the plasma cortisol level post-stress, and **(C)** the Δplasma cortisol level (cortisol post – cortisol pre-stress) of Nile tilapias. The stress applied was non-social stress through a confinement stressor. In Figure 5A, the highlighted p-values indicate a significant difference between the groups under the Tukey Test ($p < 0.05$). The absence of highlighted p-values indicates there is no significant difference between the treatments (Figure 5B and 5C; $p > 0.05$). Values are mean ± SD (N = 15).

5.4. FEED INGESTION AND GROWTH VARIABLES

The growth response variables: Final standard length ($F_{(3, 55)} = 0.233$, $p = 0.873$), final weight ($H_{(3)} = 1.09$, $p = 0.779$), average weight gain (AWG; ($H_{(3)} = 0.37$, $p = 0.946$), feed conversion (FC; ($H_{(3)} = 0.128$, $p = 0.988$), specific growth rate (SGR; $F_{(3, 55)} = 0.096$, $p = 0.962$) and condition factor (K; $H_{(3\ )}= 1.757$, $p = 0.624$) did not significant differ between the treatments (Table 2). In relation to the feed ingestion, during the first four weeks, we did not observe significant treatment effect ($F_{(3, 60)} = 0.641$, $p = 0.591$) and effect of the interaction between treatments and sampling time points ($F_{(9, 180)} = 0.892$, $p = 0.533$). However, we observed a significant effect of sampling time points ($F_{(3, 180)} = 5.304$, $p = 0.002$). Fish from all treatments ingested more feed in the week 1 compared to the week 3 and 4 ($p = 0.034$ and $p = 0.003$, respectively; Figure 6). The feed ingestion in the fifth week was analyzed apart from the other weeks, due to the manipulation of fish to the blood samples collection in this week, factor which may decrease fish feed ingestion compared to the other weeks. Therefore, we also did not observe a significant effect of the treatments in the feed ingestion of fish in the week 5 ($H_{(3)} = 3.084$, $P = 0.379$).

Table 2 – Growth response variables of Niles tilapias fed during 28 days with diets containing different CBD doses. The absence of asterisks indicates there is no significant difference between the treatments under one-way ANOVA or Kruskal-Wallis tests.

Treatments	Final standard length (cm)	Final weight (g)	AWG [1] (g)	FC [2] (g/g)	SGR [3] (%)	K [4] (%)
Control	11.04 ± 0.697	47 ± 9.024	17 ± 7.512	1.742 ± 0.895	1.242 ± 0.492	3.483 ± 0.484
CBD[5] 1	11.043 ± 0.746	47.667 ± 7.761	18 ± 7.746	1.737 ± 1.158	1.337 ± 0.534	3.476 ± 0.481
CBD 10	10.932 ± 0.512	45.286 ± 6.65	16.357 ± 6.935	1.707 ± 0.395	1.37 ± 0.369	3.503 ± 0.477
CBD 20	10.909 ± 0.794	45.333 ± 10.431	19.545 ± 8.409	1.681 ± 0.983	1.286 ± 0.489	3.454 ± 0.507

[1] AWG – Average weight gain. [2] FC – Feed conversion. [3] SGR – Specific Growth Rate. [4] K – Condition Factor. [5] CBD – Cannabidiol.

Values are mean ± SD (N = 15). No significant differences between the treatments using one-way ANOVA or Kruskal-Wallis tests.

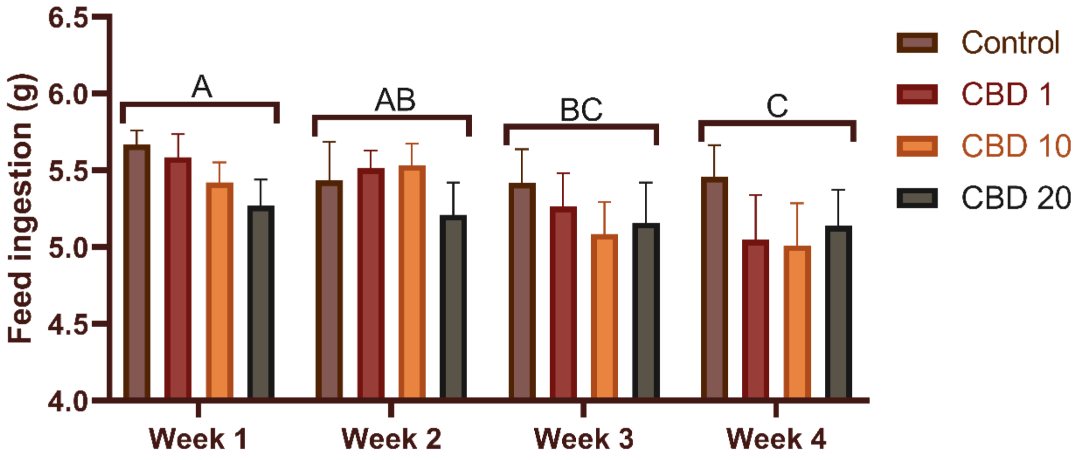

Figure 6 – Effect of diets containing different CBD doses, 0 (control), 1, 10, and 20 mg/kg on the feed ingestion of Nile tilapias. The mean feed ingestion of fish was calculated by week, for 4 weeks. The uppercase letters indicate significant differences between the weeks, unifying all treatments (Tukey test, $p < 0.05$). Values are mean ± SD (N = 15).

5.5. MORPHOLOGICAL AND HISTOLOGICAL VARIABLES OF REPRODUCTION

We observed a significant effect of CBD on the gonadosomatic index (GI; ($F_{(3, 34)} = 11.121$, $p < 0.001$; Figure 7). Fish from the CBD 10 treatment presented a higher GI than those from control ($p = 0.004$) and CBD 1 ($p = 0.031$) treatments.

Similarly, fish from CBD 20 treatment also presented a higher GI compared to fish from control and CBD 1 treatments ($p < 0.001$; Figure 7)

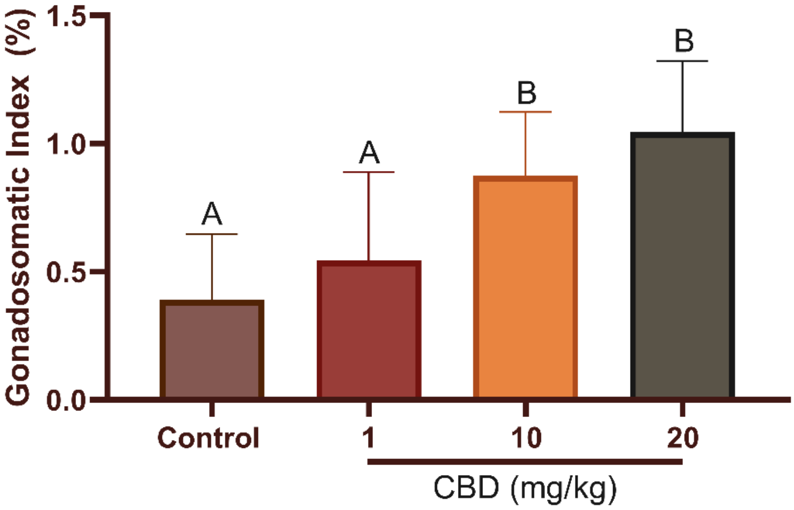

Figure 7 – Effect of diets containing different CBD doses, 0 (control), 1, 10, and 20 mg/kg on the gonadosomatic index (GI) of Nile tilapias. The uppercase letters indicate significant differences between the treatments (Tukey test, $p < 0.05$). Values are mean ± SD (N = 10).

Histomorphometric analysis revealed a significant effect of CBD on the number of spermatozoa by field ($F_{(3, 13)} = 18.588$, $p < 0.001$; Figure 8E). The CBD doses of 1 mg/kg and 10 mg/kg decreased the number of spermatozoa by field of Nile tilapias compared to the control treatment ($p < 0.001$ and $p = 0.418$, respectively). The 20 mg/kg dose also decreased the number of spermatozoa by field compared to the control fish (~40%), however, this decrease was not significant ($p = 0.63$; Figure 8).

