UNIVERSIDADE FEDERAL DE MINAS GERAIS

USO DE DIFERENTES ANESTÉSICOS PARA MANIPULAÇÃO BIOMÉTRICA E TRANSPORTE DE PEIXES DE ÁGUA DOCE

ANDRE LIMA FERREIRA

BELO HORIZONTE

ESCOLA DE VETERINÁRIA – UFMG

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Tese apresentada ao Programa de Pós-Graduação em Zootecnia da Escola de Veterinária da Universidade Federal de Minas Gerais como requisito para obtenção do grau de Doutor em Zootecnia.

Área de concentração: Produção Animal/Aquacultura.

Orientador: Dr. Ronald Kennedy Luz.
Coorientadora: Drª. Gisele Cristina Favero.

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COLEGIADO DO CURSO DE PÓS-GRADUAÇÃO EM ZOOTECNIA

FOLHA DE APROVAÇÃO

USO DE DIFERENTES ANESTÉSICOS PARA MANIPULAÇÃO BIOMÉTRICA E TRANSPORTE DE PEIXES DE ÁGUA DOCE

ANDRE LIMA FERREIRA

Tese de Doutorado defendida e aprovada, no dia **vinte e cinco de março de dois mil e vinte e dois**, pela Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Zootecnia da Universidade Federal de Minas Gerais, constituída pelos seguintes professores:

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Belo Horizonte, 25 de março de 2022.

“Os dias prósperos não vêm por acaso.

Nascem de muito trabalho e persistência.”

Henry Ford

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Dedico aos meus pais (Lucimar e Ivanda), meu irmão (Gustavo), minha cunhada (Lunara) e minha sobrinha/afilhada (Ísis Giovanna). E também a minha fiel companheira de quatro patas (Bel).

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Resumo

Procedimentos realizados nas pisciculturas, como biometria e transporte de peixes vivos podem ocasionar perturbações na homeostase dos peixes, acometendo o estresse; como consequência disso pode ocorrer depressão do sistema imunológico e até mesmo morte dos animais. Desta forma, foram realizados cinco artigos. No Artigo 1 – avaliou-se diferentes concentrações de benzocaína e mentol (0, 12,5, 25, 50, 75, 100, 125 mg L^{-1} para cada anestésico) em juvenis de *Aulonocara nyassae*. Para o teste com benzocaína, os peixes foram classificados em Juvenis I (0.74 ± 0.31 g) e Juvenis II (3.80 ± 0.92 g). Já, para o experimento de mentol foram utilizados Juvenis I (1.01 ± 0.39 g) e Juvenis II (3.73 ± 0.78 g). Foram realizados testes independentes para cada anestésico e classe de tamanho de peixe em delineamento inteiramente casualizado (DIC). Foram mensurados os tempos de indução e recuperação da anestesia e frequências ventilatória (VF) dos animais. Concentrações entre 75 e 125 mg L^{-1} de benzocaína para Juvenis I e 50 a 125 mg L^{-1} para Juvenis II são ideais. Para mentol, concentrações entre 50 e 125 mg L^{-1} podem ser usadas para ambas classes de *A. nyassae*. Logo, no Artigo 2, foram avaliadas diferentes concentrações de eugenol e mentol (0, 25, 50, 75, 100 e 125 mg L^{-1}) em dois tamanhos de juvenis de *Piaractus brachypomus*. Para o teste de eugenol, foram utilizados Juvenis I (0.87 ± 0.20 g) e Juvenis II (17.14 ± 3.27 g). Já, para o ensaio com mentol foram usados Juvenis I (0.83 ± 0.21 g) e Juvenis II (16.83 ± 2.78 g). Foram realizados testes independentes para cada classe de tamanho de peixe e anestésico, em DIC. Concentrações de eugenol entre 50 e 100 mg L^{-1} foram capazes de induzir anestesia para ambas as classes de tamanho juvenil de *P. brachypomus*, enquanto o mentol foi capaz de induzir anestesia em concentrações entre 25 e 100 mg L^{-1} para Juvenis I e entre 50 e 125 mg L^{-1} para Juvenis II. O uso de 50 mg L^{-1} de eugenol reduziu a VF durante a recuperação e prevenir aumento da glicose plasmática, tendo pouca influência nos parâmetros hematológicos e bioquímicos após biometria. O uso de 50 mg L^{-1} de mentol também reduziu a VF durante a recuperação e não causou alterações nos parâmetros no sangue que fosse prejudicial à fisiologia dos peixes. No Artigo 3 foram investigadas as propriedades físicas e químicas de nanopartículas de zeína contendo eugenol (NPZMA), no processo de anestesia de *Oreochromis niloticus* e seus efeitos na hemogasometria e sua estabilidade na água. Foram realizados quatro testes independentes em DIC. O novo método de aplicação de eugenol através de nanopartículas de zeína mucoadesivas (NPZMA) demonstrou cargas positivas e fácil adesão ao muco dos peixes. O método levou oscilações na qualidade da água durante o

período de observação (1 h), embora tenha permanecido dentro da faixa ideal para o cultivo de *O. niloticus*. O experimento 3 revelou tempos de indução semelhantes para eugenol-80 mg L^{-1}, NPZMA-80 mg L^{-1} e NPZMA-40 mg L^{-1}. O tempo de recuperação foi mais curto para NPZMA-20 mg L^{-1} e mais longo para NPZMA-80 mg L^{-1}. O experimento 4 demonstrou que as concentrações testadas não têm efeitos nas variáveis hemogasométricas. Já, no Artigo 4 foram avaliadas diferentes concentrações do óleo essencial de *Ocimum gratissimum* L. (EOOG) para anestesia e seu uso na água de transporte de juvenis de *O. niloticus* e os efeitos na frequência ventilatória, hematologia e bioquímica do sangue e estresse oxidativo. Foram realizados três experimentos independentes em DIC. Concentrações entre 90 e 150 mg L^{-1} de EOOG são recomendadas para *O. niloticus* com 40 g. O uso de 90 mg L^{-1} de EOOG preveniu aumento da glicose plasmática logo após a indução anestésica e 1 h após recuperação, porém causou alterações no sistema de defesa antioxidante aumentando as espécies reativas de oxigênio no tecido hepático e renal. O uso de 10 mg L^{-1} EOOG no transporte aumentou os valores de glicose e diminuiu hematócrito imediatamente. O transporte de *O. niloticus* com peso médio de 12 g durante 4,5 h pode ser realizado com concentração de 5 mg L^{-1} de EOOG. No Artigo 5 foram investigadas diferentes concentrações anestésicas e sedativas do óleo essencial de *Hesperozygis ringens* (EOHR) para juvenis de *Colossoma macropomum*. Foram realizados quatro ensaios independentes em DIC. Concentrações entre 150 e 450 µL L^{-1} EOHR são recomendadas para anestesia de *C. macropomum* com peso médio de 3 g. Concentrações de 15 e 30 µL L^{-1} de EOHR foram capazes de reduzir os níveis de amônia não ionizada durante o transporte de *C. macropomum* com 2 g; no entanto, seu uso em sacos plásticos por longos períodos (24 h) deve ser evitado, pois reduz os níveis de oxigênio dissolvido. Esses resultados são promissores para cadeia industrial da piscicultura; contudo estudos de anestesia para peixes que investiguem a morfologia e histologia de tecidos, além do desempenho zootécnico dos animais, se fazem necessários.

Palavras chaves: Aquacultura, Anestesia, Fisiologia de peixe, Óleo essencial de planta, Manejo estressante.

Abstract

Procedures performed in fish farms, such as biometry and transport of live fish can cause disturbances in fish homeostasis, affecting stress; as a consequence of this, depression of the immune system and even death of animals can occur. Thus, five articles were carried out. In Article 1 – different concentrations of benzocaine and menthol (0, 12.5, 25, 50, 75, 100, 125 mg L^{-1} for each anesthetic) were evaluated in juveniles of *Aulonocara nyassae*. The fish were classified as Juveniles I (0.74 ± 0.31 g) and Juveniles II (3.80 ± 0.92 g) for the benzocaine test. For the menthol experiment, Juveniles I (1.01 ± 0.39 g) and Juveniles II (3.73 ± 0.78 g) were used. Independent tests were performed for each anesthetic and fish size class in a completely randomized design (DIC). Induction and recovery times from anesthesia and ventilatory frequency (VF) of the animals were measured. Concentrations between 75 and 125 mg L^{-1} of benzocaine for Juveniles I and 50 to 125 mg L^{-1} for Juveniles II are ideal. For menthol, concentrations between 50 and 125 mg L^{-1} can be used for both classes of *A. nyassae*. Therefore, in Article 2 different concentrations of eugenol and menthol (0, 25, 50, 75, 100 and 125 mg L^{-1}) were evaluated in two sizes of juveniles of *Piaractus brachypomus*. For the eugenol test, Juveniles I (0.87 ± 0.20 g) and Juveniles II (17.14 ± 3.27 g) were used. For the menthol assay, Juveniles I (0.83 ± 0.21 g) and Juveniles II (16.83 ± 2.78 g) were used. Independent tests were performed for each fish size class and anesthetic in DIC. Eugenol concentrations between 50 and 100 mg L^{-1} were able to induce anesthesia for both juvenile size classes of *P. brachypomus*, while menthol was able to induce anesthesia at concentrations between 25 and 100 mg L^{-1} for Juveniles I and between 50 and 125 mg L^{-1} for Juveniles II. The use of 50 mg L^{-1} of eugenol was able to reduce the ventilatory rate (VF) during recovery and prevented an increase in plasma glucose, having little influence on hematological and biochemical parameters after biometry. The use of 50 mg L^{-1} of menthol also reduced VF during recovery and did not cause changes in blood parameters that were harmful to fish physiology. In Article 3, the physical and chemical properties of zein nanoparticles containing eugenol (NPZMA) in the anesthesia process of *Oreochromis niloticus* and their effects on blood gas analysis and their stability in water were investigated. Four independent tests were performed in DIC. The new method of eugenol application through mucoadhesive zein nanoparticles (NPZMA) demonstrated positive charges and easy adhesion to fish mucus. The method led to fluctuations in water quality during the observation period (1 h), although it remained within the ideal range for *O. niloticus* cultivation. Experiment 3 revealed similar

induction times for eugenol-80 mg L^{-1}, NPZMA-80 mg L^{-1} and NPZMA-40 mg L^{-1}. Recovery time was shorter for NPZMA-20 mg L^{-1} and longer for NPZMA-80 mg L^{-1}. Experiment 4 demonstrated that the concentrations tested have no effect on blood gas variables. Already, in Article 4 different concentrations of essential oil of *Ocimum gratissimum* L. (EOOG) were evaluated for anesthesia and their use in the transport water of juveniles of *O. niloticus* and their effect on VF, hematology and blood biochemistry and oxidative stress. Three independent experiments were carried out in DIC. Concentrations between 90 and 150 mg L^{-1} of EOOG are recommended for *O. niloticus* with 40 g. The use of 90 mg L^{-1} of EOOG prevented an increase in plasma glucose soon after anesthetic induction and 1 h after recovery, but caused changes in the antioxidant defense system, increasing reactive oxygen species in hepatic and renal tissue. The use of 10 mg L^{-1} EOOG in transport increased glucose values and decreased hematocrit immediately after transport. The transport of *O. niloticus* with an average weight of 12 g for 4.5 h can be carried out with a concentration of 5 mg L^{-1} of EOOG. In Article 5, different anesthetic and sedative concentrations of the essential oil of *Hesperozygis ringens* (EOHR) were investigated for juveniles of *Colossoma macropomum*. Four independent trials were carried out in DIC. Concentrations between 150 and 450 µL L^{-1} EOHR are recommended for anesthesia of *C. macropomum* with an average weight of 3 g. Concentrations of 15 and 30 µL L^{-1} of EOHR were able to reduce the levels of unionized ammonia during the transport of *C. macropomum* with 2 g; however, its use in plastic bags for long periods (24 h) should be avoided, as it reduces dissolved oxygen levels. These results are promising for the fish farming industrial chain; however, studies of anesthesia for fish that investigate the morphology and histology of tissues, in addition to the zootechnical performance of the animals, are necessary.

Key words: Aquaculture, Anesthesia, Fish physiology, Handling stress, Plant essential oil.

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FIGURE 7. Protein levels of the liver (a), gills (b), kidney, (c), and brain (d) of *Oreochromis niloticus* measured immediately after anesthetic induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after recovery. Ethanol group: 0 mg L^{-1} EOOG. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way ANOVA, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration.

FIGURE 8. Biochemical and hematological parameters of *Oreochromis niloticus* measured after transport with essential oil of *Ocimum gratissimum* (EOOG). Basal group: fish not transported. Ethanol group: 0 mg L^{-1} EOOG. Plasma glucose (a), hematocrit (b), and plasma protein (c). Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way ANOVA, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration. Asterisk indicates a significant difference from the basal group.

ARTIGO 5. Efficacy of *Hesperozygis ringens* **essential oil as an anesthetic and for sedation of juvenile tambaqui** (*Colossoma macropomum*) **during simulated transport**

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ACTH – Adrenocorticotropic hormone

ANOVA – Analysis of variance

Beats min^{-1} - Opercular beat per minute

CAT – Catalase

CEUA – Comissão de ética no uso de animais

CNS – Central nervous system

CRH - Corticotrophin releasing hormone

CV - Coefficient of variation

DIC - Completely randomized design

DNA – Deoxyribonucleic acid

EO – Essential oil

EV – Escola de Veterinária

EOOG – Essential oil *Ocimum gratissimum* L.

EOHR – Essential oil *Hesperozygis ringens*

EOLA – Essential oil *Lippia alba*

FAO – Food and Agriculture Organization of the United Nations

FDA – Food and Drug Administration

VF – Ventilatory frequency

Glu - Glucose

GPx – Glutathione peroxidase

GST – Glutathione-S-transferase

Hct - Hematocrit

LAQUA – Laboratório de Aquacultura

NPSH – Non-protein thiol

NPZMA - Mucoadhesive zein nanoparticles containing eugenol

PABA – p-aminobenzoic acid

PDI - Polydispersion index

pH – Hydrogen potential

RAS – Recirculating aquaculture system

ROS - Oxigen-reactive species

s – Seconds

SOD – Dismutase

TBARS - Thiobarbituric acid reactive substances

UFMG – Universidade Federal de Minas Gerais

US$ - Dollar

Beecf – Deficit base

cm – Centimeter

% - Percentage

ºC - Degrees Celsius

g – Gram

g dL^{-1} - Gram per deciliter

g kg^{-1} - Gram per kilo

K^{+} - Potassium

kcal kg^{-1} – Kcal per kilo

L - Liters

min – Minutes

mg L^{-1} - milligrams per liter

mg dL^{-1} - Milligrams per decilitre

mL – milliliter

$\mu L\ L^{-1}$ - microliter per liter

mm – Millimeter

mmHg - Millimeter of mercury

mmol - Millimol

$mmol\ L^{-1}$ - millimole per liter

mS - Millisiemens

MS-222 - Tricaine methanesulfonate

Na^{+} - Sodium

PCO_2 - Partial pressure of carbon dioxide

pH - Hydrogen potential

TCO_2 - Carbon dioxide rate

$HC0_3^{-}$ Bicarbonate ion

Sumário

1. INTRODUÇÃO GERAL

Segundo a FAO (2020), a produção global de peixes foi de 179 milhões de toneladas em 2018, com venda total em valor aproximado de US$ 401 bilhões, dos quais 82 milhões de toneladas, avaliadas em US$ 250 bilhões, foi proveniente da produção aquícola. A aquicultura representa 46% da produção total e 52% de pescado para consumo humano (FAO, 2020). No Brasil, a produção de peixes chegou a 841.005 toneladas no ano de 2021 (PEIXE BR, 2022). A espécie de maior produção no país é a tilápia do Nilo *Oreochromis niloticus* (PEIXE BR, 2022); a tilapicultura foi registrada em aproximadamente 110.000 fazendas de cultivo em todos os estados (IBGE, 2020). Segundo PEIXE BR (2022) os estados de maior produção de tilápia são o Paraná, com aproximadamente 182.000 toneladas (t), seguido por São Paulo com cerca de 76.140 t, Minas Gerais com 47.000 t (ocupando o terceiro lugar), Santa Catarina com 41.700 t e Mato Grosso do Sul com cerca de 34.450 t. Já, a produção de espécies nativas foi estimada em 262.370 toneladas em 2021 e maiores estados produtores foram Rondônia (59.600 t), Mato Grosso (37.000 t), Maranhão (37.000 t), Pará (24.200 t) e Amazonas (21.000 t). As conhecidas popularmente por espécies redondas, tambaqui *Colossoma macropomum*, pirapitinga *Piaractus brachypomus* e seus híbridos, como: patinga: *Piaractus mesopotamicus* x *P. brachypomus*; e tambatinga: *C. macropomum* x *P. brachypomus*) são o segundo e terceiro grupos das espécies de peixes nativos de maior produção no Brasil, respectivamente (PEIXE BR, 2022). Segundo projeções da FAO (Organização das Nações Unidas para Alimentação e Aquicultura) a produção aquícola no Brasil crescerá em 32% de 2018 até 2030 (FAO, 2020).

Outra atividade que tem ganhado destaque no cenário mundial é a aquariofilia, principalmente com o cultivo de peixes para fins de companhia e ornamentação (Faria et al., 2019). Há diferentes relatos nas culturas oriental, romana e egípcia que a criação de peixes para fins estéticos é antiga (Botelho Filho, 1990). A denominação “peixe ornamental” engloba variadas espécies de peixes com formatos, tamanhos e colorações distintas criadas em cativeiro (Alderton, 2019). A cadeia industrial de produção de peixes ornamentais movimenta mais de US$ 27 bilhões anualmente, incluindo itens como: equipamentos, acessórios, plantas ornamentais e alimentação (Ploeg, 2013). Segundo o mesmo autor, este comércio movimenta aproximadamente 1,5 bilhões de exemplares por ano, onde, Singapura é o principal exportador de peixes, com US$ 61,4 milhões, seguido pela Espanha com US$ 26,5 milhões e República Tcheca com US$ 21,7 milhões. Cerca de 60% das exportações de peixes

ornamentais são de países em desenvolvimento e o Brasil contribui com apenas 5,9% do total de exportações mundiais (Ploeg, 2013; Rossoni et al., 2014).

Diante deste aumento na cadeia industrial de peixes, tanto para consumo humano quanto para ornamentação, nos cultivos os peixes têm de lidar com diferentes situações estressantes que podem comprometer seu bem-estar, incluindo a manipulação, principalmente durante a biometria e o transporte (Pickering, 1981; Barton et al., 2002). O estresse induzido por tais práticas desencadeia alterações fisiológicas as quais podem comprometer o desempenho produtivo e reprodutivo dos peixes (Gressler et al., 2012; Souza et al., 2019a), além de poder levar a morte do animal. Nesse sentido, os anestésicos, em concentrações ideais, são apresentados como benéficos por diminuir a mobilidade dos peixes evitando possíveis danos físicos, bem como alterações hematológicas, bioquímicas e endócrinas (Ortuno et al., 2002; Souza et al., 2019b) durante diferentes procedimentos nas pisciculturas (Ross e Ross, 2008). Seus benefícios também foram descritos no transporte de peixes vivos, uma vez que diminuem a excitação dos animais (efeito sedativo), reduz a excreção de amônia e gás carbônico, como também o consumo de oxigênio e a deterioração da qualidade da água (Ross e Ross, 2008; Aydin e Barbas, 2020, Brandão et al., 2021).

Assim, o objetivo deste trabalho foi avaliar diferentes compostos anestésicos e/ou sedativos (sintético ou natural) e formas de aplicação (diluição em álcool ou nanoemulsão) nas respostas fisiológicas e metabólicas de peixes de água doce após situações de manipulação, como biometria e transporte.

2. REVISÃO BIBLIOGRÁFICA

2.1 Anestesia em peixes

A palavra “anestesia” deriva do grego antigo *an* (ausência) + *aisthesis* (sensação), que significa “ausência de sensação”. A anestesia provoca perda de sensação de todo ou parte do corpo e causa depressão da função nervosa, acometida geralmente por um fármaco, sendo, no entanto, este processo reversível (Williams e Wilkins Company, 1982).

O processo de indução anestésica engloba vários componentes, o que inclui sedação, inconsciência, amnésia e analgesia (Zahl et al., 2012). Segundo os mesmos autores, o efeito da sedação é caracterizado pela redução da sensibilidade, enquanto a anestesia geral compreende o estado de inconsciência, amnésia e também imobilização e alívio da dor. Desta

forma, um mesmo fármaco pode ser considerado sedativo e/ou anestésico, o que depende da concentração aplicada, período de exposição e, consequentemente, seu efeito no organismo animal.

Nesse sentido, segundo Ross e Ross (2008), o estágio de anestesia em peixes pode ser classificado em quatro etapas, sendo elas: estágio I – sedação: diminuição de reatividade a estímulo externo; estágio II – anestesia leve: perda parcial do equilíbrio e natação errática; estágio III – anestesia profunda: perda total do equilíbrio e interrupção da locomoção e inconsciência e estágio IV – anestesia cirúrgica: perda total de reações a estímulos táteis e movimento opercular irregular. Segundo esses mesmos autores, a recuperação da anestesia nos peixes pode ser descrita também em quatro etapas, sendo elas: estágio I pela leve recuperação do movimento opercular e início da movimentação das nadadeiras; no estágio II pelo início da recuperação do equilíbrio e leve reação a estímulos externos; no estágio III ocorre recuperação parcial do equilíbrio e dos movimentos de natação e no estágio IV é caracterizado pela recuperação total do equilíbrio e dos movimentos operculares e de natação.

As concentrações ideais de cada anestésico variam entre as espécies de peixes e seus tamanhos (Ross e Ross, 2008; Ribeiro et al., 2015; Readman et al., 2017; Tarkhani et al., 2017; Ferreira et al., 2020a), bem como da qualidade da água e de outros fatores externos (Massee et al., 1995; Gomes et al., 2011). Sua eficiência está associada à capacidade de indução rápida (até 180 s) e curto período de recuperação (até 300 s) (Keene et al., 1998; Ross e Ross, 2008).

Desta forma, um bom anestésico para peixes é aquele que além de apresentar o estado de anestesia desejado, não cause danos biológicos ao animal, ou seja, não afete negativamente seu desempenho zootécnico, reprodutivo e sobrevivência (Cunha et al., 2010; Becker et al., 2012). É importante também, que o anestésico não deixe resíduos na carne, não desencadeie alterações celulares (Roubach e Gomes, 2001, Teixeira et al., 2017), não cause danos ao operador, que seja de fácil acesso e baixo custo (Cunha et al., 2010).

Na aquacultura existem diferentes compostos anestésicos (sintéticos ou naturais) e formas de aplicação (Purbosari et al., 2019; Uehara et al., 2019; Yostawonkul et al., 2019). O uso de anestésicos injetáveis em peixes é eficiente, no entanto, requer mão de obra qualificada, o que torna um método oneroso (Ross e Ross, 2008). A forma de aplicação de anestésicos na piscicultura mais utilizada é a em banhos de imersão, na qual a absorção do fármaco ocorre através da pele, brânquias e intestino (Summerfelt e Smith, 1990; Rombout et

al., 2014). Normalmente, a maioria dos anestésicos utilizados na piscicultura necessita de prévia diluição em baixas concentrações de álcool etílico para sua diluição na água (Souza et al., 2019a; Aydin e Barbas, 2020; Brandão et al., 2021). Desta forma, a administração de medicamentos envolvendo nanopartículas consiste na otimização da solubilidade aquosa e biodisponibilidade da droga, além de minimizarem as reações de hipersensibilidade tecidual (Irache e González-Navarro, 2017; Wang et al., 2017) em relação a formulações convencionais (Torchilin, 2014). Desta forma, é de extrema importância desenvolver e implementar novas tecnologias para identificar diferentes terapêuticos solúveis em água e com isso melhorar o uso dos anestésicos e seus compostos ativos no cultivo de peixes (Aydin e Barbas, 2020). Assim, pesquisadores têm se esforçado para aplicar a nanotecnologia em estudos de anestesia para peixes. Yostawonkul et al. (2019) avaliaram o efeito de nanopartículas lipídicas para melhorar a miscibilidade aquosa do óleo de cravo e observaram diminuição significativa no tempo de indução da anestesia para tilápia do Nilo *O. niloticus*. Kheawfu et al. (2018) e Khumpirapang et al. (2017) relataram que as nanopartículas de óleo essencial de *Syzygium aromaticum* e *Alpinia galanga* induziram uma anestesia significativamente mais rápida do que suas soluções diluídas em álcool para quinguio *C. auratus* e carpa comum *Cyprinus carpio*, respectivamente. Por outro lado, um estudo com nanopartículas mucoadesivas de zeína contendo benzocaína promoveu uma anestesia mais eficaz em zebrafish *Danio rerio* (Charlie-Silva et al., 2020). Já Rodrigues et al. (2021) relataram que a nanoemulsão do óleo essencial *Nectandra grandiflora* causou maior proteção e melhorias em sua atividade farmacológica e ainda concluiram que 30 mg L^{-1} deste composto pode ser usado para sedação durante 24 h e 100 mg L^{-1} para anestesia profunda de juvenis de tilápia do Nilo *O. niloticus*.

De modo geral, os anestésicos, quando dispersos na água são absorvidos pelos peixes através das brânquias, sendo distribuídos para corrente sanguínea e chega ao sistema nervoso central (CNS). As depressões generalizadas do CNS, causadas pelos anestésicos, estão associadas a liberação de transmissores ou excitabilidade da membrana, ou também pela combinação destas ações (Ross e Ross, 2008). Segundo os mesmos autores, o mecanismo de ação dos anestésicos envolve a estabilização do alastramento de impulsos nervosos em axônios eferentes e/ou aferentes, impedimento da liberação de neurotransmissores na membrana pré-sináptica e impedimento dos sítios receptores na membrana pós-sináptica. Este fato ocasiona depressões dos centros bulbares respiratórios, que reduzem a frequência

ventilatória e o fluxo sanguíneo dos animais, o que leva os peixes a hipóxia tecidual e perda dos sentidos (Arias, 1999).

2.2 Fármacos anestésicos para peixes

O metanossulfonato de tricaína (MS-222) e a benzocaína são os anestésicos sintéticos mais utilizados na piscicultura. Contudo, apenas o MS-222 é aprovado pela Food and Drug Administration dos EUA para uso em alimentos para peixes, e os animais quando expostos ao fármaco devem ser mantidos em depuração por 21 dias antes do consumo humano (Woody et al., 2002). A benzocaína é um éster etílico do ácido p-aminobenzóico (PABA), sendo um anestésico local, usado como calmante para dores em mamíferos. A benzocaína - um anestésico sintético amplamente utilizado por ser de fácil acesso e baixo custo, não possuir ação mutagênica e poder ser rapidamente metabolizada pelos peixes (Gomes et al., 2001; Woody et al., 2002; Gontijo et al., 2003). Esse anestésico é comercializado na forma de sal cristalino e apresenta características hidrofóbicas, sendo necessária a dissolução em álcool etílico antes de ser adicionado água (Gimbo et al., 2008). Desta forma a benzocaína já foi testada como anestésico e sedativo para muitas espécies de peixes, como: tambaqui *Colossoma macropomum* (Gomes et al., 2001), quinguio *Carassius auratus* (Bittencourt et al., 2012b), guppy *Poecilia vivípara* (Bolasina et al., 2017); acará bandeira *Pterophyllum scalare* (Romaneli et al., 2018), curimba *Prochilodus lineatus* (Junior et al., 2019), pacamã *Lophiosilurus alexandri* (Ribeiro et al., 2019), lambari-bocarra *Oligosarcus argenteus* (Uehara et al., 2019) e panga *Pangasianodon hypophthalmus* (Boaventura et al., 2021b) ressaltando que as concentrações ideais variam entre as espécies citadas. Este produto pode ser usado com frequência sem afetar o desempenho produtivo e reprodutivo dos peixes (Ross e Ross, 2008).

Além dos anestésicos sintéticos, alguns óleos essenciais extraídos das plantas apresentam efeito anestésico e/ou sedativo em peixes e, dependendo da concentração, apresentam redução nas alterações bioquímicas, hematológicas e endócrinas e, consequentemente, melhoram o estado de bem-estar (Strange e Schreck 1978). Há décadas que pesquisas vêm sendo realizadas para avaliar a eficácia anestésica do OE (óleo essencial) de cravo-da-índia (*Eugenia aromatica* e *Eugenia caryophylata*) para diferentes espécies de peixes, conforme revisado por Javahery et al. (2012) e Priborsky e Velisek (2018). Diferentes estudos descreveram os efeitos do OE de cravo ou seu composto ativo (eugenol) como sendo

seguros e eficazes para a anestesia de peixes (Inoue et al., 2011; Ribeiro et al., 2015; Ribeiro et al., 2019; Aydin e Barbas, 2020; Ferreira et al., 2020b; Silva et al., 2021c). Além de sua eficácia, o OE de cravo é amplamente utilizado em manejos da piscicultura por ser um produto de baixo custo efetivo, pouco tóxico, abundante e ecologicamente correto (Mirghaed et al., 2018; Aydın et al., 2019). A literatura relata que o OE de cravo para juvenil de angelfish *P. scalare* pode ser menos prejudicial do que MS-222 e 2-fenoxietanol, e economicamente acessível para uso em larga escala (Mitjana et al., 2014). Contudo, outros estudos em peixes relataram efeitos colaterais após exposição ao eugenol, como elevação dos níveis de cortisol e glicose (Hoseini et al., 2011; Hoseini e Ghelichpour, 2012), possíveis reduções nos valores de linfócitos e aumento de neutrófilos no sangue (Gholipour et al., 2011), insuficiência ventilatória (Sladky et al., 2001) e concentrações elevadas deste fármaco podem acometer necrose branquial (Abdel-Fattah et al., 2005). E ainda, Barbas et al. (2021) afirmam que o uso de eugenol pode ser potencialmente tóxico para o cérebro dos peixes, e que protocolos que sugerem o uso amplo de eugenol para anestesia rápida ou eutanásia de peixes devem ser cuidadosamente revisados, pois levantam preocupações em termos de ética e bem-estar dos animais.

A planta alfavaca *Ocimum gratissimum* L., pertencente a familia Laminaceae, é encontrada em todas as regiões tropicais, incluindo o Brasil (Alburquerque et al., 2007). A literatura relata que o eugenol (73,6%) e *β*-bisaboleno (18,3%) são os principais componentes do óleo essencial de *O. gratissimum* L. (EOOG) (Silva et al., 2012). Desta forma, esses compostos do EOOG tem demonstrado efeito anestésico e/ou sedativo e seu uso, recomendado para manipulação biométrica e transporte de diferentes espécies de peixes como jundiá *Rhamdia quelen* (Silva et al., 2012); matrinxã *Brycon amazonicus* (Ribeiro et al., 2016), pacamã *L. alexandri* (Boaventura et al., 2020; Boaventura et al., 2021a), *C. macropomum* (Boijink et al., 2016; Chagas et al., 2021) e *O. niloticus* (Adewale et al., 2017; Ferreira et al., 2021a). Do mesmo modo, a espanta pulga *Hesperozygis ringens* é uma planta nativa do Sul do Brasil (Dolwitsch et al., 2020). A literatura relata que os compostos majoritários do OE de *H. ringens* (EOHR) são pulegone (95,18%) e limoneno (1,28%) (Toni et al. 2014) e este OE demonstrou propriedades anestésicas e sedativas para jundiá *R. quelen* (Silva et al. 2013; Toni et al., 2014; Toni et al. 2015) e tambaqui *C. macropomum* (Ferreira et al., 2022).

O mentol é uma substância (ou, composto) extraído de plantas do gênero *Mentha* (Matos, 2000; Patel et al., 2007) e também tem demonstrado efeito anestésico e sedativos para muitas espécies, tais como curimba *P. lineatus* (Junior et al., 2018), angelfish *P. scalare* (Romaneli et al., 2018), truta arco-íris *Oncorhynchus mykiss* (Teta e Kaiser, 2019), guppy *Poecilia reticulata* (Cunha et al., 2020), ciclídeo africano *A. nyassae* (Ferreira et al., 2020a), pirapitinga *Piaractus brachypomus* (Zapata-Guerra et al., 2020; Ferreira et al., 2021b) e pacamã *L. alexandri* (Ananias et al., 2022). O mentol é um produto natural (Yadegarinia et al., 2006) de baixo custo e facilmente encontrado em farmácias (Façanha e Gomes, 2005). Como revisado por Aydin e Barbas (2020), os autores descreveram que um bom anestésico para peixes deve levar em consideração as melhores relações concentrações-resposta, junto com a caracterização comportamental dos tempos de indução da anestesia e recuperação e que cause menor efeito colateral nos animais. Além disso, que garanta maior segurança tanto para o animal e manipulador (Ross e Ross, 2008), cause maior conforto e promova o bem estar de peixes durante manejo de biometria e transporte.

2.3 Hematologia, bioquímica, gasometria do sangue, estado oxidativo e frequência ventilatória de peixes frente ao uso de anestésicos

Barton e Iwana (1991) definiram estresse como uma mudança no estado fisiológico, na qual a desordem da homeostase ocorre por um agente estressor. No cultivo de peixes existem muitos estressores, tais como biometria, transporte e anestésicos (Barton, 2002; Matthews e Varga, 2012). Distúrbios fisiológicos, provocados por fatores estressantes causam desconforto nos peixes e prejudicam seu bem-estar podendo comprometer sua sobrevivência. Durante a tentativa de manter a homeostase do organismo, há consumo de energia para esse processo de restauração homeostática, como, respiração, locomoção e reparação de tecidos, sendo que esta energia poderia ser utilizada para crescimento e reprodução (Schreck, 2010).

O controle neuroendócrino em resposta ao estresse dos mamíferos é semelhante para peixes (Barton, 2000). Basicamente, dois componentes são acionados: o eixo sistema nervoso autônomo-tecido cromafim (SSCC) e o eixo hipotálamo-hipófise-tecido interrenal (HHI). Anatomicamente os peixes apresentam no rim cefálico, células cromafins e interrenais, estruturas homólogas à glândula adrenal dos mamíferos. Após segundos de contato com um estressor ocorre a ativação do SSCC que promove a liberação de catecolaminas circulantes, como as noradrenalinas e adrenalinas (Urbinati e Carneiro, 2004). Após a ativação do SSCC e

após minutos em contato com o estressor, o HHI é ativado em uma cascata de reações. Resumidamente, o sistema nervoso central (CNS), através de centros superiores, estimula os neurônios do hipotálamo a produzir o hormônio liberador de corticotrofina (CRH). Estes neurônios projetados a hipófise ativam a liberação de corticotropina (ACTH) que por fim, estimula as células interrenais a sintetizarem glicorticóides, como o cortisol (Weendelar Bonga, 1997; Weendelar Bonga, 2011).

Existem três tipos de respostas ao estresse: primária, secundária ou terciária (Barton, 2002). A resposta primária, conhecida como a resposta de alarme, envolve a ativação dos dois eixos (SSCC e HHI) e, consequente liberação de hormônios do estresse, como corticosteroides e catecolaminas (Wendelaar Bonga, 1997; Barton, 2002; Jerez-Cepa et al., 2021). Quando liberados na corrente sanguínea, os hormônios do estresse induzem respostas secundárias nos tecidos-alvo, atuando em seus receptores (Mommsen et al., 1999). Geralmente, a secreção de catecolaminas é iniciada logo após o início da resposta ao estresse e é reduzida de forma transitória (Wendelaar Bonga, 1997). Respostas secundárias dos peixes incluem alterações celulares e imunológicas, composição iônica, hematológicas, metabólicas e osmorreguladoras, como aumento dos níveis de glicose plasmática no sangue, aumento da pressão arterial e frequência ventilatória, hematócrito, atividade da lisozima e síntese de anticorpos (Wendelaar Bonga, 1997; Ortuno et al., 2002; Iwama et al., 2004; Pankhust, 2011; Jerez-Cepa et al., 2021). Estes levam a respostas terciárias, como diminuição do consumo de alimentos e consequente queda no desempenho produtivo e reprodutivo, além de deixar o animal susceptível às infecções por diversos patógenos, entre outros (Wendelaar Bonga, 1997; Barton, 2002). Diante da grande importância na cascata fisiológica do estresse, a concentração de glicose é um dos principais indicadores de estresse mensurados em peixes. Muitos pesquisadores demonstraram que manejos mal planejados (biometria e transporte de peixes) foram responsáveis por aumentar os níveis de cortisol, ativando a glicogenólise e a gliconeogênese com aumentos nos valores de glicose plasmática, como revisado por Aydin e Barbas (2020) e Souza et al. (2019). Tal fato também foi relatado para peixes expostos ao ar ou em condições de hipóxia (Henrique et al., 1998; Abdel-Tawwab et al., 2015; Li et al., 2018; Neves et al., 2020, Neves et al., 2022).

As respostas fisiológicas também podem ser avaliadas através da hematologia, as quais são empregadas como auxiliares de diagnóstico da saúde dos animais. Por meio do acompanhamento desses parâmetros é possível avaliar o perfil homeostático de peixes

submetidos a situações estressantes (Kori-Siakpere et al., 2006). As células vermelhas do sangue, compostas por eritrócitos ou hemácias, são responsáveis pelo transporte de oxigênio e gás carbônico através da hemoglobina e com o hematócrito são indicadores da capacidade de transporte de oxigênio em peixes (Tavares-Dias e Moraes, 2004). Desta forma, em peixes, quando os valores de hematócritos e hemácias estão reduzidos podem indicar sinais de anemia e comprometimento do estado de saúde (Vosyliené, 1999). Segundo Tavares-Dias e Moraes (2004), os valores de hematócrito podem sofrer oscilações quando há aumento da atividade eritropoiética do rim e baço ocasionados pelo estresse, sendo que tais alterações podem ocasionar hemoconcentração (liberação de eritrócitos pelo baço) ou hemodiluição (pode ocorrer em peixes de água doce devido a maior entrada de água por osmose ocasionado pelo aumento da VF). Muitos trabalhos demonstraram o aumento nas hemácias, hematócrito e/ou hemoglobina após condição de hipóxia e anestesia de peixes (Affonso et al., 2002; Yang et al., 2017; Ribeiro et al., 2019; Boaventura et al., 2020; Neves et al., 2020). Segundo Teixeira et al. (2017) a concentração de 300 µL L^{-1} do óleo essencial de *A. triphylla* ocasionou anestesia em tilápia do Nilo *O. niloticus*, redução nos níveis de cortisol 1 h após a anestesia e manipulação e não afetou o sabor e/ou odor na sua carne. Já, a concentração de 500 µL L^{-1} do óleo essencial de *Lippia alba* (EOLA) também foi capaz de promover a anestesia profunda de *O. niloticus* e reduzir o cortisol após manuseio (Hohlenwerger et al., 2016). Outro estudo demonstra que o uso de eugenol (50 mg L^{-1}) para anestesia profunda de pirapitinga *P. brachypomus* foi seguro e eficiente com prevenção no aumento nos níveis de glicose plasmática 1 h após manejo biométrico; e com poucas influências nos valores de hematócrito e triglicerídeos (Ferreira et al., 2021b). Da mesma forma, o uso de mentol (50 mg L^{-1}) foi eficiente para anestesia (perda de consciência) e na diminuição dos efeitos do estresse causado pelo manejo de biometria em pacamã *L. alexandri* (Ananias et al., 2022). Em contrapartida, a concentração anestésica de 50 mg L^{-1} de mentol para pirapitinga *P. brachypomus* não foi capaz de prevenir o aumento da glicose 1 h após biometria; no entanto causou mínimas oscilações nos níveis de proteína plasmática, hematócrito e colesterol (Ferreira et al., 2021b). Além disso, o uso de diferentes concentrações de eugenol e mentol para anestesia de juvenis de pirapitinga *P. brachypomus* não provocaram mudanças significativas nos valores de hemoglobina (Ferreira et al., 2021b). Por outro lado, a baixa concentração do óleo essencial de *O. gratissimum* L. (10 mg L^{-1}) adicionada à água de transporte de pacamã *L. alexandri* foi capaz de promover a sedação dos animais, reduzir o consumo de oxigênio, excreção de

amônia e prevenir o aumento dos níveis de glicose plasmática imediatamente após o transporte (Boaventura et al., 2021a). Já, a concentração de 5 mg L^{-1} do óleo essencial de *O. gratissimum* L. utilizada no transporte de tilápia do Nilo *O. niloticus* não provocou mudanças nas variáveis de qualidade da água (temperatura, pH, oxigênio dissolvido e amônia total) e causou mínimas influências nos valores de hematócrito e glicose plasmática (Ferreira et al., 2021a). Outro trabalho, demonstrou que 20 µL L^{-1} do EOLA adicionada a água de transporte de tilápia do Nilo *O. niloticus* promoveu sedação, sendo capaz de diminuir a frequência ventilatória, níveis de amônia não ionizada e de glicose dos animais (Hohlenwerger et al., 2017). Segundo Liu et al. (2022), a concentração de 40 mg L^{-1} de MS-222 adicionada a água de transporte de yellow catfish *Pelteobagrus fulvidraco* permitiu menor excreção de nitrogênio amoniacal na água.