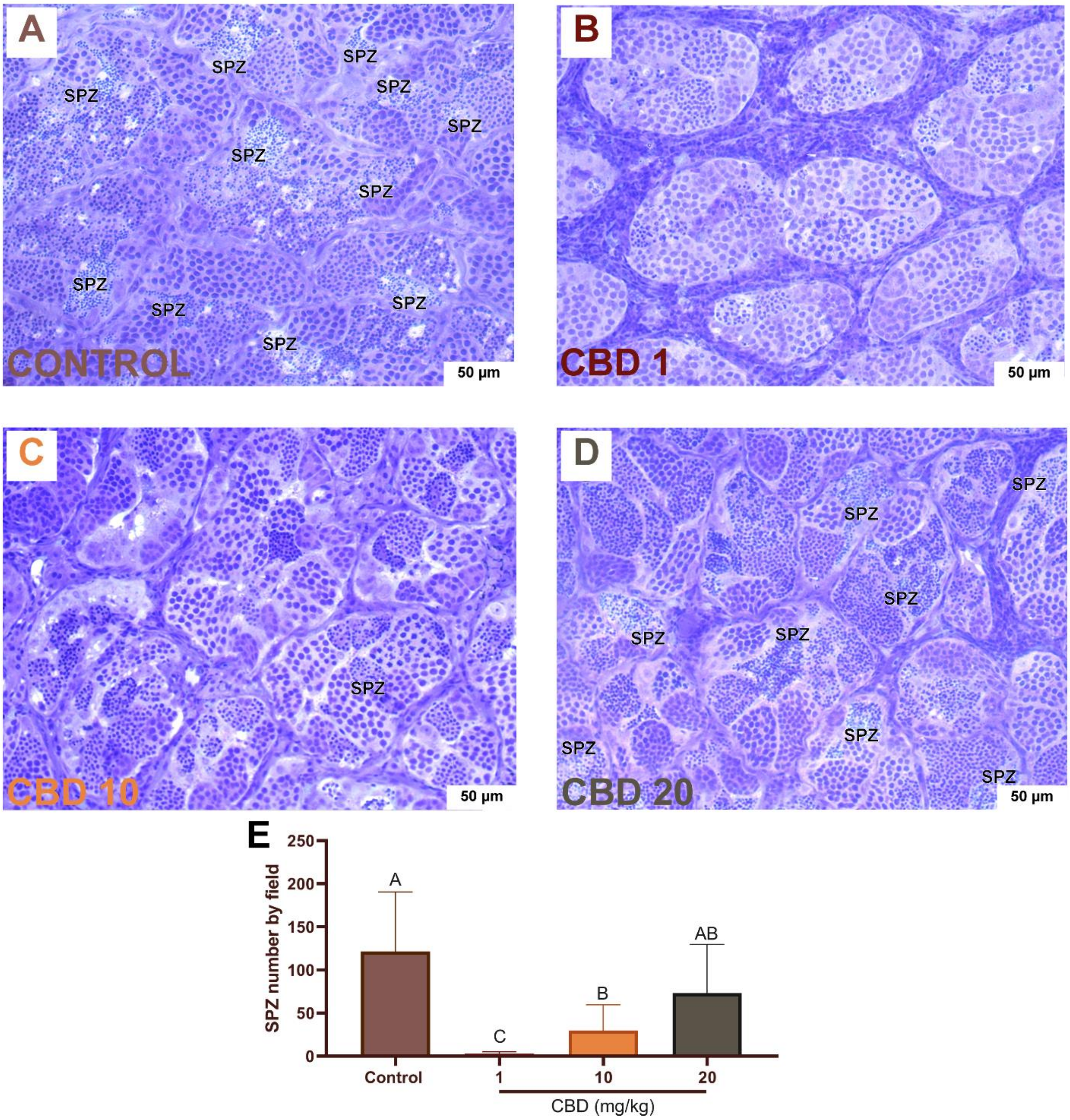

Figure 8 – Ex vivo effects of 0 (control), 1, 10, and 20 mg/kg of CBD on Nile tilapia spermatogenesis. **(A, B, C, D)** Histological sections of Nile tilapia testes following 35-days of exposure to CBD. Spermatozoa (SPZ) are indicated in each figure (scale: 50 µm). **(E)** Ex vivo effects of 0 (control), 1, 10, and 20 mg/kg of CBD on the mean SPZ number by field (total of 20 fields by fish) in testicular explant from Nile tilapias. Different uppercase letters indicate significant differences between treatments (Tukey test, $p < 0.05$) The number of SPZ was determined by morphophysiological analysis using ImageJ software (mean ± SD; N = 5).

6. DISCUSSION

In this study, we investigated the CBD effect on behavioral and morpho-physiological variables related to the welfare of Nile tilapia. We found that the 10 mg/kg dose of CBD was efficient in reducing fish aggressiveness over 28 days. Regarding the social stress, CBD was not efficient in mitigating the stress responses of fish induced by their image reflected in a mirror (social stressor). However, the 20 mg/kg dose of CBD was efficient in attenuating the VR increase induced by confinement stress (non-social stressor). Moreover, in the fifth week of the experiment, fish from CBD 10 and CBD 20 treatments presented a lower level of cortisol when not submitted to an acute stressor. None CBD dose altered feed ingestion or any growth response variable. In addition, the 10 mg/kg and the 20 mg/kg CBD doses still increased fish GI. However, even increasing the testes of fish, CBD at doses of 1 and 10 mg/kg significantly reduced the number of spermatozoa. CBD 20 treatment did not show a significant decrease in the spermatozoa number of fish; however, the group also presented a slightly reduce in spermatozoa numbers (~40%). Therefore, in general, our results demonstrated that CBD in different doses can be incorporated into the fish diet to increase animal welfare in captive and artificial environments.

The CBD efficacy in decreasing aggressiveness of animals was proved only in mammals, more specifically, in rats and dogs (CORSETTI *et al.*, 2021; HARTMANN *et al.*, 2019), but not so far in other taxa. This study was the first to show that CBD is efficient in reducing the aggressiveness of fishes. In dogs, oral CBD administration through 45 days decreased the aggressive behaviors toward humans (CORSETTI *et al.*, 2021). Regarding rats, intraperitoneal doses between 5 and 60 mg/kg of CBD decreased aggressiveness, in which the intermediate doses of 15 and 30 mg/kg were the most efficient (HARTMANN *et al.*, 2019). We observed that CBD oral administration through 28 days was also efficient in decreasing fish aggressiveness. Differently, the results found in rats, only the intermediate 10 mg/kg dose of CBD was efficient in reducing fish aggressive behavior. Lower doses (1 mg/kg) and higher doses (20 mg/kg) did not decrease the animals' aggressive behaviors. Proving that CBD decreases aggressiveness in multiple species is important to identify evolutionary conservated mechanisms and common effects of the drug between all these species. These common mechanisms and effects of CBD may probably be shared not only by the studied species but also with other species, such as humans (GERLAI, 2014). Ultimately, this can help researchers to develop CBD treatments for disorders related to aggressiveness increment in humans.

CBD decreases rats' aggressive behavior through a mechanism associated with the activation of the 5-HT1A serotoninergic receptors and CB1 endocannabinoid receptors (HARTMANN *et al.*, 2019). Although we did not have investigated the mechanism involved in CBD anti-aggressive effects in fish, probably this was similar to the mechanism described in mammals. Firstly, fish 5HT1-A receptors show high similarity to mammals' 5HT1-A receptors (KHAN; DESCHAUX, 1997; WINBERG; NILSSON, 1996), and in both taxa, the present receptor plays a role in the aggressiveness decrease (CLOTFELTER *et al.*, 2007). In addition, the endocannabinoid system is highly conserved between fish and mammals (ELPHICK, 2012; OLTRABELLA *et al.*, 2017), and the CB1 receptors show about 70% similarity between the taxa (OLTRABELLA *et al.*, 2017). The activation of the endocannabinoid system by CBD and other cannabinoids, many times triggers biphasic effects in behavioral responses, such as feeding behavior, locomotor activity, and anxiety-like behavior (REY *et al.*, 2012). Biphasic effects are characterized when a low dose of a drug trigger an effect, while higher doses did not trigger the effect, or even trigger the opposite effect, and vice-versa (REY *et al.*, 2012). Regarding the effect of CBD on anxiety-like behavior, for example, lower and intermediate doses induce anxiolytic-like responses, while higher doses result in the opposite effect, triggering anoxygenic-like responses (REY *et al.*, 2012). Probably, we found the same classic CBD biphasic effect in our results of aggressive behavior, in which an intermediate dose of 10 mg/kg decrease fish aggressiveness, while a higher dose of 20 mg/kg did not decrease.