Da mesma maneira a hemogasometria também é ferramenta utilizada como indicadora da saúde dos peixes (Barton, 2002; Tavares Dias et al., 2008). A gasometria do sangue avalia os parâmetros metabólicos (equilíbrio ácido-base) das trocas gasosas respiratórias, tais como pressão parcial de dióxido de carbono (pCO_2), pressão parcial de oxigênio (pO_2), pH sanguíneo e íon bicarbonato (HCO^-_3). Ao associar essas ferramentas pode-se inferir de forma eficaz sobre a fisiologia do peixe desencadeada pelo estresse (Barbas et al., 2016; Mattioli et al., 2019a, Neves et al., 2020). Todos esses parâmetros nos auxiliam a avaliar condições de estresse por manipulação e anestésicos (Honorato et al., 2014; Mattioli et al., 2019b; Neves et al., 2022). Recentemente foi relatado que o uso de nanopartículas de zeína contendo eugenol para tilápia do Nilo *O. niloticus* foi capaz de potencializar a anestesia e não ocasionar mudanças nas variáveis hemogasometricas desta espécie (Ferreira et al., 2020b).

Outra resposta ao estresse também conhecida é a perturbação do equilíbrio hidromineral. Segundo Mariano et al. (2009) oscilações nas variáveis dos níveis de cálcio, cloreto, potássio e sódio, podem indicar um quadro de desequilíbrio ácido-base em peixes. A adição de sedativos e anestésicos na água de transporte diminuiu a perda líquida de íons em *R. quelen* (Parodi et al., 2014, Zeppenfeld et al., 2014; Becker et al., 2016; Becker et al., 2017) e esta resposta é seguida por elevação nos valores de íons plasmáticos (Zeppenfeld et al., 2014).

Porém, condições de anestesia e hipóxia podem desencadear oscilações no estado oxidativo das células de peixes (Matés, 2000; Velisek et al., 2011; Souza et al., 2018; Baldissera et al., 2020). Estresse oxidativo é definido entre o desequilíbrio dos agentes oxidantes e antioxidantes em favor dos oxidantes (Velisek et al., 2011; Halliwell e Gutteridge,

2015). Os oxidantes são constituintes de espécies reativas de oxigênio (ROS) que podem ocasionar danos em lipídeos, proteínas e DNA (Evans e Halliwell, 1999, Morel e Barouki, 1999; Gandhi e Abramov, 2012). Já os antioxidantes são substâncias capazes de reduzir ou prevenir a oxidação de uma determinada molécula (Halliwell e Gutteridge, 2007). Em peixes, estressores físicos (captura, hipóxia, manuseio e transporte, por exemplo) desencadeiam alterações fisiológicas e metabólicas que culminam em perturbações respiratórias, cerebrais e hormonais, bem como na síntese de ROS (Barton, 2002; Xing et al., 2012; Faught et al., 2016) que afetam diretamente o sistema imunológico dos animais (Biller e Takahashi, 2018). Os peixes, na tentativa de manter a homeostase frente a um estressor, neutralizam ROS para paralisar os efeitos do estresse oxidativo e prevenir possíveis danos oxidativos atuando no sistema de defesa antioxidante (Halliwell e Gutteridge, 2015). O sistema de defesa antioxidante é composto por inibidores oxidantes não enzimáticos como o tiol não proteíco (NPSH) (Birnie-Gauvin et al., 2017) e pelas enzimas antioxidantes, como a superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa-S-transferase (GST) e catalase (CAT) (Halliwell et al., 1995; Barbosa et al., 2010), todas elas abundantes nos tecidos dos peixes (Van Der Oost et al., 2003). Desta forma, as substâncias antioxidantes enzimáticas e não enzimáticas atuam na prevenção da carbonilação de proteínas e peroxidação lipídica, que comumente são avaliadas por biomarcadores de substâncias reativas a proteína carbonila (CP) e ao ácido tiobarbitúrico (TBARS), respectivamente (Biller e Takahashi, 2018). Segundo esses mesmos autores, a presença de ROS nos tecidos (células) desencadeia uma cascata de reações bioquímicas que reduzirão a função celular devido aos danos oxidativos em carboidratos, proteínas e lipídeos, e assim podendo culminar no acúmulo de constituintes moleculares oxidados e até mesmo apoptose. Assim, pesquisadores têm utilizado diferentes anestésicos (em concentrações ideais) para anestesia e sedação de peixes com intuito de aumentar a capacidade antioxidante durante a manipulação dos animais (Souza et al., 2019a). Segundo Saccol et al. (2017) os óleos essenciais de *Myrcia sylvatica* e *Curcuma longa* L. foram eficientes para anestesia (200 e 500 µL L^{-1}) e sedação (10 e 40 µL L^{-1}) de tambaqui *C. macropomum* demonstrando diminuição dos níveis de peroxidação lipídica e aumento da atividade de enzimas antioxidantes (CAT, GPx, GST e SOD) e também no potencial antioxidante reativo total e NPSH em tecidos branquiais, cerebrais, hepáticos e renais quando comparados ao grupo controle. Avaliando o efeito anestésico de EO de *A. triphylla* e MS-222 para *R. quelen*, foi registrado que o EO de *A. triphylla* foi capaz de impedir a peroxidação

lipídica no tecido hepático, o que não foi observado para MS-222 (Gressler et al., 2014). O uso do óleo essencial de *O. gratissimum* L. (EOOG) (10 mg L^{-1}) quando adicionado a água de transporte de pacamã *L. alexandri* induziu maior proteção contra possíveis danos oxidativos (Boaventura et al., 2021a). Por outro lado, Boaventura et al. (2020) relataram que a concentração de 90 mg L^{-1} de EOOG quando utilizado para manejo de biometria promoveu anestesia profunda em pacamã *L. alexandri* e não causou danos teciduais; no entanto ocasionou alterações no sistema de defesa antioxidante com aumento de ROS no tecido hepático e cerebral e redução dos níveis de GST no cérebro. Da mesma maneira, o EOOG provocou anestesia (perda de consciência) em juvenis de tilápia do Nilo *O. niloticus*; porém com aumento de ROS no tecido hepático e renal e possíveis danos lipídicos nos rins desta espécie (Ferreira et al., 2021a).

A mensuração da frequência ventilatória (VF) é um método não invasivo que indica possíveis mudanças fisiológicas no sistema respiratório dos peixes causados por estressores agudos, como por exemplo, a manipulação e o uso de anestésicos (Alvarenga e Volpato, 1995; Barreto e Volpato, 2004). Segundo Tytler e Hawkins (1981), os anestésicos causam redução da VF das brânquias devido à exaustão dos centros bulbares respiratórios ocasionando a hipóxia em peixes. Porém, uma característica comumente visível quando os peixes são manipulados ou quando entram em contato com substâncias anestésicas é a hiperventilação (Summerfelt e Smith, 1990) que ocorre a fim de aumentar o consumo de oxigênio (Alvarenga e Volpato, 1995). No entanto, após o contato inicial com um anestésico, a VF reduz consideravelmente (Becker et al., 2012). Segundo Souza et al. (2019b) a concentração de 4 µL L^{-1} de propofol provocou anestesia geral em tambaqui *C. macropomum* apresentando propriedades miorrelaxantes, diminuição na frequência cardíaca e capacidade ventilatória (intensidade e frequência de batimentos operculares) desta espécie. Em contrapartida, outro estudo descreve que não foi possível estabelecer uma relação direta entre o aumento das concentrações de benzocaína e mentol e redução da VF para duas classes de tamanhos de juvenis do ciclídeo africano *A. nyassae* (Ferreira et al., 2020a). Este mesmo comportamento foi também observado para juvenis de tambaqui *C. macropomum* anestesiados com diferentes concentrações dos óleos essenciais de *L. alba* e *L. origanoides* (Silva et al., 2019). No entanto, o uso de eugenol e mentol (50 mg L^{-1} para ambos anestésicos) não causou diferenças significativas na VF durante a indução da anestesia em duas classes de tamanhos de pirapitinga *P. brachypomus*; porém durante a recuperação a VF reduziu

consideravelmente em ambos anestésicos e tamanhos de peixes avaliados (Ferreira et al., 2021b). Segundo Becker et al. (2018) as diferentes concentrações de óleo essencial de *L. alba* e *L. origanoides* para a anestesia de jundiá *R. quelen* não provocaram influências significativas na VF dos animais em relação ao grupo controle. A literatura menciona que o aumento ou diminuição geral na resposta da VF ao utilizar anestésicos pode ser explicada por respostas espécie-específicas (Becker et al., 2018; Ferreira et al., 2020a). Desta forma, estabelecer diferentes protocolos de anestesia que proporcionem maior segurança para o animal durante a biometria e transporte de peixes vivos e principalmente que garantem o bem estar animal, se fazem necessários.

2.4 Manipulação biométrica e transporte de peixes frente ao uso de anestésicos

Em produções intensivas de peixes, o estresse dos animais é uma condição praticamente inevitável (Iwama, 1993) que muitas vezes pode ser desencadeado pela prática biométrica e transporte, por exemplo (Barton, 2002). Todo processo de produção necessita de acompanhamento que permita avaliar o crescimento e saúde dos peixes durante o ciclo de cultivo (Lima et al., 2013). Desta forma, o manejo de biometria (mensuração do comprimento e peso) é comumente realizada em pisciculturas (Ostrensky e Boeger, 1998) e centros de pesquisas. Segundo Lima et al. (2013) a biometria de peixes deve ser realizada de forma cuidadosa e rápida, a fim de não causar injurias físicas aos animais e também garantir menor tempo de exposição ao ar. Além disso, é desejável que este manejo seja realizado a cada 15 dias ou mensalmente; pois assim permitirá melhor ajuste na alimentação dos animais.

Já o transporte de peixes vivos é uma prática de grande entrave na cadeia produtiva da piscicultura e muitos fatores devem ser levados em consideração, como a densidade de estocagem (Ibrahim et al., 2015; FAO, 2016; Espinoza-Ramos et al., 2021), concentrações de oxigênio e de dióxido de carbono (Sampaio e Freire, 2016), período de jejum (Kubitza, 1997), duração do transporte (Luz et al., 2013; Paranhos et al., 2021). Tais procedimentos (biometria e transporte), se não realizados corretamente, podem causar sérios danos ao ciclo de produção de peixes e até mesmo a morte dos animais.

Com isso, diferentes anestésicos, têm sido testados para completa imobilização animal, por trazerem mais segurança (tanto para o animal quanto para o colaborador) em diversos manejos adotados na piscicultura (Ross e Ross, 2008; Ribeiro et al., 2013; Ferreira et al., 2021a, Brandão et al., 2021). Recentemente foi relatado que diferentes concentrações de

eugenol e mentol para anestesia de pirapitinga *P. brachypomus* seguida de manuseio biometrico, não provocaram mortalidade após 48 h de recuperação, e todos os peixes retomaram a alimentação dentro de 24 horas após o teste (Ferreira et al., 2021b). Da mesma maneira, não foi observada mortalidade após a anestesia com diferentes concentrações de mentol para pacamã *L. alexandri* após manejo biométrico (Ananias et al., 2022). Em contrapartida, a anestesia profunda com benzocaína para o ciclídeo africano *A. nyassae* promoveu uma sobrevivência > 90%. Este conjunto de trabalhos são importantes para cadeia industrial da piscicultura e novas tecnologias devem ser investigadas para melhorar a aplicação de anestésicos na aquicultura. Além disso, estudos que avaliem o desempenho zootécnico dos peixes após testes de anestesia, se fazem necessário.

2.5 Espécies estudadas

2.5.1 Oreochromis niloticus

A tilápia do Nilo *O. niloticus* pertencente à família Cichlidae, é a terceira espécie de água doce mais produzida mundialmente (FAO, 2020). No Brasil, a tilápia do Nilo é a espécie mais produzida no país, representando 63,5% da sua produção total de peixes de cultivo (PEIXE BR, 2022). Algumas características desta espécie favorecem o seu cultivo, como hábito alimentar onívoro, rápido crescimento, melhor conversão alimentar e boa qualidade da carne (Coward e Bromage, 2000; Asad et al., 2010; Sayed e Moneeb, 2015). Devido ao aprimoramento das técnicas para produção de tilápia do Nilo *O. niloticus*, essa espécie continua em destaque devido ao seu potencial econômico e além disso é considerada modelo para estudos sobre a biologia de ciclídeos (Fujimura e Okada, 2007) e de anestesia (Ribeiro et al., 2015; Ferreira et al., 2020b, Ferreira et al., 2021a). No Brasil, além da produção de tilápia do Nilo *O. niloticus*, o cultivo de peixes nativos tem contribuído para aumento da produção no país (PEIXE BR, 2022).

2.5.2 Colossoma macropomum

O tambaqui *C. macropomum* (Cuvier, 1816) é uma espécie de peixe migratório nativo das bacias dos rios Amazonas e Orinoco (Araujo Lima e Goulding, 1997; Reis, 2003), pertencente à superclasse Actinopterygii, ordem Characiformes e família Serrasalminae (Buckup et al., 2007). Seu consumo é apreciado no Brasil, Colômbia, Venezuela e Peru

(Woynárovich e Anrooy, 2019). A produção brasileira dessa espécie perde apenas para tilápia do Nilo *O. niloticus* (PEIXE BR, 2022). O tambaqui *C. macropomum* é considerado um animal rústico com crescimento rápido (Araujo Dairik et al. 2018), de hábito alimentar onívoro com tendência de zooplanctófago na fase larval e de frugívoro na fase adulta (Saint-Paul, 1984) e fácil manejo (Araujo Lima e Goulding, 1997), o que o tornou popular para a aquicultura (Gomes e Baldisserotto, 2019). Esta espécie é conhecida por sua alta resistência a hipóxia (Neves et al., 2020; Neves et al., 2022) e isso se deve ao fato da sua região de origem possuir baixa concentração de oxigênio dissolvido na água (Morais e O'Sullivan, 2017).

Muitos centros de pesquisas e produtores têm se esforçado para promover o papel tecnológico para a criação de tambaqui *C. macropomum* em cativeiro, o que inclui estudos em requerimento de proteína após restrição alimentar (Santos et al., 2012), densidade de estocagem para cultivo em tanques rede (Silva e Fujimoto, 2015), densidade de estocagem (Lima et al., 2019); condições de hipóxia (Neves et al., 2020), exposição ao ar (Neves et al., 2022), níveis de proteína na ração (Sousa et al., 2016), restrição alimentar (Assis et al., 2020) e condições de alimentação automática (Guilherme et al., 2021). Recentemente, resultados importantes foram descritos para produção de larvas de tambaqui *C. macropomum* (Santos et al., 2020; Santos et al., 2022), cultivo de juvenis (Boaventura et al., 2021c, Silva et al., 2021a) e fase de crescimento (Santos et al., 2021) em sistemas de recirculação de água. Além da sua importância comercial, o tambaqui *C. macropomum* tem sido usado como modelo biológico em programas de biomonitoramento (Corrêa et al., 2007; Marcuschi et al., 2010; Braz-Mota et al., 2015) e quanto ao uso de anestésicos como revisado por Brandão et al. (2021).

2.5.3 Piaractus brachypomus

A pirapitinga *P. brachypomus* (Cuvier, 1818) é uma espécie onívora de água doce pertencente à ordem Characiformes, que geralmente é encontrada naturalmente no Rio Orinoco, Bacias do Amazonas e do Prata (Paraná-Paraguai) (Schultz, 1944). Esta espécie apresenta importância comercial com destaque em países da América Central e América do Sul (Nascimento et al., 2010; Jorge et al., 2018). No Brasil, a produção desta espécie apresenta destaque na produção de peixes nativos (PEIXE BR, 2022). Diferentes estudos têm buscado promover melhorias no cultivo desta espécie de interesse comercial, tais como, no manejo reprodutivo (Nascimento et al., 2010; Chaves-Moreno et al., 2012), nutricional

(Guimarães e Martins, 2015), frequência alimentar (Favero et al., 2022) e anestésicos (Zapata-Guerra et al., 2020; Ferreira et al., 2021b).

2.5.4 Aulonocara nyassae

A ordem dos Perciformes inclui um terço de todas as espécies de peixes e apresenta grande diversidade entre os vertebrados (Helfman et al., 2009). A família Cichlidae é a segunda maior dentre a ordem Perciformes com cerca de 1.900 espécies (Weyl et al., 2010). Essas espécies de ciclídeos estão distribuídas na África, América Central e do Sul, Oriente Médio e parte do Sudeste asiático (Berra, 2001).

Um dos maiores lagos da África, o lago Malawi, apresenta grande biodiversidade de espécies de peixes registradas (Fryer e Iles, 1972; Weyl et al., 2010). Neste lago, foram encontradas 800 a 1000 espécies de ciclídeos (Snoeks, 2004) dentre elas as do gênero *Aulonocara* (Konings, 1995). As espécies de peixes deste gênero estão entre as mais comercializadas na aquariofilia ornamental nos últimos anos (Schwalbe et al., 2012).

A espécie *A. nyassae* (Regan, 1922), conhecida popularmente como *African peacock*, *blue cichlid*, *emperor cichlid* e *peacock blue cichlid* é encontrada em locais arenosos (Konings, 1995). Segundo o mesmo autor, os machos apresentam coloração mais forte azul-escuro na qual demonstram o comportamento territorialista da espécie, o que chama a atenção dos aquaristas. Devido ao seu interesse comercial, pesquisadores têm se dedicado em promover o cultivo desta espécie em cativeiro, como pode observar através de trabalhos relacionados com a produção de juvenis (Karslı, 2021), reprodução (Silva et al., 2021b) e uso de anestésicos (Ferreira et al., 2020a). Contudo, há carências de informações na literatura sobre as concentrações recomendadas (anestésicos sintéticos ou naturais) para a manipulação e transporte desta espécie.

3. OBJETIVOS

3.1 GERAL

Avaliar as respostas fisiológicas e metabólicas de peixes de água doce expostos a diferentes compostos anestésicos e/ou sedativos (sintético ou natural) e forma de aplicação (diluição em álcool ou nanoemulsão) para manejo de biometria e transporte.

3.2 ESPECÍFICOS

- Avaliar a sobrevivência e retorno da alimentação dos peixes em todos os experimentos.

Artigo 1

- Avaliar diferentes concentrações de benzocaína e mentol para os tempos de indução e recuperação e frequência ventilatória durante o processo de anestesia, em duas classes de tamanhos de juvenis do ciclídeo africano *A. nyassae*.

Artigo 2.

- Avaliar diferentes concentrações de eugenol e mentol para os tempos de indução e recuperação e frequência ventilatória durante o processo de anestesia, em duas classes de tamanhos de juvenis de pirapitinga *P. brachypomus.*
- Avaliar o efeito anestésico de eugenol e mentol em juvenis de pirapitinga *P. brachypomus* nas variáveis sanguíneas (hematológicas e bioquímicas) após biometria.

Artigo 3

- Avaliar os parâmetros físico-químicos das características nanopartículas de zeína mucoadesivas contendo eugenol (NPZMA) e sua composição físico-química.
- Avaliar diferentes concentrações de nanopartículas de zeína mucoadesivas (NPZMA) contendo eugenol para o tempo de indução e recuperação de tilápia do Nilo *O. niloticus*.
- Avaliar o efeito de NPZMA sob os parâmetros de qualidade da água.
- Avaliar o efeito anestésico de NPZMA sob as variáveis de gasometria do sangue.