To be in good welfare conditions, animals must express natural behaviors in captive environments (FRASER *et al.*, 1997; VON KEYSERLINGK; WEARY, 2017). The Nile tilapia is an aggressive cichlid and one of the most farmed fish in the world (ALVARENGA; VOLPATO, 1995; FAO, 2020). Thus, one of the conditions for the species to be considered at a good welfare level in aquaculture environments, is that fish must present aggressive behaviors in the stock tanks since it is a natural component of their natural behavior. However, the environmental conditions in aquaculture tanks, mainly the high stock densities which fish are subject, are responsible for intensifying the aggressive behavior of many species to an unnatural level (GONÇALVES-DE-FREITAS *et al.*, 2019). This leads to an increase in social stress and fish injuries, shrinking the adaptive value of the aggressive behavior in the current environment. As a consequence of all these effects together, there is a decrease in fish welfare (GONÇALVES-DE-FREITAS *et al.*, 2019). Then, the reduction of aggressive behaviors observed in the CBD 10 treatment is important to mitigate the effects of the aquaculture environments on Nile tilapia behavior and maintain fish aggressiveness level in captivity close to the level observed in natural environments. This effect is important to preserve the adaptive value of the aggressive behavior for the species in artificial environments, and consequently, increase Nile tilapia welfare.

Regarding the social stress, surprisingly, we observed that in the first week of receiving the treated diet, fish from the CBD 10 group increased their VR pre and post-stress (Figure 3A and 3B). In the next weeks of the experiment, the VR values of the group returned to the baseline level. The VR is a reliable indicator of stress (BARRETO; VOLPATO, 2011) and anxiety-like effects on fishes (MAXIMINO *et al.*, 2010; YOSHIDA, 2022) since it was closely related to the increment of the metabolic rate, which by your turn increases in stressful situations (BARRETO; VOLPATO, 2011; MOMMSEN; VIJAYAN; MOON, 1999) and anxiety-like situations (MAXIMINO *et al.*, 2010; YOSHIDA, 2022). The exposition to new environments and drugs are two experiences that induce an anxiogenic-like effect in fish and consequently increase their VR (YOSHIDA, 2022). Although we have not carried out any test to analyze the CBD effect on fish anxiety-like responses, the previous results described may be related not only to a stress response but also to a possible anxiogenic-like effect of CBD, since fish increased their VR independent of the presence or not of an acute stressor. Some cannabinoids show an anxiogenic effect a few days after their administration, but this effect seems to attenuate over time (CABERLOTTO *et al.*, 2004; HILL; GORZALKA, 2006). The CBD intraperitoneal acute administration was reported to decrease anxiety-like behavior in fish and mammals (NAZARIO et al., 2015; SCHIER et al., 2012). However, similar to our results, a chronicle of short-term CBD administration (11 days) results in an anxiogenic-like effect in rats (ELBATSH *et al.*, 2012). Thus, further studies are necessary to detangle whether the chronicle CBD administration can present an anxiogenic-like effect in the first days of its application, as well as other cannabinoids.

CBD did not significantly alter fish VR variation (ΔVR) after a social stressor (Figure 3C). It is important to highlight that even did not find any significant effect ($p = 0.84$), we observed a minor effect, since CBD groups seem to present a pattern over the experiment. All CBD treatments had a lower VR variation when exposed to a social stressor compared to the control treatment, which presents a constant ΔVR through all experimental periods (Figure 3C). Further studies are necessary to elucidate whether this minor effect observed in our study can be a real significant effect.

There are some divergences in the CBD effect on stress attenuation. Some studies showed that CBD decreases stress based on heart rate levels (RESSTEL *et al.*, 2009) and cortisol levels (HURD *et al.*, 2019) in dogs and humans respectively. However, some studies demonstrated that CBD is not efficient in decreasing cortisol levels in humans (APPIAH-KUSI *et al.*, 2020; ZUARDI *et al.*, 1993) and dogs (MORRIS *et al.*, 2020). This divergence can be related to an inefficient dose of the drug for the desired effect or can be related to the choice of an inadequate administration-via or administration-time (MILLAR *et al.*, 2018), or even though, can be due to a different effect of CBD depending on the stressor type applied (e.g., social or non-social).

We observed that the higher the CBD dose, the lower was the increase in the VR (ΔVR) in response to non-social stress (Figure 4C). The dose of 20 mg/kg significantly reduce the VR increase in response to the confinement stress compared to the control group. Interestingly, here the CBD effect was dose-dependent and not biphasic. The CBD can promote both types of responses in organisms (EWING *et al.*, 2019; PANDELIDES *et al.*, 2020). Therefore, CBD was efficient in attenuating the VR increase in response to a non-social stressor but was not efficient in response to a social stressor. One possible explanation for this result could be the time that fish were exposed to the CBD diet. The non-social stress was applied only in the fifth week, while the social stressor was applied during the first four experimental weeks. Studies showed that a long-term CBD exposition (28 days – 10 mg/kg) had an anxiolytic-like and antidepressant effect in rats exposed to chronic unpredictable mild stress (GÁLL *et al.*, 2020). Thus, the long-term CBD administration may also affect the attenuation of some stress responses, such as the VR in fish.

Besides the time of exposition to CBD, the type of stressor, or in other words, whether it was a social or non-social stressor, may be influenced by the differences in the stress-attenuation effect of the drug. In rats, CBD doses of 10 and 20 mg/kg were efficient in reducing physiological responses (namely, an increase in heart rate) to a non-social stressor (restraint stress (RESSTEL *et al.*, 2009)). Similarly, in humans, CBD 10 mg/kg dose decreased the cortisol level in response to a non-social stressor (drug induction (HURD *et al.*, 2019)). However, the same CBD dose was ineffective in attenuating the cortisol increase in response to a social stressor ("trier social stress test") in humans patients with a high risk of psychosis (APPIAH-KUSI *et al.*, 2020). Moreover, seems that CBD stress-attenuation effect do not depend only on the type of stressor applied to animals, and the chosen dose also plays an important role in the effect, since a dose of 1.4 mg/kg did not reduce the cortisol levels in response to a non-social stressor in dogs (fireworks (MORRIS *et al.*, 2020)). Therefore, further studies are necessary to unravel whether indeed CBD attenuates only the stress responses to non-social stressors and does not attenuate the responses to social stressors. In addition, it is important to investigate the appropriate dosage for each stressor type.

Even the VR is a reliable secondary stress biomarker in fish (BARRETO; VOLPATO, 2011), other physiological stress responses can be evaluated, such as other secondary stress responses (e.g., blood glucose), and the main stress hormone in fish, cortisol (ANTUNES *et al.*, 2021). To have a broader evaluation of the stress response in an animal, it is recommended to evaluate the maximum of the stress responses possible (GONÇALVES-DE-FREITAS *et al.*, 2019; STOLTE *et al.*, 2008). In this sense, we decided to measure also the increasement of cortisol levels in response to non-social stress. The stress-induced cortisol level and the Δcortisol were not altered by the exposure to CBD. Similarly, Mortuza et al.(MORTUZA; MASON; MUSTAFA, 2021) found that CBD did not significantly decrease other stress biomarkers, such as glucose, hematocrit, and plasma protein in stressed and non-stressed Nile tilapias. Although Mortuza et al.(MORTUZA; MASON; MUSTAFA, 2021) did not find significant effects, CBD groups that received about 1 mg/kg of CBD per day for only three days, slightly reduced some of the secondary stress responses measured in their study.

Cortisol plays a role in the increase of metabolism in fish (BARTON; SCHRECK, 1986; DAVIS; SCHRECK, 1997). The VR also increases with the metabolic rate increase (BARRETO; VOLPATO, 2004; CLAIREAUX; LAGARDÈRE, 1999). Although these two stress markers present a positive correlation in fish (BARRETO; VOLPATO, 2004), cortisol alone after stress is unlikely to increase the metabolic rate, a process which depends on other factors, such as the whole-body lactate for example (BARRETO; VOLPATO, 2004; DAVIS; SCHRECK, 1997). Thus, the decrease in the ΔVR in response to the confinement stress found in our results may be related to other mechanisms that are not the stress-induced cortisol levels, such as other secondary stress biomarkers. Although we did not observe any effect of CBD on the stress-induced cortisol levels and the Δcortisol, we found that in the fifth week CBD substantially decreased the baseline cortisol levels.