Artigo 4

- Avaliar o efeito anestésico do EOOG e seu uso na água de transporte de juvenis de tilápia do Nilo *O. niloticus.*
- Avaliar diferentes concentrações do EOOG para os tempos de indução e recuperação de juvenis de tilápia do Nilo *O. niloticus*; além da frequência ventilatória durante o processo de anestesia.
- Avaliar o efeito anestésico de EOOG de juvenis de tilápia do Nilo *O. niloticus* sob as variáveis hematológicas e bioquímicas do sangue e estresse oxidativo após manipulação biométrica.
- Avaliar diferentes concentrações de EOOG para o transporte (4.5 h) sob os parâmetros físico-químicos de qualidade da água.
- Avaliar a hematologia e bioquímica do sangue de juvenis de tilápia do Nilo *O. niloticus* transportadas com uso do EOOG.

Artigo 5.

- Avaliar diferentes concentrações de EOHR para o tempo de indução anestésica e recuperação, frequência ventilatória em pequenos juvenis de tambaqui *C. macropomum*.
- Avaliar diferentes concentrações de EOHR para dois períodos de transporte simulado de pequenos juvenis de tambaqui *C. macropomum*, sob os parâmetros físico-químicos de qualidade da água.
- Avaliar as diferentes concentrações do EOHR na água de transporte sob os efeitos na excreção de amônia dos animais.
- Avaliar diferentes concentrações de EOHR para transporte simulado durante 24 h, porém sem peixes.

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5.0 ARTIGO 1.

Benzocaine and menthol as anesthetics for the African cichlid *Aulonocara nyassae*

Running Head

Anesthetics for *Aulonocara nyassae*

Artigo publicado no periódico Aquaculture International
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Abstract

The objective of this study was to investigate the effects of benzocaine and menthol on anesthesia of *Aulonocara nyassae* juveniles. Two size classes of fish were used in trials with benzocaine: Juveniles I - 70 fish of 0.74 ± 0.31 g (39.41 ± 7.48 mm); and Juveniles II - 70 fish of 3.80 ± 0.92 g (76.58 ± 9.83 mm). The fish used for trials with menthol were: Juveniles I - 70 fish of 1.01 ± 0.39 g (50.39 ± 12.75 mm) and Juveniles II - 70 fish of 3.73 ± 0.78 g (64.94 ± 8.98 mm). Seven concentrations - 0, 12.5, 25.0, 50.0, 75.0, 100.0, 125.0 mg L^{-1} - of each anesthetic (benzocaine and menthol) were tested on each size class of fish (n = 10 fish per size class and anesthetic concentration). Thus, anesthesia induction time and recovery were evaluated. Concentrations of 12.5 and 25 mg L^{-1} of benzocaine did not lead to a deep stage of anesthesia in the animals, however, the other concentrations presented anesthetic effect. For menthol, the concentration of 12.5 mg L^{-1} also did not anesthetize the fish. However, the other doses were effective and safe. The recommended anesthetic concentrations provided induction and recovery times within the limits considered ideal for fish. Concentrations between 75 and 125 mg L^{-1} of benzocaine for Juvenile I and 50 to 125 mg L^{-1} for Juvenile II are recommended. For menthol, concentrations between 50 and 125 mg L^{-1} can be used for both size classes of *A. nyassae*.

Key words: Anesthesia, handling stress, ornamental fish, physiology, ventilation frequency

1. Introduction

Handling and transport are stressful procedures involved in the management and trading of ornamental fish (Pickering 1981; Barton 2002; Romaneli et al. 2018; Oliveira et al. 2019). Exposure to stressors trigger physiological changes that may compromise fish health and survival (Wendelaar Bonga 1997). Blood glucose and cortisol concentrations are often employed as stress indicators (Morgan and Iwama 1997; Barton 2002) and observations of ventilation frequency can provide indications of changes in metabolic rate following exposure to a stressor (Summerfelt and Smith 1990; Alvarenga and Volpato 1995; Toni et al. 2014; Becker et al. 2018).

The use of anesthetics in effective doses promotes safety for both fish and the handler during different management practices adopted in pisciculture (Ross and Ross, 2008; Weber, 2011; Ribeiro et al. 2015), and can promote animal welfare and increased survival of fish. The concentration and efficacy required for anesthetic induction by a drug are related to fish size and species (Woody et al. 2002; Ross and Ross 2008; Tarkhani et al. 2017), which makes it necessary to validate an anesthetic before its use (King 2009). The choice of a particular anesthetic is associated with its legal implications, economic viability, ease of application and low risk to animals and handlers (Padua et al. 2012). Ideally, anesthetic induction time should not exceed 180 s, while recovery time should not exceed 300 s (Keene et al. 1998; Ross and Ross 2008).

Benzocaine is a synthetic anesthetic that is widely used because it is easily accessible and inexpensive, has no mutagenic action and can be rapidly metabolized by fish (Woody et al. 2002; Gontijo et al. 2003). This product can be used frequently without affecting productive and reproductive performance of fish and is environmentally friendly (Okamoto et al. 2009; Okamura et al., 2010).

Menthol, which is an essential oil extracted from plants of the genus *Mentha* (Patel et al. 2007), is another alternative anesthetic that has been used effectively for many fish species, such as rainbow trout, *Oncorhynchus mykiss* (Teta and Kaiser 2019); curimba, *Prochilodus lineatus* (Junior et al. 2018); and the angelfish *Pterophyllum scalare* (Romaneli et al. 2018). Menthol is easily found in pharmacies, is inexpensive (Facanha and Gomes 2005) and is a safe and natural product (Yadegarinia et al. 2006).

Species of the genus *Aulonocara* are the most popular ornamental fish among African cichlids, and have had great international commercialization in recent years (Schwalbe et al.

2012). The Blue cichlid *Aulonocara nyassae* originates from Lake Malawi and is of ornamental interest due to its "*blue orchid*" coloration that catches the attention of aquarists. Nonetheless, no data are available on the effects of benzocaine and menthol as anesthetics for the species.

Therefore, the present study aimed to investigate the effects of benzocaine and menthol on anesthesia of *A. nyassae* juveniles, as well as on ventilation frequency during anesthetic induction and recovery.

2 Material and Methods

The experiments were carried out at Laboratório de Aquacultura of the Escola de Veterinária, Universidade Federal de Minas Gerais, under registration number 326/2019 of Comissão de Ética no Uso de Animais.

Fish of *A. nyassae* were produced and cultivated in six rectangular tanks with 42 L useful volume, at a density 50 fish $tank^{-1}$, in a water recirculation system with temperature maintained at 27.21 ± 0.09 ºC, pH around 7.08 ± 0.06 (measured by Hanna HI98130 multiparameter probe), dissolved oxygen of 6.81 ± 0.58 mg L^{-1}, (Water Quality Meter AK87 oximeter) and total ammonia of 0.53 ± 0.02 mg L^{-1}, (Alfakit Labcon Test colorimetric kit). The animals were fed 1.7 mm diameter commercial feed containing 460.0 g kg^{-1} crude protein, 80 g kg^{-1} ether extract, 140 g kg^{-1} mineral matter, 20 g kg^{-1} calcium and 15 g kg^{-1}, as reported by the manufacturer, three times a day (09:00, 12:00 and 15:00 hours) until apparent satiety. The water of the system was changed once a week with 50% renewal of its useful volume. Fish were fasted for 24 h prior to testing.

The anesthetics tested were benzocaine (ethyl 4-aminobenzoate 99%, Sigma Aldrich®, SLBH1225V) and menthol (99% CRQ-1006310100). Two size classes of fish were used in trials with benzocaine: Juveniles I - 70 fish of 0.74 ± 0.31 g (39.41 ± 7.48 mm); and Juveniles II - 70 fish of 3.80 ± 0.92 g (76.58 ± 9.83 mm). The fish used for trials with menthol were: Juveniles I - 70 fish of 1.01 ± 0.39 g (50.39 ± 12.75 mm); and Juveniles II - 70 fish of 3.73 ± 0.78 g (64.94 ± 8.98 mm). Seven concentrations - 0 (control), 12.5, 25.0, 50.0, 75.0, 100.0, 125.0 mg L^{-1} - of each anesthetic (benzocaine and menthol) were tested on each size class of fish. Trials consisted of 10 replicates for each size class and concentration of anesthetic, with individual fish acting as independent replicates. The experiments were

conducted independently for each anesthetic and size class of fish, using a completely randomized design. Anesthetic concentrations were prepared by dilution in five mL ethyl alcohol (98.1% purity), and the control consisted of ethyl alcohol without anesthetic (Ribeiro et al., 2013).

For analysis of anesthesia induction and recovery, fish were randomly captured one at a time and placed in a 1 L beaker containing the concentration of anesthetic to be tested. Control animals were observed for 10 min. Anesthetic induction time was evaluated using a digital timer (Taksun Ts1809). The behavioral characteristics of the deep anesthesia used in this study followed the recommendations of Ross and Ross (2008), and were basically the loss of equilibrium and absence of swimming. Throughout the anesthetic induction process, the ventilation frequency (VF) of the animals was evaluated by counting the number of opercular beats for per minute adapted from Alvarenga and Volpato (1995). Once fish reached deep anesthesia they were weighed on an analytical balance (Marte AD5002) and measured for length using a digital caliper (Starret® 799). The fish were then transferred individually to a 1 L beaker with clean water (from the cultivation system itself) for anesthetic recovery, which was timed and VF measured. The animals were considered recovered when they presented reflexes to external stimuli and normal swimming balance (Ross and Ross 2008).

After the experiments, fish of each size class and anesthetic were pooled and kept in 28 L tanks in a recirculating aquaculture system to observe the return to appetite and record survival after 24 h. The fish were fed three times a day until apparent satiety.

Data were tested for normality by the Shapiro Wilk test and homoscedasticity of variances by the Leven test, followed by ANOVA. Regression analyses were performed to better fit the model ($P < 0.05$). Data were analyzed using R software version 3.5.2.

3 Results

Benzocaine

Benzocaine concentrations of 0, 12.5 and 25.0 mg L^{-1} did not induce deep anesthesia in the fish of either size class. For the other tested concentrations, induction time had a quadratic effect ($P < 0.05$) for Juvenile I (Fig. 1a), with an estimated inflection point at 108

mg L^{-1} (41.87 s). Recovery time for Juvenile I also presented a quadratic effect ($P < 0.05$) among the studied concentrations with a maximum point at 68 mg L^{-1} (118.93 s) (Fig. 1b).

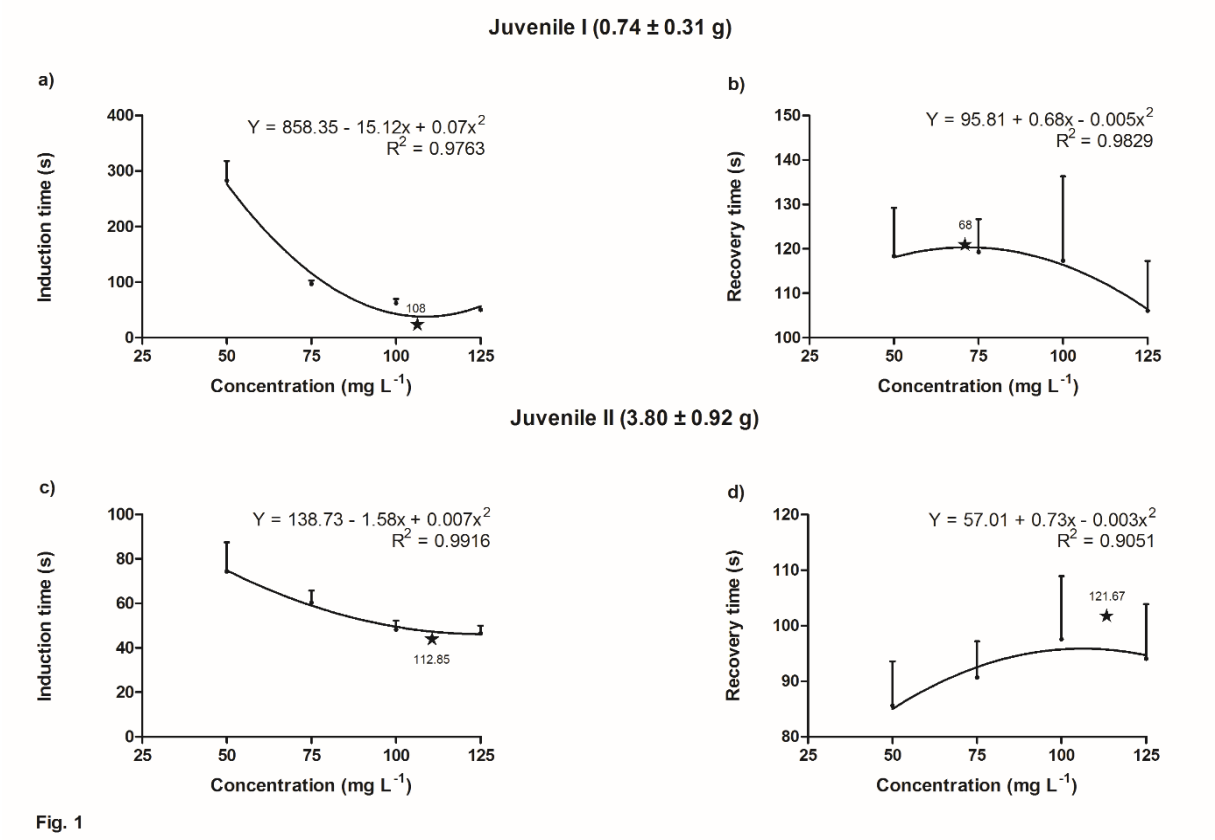

Fig. 1 Values (mean ± standard error) of time (seconds) of induction (a, c) and recovery of anesthesia (b, d) in two size classes of *Aulonocara nyassae*, submitted to different benzocaine concentrations. Star represents value estimated by the equation derived.

A quadratic effect ($P < 0.05$) was also observed for induction time for the Juvenile II size class, with the estimated minimum point being at 112.85 mg L^{-1}, while induction time ranged from 48.20 to 76.40 s (Fig. 1c). Recovery time also had a quadratic effect ($P < 0.05$) among concentrations for these fish, with the maximum point at 121.67 mg L^{-1} (101.41 s) (Fig. 1d). Increasing doses of benzocaine were accompanied by increasing recovery times for the Juvenile II size class.

Ventilation frequency during induction of Juvenile I presented a quadratic effect ($P < 0.05$) among the studied concentrations, with the estimated minimum point being at 110 mg L^{-1} (50.63 beats min^{-1}) (Fig. 2a). During recovery, the VF for Juvenile I also had a quadratic

effect ($P < 0.05$) among evaluated concentrations, with a minimum point at 66.67 mg L^{-1} and a range of 72.11– 83.53 beats min^{-1} (Fig. 2b).

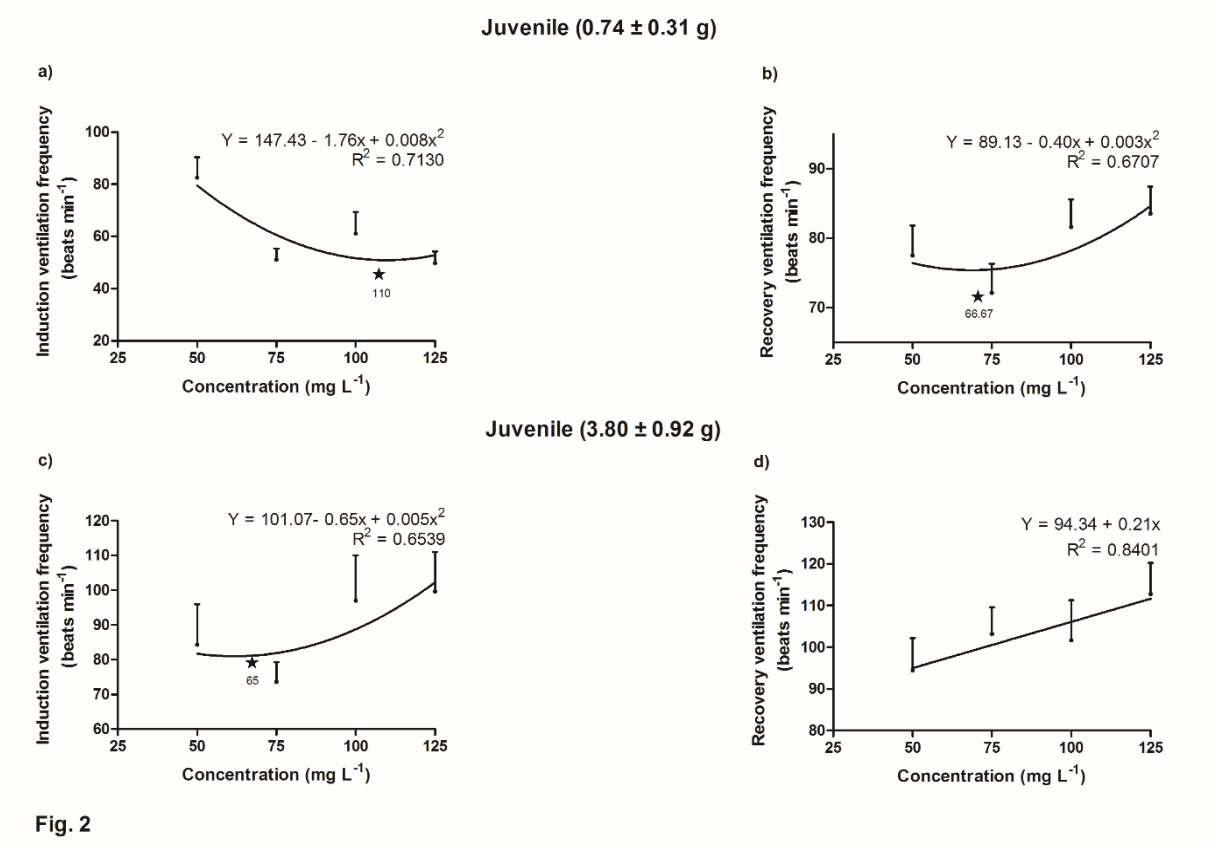

Fig. 2 Values (mean ± standard error) ventilation frequency (opercular beats per minute) during induction (a, c) and recovery of anesthesia (b, d) in two size classes of *Aulonocara nyassae*, submitted to different benzocaine concentrations. Star represents value estimated by the equation derived.

Ventilation frequency during induction of Juvenile II presented a quadratic effect among the evaluated concentrations ($P < 0.05$) with the minimum point at 65 mg L^{-1} (79.94 beats min^{-1}) (Fig. 2c). During recovery, the VF for Juvenile II had a direct linear effect ($P < 0.05$) among the studied concentrations and a range of 94.47 – 112.75 beats min^{-1} (Fig. 2d).

Survival for Juvenile I was > 90% at 24 hours after the end of the experiment and presented a linear effect ($P < 0.05$) among the evaluated concentrations with the estimated equation $Y = 109.0 - 0.16x$, $R^2 = 0.80$, and all surviving animals feeding normally. Survival for Juvenile II was 100% 24 hours after the end of the experiment for all benzocaine concentrations, with all animals resuming feeding.

Menthol

Menthol concentrations of 0, 12.5 mg L^{-1} did not induce deep anesthesia in the fish of either size class. For the other concentrations, induction time for Juvenile I had a quadratic effect ($P < 0.05$), with an estimated inflection point of 122.25 mg L^{-1} (11.78 s) (Fig. 3a). Recovery time for Juvenile I also had a quadratic effect ($P < 0.05$) with a maximum point of 40 mg L^{-1} (91.98 s) (Fig. 3b).

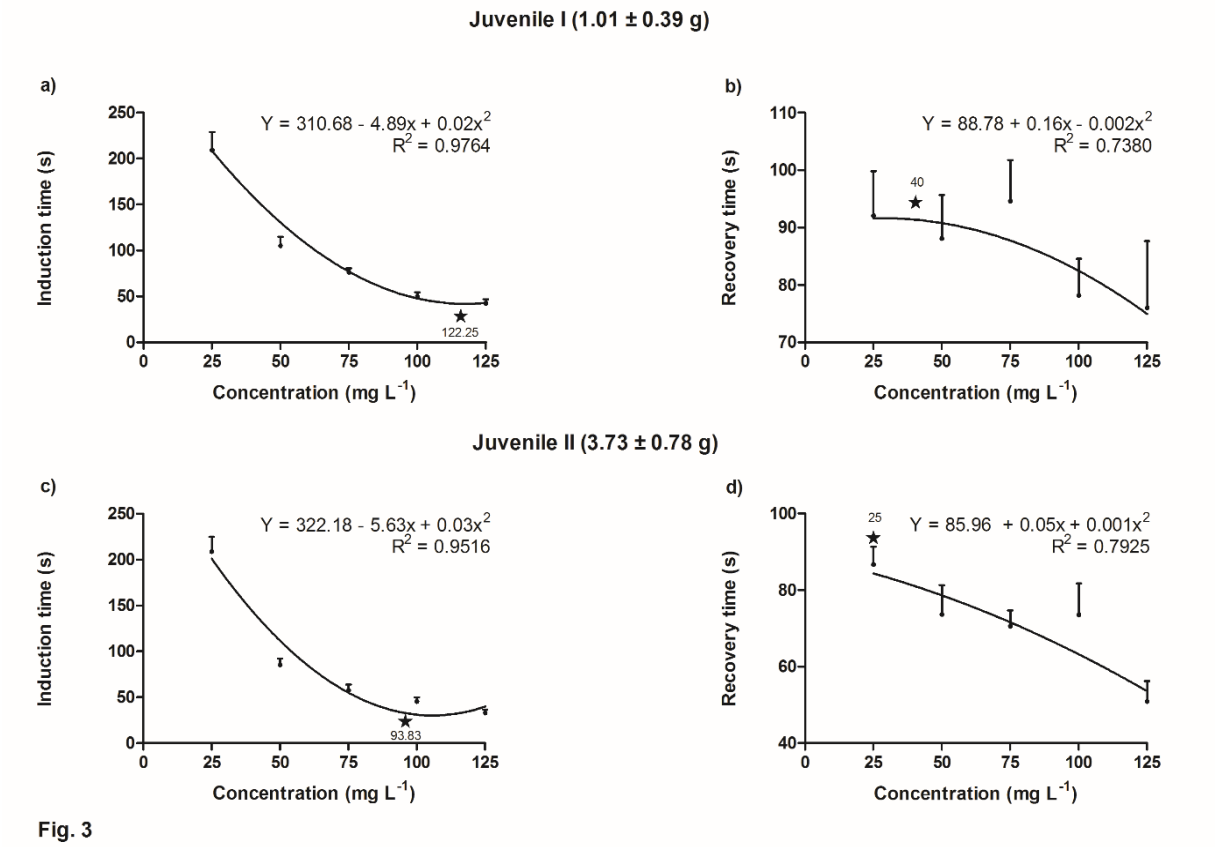

Fig. 3

Fig. 3 Values (mean ± standard error) of time (seconds) of induction (a, c) and recovery of anesthesia (b, d) in two size classes of *Aulonocara nyassae*, submitted to different menthol concentrations. Star represents value estimated by the equation derived.

The induction time for Juvenile II also had a quadratic effect ($P < 0.05$) among the different menthol concentrations, with the minimum at 93.83 mg L^{-1} (58.02 s) (Fig. 3c). The

recovery time for Juvenile II had a quadratic effect ($P < 0.05$) among the studied concentrations with a maximum point of 25 mg L^{-1} and a range of 50.90 – 86.70 s (Fig. 3d).

Ventilation frequency for Juvenile I during induction had a direct linear effect ($P < 0.05$) among the concentrations and ranged 44.52 – 169.78 beats min^{-1} (Fig. 4a). For the same size class, VF during recovery had a quadratic effect ($P < 0.05$) among the concentrations with a minimum point of 47.50 mg L^{-1} (70.20 beats min^{-1}) (Fig. 4b).

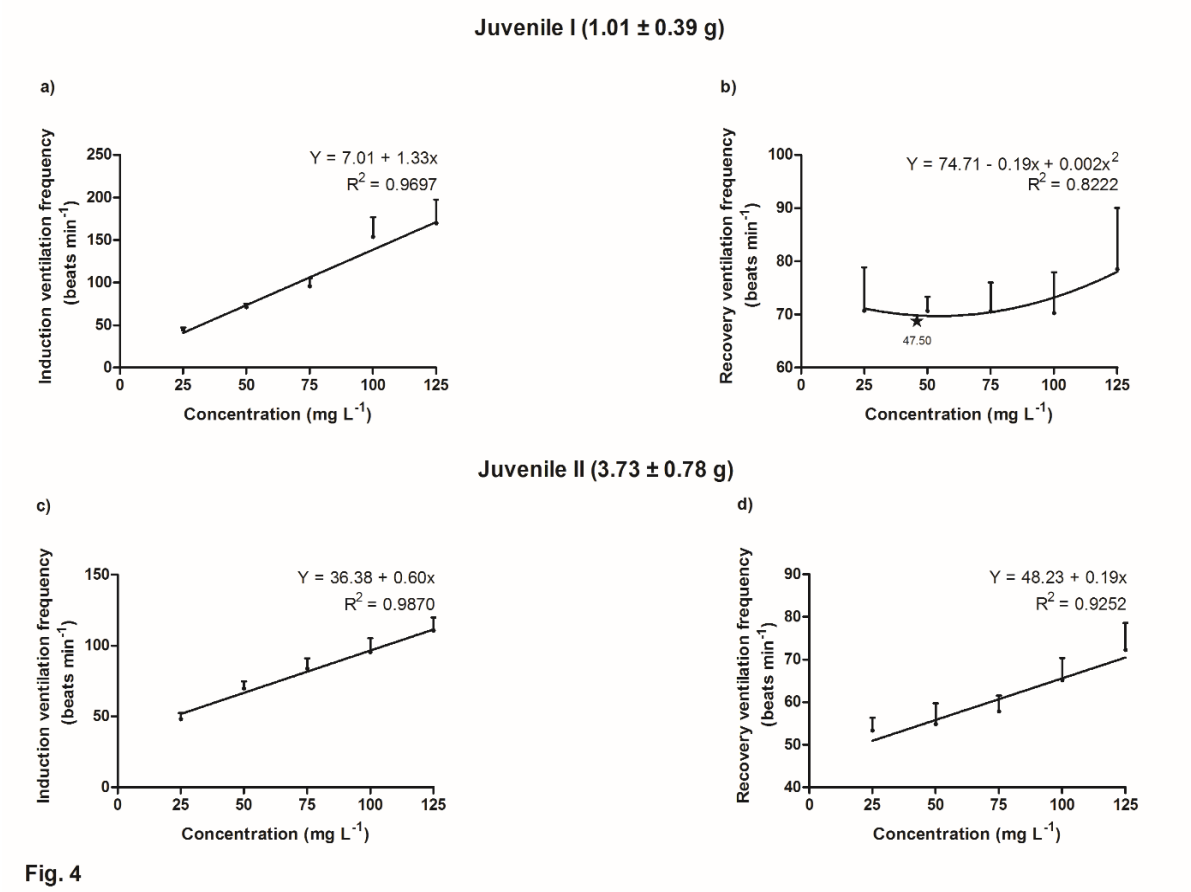

Fig. 4 Values (mean ± standard error) ventilation frequency (opercular beats per minute) during induction (a, c) and recovery of anesthesia (b, d) in two size classes of *Aulonocara nyassae*, submitted to different menthol concentrations. Star represents value estimated by the equation derived.

Ventilation frequency for Juvenile II during induction had a direct linear effect ($P < 0.05$) among concentrations ranging 48.13 – 110.70 beats min^{-1} (Fig. 4c). For the same size class, VF during recovery also had direct linear effect ($P < 0.05$) among concentrations (Fig. 4d). Survival for Juvenile I and Juvenile II size classes was 100% at all concentrations evaluated after 24 hours of recovery, and all animals resumed feeding.

4 Discussion

Benzocaine and menthol proved to be efficient for anesthesia of *A. nyassae.* Concentrations were found with induction times of less than 180 s and recovery times less than 300 s, as recommended by Keene et al. (1998) and Ross and Ross (2008).

Benzocaine and menthol are anesthetics that require dilution in ethanol; however, this solvent has no induction effect and does not cause mortality in fish at low concentration (Junior et al. 2018, Ribeiro et al. 2019, Teta and Kaiser 2019), as also observed for fish in the control group of the present study.

In the benzocaine experiment, anesthetic efficiency for the Juvenile I size class was between the concentrations of 75 and 125 mg L^{-1}, while for Juvenile II it was between 50 and 125 mg L^{-1}; then providing induction and recovery times within the limits considered ideal for fish (Keene et al. 1998 and Ross and Ross 2008). During the process of anesthesia, and until a complete loss of swimming balance is achieved, anesthetic-related corticosteriods and catecholamines are released. Therefore, the faster that immobilization can occur, the less of these metabolites will be released in the organism (Rothwell et al. 2005). Thus, benzocaine concentrations of 108 and 112.85 mg L^{-1} were calculated for Juvenile I and Juvenile II size classes, respectively. Values close to the concentrations of benzocaine indicated in this study have been reported for some species. Concentrations of 87.5 and 100 mg L^{-1} were recommended for common carp, *Cyprinus carpio*, weighing an average 1.9g (Bittencourt et al. 2012a), whereas concentrations ranging 110 – 160 mg L^{-1} were recommended for angelfish, *P. scalare*, weighing an average of 16g (Romaneli et al. 2018). Ideal concentrations of benzocaine for juvenile pacamã, *Lophiosilurus alexandri*, with an average weight of 214g, were found to range 60 – 120 mg L^{-1} (Ribeiro et al. 2019). This variation in the concentration of a given anesthetic is related to fish size and species (Ross and Ross 2008; Fernandes et al. 2016; Tarkhani et al. 2017), as observed in the present study.

High survival (> 90%) was observed for the Juvenile I size class 24 hours after the benzocaine experiment. In addition, the surviving fish resumed feeding during this period. The recorded mortality was not associated with anesthetic toxicity, but rather with fights (bites) observed among animals in experimental units. Survival was 100% for the Juvenile II size class, and all animals fed normally after 24 hours. Benzocaine is a safe synthetic

anesthetic for fish and can be rapidly metabolized (Gontijo et al. 2003; Okamoto et al. 2009; Okamura et al. 2010), which may explain the high survival rates observed after its use. Junior et al. (2019) evaluated different concentrations of benzocaine for juvenile curimba, *P. lineatus*, and observed a survival rate of > 90%, 96-hours after the end of the study. However, for juvenile pacamã, *L. alexandri*, benzocaine concentrations of 60, 120, 240 and 480 mg L^{-1} also did not cause mortality in fish 24 hours after the test (Ribeiro et al. 2019), demonstrating its efficiency for different species.

The present study demonstrated that the concentration of 25 mg L^{-1} of menthol promoted deep anesthesia for Juvenile I and Juvenile II size classes, but it took longer than 180 s. For the other concentrations evaluated, however, induction was reached within 180 s, and recovery times were less than 300 s, as suggested by Keene et al. (1998) and Ross and Ross (2008), with good anesthetic efficiency at concentrations of 122.25 mg L^{-1} and 93.83 mg L^{-1} for Juvenile I and Juvenile II, respectively. Hoshiba et al. (2015) evaluated the anesthetic effect of menthol for juveniles of the platy *Xiphophorus maculatus* weighing an average of 0.168g and found that concentrations between 100 and 250 mg L^{-1} had a safe effect. Similarly, menthol concentrations of 150 to 250 mg L^{-1} for the angelfish, *P. scalare*, weighing 16g (Romaneli et al. 2018) and of 50 to 125 mg L^{-1} for lambari, *Oligosarcus argenteus*, weighing an average of 11.3g (Uehara et al. 2019), were also found to be suitable for anesthesia management, which are close to the concentrations found in the present study.

In the menthol experiment, survival was 100% after 24 hours for both size classes of *A. nyassae* and all concentrations evaluated, with all animals resuming feeding. This high survival may be related to the product because it is natural and safe (Yadegarinia et al. 2006). Menthol concentrations of 50, 100, 150, 200 and 250 mg L^{-1} did not cause mortality for the platy *X. maculatus* (Hoshiba et al. 2015). Evaluating different concentrations of menthol for juvenile curimba, *P. lineatus*, Junior et al. (2018) observed high survival (> 90%) after 96 hours of anesthetic recovery with all animals resuming feeding.

The present study was not able to establish a direct relationship between increasing benzocaine and menthol concentrations and reduced VF for the Juvenile I and Juvenile II size classes of *A. nyassae*, as was also observed by Silva et al. (2019). Ventilation frequency is a useful parameter for understanding how fish physiology responds to anesthetics (Alvarenga and Volpato 1995; Becker et al. 2012). According to Toni et al. (2014), increased VF was observed for the catfish *Rhamdia quelen* after some minutes of exposure to essential oils of

Hesperozygis ringens and *Lippia alba*. This hyperventilation is common in fish and is associated with increased oxygen consumption during anesthesia (Summerfelt and Smith 1990). Generally, after initial contact with an anesthetic, VF values decrease considerably (Becker et al. 2012). In contrast, Becker et al. (2018) did not observe any differences in VF values for the catfish *R. quelen* exposed to essential oils of *L. alba* and *L. origanoides* compared to the control group. According to these authors, difficulty in establishing a general increase or decrease response of VF when using anesthetics can be explained by species-specific responses, which is also apparent from the present study.

5 Conclusion

Benzocaine and menthol proved to be effective anesthetics for juvenile *A. nyassae.* The recommended concentrations of benzocaine are between 75 to 125 mg L^{-1} for Juvenile I and between 50 to 125 mg L^{-1} for Juvenile II size classes. For menthol, concentrations between 50 and 125 mg L^{-1} can be used for both juvenile size classes.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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6.0 ARTIGO 2

Anesthesia with eugenol and menthol for *Piaractus brachypomus* (Cuvier, 1818): Induction and recovery times, ventilation frequency and hematological and biochemical responses

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Abstract

This work evaluated the use of eugenol and menthol as anesthesia for juvenile *Piaractus brachypomus*. Experiment I determined the times for anesthesia induction and recovery using different concentrations of eugenol and menthol (0, 25, 50, 75, 100 and 125 mg L^{-1}), as well as their effects on ventilation frequency (VF) for two size classes: Juveniles I - 0.87 ± 0.20 g and Juveniles II - 17.14 ± 3.27 g for eugenol and Juveniles I - 0.83 ± 21 g and Juveniles II - 16.83 ± 2.78 g for menthol. Based on data from Experiment I, Experiment II evaluated the effects of 0, 25 (beyond the recommended times for induction and recovery) and 50 mg L^{-1} (within the recommended times for induction and recovery) of eugenol and menthol (independent tests) on hematological and biochemical parameters of fish measured at two blood collection times (1 h post-induction and 24 h post-recovery). Sixty juveniles weighing 16.92 ± 3.34 g and 60 juveniles weighing 18.49 ± 3.07 g were used to evaluate eugenol and menthol, respectively. Survival was 100% at 24 h after the end of Experiment I. Eugenol concentrations between 50 and 100 mg L^{-1} were able to induce deep anesthesia for both juvenile size classes, while menthol was able to induce deep anesthesia at concentrations between 25 and 100 mg L^{-1} for Juveniles I and between 50 and 125 mg L^{-1} for Juveniles II. The use of 50 mg L^{-1} of eugenol was able to reduce VF during recovery and prevent an increase in plasma glucose while having little influence on hematological and biochemical parameters after handling. The use of 50 mg L^{-1} of menthol also reduced VF during recovery and did not cause changes in blood parameters that would be harmful to fish physiology.

Key words: Pirapitinga, Welfare, Handling, Essential oils

1. Introduction

Routine procedures performed in fish farms and handling for experimental sampling are necessary in aquaculture (Cunha et al., 2010; Oliveira et al., 2019; Ribeiro et al., 2019). These managements, if performed incorrectly, can damage animals (Gholipourkanani and Ahadizadeh, 2013; Bowker et al., 2019) and increase susceptibility to infectious diseases (Gimbo et al., 2008) and even cause death. Thus, anesthesia has been an effective management to minimize stress or physical damage during various aquaculture procedures (Velisek et al., 2011; Javahery et al., 2012; Teixeira et al., 2017; Bahi et al., 2018). Anesthetics are chemical or physical agents that first induce a calming effect in fish, which is followed by the loss of mobility, balance and consciousness and the occurrence of analgesia (Summerfelt and Smith, 1990; Ross and Ross, 2008).

Some essential oils extracted from plants have anesthetic and/or sedative effects on fish and, depending on the concentration, their chemical composition and chemotype have been shown to reduce biochemical, hematological and endocrine alterations, thus improving animal welfare status (Strange and Schreck, 1978; Souza et al., 2019a; Boaventura et al., 2020; Boaventura et al., 2021a). In this sense, eugenol is the most commonly used plant anesthetic in aquaculture (Vidal et al., 2008; Aydın and Barbas, 2020). Eugenol is one of the ingredients of clove oil, which is a distillate of parts of plants of the genus *Eugenia* (*Eugenia caryophyllat*a and *Eugenia aromatica*) (Lee and Shibamoto, 2001). Its anesthetic effect was demonstrated for *Piaractus brachypomus* with an average weight of 256 g (Martins et al., 2014), and for other species, such as Nile tilapia, *Oreochromis niloticus* (Simões et al., 2011; Ribeiro et al., 2015); pacu, *Piaractus mesopotamicus* (Rotili et al., 2012); and pacamã, *Lophiosilurus alexandri* (Ribeiro et al., 2013; Ribeiro et al., 2019). Another alternative agent that has been used in fish anesthesia is menthol, an essential oil extracted from plants of the genus *Mentha* (Matos, 2000; Patel et al., 2007). This compound demonstrated efficacy in the anesthesia of *P. brachypomus* with an average weight of 2 g (Zapata Guerra et al., 2020), and for other species, such as tambaqui, *Colossoma macropomum* (Façanha and Gomes, 2005); pacu, *P. mesopotamicus* (Gonçalves et al., 2008); Nile tilapia, *O. niloticus* (Teixeira et al., 2011); curimba, *Prochilodus lineatus* (Medeiros et al., 2018); and lambari-bocarra, *Oligosarcus argenteus* (Uehara et al., 2019).

However, the ideal concentration of a given anesthetic can vary according to species and animal size (Ross and Ross, 2008; Teixeira et al., 2011; Ribeiro et al., 2013; Ribeiro et al., 2015; Tarkhani et al., 2017). In addition, anesthetic efficiency for fish is associated with a short anesthesia induction time (<180 seconds) and a fast recovery time (<300 seconds), as suggested by Keene et al. (1998) and Ross and Ross (2008). Thus, it is necessary to validate an anesthetic before its use (King, 2009).

Pirapitinga, *P. brachypomus*, is a freshwater species belonging to the order Characiformes, that is generally found naturally in the Orinoco, Amazonas and La Plata (Paraná-Paraguai) basins (Schultz, 1944). The species has commercial importance with prominence in Central America, Brazil, Colombia, Peru and Venezuela (Nascimento et al., 2010; Jorge et al., 2018). However, there is a lack of published studies on the effects of eugenol and menthol for this species, including on hematological and biochemical responses after handling stress.

Thus, the objective of this work was to evaluate different concentrations of eugenol and menthol for induction and recovery of two size classes of juvenile *P. brachypomus*, as well as their effects on ventilation frequency and hematological and biochemical parameters.

2. Material and methods

The experiments were performed at Laboratório de Aquacultura (Laqua) of the Escola de Veterinária (EV), Universidade Federal de Minas Gerais (UFMG), and the methodology was approved by the Comitê de Ética no Uso de Animais, Process 159/2020.

2.1 Animals and environmental acclimation

Juvenile *P. brachypomus* were previously acclimatized for two weeks in water basins with a useful volume of 32 L, in a water recirculation system (RAS) at a density of 10 fish basin^{-1}. Smaller juveniles were fed commercial mash containing 460.0 g kg^{-1} crude protein, 80 g kg^{-1} ether extract, 140 g kg^{-1} mineral matter, 20 g kg^{-1} calcium, and 15 g kg^{-1} phosphorus, as reported by the manufacturer, two times a day (09:00 and 15:00 h) until apparent satiety. Larger juveniles were fed 2.6-mm diameter commercial feed containing 360.0 g kg^{-1} crude protein, 70 g kg^{-1} ether extract, 140 g kg^{-1} mineral matter, 10 g kg^{-1} calcium and 6 g kg^{-1} phosphorus, twice a day (09:00 and 15:00 h) until apparent satiety. The water of the RAS was renewed twice a week at 30% of its useful volume and water quality parameters

were measured three times a week, before the first feeding, and their values expressed as mean ± standard deviation. For the culture system of younger juveniles, the temperature remained at 26.93 ± 0.85 ºC, pH between 7.38 ± 0.25 (measured with a Hanna HI98130 multi-parameter probe), dissolved oxygen at 8.56 ± 0.43 mg L^{-1}, (EcoSense® DO200A oximeter), and total ammonia at 0.11 ± 0.06 mg L^{-1} (Alfakit Labcon Test colorimetric kit). As for the culture system of the larger juveniles, the temperature remained around 26.41 ± 0.97 ºC, pH between 7.57 ± 0.24, dissolved oxygen at 10.51 ± 0.34 mg L^{-1} and total ammonia at 0.17 ± 0.09 mg L^{-1}. The fish were fasted 24 h before the tests. The anesthetics used in the experiments were eugenol (99% purity, Escama Forte®) and menthol (99% purity, CRQ-1006310100). Anesthetic concentrations were prepared by dilution in 5 mL ethyl alcohol (98.1% purity), and the control consisted of ethyl alcohol without anesthetic (Ribeiro et al., 2015).