The decrease of the baseline cortisol levels induced by CBD is promisor to the welfare improvement of captive fish. The lower baseline cortisol levels can be beneficial to organism functioning, since higher cortisol levels lead to the impairment of the reproduction system (SCHRECK; CONTRERAS-SANCHEZ; FITZPATRICK, 2001), depress immunity (POTTINGER, 2008), impair the growth and organs development (FERNANDES; VOLPATO, 1993; SOPINKA *et al.*, 2016), among other negative effects on the organism functioning (SOPINKA *et al.*, 2016). However, the cortisol increasing as a stress response, is also adaptive to animals, helping them cope adequately with environmental challenges and improving their survival chance and their fitness (WENDELAAR BONGA, 1997). Our results showed that CBD decrease only the baseline cortisol hormone concentration. Thus, when fish treated with CBD are submitted to an acute stressor, they increase their cortisol level similar to fish not submitted to CBD, showing an adequate stress response that allows them to present the expected physiological responses and behavior alterations to cope with the stressor. From an integrative welfare perspective, this is important, since CBD may improve the functionality of fish organisms in a rearing condition without any acute stressor. Meanwhile, whether a stressful event occurs, fish can trigger a normal stress response, which may allow them to express their natural behavior to cope with a stressor in captivity. For example, in a risky situation, such as the presence of a threat, chasing by a net, or in an intraspecific territorial fight, fish will present a normal increase in cortisol levels, which can allow the appropriate behavior responses for each stressful situation.

In addition to playing a role in the regulation of the aggressiveness and stress axis (PACHER; BÁTKAI; KUNOS, 2006), the endocannabinoid system also plays a role in the regulation of body weight gain and food intake (DI MARZO, 2008; IGNATOWSKA-JANKOWSKA; JANKOWSKI; SWIERGIEL, 2011; PERTWEE, 2009). Thus, cannabinoids such as CBD that modulate this system can affect growth variables and animals' food intake (GÁLL *et al.*, 2020; IGNATOWSKA-JANKOWSKA; JANKOWSKI; SWIERGIEL, 2011). Some studies showed that CBD alters the body weight gain in rats, however, the results are divergent, since CBD can increase the body weight gain (GÁLL *et al.*, 2020), did not affect it (OSBORNE *et al.*, 2017), or even decrease the body weight gain (IGNATOWSKA-JANKOWSKA; JANKOWSKI; SWIERGIEL, 2011). Regarding fish, females zebrafish exposed to CBD at the larval stage had a reduction of their size (weight and length) in adulthood, while males did not present any alteration in their size (PANDELIDES *et al.*, 2020). Similarly, in our results, juvenile males of Nile tilapia supplemented with different CBD doses did not show alterations in any of the growth response variables measured, nor in the food intake. The food intake only decreased in the last experimental weeks but for all experimental treatments. First of all, it is relevant to highlight that diet supplemented with CBD was not unpalatable for fish, validating this CBD-administration method for the taxon. Moreover, it is important to emphasize that CBD supplementation improves fish welfare and did not reduce their body weight gain, which is beneficial for both, animals and fish farmers since CBD can provide a better quality of life in captivity environments for Nile tilapias, and also maintain the same level of meat production for the fish farmers.

S to our results, a CBD dose of 10 mg/kg did not affect the weight gain in rats (OSBORNE *et al.*, 2017). However, (GÁLL *et al.*, 2020) observed that this same dose increased the body weight gain and food intake of sucrose in rodents. (GÁLL *et al.*, 2020) exposed the rats to a chronic unpredictable mild stress for 28 days. The chronic stress decreases the feed intake and the weight gain (MARTÍ; MARTÍ; ARMARIO, 1994), thus, probably the weight gain induced by CBD may be related to some effect of the drug in the attenuation of the chronic stress (GÁLL *et al.*, 2020).

In our study, we did not apply any chronic stress to fish, only exposed the to some acute stressors. Considering that CBD did not affect any growth variable and food intake in the presence of only acute stressors in fish, and can induce an increase in the food intake and body weight gain in the presence of chronic stress in rats (GÁLL *et al.*, 2020), the supplementation of fish diet with CBD may bring even better results in real aquaculture conditions. In aquaculture tanks, fish are subjected to some chronic stressors, such as high stock densities, and several acute stressors, such as the processes of grading, and slaughter (CONTE, 2004). Further studies are necessary to elucidate whether, in a real aquaculture condition, where animals are exposed to the chronic stress of high densities, the CBD can increase feed intake and body weight gain in fish.

Although we did not observe any CBD effect on growth variables of Nile tilapias, the 10 and 20 mg/kg doses increased the gonadosomatic index (GI) of fish. The GI is a macroscopic measure used to estimate the gonadal maturation in several fish species (FLORES *et al.*, 2019; VITALE; SVEDÄNG; CARDINALE, 2006; ZEYL; LOVE; HIGGS, 2014), being also welfare indicative since high values of GI means that animals are in good rearing conditions (NOBLE *et al.*, 2018). A factor that impairs gonadal development and consequently decreases the GI is the high cortisol level (CONSTEN *et al.*, 2001). In our study, Nile tilapias treated with high doses of CBD (10 and 20 mg/kg) presented a decrease in their baseline cortisol level in the fifth experimental week. The cortisol level has not been accessed before, so we do not know whether the hormone level was already low in the first four experimental weeks. Our hypothesis is that maybe, the baseline cortisol level was already low during the first experimental weeks, which leads to a higher development of the fish testes. Additional research can elucidate what is the minimum time necessary to CBD decrease the baseline cortisol, and then confirm or reject this hypothesis.

The corticosteroid hormones, such as cortisol, are not the only hormones that affect the testis's development, sexual hormones such as testosterone, progesterone, and estradiol also play an important role in it (SCHOLZ; KLÜVER, 2009). Carvalho et al. (CARVALHO *et al.*, 2022) showed that CBD orally administrated for 34 days did not alter the level of the sexual hormone in rats, and consequently the gonadal development. The authors of this study did not measure the level of any corticosteroid hormones in rats. In rats and mice, the main corticosteroid hormone that regulates the stress responses is corticosterone (GONG *et al.*, 2015), differently in fish and humans, in which cortisol lead a key role in the stress axis regulation (ANTUNES *et al.*, 2021; RUSSELL; LIGHTMAN, 2019). Therefore, due to these differences in the stress axis between the species, maybe in rats and mice, CBD did not have the same stress-attenuation effect observed in fish and consequently did not have the same effect on the testis's development. However, in our study, we also did not measure any sexual hormones of fish. There are also some differences in the reproductive system and its endocrine regulation between fish and mammals (NISHIMURA; TANAKA, 2014; WILHELM; PALMER; KOOPMAN, 2007). Therefore, we did not discard that the gonadal development promoted by CBD in our study may also have been influenced by any effect of the drug on the sexual hormones of fish. We encourage evolutionary comparative studies to verify the CBD effects on the stress axis and reproductive system of different vertebrates. This will help to unravel the exact underlying mechanisms of the drug in the body systems, which can be very useful for medicinal purposes, animal behavior improvement, and other promising applications of CBD.

Although there is an increase in the testes size of the Nile tilapias treated with CBD, it was not accompanied by an increase in the number of spermatozoa. Fish treated with 1 and 10 mg/kg of CBD presented fewer spermatozoa compared to the control fish. In particular, the 1 mg/kg dose drastically decreased the number of spermatozoa cells (Figure 8E). The endocannabinoid system plays a key role in the regulation of spermatogenesis (GRIMALDI *et al.*, 2009). The CB2 receptors are expressed in all the cell stages of spermatogenesis, and it was suggested that their activation by endocannabinoids, such as the anandamide, is related to the regulation of the mitotic cells of the spermatogenesis (Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss miceCARVALHO *et al.*, 2018). Meanwhile, another target receptor of anandamide, the transient receptor potential cation channel subfamily V member 1 receptors (TRPV1), are highly expressed in the meiotic stage, and possibly are involved in the control of this spermatogenesis stage (Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss miceCARVALHO *et al.*, 2018). The CBD promotes an increase of anandamide, blocking the fatty acid amide hydrolase enzyme (FAAH), which is responsible for degrading the endocannabinoid. This increase of anandamide leads to high activation of CB2 receptors (Chronic cannabidiol exposure promotes functional impairment in sexual behavior and fertility of male miceCARVALHO *et al.*, 2018; CARVALHO; ANDERSEN; MAZARO-COSTA, 2020). However, CBD can also block the CB2 receptors or activate the TRPV1 receptors, depending on the dose (Chronic cannabidiol exposure promotes functional impairment in sexual behavior and fertility of male miceCARVALHO *et al.*, 2018; CARVALHO; ANDERSEN; MAZARO-COSTA, 2020). The CBD action mechanism described above can help us to have a better understanding of many of their biphasic effects in the organism since depending on the dose, the drug can activate one or another receptor (Chronic cannabidiol exposure promotes functional impairment in sexual behavior and fertility of male miceCARVALHO *et al.*, 2018; HARTMANN *et al.*, 2019). For example, low doses of CBD inhibit sexual behavior, by indirect activation of CB1 receptors, while higher doses improve it, blocking CB1 receptors or activating TRPV1 (Chronic cannabidiol exposure promotes functional impairment in sexual behavior and fertility of male miceCARVALHO *et al.*, 2018). Therefore, different doses of the drug can cause distinct disturbances on the spermatogenesis axis (Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss miceCARVALHO *et al.*, 2018; CARVALHO; ANDERSEN; MAZARO-COSTA, 2020). In our results, we observed that low doses of CBD decrease substantially the number of spermatozoa, and higher doses promote a smaller decrease in it (Figure 8). We did not analyze the effect of CBD on other stages of spermatogenesis, such as cells pre-meiotic. Maybe, smaller doses such as the 1 mg/kg dose led to an increase of pre-meiotic cells and a decrease in the post-meiotic cells, while higher doses could have the opposite effect. Further studies are necessary to elucidate how the CBD biphasic effect regulates the entire spermatogenesis.