Experiment 1. Anesthetic effects of eugenol and menthol on juvenile *Piaractus brachypomus*

Two juvenile sizes classes were used in the eugenol experiment: Juveniles I - 60 fish weighing 0.87 ± 0.20 g (36.48 ± 2.82 mm); and Juveniles II - 60 fish weighing 17.14 ± 3.27 g (97.93 ± 6.77 mm). Two juvenile size classes were also used in the menthol experiment: Juveniles I - 60 fish weighing 0.83 ± 0.21 g (36.61 ± 3.39 mm); and Juveniles II - 60 fish weighing 16.83 ± 2.78 g (98.74 ± 5.76 mm). Six concentrations of each anesthetic (eugenol and menthol) were evaluated for each size class — 0 (control - ethyl alcohol only), 25, 50, 75, 100 and 125 mg L^{-1}. The experiments were conducted independently for each size class and anesthetic, using a completely randomized design with 10 animals for each concentration, with each animal being considered a replicate.

Most anesthesia protocols in the literature have shown that deep anesthesia and sedation is suitable for the handling and transport of fish (Souza et al., 2019; Aydin and Barbas, 2020). Thus, for analysis of anesthesia induction and recovery, fish were fasted and then randomly captured one at a time and placed in a 1-L beaker containing the anesthetic and concentration to be evaluated. Fish in the control group were observed for 10 min (Ferreira et al., 2020). Anesthesia induction time was measured with a digital timer (Akson AK68) and the animals were considered anesthetized when they reached the stage of deep anesthesia,

which was basically characterized by the absence of swimming and loss of balance and consciousness (Small, 2003; Ross and Ross, 2008). The effect of analgesia was not evaluated in this study. The ventilation frequency (VF) of the fish was evaluated from the beginning of induction anesthesia by counting the number of opercular beats per minute (adapted from Alvarenga and Volpato 1995). After reaching deep anesthesia, the fish were weighed on an analytical balance (Marte AD5002) and measured with a digital caliper (Starret® 799), a procedure (biometric handling) that took about 40 seconds. The fish were then transferred individually to a 1-L beaker with clean water (from the RAS itself) for anesthetic recovery, during which VF was also evaluated. Fish were considered recovered when they showed movements and normal swimming balance (Small, 2003; Ross and Ross, 2008).

At the end of the experiment, the fish of each size class and anesthetic were allocated to their original experimental units to observe their return to feeding and survival after 48 h. During this period the animals were fed twice a day until apparent satiety.

Experiment 2. Hematological and biochemical responses after handling of juvenile *Piaractus brachypomus* anesthetized with eugenol and menthol

Based on the results of Experiment 1 for the larger juveniles (Juveniles II size classes), new tests were performed using the concentrations of 0 (control ethyl alcohol only), 25 and 50 mg L^{-1} of eugenol and menthol. The concentrations of 25 and 50 mg L^{-1} were chosen because they were beyond and within, respectively, the induction and recovery times considered ideal for fish (Keene et al., 1998; Ross and Ross, 2008). In this experiment, 60 juveniles with an average weight of 16.92 ± 3.34 g (99.98 ± 7.52 mm) and 60 juveniles with an average weight of 18.49 ± 3.07 g and (101.50 ± 5.81 mm) were used for the independent tests with eugenol and menthol, respectively. All fish were fasted for 24 h and tests were performed independently for each anesthetic (eugenol and menthol) using a completely randomized design with each fish being considered a replicate. The procedures were the same as those described for Experiment 1. However, in this experiment blood samples were collected from 10 fish for each concentration and anesthetic at 1 h post-induction and 24 h post-recovery, using a completely randomized design in a factorial arrangement (3 x 2). For blood collection, the fish were captured and restrained with a damp cloth while approximately 500 μL of blood was collected by caudal puncture using heparinized syringes.

Blood aliquots were used to measure hematocrit (%) and hemoglobin (g dL^{-1}). Hematocrit was measured by centrifuging capillary tubes at 10,000-rpm (Microline-Laborline®) for 10 minutes, as described by Goldenfarb et al. (1971). Hemoglobin was analyzed by adding and homogenizing 10 μL of blood in 2 mL of working reagent (Bioclin®), followed by reading on a UV/Vis spectrophotometer (Biochrom Libra S21 – S22). Biochemical analyses were performed by centrifuging blood at 4,000 rpm for 10 min. Plasma protein (g dL^{-1}) was determined using a Goldberg manual refractometer, while plasma glucose (mg dL^{-1}), triglyceride (mg dL^{-1}) and cholesterol (mg dL^{-1}) were determined using commercial kits (Bioclin®), followed by reading on a UV/Vis spectrophotometer (Biochrom Libra S22).

2.2 Statistical analysis

Data were tested for normality (Shapiro Wilk) and homoscedasticity of variances (Levene Test). Anesthesia induction and recovery times were submitted to ANOVA followed by applying regression analyses to better adjust the model (P <0.05). Blood variables were submitted to two-way ANOVA followed by Tukey's post-test (P <0.05). Data analyses were performed with R and SAS software.

3. Results

Experiment I

Eugenol

Induction time for the Juveniles I size class showed a linear response plateau effect (P <0.05) among eugenol concentrations (Fig. 1A), with an inflection point at 72.90 mg L^{-1}, beyond which induction time was constant. Recovery time for Juveniles I showed a direct linear effect (P <0.05) among concentrations (Fig. 1B) and ranged 155-368 seconds.

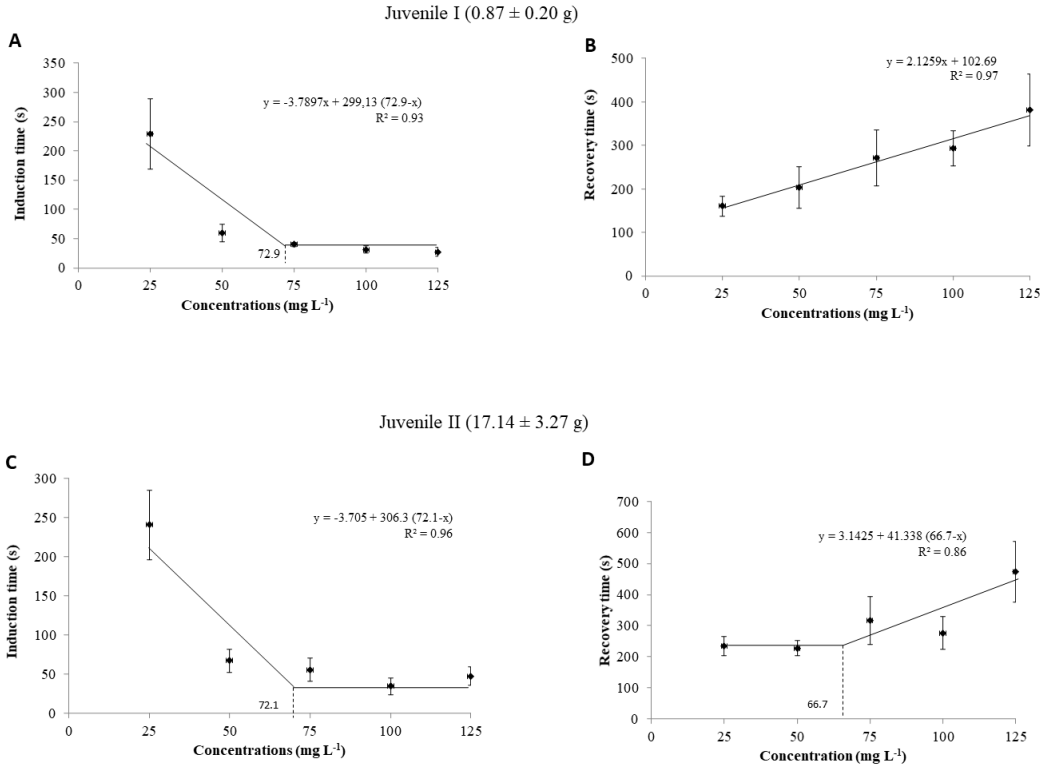

Figure 1. Time required for anesthesia induction (A, C) and recovery (B, D) for juvenile *Piaractus brachypomus* using different concentrations eugenol.

Induction time for Juveniles II showed a linear response plateau effect (P <0.05) among eugenol concentrations (Fig. 1C), with an inflection point at 72.10 mg L^{-1}, beyond which induction time was constant. The recovery time for Juveniles II also showed a linear response plateau effect (P <0.05) among eugenol concentrations (Fig. 1D), with an inflection point at 66.70 mg L^{-1}.

Eugenol concentrations had no effect on VF during induction of Juveniles I (P> 0.05) (Table 1). During recovery of Juveniles I, VF exhibited a quadratic effect among the evaluated concentrations (P <0.05) with a maximum at 70.95 mg L^{-1} (56.63 beats min^{-1}). Eugenol concentrations had no effect on VF during induction of Juveniles II (P> 0.05) (Table 1). During recovery of Juveniles II, VF exhibited a quadratic effect among the evaluated concentrations (P <0.05) with a maximum at 65.02 mg L^{-1} (80.93 beats min^{-1}).

Table 1. Values (mean ± standard deviation) for ventilation frequency during anesthesia using different concentrations of eugenol and two size classes of *Piaractus brachypomus*

	Juvenile I (0.87 ± 0.20 g)	
Concentration of Eugenol (mg L^{-1})	Induction ventilation frequency (beats min^{-1})	Recovery ventilation frequency (beats min^{-1})*
25	77.89 ± 14.54	51.81 ± 13.48
50	90.48 ± 19.07	49.10 ± 13.20
75	84.39 ± 20.53	55.16 ± 8.63
100	96.17 ± 24.91	61.39 ± 11.88
125	95.70 ± 30.08	42.02 ± 12.16
Equation	ns	$y = -0.0038x^2 + 0.5392x + 37.502$
R^2	-	0.4063
Inflection point	-	70.95
	Juvenile II (17.14 ± 3.27 g)	
Concentration of Eugenol (mg L^{-1})	Induction ventilation frequency (beats min^{-1})	Recovery ventilation frequency (beats min^{-1})*
25	74.37 ± 10.49	67.70 ± 6.59
50	85.38 ± 17.61	74.13 ± 15.59
75	89.06 ± 16.41	68.44 ± 11.23
100	83.95 ± 18.95	67.57 ± 13.43
125	87.98 ± 18.17	48.16 ± 17.35
Equation	ns	$y = -0.0092x^2 + 1.1964x + 42.037$
R^2	-	0.9314
Inflection point	-	65.02

ns: no significant. *Significance denoted as ($P < 0.05$).

Survival for both classes of juveniles was 100% for all eugenol concentrations after 48 h of recovery, and all animals resumed feeding within 24 h after testing.

Menthol

Induction time for the Juveniles I size class showed a linear response plateau effect (P <0.05) among concentrations (Fig. 2A), with an inflection point at 72.7 mg L^{-1}, beyond which induction time was constant. Recovery time for Juveniles I showed a quadratic effect among concentrations (P <0.05) (Fig. 2B) with a minimum at 41 mg L^{-1} (122 seconds).

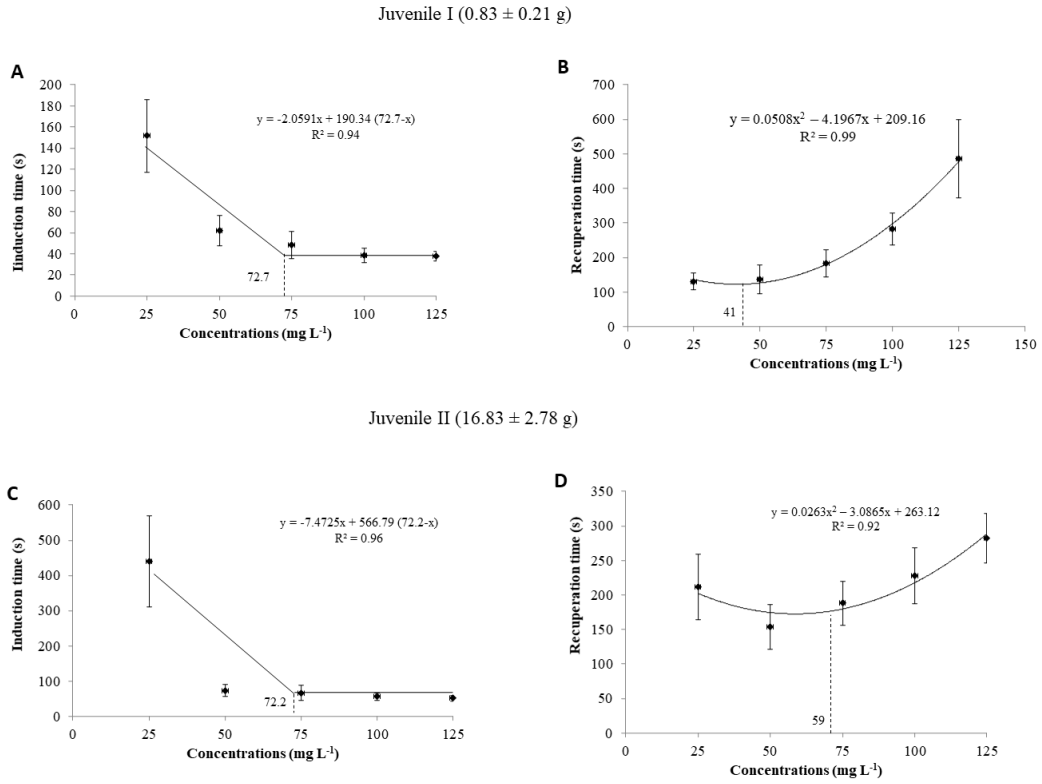

Figure 2. Time required for anesthesia induction (A, C) and recovery (B, D) for juvenile *Piaractus brachypomus* using different concentrations menthol.

Induction time for Juveniles II showed a linear response plateau effect (P <0.05) among menthol concentrations (Fig. 2C), with an inflection point at 72.2 mg L^{-1}. Recovery

time for Juveniles II showed a quadratic effect among concentrations (P <0.05) (Fig. 2D) with a minimum at 59 mg L^{-1} of menthol (172 seconds).

Menthol concentrations had no effect on VF during induction of Juveniles I (P> 0.05) (Table 2). During recovery of Juveniles I, VF exhibited a quadratic effect among the evaluated concentrations (P <0.05) with a maximum at 71.23 mg L^{-1} (64.80 beats min^{-1}). Menthol concentrations had no effect on VF during induction of Juveniles II (P> 0.05) (Table 2). During recovery of Juveniles II, VF exhibited a quadratic effect among the evaluated concentrations (P <0.05) with a maximum at 59.86 mg L^{-1} (66.46 beats min^{-1}).

Table 2. Values (mean ± standard deviation) for ventilation frequency during anesthesia using different concentrations of methanol and two size classes of *Piaractus brachypomus.*

	Juvenile I (0.83 ± 0.21 g)	
Concentration of Menthol (mg L^{-1})	Induction ventilation frequency (beats min^{-1})	Recovery ventilation frequency (beats min^{-1})*
25	71.60 ± 13.35	63.20 ± 13.17
50	93.20 ± 18.02	64.78 ± 11.77
75	83.80 ± 18.95	68.20 ± 10.48
100	99.13 ± 20.07	61.50 ± 13.83
125	97.78 ± 21.63	46.40 ± 9.85
Equation	ns	$y = -0.0037x^2 + 0.5271x + 46.032$
R^2	-	0.7536
Inflection point	-	71.23
	Juvenile II (16.83 ± 2.78 g)	
Concentration of Menthol (mg L^{-1})	Induction ventilation frequency (beats min^{-1})	Recovery ventilation frequency (beats min^{-1})*
25	57.49 ± 12.71	55.24 ± 10.22
50	76.49 ± 16.63	66.69 ± 9.35
75	74.73 ± 20.68	64.32 ± 14.00
100	84.30 ± 18.29	62.34 ± 10.45
125	81.83 ± 24.07	59.39 ± 10.43
Equation	ns	$y = -0.0046x^2 + 0.5507x + 49.976$
R^2	-	0.8276
Inflection point	-	59.86

ns: no significant. *Significance denoted as ($P < 0.05$).

Survival for both classes of juveniles was 100% for all menthol concentrations after 48 h of recovery, and all animals resumed feeding within 24 h after testing.

Experiment II

Hematology and blood biochemistry

Eugenol

There was no interaction between eugenol concentration and blood collection time ($P > 0.05$) (Table 3) for all evaluated variables. There were also no differences among concentrations and between collection times for hemoglobin, protein and cholesterol ($P > 0.05$). Hematocrit values did not differ significantly ($P > 0.05$) among eugenol concentrations. However, higher hematocrit values were observed 1 h post-anesthesia, which reduced after 24 h of recovery ($P < 0.05$). Glucose was highest for juveniles submitted to 0 mg L^{-1} of eugenol (control) ($P < 0.05$) and lowest for 50 mg L^{-1}. Glucose levels did not differ significantly between collection times ($P > 0.05$). Fish anesthetized with 50 mg L^{-1} of eugenol had lower triglyceride values while higher values were observed for 25 mg L^{-1} ($P < 0.05$). Triglyceride levels did not differ significantly between collection times ($P > 0.05$).

Table 3. Values (mean ± standard deviation) of hematological and biochemistry parameters at two blood collection times for *Piaractus brachypomus* submitted to different concentrations of eugenol.

		Hemoglobin (g dL^{-1})	Hematocrit (%)	Protein (g dL^{-1})	Glucose (mg dL^{-1})	Triglycerides (mg dL^{-1})	Cholesterol (mg dL^{-1})
Concentrations (mg L^{-1})	0 (Control)	6.59 ± 0.80	11.38 ± 1.69	6.30± 1.43	80.40 ± 7.07 a	126.02 ± 23.72 ab	129.24 ± 25.54
	25	6.57 ± 0.78	11.39 ± 5.18	6.26 ± 0.85	76.79 ± 8.59 ab	139.18 ± 24.85 a	120.5 ± 12.65
	50	6.67 ± 0.67	9.75 ± 2.25	5.63 ± 0.38	70.64 ± 8.59 b	111.69 ± 20.52 b	120.49 ± 17.98
Collection time (h)	1	6.81± 0.73	12.5 ± 3.85 a	5.73 ± 0.29	77.32 ± 9.68	128.14 ± 30.45	127.63 ± 20.95
	24	6.40 ± 0.70	9.17 ± 1.59 b	6.39 ± 1.32	74.56 ± 8.03	123.12 ± 19.03	119.20 ± 17.16
Overall average		6.61± 0.75	10.84 ± 3.04	6.06 ± 0.81	75.94 ± 8.85	125.63 ± 24.74	123.42 ± 29.53
P-value Concentrations (A)		0.94	0.42	0.22	0.02	0.01	0.46
P-value Collection time (B)		0.11	0.009	0.07	0.32	0.50	0.21
P-value Interaction (A x B)		0.98	0.12	0.06	0.60	0.09	0.90
CV (%)		11.45	25.74	14.00	10.84	17.73	16.05

Means followed by different letters in a column differ by Tukey test (P <0.05); CV = Coefficient of variation.

Menthol

There was no interaction between menthol concentration and blood collection time ($P > 0.05$) (Table 4) for all evaluated variables. There were also no differences among concentrations and between collection times for hemoglobin and triglycerides ($P > 0.05$). However, fish submitted to 25 mg L^{-1} of menthol had higher hematocrit values than did those submitted to 0 and 50 mg L^{-1} ($P < 0.05$), but with no difference between collection times ($P > 0.05$). Plasma protein and cholesterol levels did not differ significantly ($P > 0.05$) among menthol concentrations. However, lower values for these variables were observed at 1 h post-anesthesia, which increased after 24 h ($P < 0.05$). Plasma glucose levels were higher 1 h post-anesthesia and reduced after 24 h ($P < 0.05$).