Other studies did not find a CBD effect on the number of spermatozoa, as our results (CARVALHO *et al.*, 2022), however, they reported other reproductive toxicity CBD effects on the male reproductive system in a large number of animals (sea urchins (SCHUEL *et al.*, 1987); rats (CARVALHO; ANDERSEN; MAZARO-COSTA, 2020); and zebrafish (PANDELIDES *et al.*, 2020), such as deregulation on spermatogenesis, impairment on sperm quality and motility (Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss miceCARVALHO *et al.*, 2018, 2022; PANDELIDES *et al.*, 2020), and also reduction in sperm fertility (SCHUEL *et al.*, 1987). However, there is an active debate about the reversibility or not of reproductive toxicity effects of CBD on the male reproductive system, having divergences about it (CARVALHO; ANDERSEN; MAZARO-COSTA, 2020). Some authors suggest that an important factor that can lead to reversible or irreversible effects of CBD on the reproductive system is the period in which animals are exposed to the drug (CARVALHO; ANDERSEN; MAZARO-COSTA, 2020). There was suggested that exposures during the gonadal development can lead to irreversible and long-term effects (CARTY *et al.*, 2019; CARVALHO; ANDERSEN; MAZARO-COSTA, 2020), while exposures after this period lead to reversible effects (Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss miceCARVALHO *et al.*, 2018; CARVALHO; ANDERSEN; MAZARO-COSTA, 2020). Therefore, to use CBD as a tool to improve animal welfare, we have to take into count these toxicity effects on the males' reproductive system and also elucidate better whether these effects are reversible or irreversible, and at what age the use of CBD is safer to the male reproductive system.

Finally, the stress and aggressiveness reduction, besides the increase in the GI reflects an improvement in fish welfare. The stress level is a classic and highly validated welfare indicator (MARTINS *et al.*, 2012), and its reduction leads to an improvement in fish's quality of life in artificial environments. About aggressiveness, aggressive interactions are a natural component of the behavior of many social species of fish, including the Nile tilapia (ALVARENGA; VOLPATO, 1995). However, the aquaculture environment conditions increase the number of aggressive behaviors to an exacerbated level, in which it is no longer adaptive, leading to an increase in bodily injuries and the social stress of fish (GONÇALVES-DE-FREITAS *et al.*, 2019). Therefore, the aggressiveness reduction of fish in aquaculture environments can act as an adjustment mechanism, regulating the aggressive behavior of the Nile tilapia to a level considered natural for the species, restoring the adaptive value of this behavior in the current environment, and ultimately improving fish welfare. Regarding the GI, it also has been considered a relevant welfare indicator, since high values of GI indicate good rearing conditions that allows a better gonadal development (NOBLE *et al.*, 2018).

7. CONCLUSIONS

Therefore, we conclude that CBD can be offered in fish diets to improve their welfare in aquaculture and captivity environments. The 10 mg/kg dose reduced fish aggressive behavior, reduced non-social stress and baseline cortisol levels, and also improved the GI. However, it is important to take into account that this CBD dose significantly reduced the spermatozoa number of fish, and it is necessary to caution when using it to improve the welfare of Nile tilapias reared for reproduction purposes. The 20 mg/kg dose presented the best results in the reduction of non-social stress, reducing baseline cortisol levels, and in increasing GI. This dose did not significantly reduce the number of spermatozoa, however, the CBD 20 fish presented ~40% fewer spermatozoa than the control fish. So, it is also necessary to have some caution when using CBD at this dosage to improve fish welfare, mainly when fish are reared for reproduction purposes. Therefore, CBD at 20 mg/kg can be a promising tool to improve the welfare of non-aggressive social fish species, while CBD at 10 mg/kg dose can be used to improve the welfare of aggressive fish species, such as the Nile tilapia. This improvement of fish welfare by the CBD administration is accompanied by a non-reduction in fish growth. This can be beneficial for the animals, which presents a better quality of life, and also for the fish farmers, who can keep the same level of fish production that they will have using feed without CBD, with the advantage of producing animals with a better-added value due to the rearing conditions. Our study was the first to explore the potential of CBD effects as a tool to improve the welfare of captivity and farming animals, being effective in fish. Currently, CBD is not a cheap resource, but the low concentrations used in our study already presented promising results. Besides, with the constant increase in cannabis acceptance by society, accompanied by the trend of the legalization and farming of the plant in many countries, CBD prices may decrease in a near future (MORTUZA; MASON; MUSTAFA, 2021). However, further studies focusing on the CBD effect on the quality of fish meat, as well as whether there is an accumulation of the drug in the meat and the water are necessary before the implementation of this welfare improvement method in aquacultures. We encourage researchers to investigate the efficacy of the CBD in the welfare improvement of other captivity and farming animals.

REFERENCES

ALMEIDA, Valéria *et al.* Cannabidiol exhibits anxiolytic but not antipsychotic property evaluated in the social interaction test. **Progress in Neuro-Psychopharmacology and Biological Psychiatry**, v. 41, p. 30–35, 2013.

ALVARENGA, Cláudia Maria Domingues; VOLPATO, Gilson Luiz. Agonistic profile and metabolism in alevins of the Nile tilapia. **Physiology & Behavior**, v. 57, n. 1, p. 75–80, 1995.

AMOAKO, A. A. *et al.* Anandamide modulates human sperm motility: Implications for men with asthenozoospermia and oligoasthenoteratozoospermia. **Human Reproduction**, v. 28, n. 8, p. 2058–2066, 2013.

ANTUNES, Diogo F. *et al.* Early social experience has life-long effects on baseline but not stress-induced cortisol levels in a cooperatively breeding fish. **Hormones and Behavior**, v. 128, n. July 2020, p. 104910, 2021.

APPIAH-KUSI, E. *et al.* Effects of short-term cannabidiol treatment on response to social stress in subjects at clinical high risk of developing psychosis. **Psychopharmacology**, v. 237, n. 4, p. 1121–1130, 2020.

ARCHARD, Gabrielle A.; BRAITHWAITE, Victoria A. Variation in aggressive behaviour in the poeciliid fish Brachyrhaphis episcopi: Population and sex differences. **Behavioural Processes**, v. 86, n. 1, p. 52–57, 2011.

BARRETO, Rodrigo Egydio *et al.* Aggressive behaviour traits predict physiological stress responses in Nile tilapia (Oreochromis niloticus). **Marine and Freshwater Behaviour and Physiology**, v. 42, n. 2, p. 109–118, 2009.

BARRETO, Rodrigo Egydio; VOLPATO, Gilson Luiz. Caution for using ventilatory frequency as an indicator of stress in fish. **Behavioural Processes**, v. 66, n. 1, p. 43–51, 2004.

BARRETO, Rodrigo E.; VOLPATO, Gilson L. Ventilation rates indicate stress-coping styles in Nile tilapia. **Journal of Biosciences**, v. 36, n. 5, p. 851–855, 2011.

BARRETO, Rodrigo Egydio; VOLPATO, Gilson Luiz. Ventilatory frequency of Nile tilapia subjected to different stressors. **Journal of Experimental Animal Science**, v. 43, n. 3, p. 189–196, 2006.

BARTON, B. A.; SCHRECK, C. B. Metabolic Cost of Acute Physical Stress in Juvenile Steelhead. **Transactions of the American Fisheries Society**, v. 116, n. 2, p. 257–263, 1986.

BATTISTA, Natalia *et al.* Regulation of female fertility by the endocannabinoid system. **Human Fertility,** v. 10, n. 4, p. 207-216, 2007.

BILLARD, R; BRY, C; GILLET, C. Stress, environment and reproduction in teleost fish. *In*: PICKERING, A.D. (org.). **Stress and Fish**. London: Academic Press, 1981. p. 185–208. *E-book*. Disponível em: https://hal.archives-ouvertes.fr/hal-01600274. Acesso em: 3 mar. 2021.

BISOGNO, Tiziana *et al.* Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. **British Journal of Pharmacology**, v. 134, n. 4, p. 845–852, 2001.

BROOM, Donald M. A History of Animal Welfare Science. **Acta Biotheoretica**, v. 59, n. 2, p. 121–137, 2011.

BURSTEIN, Sumner. CBD Receptor Binding Signaling events Downstream events. **BIOORGANIC & MEDICINAL CHEMISTRY**, n. January, 2015.

CABERLOTTO, Laura *et al.* Corticotropin-Releasing Hormone (CRH) mRNA Expression in Rat Central Amygdala in Cannabinoid Tolerance and Withdrawal: Evidence for an Allostatic Shift?. **Neuropsychopharmacology**, v. 29, n. 1, p. 15–22, 2004.

CAMARGO-DOS-SANTOS, Bruno *et al.* The impact of catch-and-release on feeding responses and aggressive behavior in Nile tilapia (Oreochromis niloticus). **Marine and Freshwater Behaviour and Physiology**, v. 54, n. 3, p. 133–148, 2021.

CAMPOS, Alline Cristina; FERREIRA, Rogério Frederico; GUIMARÃES, Francisco Silveira. Cannabidiol blocks long-lasting behavioral consequences of predator threat stress : Possible involvement of 5HT1A receptors. v. 46, 2012.

CARTY, Dennis R. *et al.* Multigenerational consequences of early-life cannabinoid exposure in zebrafish. **Toxicology and Applied Pharmacology**, v. 364, n. December 2018, p. 133–143, 2019.

CARVALHO, Renata K. *et al.* Chronic cannabidiol exposure promotes functional impairment in sexual behavior and fertility of male mice. **Reproductive Toxicology**, v. 81, p. 34–40, 2018.

CARVALHO, Renata K. *et al.* Chronic exposure to cannabidiol induces reproductive toxicity in male Swiss mice. **Journal of Applied Toxicology**, v. 38, n. 9, p. 1215–1223, 2018.

CARVALHO, Renata K. *et al.* Decreasing sperm quality in mice subjected to chronic cannabidiol exposure: New insights of cannabidiol-mediated male reproductive toxicity. **Chemico-biological interactions**, v. 351, p. 109743, 2022, 2022.

CARVALHO, Renata K.; ANDERSEN, Monica L.; MAZARO-COSTA, Renata. The effects of cannabidiol on male reproductive system: A literature review. **Journal of Applied Toxicology**, v. 40, n. 1, p. 132–150, 2020.

CECCONI, Sandra *et al.* Role of Major Endocannabinoid-Binding Receptors during Mouse Oocyte Maturation. **International Journal of Molecular Sciences**, v. 20, n. 12, p. 2866, 2019.

CLAIREAUX, G.; LAGARDÈRE, J. P. Influence of temperature, oxygen and salinity on the metabolism of the European sea bass. **Journal of Sea Research**, v. 42, n. 2, p. 157–168, 1999.

CLOTFELTER, Ethan D. *et al.* Serotonin decreases aggression via 5-HT1A receptors in the fighting fish Betta splendens. **Pharmacology Biochemistry and**

Behavior, v. 87, n. 2, p. 222–231, 2007.

CONSTEN, D. *et al.* Long-term cortisol treatment inhibits pubertal development in male common carp, Cyprinus carpio L. **Biology of Reproduction**, v. 64, n. 4, p. 1063–1071, 2001.

CONTE, F. S. Stress and the welfare of cultured fish. *In*: , 2004. **Applied Animal Behaviour Science**, v. 86, n. 3-4, p. 205-223, 2004.

CORSETTI, Sara *et al.* Cannabis sativa L. may reduce aggressive behaviour towards humans in shelter dogs. **Scientific Reports 2021 11:1**, v. 11, n. 1, p. 1–10, 2021.

CUNHA, Jomar M. *et al.* Chronic administration of cannabidiol to healthy volunteers and epileptic patients. **Pharmacology**, 1980.

DAVIS, L.E.; SCHRECK, C.B. The energetic response to handling stress in juvenile coho salmon. **Transactions of the American Fisheries Society**, v. 126, n. 2, p. 248–258, 1997.

DESJARDINS, Julie K.; FERNALD, Russell D. What do fish make of mirror images?. **Biology Letters**, v. 6, n. 6, p. 744–747, 2010.

DEVINSKY, Orrin *et al.* Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. **Epilepsia**, v. 55, n. 6, p. 791–802, 2014.

DI MARZO, Vincenzo. Targeting the endocannabinoid system: To enhance or reduce?, **Nature reviews Drug discovery**, v. 7, n. 5, p. 438-455, 2008..

DUNCAN, I.J. A concept of welfare based on feelings. *In*: BENSON, G. J.; ROLLIN, B. E. (org.). **The Well-Being of Farm Animals: Challenges and Solutions**. Ames, IA, USA: Blackwell, 2004. p. 85–101. *E-book*. Disponível em: https://books.google.com.br/books?hl=pt-BR&lr=&id=74BPNxkaZoAC&oi=fnd&pg=PA85&dq=A+concept+of+welfare+based+on+feelings.+Pages+85–101+in+The+Well-Being+of+Farm+Animals:+Challenges+and+Solu-+tions.&ots=nAR4heKJgB&sig=zPRdpDmNUPkunFoPHdBQ1flZHv4. Acesso em: 13 jan. 2022.

ELBATSH, Maha M. *et al.* Anxiogenic-like effects of chronic cannabidiol administration in rats. **Psychopharmacology**, v. 221, n. 2, p. 239–247, 2012.

ELLIS, T. *et al.* The relationships between stocking density and welfare in farmed rainbow trout. **Journal of Fish Biology**, v. 61, n. 3, p. 493–531, 2002.

ELPHICK, Maurice R. **The evolution and comparative neurobiology of endocannabinoid signalling**. Philosophical Transactions of the Royal Society B: Biological Sciences, v. 367, n. 1607, p. 3201-3215, 2012.

EWING, Laura E. *et al.* Hepatotoxicity of a Cannabidiol-Rich Cannabis Extract in the Mouse Model. **Molecules**, v. 24, n. 9, p. 1694, 2019.

FALLAH, Hamideh P. *et al.* Effects of gonadotropin-inhibitory hormone on early and late stages of spermatogenesis in ex-vivo culture of zebrafish testis. **Molecular and Cellular Endocrinology**, v. 520, p. 111087, 2021.

FALLAH, Hamideh P. *et al.* Paracrine/autocrine control of spermatogenesis by gonadotropin-inhibitory hormone. **Molecular and Cellular Endocrinology**, v. 492, n. March, p. 110440, 2019.

FAO. **The State of Food and Agriculture 2020**. Rome: FAO, 2020.

FERNANDES, Marisa de Oliveira; VOLPATO, Gilson Luiz. Heterogeneous growth in the Nile tilapia: Social stress and carbohydrate metabolism. **Physiology & Behavior**, v. 54, n. 2, p. 319–323, 1993.

FLORES, Andrés *et al.* Accuracy of gonadosomatic index in maturity classification and estimation of maturity ogive. **Fisheries Research**, v. 210, n. July 2018, p. 50–62, 2019.

FRASER, D. *et al.* A scientific conception of animal welfare that reflects ethical concerns. **Animal Welfare**, v. 6, n. 3, p. 187–205, 1997.

FREEMAN, Abigail M. *et al.* How does cannabidiol (CBD) influence the acute effects of delta-9-tetrahydrocannabinol (THC) in humans? A systematic review. **Neuroscience & Biobehavioral Reviews**, v. 107, p. 696-712, 2019.

GÁLL, Zsolt *et al.* Effects of Chronic Cannabidiol Treatment in the Rat Chronic Unpredictable Mild Stress Model of Depression. **Biomolecules**, v. 10, n. 5, p. 801, 2020.