Table 4. Values (mean ± standard deviation) of hematological and biochemistry parameters at two blood collection times for *Piaractus brachypomus* submitted to different concentrations of menthol.

		Hemoglobin ($g\ dL^{-1}$)	Hematocrit (%)	Protein ($g\ dL^{-1}$)	Glucose ($mg\ dL^{-1}$)	Triglycerides ($mg\ dL^{-1}$)	Cholesterol ($mg\ dL^{-1}$)
Concentrations							
($mg\ L^{-1}$)	0 (Control)	7.27 ± 1.01	10.13 ± 1.81 b	5.70 ± 0.81	75.36 ± 7.11	121.97 ± 31.59	125.20 ± 17.96
	25	7.43 ± 0.90	15.25 ± 4.41 a	5.60 ± 0.38	78.11 ± 8.15	109.99 ± 18.61	123.48 ± 11.12
	50	7.16 ± 0.57	10.00 ± 3.96 b	5.51 ± 0.30	74.74 ± 8.75	129.54 ± 15.97	128.22 ± 15.86
Collection time (h)	1	7.22 ± 0.69	12.83 ± 3.42	5.23 ± 0.29 b	81.20 ± 6.83 a	120.64 ± 26.20	120.61 ± 13.26 b
	24	7.35 ± 0.97	10.75 ± 4.58	5.98 ± 0.43 a	70.94 ± 5.24 b	120.36 ± 22.03	130.67 ± 15.17 a
Overall average		7.28 ± 0.83	11.79 ± 4.00	5.61± 0.36	76.07 ± 6.02	120.50 ± 24.12	125.64 ± 14.22
P-value Concentrations (A)		0.75	0.0078	0.56	0.38	0.12	0.70
P-value Collection time (B)		0.64	0.14	<0.0001	<0.0001	0.97	0.03
P-value Interaction (A x B)		0.77	0.71	0.09	0.84	0.19	0.09
CV (%)		12.08	28.32	6.18	8.20	19.04	11.04

Means followed by different letters in a column differ by Tukey test (P <0.05); CV = Coefficient of variation.

4. Discussion

Experiment I found the use of eugenol and menthol to be efficient and safe for the anesthesia of juvenile *P. brachypomus* of different sizes, with induction and recovery times within acceptable limits for fish and minimal effects on hematological and biochemical parameters. These substances were also shown to be effective anesthetics (for loss consciousness) for individuals of this same species weighing 2 g (Zapata Guerra et al., 2020) and 256 g (Martins et al., 2014), which demonstrates good effects of these drugs for this species.

As anesthetics, these substances require dilution in ethanol; nonetheless, in low concentrations this solvent has no inducing effect and does not cause mortality in fish (Ribeiro et al., 2013; Teta and Kaiser, 2019; Ferreira et al., 2020), as also observed for fish in the control group of the present study. The survival of juvenile *P. brachypomus* at 48 post-anesthesia was 100% for both anesthetics and all tested concentrations and all animals resumed feeding. Working with *P. brachypomus* with an average weight of 256 g, Martins et al. (2014) also recorded 100% survival after anesthesia using eugenol in concentrations from 50 to 75 mg L^{-1}. Eugenol also did not cause mortality when evaluated for *Oreochromis niloticus* (Ribeiro et al., 2015), *Brycon amazonicus* (Barbosa et al., 2007) and *L. alexandri* (Ribeiro et al., 2019), demonstrating efficiency for different species. However, high concentrations of eugenol, such as 250 mg L^{-1}, can cause necrosis in the gills of fish (Abdel-Fattah et al., 2005). Zapata Guerra et al. (2020) found that menthol (50 mg L^{-1}) was able to prevent the activation of the stress response in *P. brachypomus*, when compared to eugenol, and without mortality. Menthol has also shown anesthetic efficacy for other species, such as *Prochilodus lineatus* (Medeiros et al., 2018), *Poecilia reticulata* (Cunha et al., 2020) and *Aulonocara nyassae* (Ferreira et al., 2020). Eugenol and menthol also did not cause mortality when used as anesthetics for *P. mesopotamicus* (Gonçalves et al., 2008) and *Oligosarcus argenteus* (Uehara et al., 2019). However, continuous periods of exposure (> 6 min) to high concentrations of menthol above 100 mg L^{-1} can alter receptors and, consequently, cause mortality in fish (Pereira da Silva et al., 2014). In general, the use of natural plant-based anesthetics has shown benefits in mitigating the effects of stress caused by the handling and transport of fish (Souza et al., 2019; Boaventura et al., 2020; Boaventura et al., 2021a).

However, anesthetic composition and, consequently, its effects may vary according to the variety of the plant and the climate, place of collection, plant part from which the EO was extracted and plant chemotype (Souza et al., 2019).

According to Marking and Meyer (1985) and Keene et al. (1998), an ideal anesthetic for fish should induce anesthesia within 180 seconds and promote recovery within 300 seconds. Based on these recommendations, the use of eugenol in the present study, for both size classes of juvenile *P. brachypomus*, indicates concentrations between 50 and 100 mg L^{-1}. Despite the size difference, these concentrations are close to those found for *P. brachypomus* weighing 256 g (50 to 75 mg L^{-1}) (Martins et al., 2014). Variation found in the ideal eugenol concentration for *L. alexandri* weighing 171 g (80 to 160 mg L^{-1}) (Ribeiro et al., 2019), *O. niloticus* weighing 5 g (75 mg L^{-1}) (Vidal et al., 2008) and *O. argenteus* weighing 11 g (50 to 75 mg L^{-1}) (Uehara et al., 2019) emphasizes the need for assessing each species at different stages of development. For the use of menthol, the concentrations indicated for the Juveniles I size class were between 25 and 100 mg L^{-1}, while for Juveniles II they were between 50 and 125 mg L^{-1}. Despite the size difference, these concentrations were close to that found for *P. brachypomus* weighing 2 g (50 mg L^{-1}) (Zapata Guerra et al., 2020), and for other species such as *P. mesopotamicus* weighing 110 g (100 mg L^{-1}) (Gonçalves et al., 2008), *A. nyassae* weighing 1 g (50 to 125 mg L^{-1}) (Ferreira et al., 2020) and *O. argenteus* weighing 11 g (50 to 100 mg L^{-1}) (Uehara et al., 2019).

Measuring VF is a non-evasive method that can be useful in understanding the physiology of fish when using anesthetics (Alvarenga and Volpato, 1995). The different concentrations of the evaluated anesthetics (eugenol and menthol) had no significant effects on VF during induction of juvenile *P. brachypomus*. During recovery, however, the concentration of 50 mg L^{-1} for both anesthetics caused a reduction in VF for the different classes of juvenile *P. brachypomus*, which corresponds to the minimum concentration that should be recommended for this species. Thus, it was possible to immobilize the animal in a safe and quiet manner (for the fish and the manipulator) for biometric handling. Hajek (2010) did not observe differences in ventilation disturbances for the common carp, *Cyprinus carpio* L., during different periods of anesthesia with tea tree oil (0.5 mL L^{-1}), however, weak, irregular ventilatory movements were observed during the first minutes of recovery, after which animals regained a continuous breathing pattern with increasing ventilation frequency.

Some researchers describe the behavior of VF responses of fishes with the use of anesthetics as potentially being related to the specific characteristics of each species. According to Silva et al. (2019), juvenile *C. macropomum* in deep anesthesia with the use of essential oil of *Lippia alba* EOLA-C (chemotype citral) presented the lowest VF at 50 µL L^{-1}, followed by 100 to 200 µL L^{-1}. However, sedation with essential oil of *L. alba* EOLA-L (chemotype linalool) did not change the VF of fish. Furthermore, we recently observed in another study of ours that the VF of the African cichlid *A. nyassae* in deep anesthesia showed different behavioral responses according to anesthetic (benzocaine and menthol) and animal size (1 and 4 g) (Ferreira et al., 2020), which helps to understand the results of the present study.

Experiment II found that the use of eugenol led to an increase in hematocrit for *P. brachypomus* 1 h post-induction. Menthol, on the other hand, led to lower hematocrit values for fish anesthetized with 50 mg L^{-1} and the control group. These findings may be associated with acute or induced stress (McDonald and Milligan, 1997), hypoxia caused by anesthesia (Hill and Forster, 2004) and/or or hemoconcentration or hemodilution caused by osmoregulatory mechanisms (Houston et al., 1996). The anesthetic or sedative effects of eugenol and menthol also caused different changes to hematocrit values for other species (Navarro et al., 2016; Ribeiro et al., 2019), but returned to normal 24 h after handling in the present study.

The use of eugenol did not cause changes in hemoglobin values, which was also observed when menthol was used during handling of the same species. This finding was also observed during eugenol anesthesia of adult *P. brachypomus* (Sladky et al., 2001), as well as for other species (Velisek et al., 2005; Pádua et al., 2012).

The highest plasma glucose values observed at 1 h post-anesthesia with eugenol were for the fish of the control group. Fish in stressful situations release catecholamines and corticosteroids that activate the processes of gluconeogenesis and glycogenolysis, which mobilize and increase glucose in the bloodstream for the body to escape or adjust to the new physiological conditions imposed by the environment (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Iwama et al., 2004; Pankhurst, 2011). However, hyperglycemia was also observed at 1 h post-induction for fish anesthetized with 25 mg L^{-1} of eugenol and may be related to increased agitation and hypoxia of the fish. However, glucose values tend to return to normal levels (Teixeira et al., 2017; Boaventura et al., 2020), as observed after 24 h of

recovery in the present study. The 50 mg L^{-1} concentration of eugenol was able to reduce the effects of handling stress. As for menthol, plasma glucose showed lower values after 24 h of recovery. This finding can be understood as the result of triggering glycogenolysis and increasing plasma glucose during the first management with fish. It could also be explained as the result of the fasting time to which the fish were submitted, which may have reduced glycogen stock and caused glycemia, as also observed by Ribeiro et al. (2019).

The lowest concentration of triglycerides was at 1 h post-induction for fish anesthetized with 50 mg L^{-1} of eugenol. This finding can be attributed to a change in the mobilization and circulation of lipids between the liver and other tissues related to the synthesis of cholesterol and phospholipids (Jun et al., 2015). However, these results are similar to those for the control group (unanesthetized fish), which helps to explain why blood cholesterol levels did not differ among the different concentrations and the assessed times. Thus, it can be inferred that the management of biometrics associated with anesthesia with eugenol had no effect on the metabolism of energy, carbohydrates and lipids, indicating that responses to stress were prevented, attenuated or unmodified in the juvenile *P. brachypomus* weighing approximately 17 g.

Plasma protein values were higher at 24 h of recovery from anesthesia with menthol. The main fractions of these proteins are albumin and globulins. In addition, the release of the hormone cortisol may increase albumin (Cunha et al., 2000). Therefore, the possible increase of this hormone may have caused changes in plasma protein concentrations since, according to Mommsen et al. (1999), glucorticoids, such as cortisol, stimulate gluconeogenesis (increases blood glucose) and the catabolic activity of proteins.

An increase in cholesterol was observed at 24 h post-recovery from anesthesia with menthol. This compound is a lipid steroid found in cell membranes of animals, is transported by blood plasma and acts as a precursor in the synthesis of cortisol (Saccol et al., 2017). As observed by Bolasina (2006), increased cholesterol, together with high levels of cortisol, in the Brazilian codling *Urophycis brasiliensis* exposed to benzocaine could be associated with an increase in cortisol synthesis. Thus, the management of biometrics with the use of menthol had minimal effects on the hemato-biochemical parameters of juvenile *P. brachypomus* weighing approximately 19 g.

5. Conclusions

Eugenol concentrations between 50 and 100 mg L^{-1} can be used for both of the studied size classes of juvenile *P. brachypomus*, and menthol concentrations between 25 and 100 mg L^{-1} for the Juveniles I size class and between 50 and 125 mg L^{-1} for the Juvenile II size class, to cause anesthesia within induction (< 180 s) and recovery (< 300 s) time intervals considered ideal for fish. The use of 50 mg L^{-1} eugenol prevented higher levels of plasma glucose and with minimal influences on other biochemical and hematological variables after handling. Furthermore, the use of 50 mg L^{-1} of menthol did not cause changes in blood parameters that would be harmful to the physiology of the fish. Additional studies analyzing the analgesic effect of these anesthetics will allow a better understanding of their efficiency for *P. brachypomus.*

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7. ARTIGO 3

Chitosan-coated zein nanopoarticles containing eugenol potentiates anesthesia in Nile tilapia

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Abstract

This study aimed to investigate the physical and chemical properties of mucoadhesive zein nanoparticles containing eugenol (NPZMA) and to evaluate their anesthetic effect in immersion baths, their stability in water and their effects on hemogasometric parameters of juvenile *Oreochromis niloticus.* Four experiments were performed: Experiment 1 characterized the nanoparticles. Experiment 2 evaluated the stability of NPZMA in water using an *in vitro* test with the following treatments: eugenol-80 mg L^{-1}; NPZMA–80 mg L^{-1}; NPZMA–40 mg L^{-1}, NPZMA–20 mg L^{-1}, water *in natura* and control (ethyl alcohol was added to the water at a proportion of 1:10 V/V). Experiment 3 tested the same treatments for time of anesthesia induction and recovery. Experiment 4 tested basal (group of non-anesthetized fish), eugenol-80 mg L^{-1} and NPZMA-40 mg L^{-1} for hemogasometric variables after two minutes of exposure. The new method for applying eugenol through mucoadhesive zein nanoparticles presented here exhibited positive loads and successful adherence to fish mucus. The method led to changes in water quality during the 60-minute observation period, although it remained within the ideal range for *O. niloticus*. Experiment 3 revealed similar induction times for eugenol-80 mg L^{-1}, NPZMA-80 mg L^{-1} and NPZMA-40 mg L^{-1} ($P>0.05$). Recovery time was shortest for NPZMA-20 mg L^{-1} and longest for NPZMA-80 mg L^{-1} ($P<0.05$). Experiment 4 found the concentrations tested to have no effects on hemogasometric variables ($P>0.05$). The results demonstrate that mucoadhesive nanoparticles reduced the use of eugenol by 50 %, as observed for NPZMA-40 mg L^{-1}. Furthermore, this new form of eugenol application based on mucoadhesive zein nanoparticles showed good stability in water and did not alter hemogasometric parameters of juvenile *O. niloticus*.

Key words: Clove oil, Handling stress, Hemogasometric, Induction and recovery, *Oreochromis niloticus*

1. Introduction

Several procedures adopted in fish farming require anesthesia, such as vaccination, collection of biological material, tag implantation, classification and reproduction (Ross and Ross, 2008; Romaneli et al., 2018; Durhack et al., 2020). The use of anesthetics in fish farming provides benefits to both fish and handlers due to the immobilization of the animal, and thus can reduce possible suffering of fish (Waterstrat and Pinkham, 2005; Javahery et al., 2012; Ribeiro et al., 2015). Commercially available chemicals have been used for such anesthetic procedures; however, these products are often administered in incorrect concentrations, resulting in animal toxicity and product waste (Roubach and Gomes, 2001). The use of plant extracts as pharmacological alternatives offers positive effects such as lower cost, faster degradation in the environment and lower levels of residues (Inoue et al., 2003). Several studies based on plant extracts as sustainable alternatives for fish farming have been carried out in recent years, such as with essential oil of *Aniba rosaeodora* and *Aniba parviflora* (Baldisserotto et al., 2018), of *Aloysia triphylla* (Becker et al., 2017) and of *Ocimum gratissimum* (Boijink et al., 2016), among others. Special attention has been given to the use of clove extract, which contains eugenol as an active ingredient, as an anesthesia for fish (Cunha et al., 2010; Mirghaed et al., 2018; Ribeiro et al., 2013, Ribeiro et al., 2019). Clove oil is obtained from the distillation of the plant *Eugenia caryophyllata*, which represents about 95 % of all the clove essential oil in India (Hoseini et al., 2015) and is assured in the list of materials listed by the US Food and Drug Administration (FDA) (Ross and Ross, 2008). Despite its effectiveness, its use is still limited due to low bioavailability; eugenol has low water solubility, which results in poor and erratic absorption after administration (Yostawonkul et al., 2019).

Anesthetics can be applied in different ways. The use of injectable anesthetics in fish farming is effective but requires qualified labor, making it an onerous method (Ross and Ross, 2008). With a bath/immersion anesthesia system, the most adopted type of system, drug absorption occurs through the gills, skin and intestine (Rombout et al., 2014). However, the biggest obstacle of these two methods of anesthesia is the form of exposure to the drug and is absorption, which varies with concentration, and thus can lead to decreased bioavailability of the drug (Charlie-Silva et al., 2018). A recent study with nanoparticles containing clove oil

revealed potent anesthetic and antibacterial effects for Nile tilapia, *Oreochromis niloticus* (Yostawonkul et al., 2019).

The administration of drugs using nanoparticles involves the optimization and bioavailability of drugs, in addition to reducing their toxic effects (Irache and González-Navarro, 2017; Wang et al., 2017), relative to conventional formulations (Torchilin, 2014). Zein nanoparticles coated with chitosan are mucoadhesive and thus have the ability to adhere to the external mucosa by interacting with the fish's mucus through its different through its different loads (Bernkop-Schnürch et al., 2004; Jiménez-Fernández et al., 2014; El-Naby et al. 2019). This property increases drug permeability due to reorganization of mucosal intercellular junctions and interference with lipid deposition (Sosnik and Raskin, 2015), which in turn improves the solubility of drugs that are poorly soluble in water (Tang et al., 2011) and modulates their release slowly.

Thus, the objective of the present work was to investigate the physical and chemical properties of zein nanoparticles coated with chitosan containing eugenol, as well as to evaluate their anesthetic effect in immersion baths, their stability in water and their effects on hemogasometric parameters of juvenile Nile tilapia, *O. niloticus*.

2. Materials and Methods

Animals and environmental acclimatization

The experiments were conducted at Laboratório de Aquacultura of Escola de Veterinária, Universidade Federal de Minas Gerais, and received approval under protocol number 56/2020 of Comissão de Ética no Uso de Animais (CEUA).

Juvenile *O. niloticus* were maintained in six rectangular tanks, with a useful volume of 100 L each and a density of 4.12 g per liter, in a water recirculation system. Temperature was maintained at 27.84 ± 0.56 ºC (mean ± standard deviation) and pH at around 7.15 ± 0.14, both measured with a multiparameter probe (Hanna HI98130), while dissolved oxygen was maintained at 6.80 ± 0.26 mg L^{-1}, measured with an oximeter (EcoSense® DO200A) (mean values ± standard deviation). The photoperiod was 12 h of light and 12 h hours of dark. Fish were fed twice a day (09:00 and 15:00 h) for two weeks with commercial feed (4 mm) containing 3200 kcal kg^{-1} of digestible energy, 320 g kg^{-1} crude protein, 70 g kg^{-1} ether

extract, 130 g kg^{-1} mineral matter and 350 mg kg^{-1} vitamin C, as informed by the manufacturer (Supra).

Experiment I: Preparation, characterization and stability of mucoadhesive zein nanoparticles

Mucoadhesive zein nanoparticles containing eugenol (NPZMA) were prepared using the antisolvent precipitation method described by Luo et al. (2011), with some modifications. The main differences from Lou et al. (2011) were: the use of a zein solution (2 % W/V) prepared in an ethanolic solution (85 % V/V) under stirring overnight; submisison of the zein solution to a hot water bath at 75 °C for 15 min; cooling the solution at room temperature and filtering through a 0.45 μm membrane (Millipore); and injecting the resulting zein nanoparticle suspension into a stirred chitosan solution (0.5 % W/V) for 24 h for polysaccharide coating.

Nanoparticle morphology

The surface morphology of the nanoparticles was analyzed by scanning electron microscopy (SEM) using suspensions diluted by a factor of 2,000 and with samples mounted on aluminum stubs and sputtered with gold. Micrographs were obtained at an accelerating voltage of 10 kV with a JEOL J SEM-6340 field-emission scanning electron microscope.