GERLAI, Robert. Fish in behavior research: Unique tools with a great promise!. **Journal of Neuroscience Methods**, v. 234, p. 54–58, 2014.

GIAQUINTO, PercÍlia Cardoso; VOLPATO, Gilson Luiz. Chemical Communication, Aggression, and Conspecific Recognition in the Fish Nile Tilapia. **Physiology & Behavior**, v. 62, n. 6, p. 1333–1338, 1997.

GONÇALVES-DE-FREITAS, Eliane *et al.* Social behavior and welfare in Nile Tilapia. **Fishes**, v. 4, n. 2, p. 1–14, 2019.

GONG, Shuai *et al.* Dynamics and Correlation of Serum Cortisol and Corticosterone under Different Physiological or Stressful Conditions in Mice. **PLOS ONE**, v. 10, n. 2, p. e0117503, 2015.

GRIMALDI, Paola *et al.* The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. **Proceedings of the National Academy of Sciences**, v. 106, n. 27, p. 11131–11136, 2009.

HARTMANN, Alice *et al.* Cannabidiol attenuates aggressive behavior induced by social isolation in mice : Involvement of 5-HT1A and CB1 receptors. **Progress in Neuropsychopharmacology & Biological Psychiatry**, v. 94, n. October 2018, p. 109637, 2019.

HILL, Matthew N.; GORZALKA, Boris B. Increased sensitivity to restraint stress and novelty-induced emotionality following long-term, high dose cannabinoid exposure. **Psychoneuroendocrinology**, v. 31, n. 4, p. 526–536, 2006.

HOTTA, Takashi; KOMIYAMA, Shiho; KOHDA, Masanori. A social cichlid fish failed to pass the mark test. **Animal Cognition**, v. 21, n. 1, p. 127–136, 2018.

HURD, Yasmin L. *et al.* Cannabidiol for the reduction of cue-induced craving and anxiety in drug-abstinent individuals with heroin use disorder: A double-blind randomized placebo-controlled trial. **American Journal of Psychiatry**, v. 176, n. 11, p. 911–922, 2019.

HUSSAIN, Shaun A. *et al.* Perceived efficacy of cannabidiol-enriched cannabis extracts for treatment of pediatric epilepsy: A potential role for infantile spasms and Lennox-Gastaut syndrome. **Epilepsy and Behavior**, v. 47, p. 138–141, 2015.

IFFLAND, Kerstin; GROTENHERMEN, Franjo. An Update on Safety and Side Effects of Cannabidiol: A Review of Clinical Data and Relevant Animal Studies. **Cannabis and Cannabinoid Research**, v. 2, n. 1, p. 139–154, 2017.

IGNATOWSKA-JANKOWSKA, Bogna; JANKOWSKI, Maciej M.; SWIERGIEL, Artur H. Cannabidiol decreases body weight gain in rats: Involvement of CB2 receptors. **Neuroscience Letters**, v. 490, n. 1, p. 82–84, 2011.

JENSEN, Hannah M. *et al.* Cannabidiol effects on behaviour and immune gene expression in zebrafish (Danio rerio). **PLoS ONE**, v. 13, n. 7, p. 1–11, 2018.

KEELEY, Ernest R. An experimental analysis of territory size in juvenile steelhead trout. **Animal Behaviour**, v. 59, n. 3, p. 477–490, 2000.

KHAN, Naim A.; DESCHAUX, Pierre. Role of serotonin in fish immunomodulation. **Journal of Experimental Biology**, v. 200, n. 13, p. 1833–1838, 1997.

KOHDA, Masanori *et al.* If a fish can pass the mark test, what are the implications for consciousness and self-awareness testing in animals?. **PLOS Biology**, v. 17, n. 2, p. e3000021, 2019.

LEAL, Esther *et al.* Stress-induced effects on feeding behavior and growth performance of the sea bass (Dicentrarchus labrax): A self-feeding approach. **Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology**, v. 181, n. 8, p. 1035–1044, 2011.

MARTÍ, Octavi; MARTÍ, Joaquín; ARMARIO, Antonio. Effects of chronic stress on food intake in rats: Influence of stressor intensity and duration of daily exposure. **Physiology and Behavior**, v. 55, n. 4, p. 747–753, 1994.

MARTINS, Catarina I.M. *et al.* Behavioural indicators of welfare in farmed fish. **Fish Physiology and Biochemistry**, v. 38, n. 1, p. 17–41, 2012.

MARTINS, Catarina I.M. *et al.* Is growth retardation present in Nile tilapia Oreochromis niloticus cultured in low water exchange recirculating aquaculture systems?. **Aquaculture**, v. 298, n. 1–2, p. 43–50, 2009.

MAXIMINO, Caio *et al.* Scototaxis as anxiety-like behavior in fish. **Nature Protocols 2010 5:2**, v. 5, n. 2, p. 209–216, 2010.

MECHOULAM, Raphael; HANUŠ, Lumír. Cannabidiol: An overview of some chemical and pharmacological aspects. Part I: Chemical aspects. **Chemistry and Physics of Lipids**, v. 121, n. 1–2, p. 35–43, 2002.

MECHOULAM, Raphael; PARKER, Linda A.; GALLILY, Ruth. Cannabidiol: An Overview of Some Pharmacological Aspects. **The Journal of Clinical Pharmacology**, v. 42, n. S1, p. 11S-19S, 2002.

MILLAR, Sophie A. *et al.* A systematic review on the pharmacokinetics of cannabidiol in humans. **Frontiers in Pharmacology**, v. 9, n. NOV, 2018.

MOMMSEN, Thomas P.; VIJAYAN, Mathilakath M.; MOON, Thomas W. **Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation**. [*S. l.*]: Springer, 1999.

MOREIRA, Fabrício A.; AGUIAR, Daniele C.; GUIMARÃES, Francisco S. Anxiolytic-like effect of cannabidiol in the rat Vogel conflict test. **Progress in Neuro-Psychopharmacology and Biological Psychiatry**, v. 30, n. 8, p. 1466–1471, 2006.

MORRIS, Elizabeth M. *et al.* The Impact of Feeding Cannabidiol (CBD) Containing Treats on Canine Response to a Noise-Induced Fear Response Test. **Frontiers in Veterinary Science**, v. 7, n. September, p. 1–13, 2020.

MORTUZA, Asif; MASON, Lindee; MUSTAFA, Ahmed. Effects of CBD (Cannabidiol) on the haematological parameters of Nile tilapia (Oreochromis niloticus, Linnaeus) under acute stress in vivo. **Aquaculture Research**, v. 52, n. 11, p. 5375–5382, 2021.

NAZARIO, Luiza Reali *et al.* Reprint of “caffeine protects against memory loss induced by high and non-anxiolytic dose of cannabidiol in adult zebrafish (Danio rerio)”. **Pharmacology Biochemistry and Behavior**, v. 139, p. 134–140, 2015.

NISHIMURA, Toshiya; TANAKA, Minoru. Gonadal Development in Fish. **Sexual Development**, v. 8, n. 5, p. 252–261, 2014.

NOBLE, Chris *et al.* **Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare**. Tromsø, Norway: Nofima, 2018. *E-book*. Disponível em: http://hdl.handle.net/11250/2575780. Acesso em: 5 mar. 2021.

O’SULLIVAN, Saoirse Elizabeth. An update on PPAR activation by cannabinoids. **British Journal of Pharmacology**, v. 173, n. 12, p. 1899–1910, 2016.

OLTRABELLA, Francesca *et al.* Role of the endocannabinoid system in vertebrates: Emphasis on the zebrafish model. **Development Growth and Differentiation**, v. 59, n. 4, p. 194–210, 2017.

OSBORNE, Ashleigh L. *et al.* Improved Social Interaction, Recognition and Working Memory with Cannabidiol Treatment in a Prenatal Infection (poly I:C) Rat Model. **Neuropsychopharmacology**, v. 42, n. 7, p. 1447–1457, 2017.

PACHER, Pál; BÁTKAI, Sándor; KUNOS, George. The endocannabinoid system as an emerging target of pharmacotherapy. **Pharmacological reviews**, v. 58, n. 3, p. 389-462, 2006.

PANDELIDES, Zacharias *et al.* Developmental exposure to cannabidiol (CBD) alters longevity and health span of zebrafish (Danio rerio). **Geroscience**, v. 42, n. 2, p. 785-800, 2020.

PERTWEE, Roger G. Emerging strategies for exploiting cannabinoid receptor agonists as medicines. **British Journal of Pharmacology**, v. 156, n. 3, p. 397–411, 2009.

POTTINGER, T. G. **Fish Welfare**. Oxford, UK: Blackwell Publishing Ltd, 2008. *E-book*. Disponível em: https://books.google.com.br/books?hl=pt-BR&lr=&id=-NNlWxyXPIYC&oi=fnd&pg=PA32&dq=Fish+Welfare+pottinger&ots=ed7lkI7s_d&sig=A1RJVEnvti7yc48bx_73StccCpM#v=onepage&q=Fish Welfare pottinger&f=false.

RAFAEL DE MELLO SCHIER, Alexandre *et al.* Revista Brasileira de Psiquiatria Cannabidiol, a Cannabis sativa constituent, as an anxiolytic drug. **Official Journal of the Brazilian Psychiatric Association**, v. 34, n. Supl1, p. 104–117, 2012.

REDDON, Adam R. *et al.* Effects of isotocin on social responses in a cooperatively breeding fish. **Animal Behaviour**, v. 84, n. 4, p. 753–760, 2012.

REDDON, Adam R.; BALSHINE, Sigal. Lateralization in response to social stimuli in a cooperatively breeding cichlid fish. **Behavioural Processes**, v. 85, n. 1, p. 68–71, 2010.

RESSTEL, Leonardo B.M. *et al.* 5-HT 1A receptors are involved in the cannabidiol-induced attenuation of behavioural and cardiovascular responses to acute restraint stress in rats. **British Journal of Pharmacology**, v. 156, n. 1, p. 181–188, 2009.

REY, Alejandro Aparisi *et al.* Biphasic effects of cannabinoids in anxiety responses: CB1 and GABA B receptors in the balance of gabaergic and glutamatergic neurotransmission. **Neuropsychopharmacology**, v. 37, n. 12, p. 2624–2634, 2012.

RODRIGUES, Maira S. *et al.* Interaction between thyroid hormones and gonadotropin inhibitory hormone in ex vivo culture of zebrafish testis: An approach to study multifactorial control of spermatogenesis. **Molecular and Cellular Endocrinology**, v. 532, p. 111331, 2021..

ROS, Albert F.H.; BECKER, Klaus; OLIVEIRA, Rui F. Aggressive behaviour and energy metabolism in a cichlid fish, Oreochromis mossambicus. **Physiology and Behavior**, v. 89, n. 2, p. 164–170, 2006.

RUSSELL, Georgina; LIGHTMAN, Stafford. The human stress response. **Nature Reviews Endocrinology 2019 15:9**, v. 15, n. 9, p. 525–534, 2019.

RUSSO, Ethan B. *et al.* Agonistic properties of cannabidiol at 5-HT1a receptors. **Neurochemical Research**, v. 30, n. 8, p. 1037–1043, 2005.

RYBERG, E *et al.* The orphan receptor GPR55 is a novel cannabinoid receptor. **British Journal of Pharmacology**, v. 152, n. 7, p. 1092–1101, 2007.

SAOUD, I. Patrick *et al.* Effect of cannabis oil on growth performance, haematology and metabolism of Nile Tilapia Oreochromis niloticus. **Aquaculture Research**, v. 49, n. 2, p. 809–815, 2018.

SARAIVA, João L.; ARECHAVALA-LOPEZ, Pablo. Welfare of fish—no longer the elephant in the room. **Fishes**, v. 4, n. 3, p. 1–3, 2019.

SARTIM, A. G.; GUIMARÃES, F. S.; JOCA, S. R.L. Antidepressant-like effect of cannabidiol injection into the ventral medial prefrontal cortex-Possible involvement of 5-HT1A and CB1 receptors. **Behavioural Brain Research**, v. 303, p. 218–227, 2016.

SCHOLZ, S.; KLÜVER, N. Effects of Endocrine Disrupters on Sexual, Gonadal Development in Fish. **Sexual Development**, v. 3, n. 2–3, p. 136–151, 2009.

SCHRECK, Carl B.; CONTRERAS-SANCHEZ, Wilfrido; FITZPATRICK, Martin S.

Effects of stress on fish reproduction, gamete quality, and progeny. **Reproductive Biotechnology in Finfish Aquaculture**, p. 3–24, 2001.

SCHUEL, H. *et al.* Cannabinoids reduce fertility of sea urchin sperm. **Biochemistry and Cell Biology**, v. 65, n. 2, p. 130–136, 1987.

SCHULZ, Rüdiger W. *et al.* Spermatogenesis in fish. **General and Comparative Endocrinology**, v. 165, n. 3, p. 390–411, 2010.

SOPINKA, Natalie M. *et al.* Stress Indicators in Fish. **Fish Physiology**, v. 35, p. 405–462, 2016.

STEGER, Richard W. *et al.* Effects of Psychoactive and Nonpsychoactive Cannabinoids on the Hypothalamic-Pituitary Axis of the Adult Male Rat. **Pharmacology Biochemistry and Behavior**, v. 37, n. 2, p. 299-302, 1990.

STOLTE, Ellen H. *et al.* Corticosteroid receptors involved in stress regulation in common carp, Cyprinus carpio. **Journal of Endocrinology**, v. 198, n. 2, p. 403–417, 2008.

STRYKOWSKI, Jennifer L.; SCHECH, Joseph M. Effectiveness of recommended euthanasia methods in larval zebrafish (Danio rerio). **Journal of the American Association for Laboratory Animal Science**, v. 54, n. 1, p. 81–84, 2015.

TOVO-NETO, Aldo *et al.* Cortisol directly stimulates spermatogonial differentiation, meiosis, and spermiogenesis in Zebrafish (Danio rerio) testicular explants. **Biomolecules**, v. 10, n. 3, 2020.

VITALE, F.; SVEDÄNG, H.; CARDINALE, M. Histological analysis invalidates macroscopically determined maturity ogives of the Kattegat cod (Gadus morhua) and suggests new proxies for estimating maturity status of individual fish. **ICES Journal of Marine Science**, v. 63, n. 3, p. 485–492, 2006.

VON KEYSERLINGK, M. A.G. *et al.* Invited review: The welfare of dairy cattle—Key concepts and the role of science. **Journal of Dairy Science**, v. 92, n. 9, p. 4101–4111, 2009.

VON KEYSERLINGK, Marina A.G.; WEARY, Daniel M. A 100-Year Review: Animal welfare in the Journal of Dairy Science—The first 100 years. **Journal of Dairy Science**, v. 100, n. 12, p. 10432–10444, 2017.

WENDELAAR BONGA, Sjoerd E. The stress response in fish. **Physiological Reviews**, v. 77, n. 3, p. 591–625, 1997.

WILHELM, Dagmar; PALMER, Stephen; KOOPMAN, Peter. Sex determination and gonadal development in mammals. **Physiological Reviews**, v. 87, n. 1, p. 1–28, 2007.

WINBERG, S; NILSSON, G. Multiple high-affinity binding sites for. **Journal of Experimental Biology**, v. 199, n. 11, 1996.

WINBERG, S; OVERLI, O; LEPAGE, O. Suppression of aggression in rainbow trout (Oncorhynchus mykiss) by dietary L-tryptophan. **J Exp Biol**, v. 204, n. Pt 22, p. 3867-76., 2001.

XIN, Ning *et al.* Effects of prednisolone on behavior and hypothalamic–pituitary–interrenal axis activity in zebrafish. **Environmental Toxicology and**

Pharmacology, v. 75, n. March 2019, 2020.

YOSHIDA, Masayuki. Recording the ventilation activity of free-swimming zebrafish and its application to novel tank tests. **Physiology & Behavior**, v. 244, p. 113665, 2022.

ZANELATI, T. V. *et al.* Antidepressant-like effects of cannabidiol in mice: Possible involvement of 5-HT 1A receptors. **British Journal of Pharmacology**, v. 159, n. 1, p. 122–128, 2010.

ZEYL, Jeffrey N.; LOVE, Oliver P.; HIGGS, Dennis M. Evaluating gonadosomatic index as an estimator of reproductive condition in the invasive round goby, Neogobius melanostomus. **Journal of Great Lakes Research**, v. 40, n. 1, p. 164–171, 2014.

ZUARDI, A. W. *et al.* Effects of ipsapirone and cannabidiol on human experimental anxiety. **Journal of Psychopharmacology**, v. 7, n. 1, p. 82–88, 1993.