Colloidal parameters of nanoparticles, mean diameter, polydispersity index (PDI) and zeta potential

Mean diameter and PDI of the nanoparticles were determined using the dynamic light scattering technique, while zeta potential was determined by microelectrophoresis. These measurements were performed at 25 °C by diluting nanoparticle suspensions with purified water using a Zetasizer Nano ZS 90 particle analyzer (Malvern) with a fixed angle of 90 degrees. The size distribution was given by the PDI. Results are expressed as the average of three determinations (Melo et al., 2016; Melo et al., 2018). The physico-chemical characteristics were evaluated over time at 0, 20, 40 and 60 days.

Nanoparticle tracking analysis (NTA)

Nanoparticle concentration and size distribution were determined by employing the NTA technique. Suspensions were diluted by a factor of 10,000 and run in triplicate for each

sample in a NanoSight LM10 instrument (Malvern) using a volumetric cell and a 532 nm laser wavelength CMOS camera for image acquisition. Videos of Brownian motion were analyzed with NanoSight 2.3 software. Each replicate consisted of five measurements with about 2,000 particles counted in each analysis (Melo et al., 2016).

Experiment II: Effects of NPZMA on the physico-chemical parameters of water

An *in vitro* test was carried out to characterize the stability of NPZMA in water. Five beakers (1 L) were distributed to each of the following treatments: eugenol-80 mg L^{-1} (Ribeiro et al., 2015); NPZMA-80 mg L^{-1}; NPZMA-40 mg L^{-1} and NPZMA-20 mg L^{-1}, water *in natura* and control (ethyl alcohol was added to the water at a proportion of 1:10 V/V). Physico-chemical parameters of the water were measured at 0, 3, 5, 15, 30, 45 and 60 minutes using a mutiparameter probe (Hanna HI98130) for temperature (ºC) and pH and an oximeter (EcoSense® DO200A) for dissolved oxygen (mg L^{-1}).

Experiment III: Anesthetic effect of NPZMA on juvenile *O. niloticus*

Anesthesia induction was analyzed using 32 juvenile *O. niloticus,* with a mean biomass of 42.23 ± 6.69 g and a mean total length of 12.90 ± 1.10 cm. The animals were fasted for 24 hours prior to the experiment. The fish were then transferred individually to beakers containing 1,000 mL of water (n=8 for each concentration) and exposed the following treatments: eugenol-80 mg L^{-1} (control) (Ribeiro et al., 2015); NPZMA-80 mg L^{-1}; NPZMA-40 mg L^{-1} and NPZMA-20 mg L^{-1} in a completely randomized design. For the control group (eugenol-80 mg L^{-1}), ethyl alcohol (1:10) was added to dilute the clove oil. Anesthetic induction time was measured using a digital stopwatch. The fish were considered anesthetized when opercular beats slowed and there was no reflex to external stimuli (Ross and Ross, 2008). For the process of recovery, each animal was relocated in 1,000 mL beakers containing clean water. Recovery time was considered from the moment of transfer to total recovery of the fish. The animals were considered recovered when they presented normal balance and reactions to external stimuli (Ross and Ross, 2008). At the end of the experiment, the fish

from each treatment were relocated to their experimental units to observe survival for 24 hours.

Experiment IV: Hemogasometric analysis of juvenile *O. niloticus* submitted to different forms of eugenol application

Based on the results of Experiment III, 15 juvenile *O. niloticus* with a mean biomass of 103.37 ± 18.51 g and a mean total length of 17.93 ± 1.13 cm were submitted to the following treatments: basal (group of non-anesthetized fish), eugenol-80 mg L^{-1} and NPZMA-40 mg L^{-1}. For the basal and eugenol-80 mg L^{-1} treatments, ethyl alcohol was added to the water at a proportion of 1:10 (V/V).

The fish were transferred to 1,000 mL beakers (n=5 for each treatment) and after two minutes of being exposed to the appropriate concentrations had blood samples collected by caudal puncture using 3-ml heparinized syringes. The studied variables were: sodium (Na^+ - mmol L^{-1}), potassium (K^+ - mmol L^{-1}), carbon dioxide rate (TCO_2 - mmol L^{-1}), glucose (Glu – mg dL^{-1}), hematocrit (Hct % PCT), hydrogen potential (pH), partial pressure of carbon dioxide (PCO_2 - mmHg), bicarbonate ion (HCO_3^- mmol L^{-1}), base deficit (Beecf - mmol L^{-1}) and hemoglobin (g dL^{-1}). The blood samples were evaluated immediately after collection using an i-STAT $EC8^+$ kit and an i-STAT-$EC8^+$ analyzer (*i-STAT*®).

Statistical analysis

Homogeneity of variances and normality of the data were tested by the Levene-test and the Shapiro-Wilk test, respectively. Two-way ANOVA followed by Tukey's post-test ($P<0.05$) were performed for water quality variables. One-way ANOVA followed by Tukey's post-test ($P<0.05$) were performed for time of induction and recovery and hemogasometry ($P<0.05$). Data analysis was performed using R version 3.5.2 - "Eggshell Igloo" Copyright©.

3. Results

Experiment I

The NPZMA were found to be spherical with no apparent clusters, and with a mean diameter of 190 nm, a PDI of 0.24 ± 0.01 and a zeta potential of 42 mV (Table 1, Fig. 1).

Table 1. Physico-chemical characteristics of mucoadhesive zein nanoparticles containing eugenol: polydispersion index (PDI), particle size and zeta potential

	Times (days)			
	0	20	40	60
PDI index	0.24 ± 0.01	0.23 ± 0.01	0.20 ± 0.01	0.28 ± 0.02
Size (nm)	100	110	140	190
Zeta potential (mV)	40	38	36	42

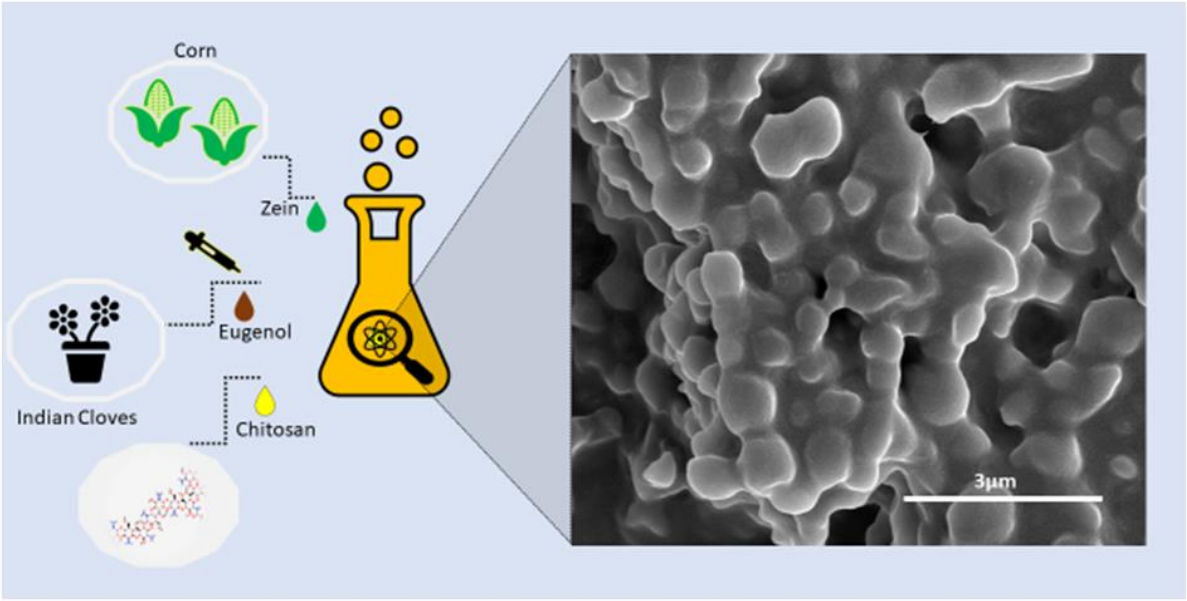

Figure 1. Scanning electron microscopy (SEM) was used to analyze the morphology of NPZMA. The nanoparticles were diluted 1:5000, mounted on plastic coverslips (Thermanox), metalized with carbon in an evaporator (Modelo Hitachi HUS4G) and analyzed by SEM with secondary electrons in a backscattered electronic image.

Experiment II

An interaction ($P<0.05$) was observed among different treatments over time for the water quality variables of temperature and pH (Table 2).

Table 2. Means and standard deviations for the physical parameters of water quality over time after in vitro testing among the different forms of eugenol application.

		Water quality parameters		
		Temperature (ºC)	pH	Dissolved oxygen (mg L^{-1})
Treatments	Eugenol-80 mg L^{-1}	26.56 ± 1.22	7.39 ± 0.12	6.38 ± 0.22b
	NPZMA-80 mg L^{-1}	26.03 ± 0.99	6.84 ± 0.15	6.43 ± 0.31b
	NPZMA-40 mg L^{-1}	25.78 ± 1.03	6.85 ± 0.09	6.35 ± 0.15 b
	NPZMA-20 mg L^{-1}	25.8 ± 1.02	6.94 ± 0.06	6.49 ± 0.26ab
	Water *"in natura"*	27.04 ± 0.71	7.21 ± 0.05	6.67 ± 0.2a
	Control	27.18 ± 0.55	6.99 ± 0.10	6.56 ± 0.07ab
Time (minutes)	0	27.52 ± 0.34	7.14 ± 0.23	6.51 ± 0.37
	3	27.33 ± 0.51	6.99 ± 0.23	6.54 ± 0.23
	5	27.11 ± 0.51	7.00 ± 0.20	6.43 ± 0.20
	15	26.41 ± 0.70	6.97 ± 0.24	6.48 ± 0.25
	30	25.87 ± 0.71	7.04 ± 0.21	6.49 ± 0.20
	45	25.36 ± 0.78	7.08 ± 0.23	6.49 ± 0.22
	60	25.18 ± 0.82	7.06 ± 0.21	6.41 ± 0.27
Overall average		26.39 ± 0.62	7.04 ± 0.23	6.47 ± 0.25
P-value Treatments (A)		<0.0001	<0.0001	0.0002
P-value Time (B)		<0.0001	<0.0001	0.7017
P-value Interaction (A x B)		<0.0001	<0.0001	0.7270
CV (%)		0.56	0.86	3.69

The *P-values* refers to Tukey's test (P<0.05); Means followed by different letters differ significantly according to Tukey's test (P<0.05); CV (%) = Coefficient of variation.

The water temperature of the different treatments differed (P<0.05) and decreased over time (Table 3).

Table 3. Breakdown of the interaction (mean ± standard deviation) between different forms of eugenol application over time and temperature (ºC).

	Treatments					
Time (minutes)	Eugenol- 80 mg L-1	NPZMA-80 mg L^{-1}	NPZMA-40 mg L^{-1}	NPZMA-20 mg L^{-1}	Water "*in natura*"	Control
0	27.77 ± 0.21^{aA}	27.33 ± 0.21^{bA}	27.17 ± 0.12^{bA}	27.17 ± 0.12^{bA}	27.80 ± 0.10^{aA}	27.90 ± 0.10^{aA}
3	27.87 ± 0.15^{aA}	26.97 ± 0.15^{bB}	26.80 ± 0.17^{bB}	26.80 ± 0.17^{bB}	27.83 ± 0.06^{aA}	27.70 ± 0.10^{aAB}
5	27.63 ± 0.38^{aA}	26.80 ± 0.10^{bB}	26.57 ± 0.12^{bB}	26.57 ± 0.12^{bB}	27.60 ± 0.10^{aA}	27.50 ± 0.10^{aBC}
15	26.77 ± 0.32^{bB}	25.90 ± 0.26^{cC}	25.67 ± 0.06^{cC}	25.73 ± 0.06^{cC}	27.13 ± 0.06^{aB}	$27.27 \pm 0.15a^{C}$
30	25.90 ± 0.26^{bC}	25.57 ± 0.06^{bC}	25.13 ± 0.06^{cD}	25.13 ± 0.06^{cD}	26.63 ± 0.06^{aC}	26.87 ± 0.15^{aD}
45	25.13 ± 0.21^{cD}	24.90 ± 0.10^{cdD}	24.67 ± 0.06^{dE}	24.70 ± 0.00^{dE}	26.17 ± 0.06^{bD}	26.60 ± 0.10^{aDE}
60	24.87 ± 0.15^{bD}	24.73 ± 0.15^{bcD}	24.47 ± 0.06^{cE}	24.47 ± 0.06^{cE}	26.13 ± 0.15^{aD}	26.40 ± 0.10^{aE}

Means followed by different lowercase letters in the same row differ significantly according to Tukey's test (P<0.05). Means followed by different capital letters in the same column differ significantly according to Tukey's test (P<0.05).

Decreases in pH were observed over time (P<0.05) for eugenol-80 mg L^{-1}, NPZMA-80 mg L^{-1}, NPZMA-40 mg L^{-1} and control, and among the evaluated treatments. However, the water of the *in natura* and NPZMA-20 mg L^{-1} treatments also had different pH values compared to the other treatments, but the values remained stable over time (Table 4).

Table 4. Breakdown of the interaction (mean ± standard deviation) between the different forms of eugenol application over time and pH

Time (minutes)	Treatments					
	Eugenol-80 mg L^{-1}	NPZMA-80 mg L^{-1}	NPZMA-40 mg L^{-1}	NPZMA-20 mg L^{-1}	Water *"in natura"*	Control
0	7.56 ± 0.17^{aA}	7.11 ± 0.16^{bcA}	6.96 ± 0.03^{dA}	6.96 ± 0.03^{dA}	7.24 ± 0.04^{bA}	7.01 ± 0.01^{cdA}
3	7.33 ± 0.06^{aCD}	6.73 ± 0.07^{cBC}	6.89 ± 0.06^{bA}	6.89 ± 0.06^{bA}	7.24 ± 0.04^{aA}	6.84 ± 0.06^{bcB}
5	7.25 ± 0.01^{aD}	6.78 ± 0.08^{bBC}	6.92 ± 0.05^{bA}	6.92 ± 0.05^{bA}	7.27 ± 0.03^{aA}	6.85 ± 0.05^{bB}
15	7.32 ± 0.04^{aCD}	6.72 ± 0.09^{cdC}	6.69 ± 0.06^{dB}	6.86 ± 0.06^{cA}	7.17 ± 0.05^{bA}	7.06 ± 0.01^{bA}
30	7.39 ± 0.04^{aBCD}	6.85 ± 0.11^{deBC}	6.81 ± 0.04^{eAB}	6.96 ± 0.03^{cdA}	7.16 ± 0.06^{bA}	7.06 ± 0.03^{bcA}
45	7.49 ± 0.06^{aAB}	6.87 ± 0.08^{deB}	6.84 ± 0.05^{eA}	7.00 ± 0.05^{cdA}	7.21 ± 0.06^{bA}	7.07 ± 0.01^{bcA}
60	7.41 ± 0.03^{aBC}	6.83 ± 0.06^{dBC}	6.85 ± 0.04^{dA}	7.01 ± 0.05^{cA}	7.20 ± 0.06^{bA}	7.06 ± 0.02^{bcA}

Means followed by different lowercase letters in the same row differ significantly according to Tukey's test ($P<0.05$). Means followed by different capital letters in the same column differ significantly according to Tukey's test ($P<0.05$).

Experiment III

The time for the eugenol-80 mg L^{-1} treatment to induce deep anesthesia was similar to that for NPZMA-80 mg L^{-1} and NPZMA-40 mg L^{-1} ($P>0.05$) (Fig. 2A). It should be noted that when the nanoparticles were associated with eugenol, the inducing effect and induction time were greater for the NPZMA-20 mg L^{-1} ($P<0.05$). In addition, fish subjected to the test with mucoadhesive nanoparticles were less responsive, compared to the eugenol-80 mg L^{-1}, and without movements.

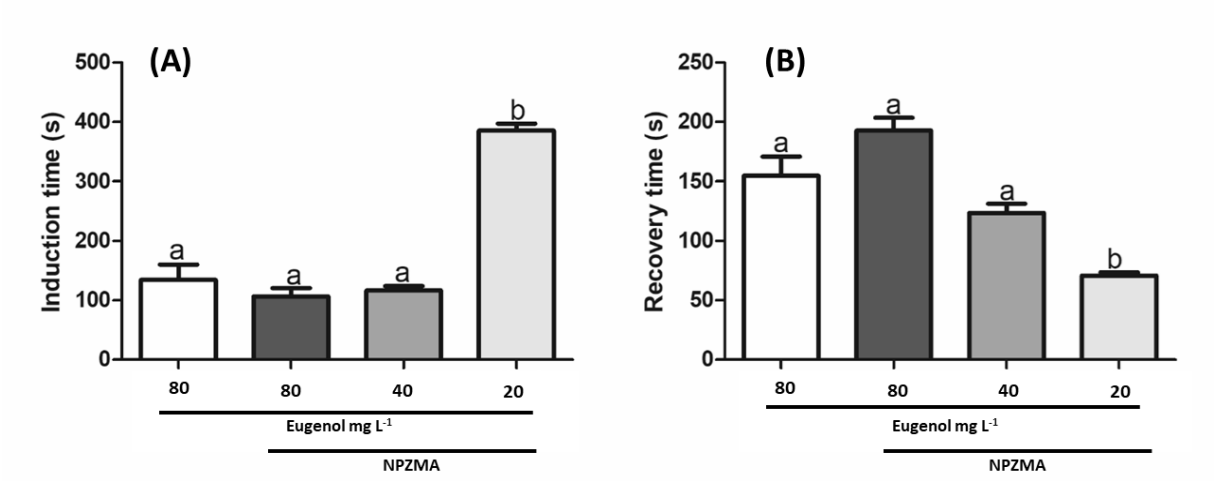

Figure 2. Time for anesthesia induction (A) and recovery (B) (mean ± standard deviation) of juvenile *O. niloticus* submitted to different forms of eugenol application. Means followed by different letters differ significantly according to Tukey's test ($P<0.05$).

The time period for anesthetic recovery was shortest for the NPZMA-20 mg L^{-1} treatment and longest for NPZMA-80 mg L^{-1} (Fig. 2B) ($P<0.05$).

Experiment IV

There were no differences in hemogasometric variables (Na^+ mmol L^{-1}, K^+ mmol L^{-1}, TCO_2 mmol L^{-1}, glucose mg dL^{-1}, hematocrit % PCT, pH, PCO_2, HCO^-_3, Beecf mmol L^{-1},

AnGap mmol L^{-1} and hemoglobine g dL^{-1}), after exposure to eugenol-80 mg L^{-1} and NPZMA-40 mg L^{-1} and basal (P>0.05) (Table 5).

Table 5. Hemogasometric values (mean ± standard deviation) for sodium (Na^+ - mmol L^{-1}), potassium (K^+ - mmol L^{-1}), carbon dioxide rate (TCO_2 - mmol L^{-1}), glucose (Glu – mg dL^{-1}), hematocrit (Hct % PCT), hydrogen potential (pH), partial pressure of carbon dioxide (PCO_2 - mmHg), bicarbonate ion (HCO_3^- mmol L^{-1}), base deficit (Beecf - mmol L^{-1}) and hemoglobin (g dL^{-1}) for blood samples of *O. niloticus* submitted to different forms of eugenol application

Parameters	Treatments			Overall average	CV (%)	*P-value*
	Basal	Eugenol-80 mg L^{-1}	NPZMA-40 mg L^{-1}			
Na (mmol L^{-1})	153.75 ± 6.55	155.60 ± 2.07	157.60 ± 4.16	155.79 ± 4.37	2.84	0.4541
K (mmol L^{-1})	5.03 ± 1.87	4.04 ± 0.15	4.30 ± 0.37	4.41 ±1.02	22.80	0.3624
TCO_2 (mmol L^{-1})	6.00 ± 0.00	5.60 ± 1.14	6.00 ± 0.71	5.86 ±0.77	13.81	0.6842
Glu (mg dL^{-1})	30.00± 7.87	35.60 ± 8.02	42.20 ± 20.64	36.36 ± 13.82	38.44	0.4504
Hct (% PCT)	16.00 ± 0.82	18.80 ± 2.39	19.80 ± 4.44	18.36 ± 3.25	16.72	0.2132
pH	7.18 ± 0.06	7.15 ± 0.06	7.12 ± 0.08	7.15 ± 0.07	0.97	0.4502
PCO_2 (mmHg)	15.48 ± 2.80	16.06 ± 1.60	17.32 ± 3.38	16.34 ± 2.60	16.46	0.5838
HCO^-_3 (mmol L^{-1})	5.70 ± 0.28	5.52 ± 0.56	5.58 ± 0.68	5.59 ± 0.51	9.88	0.8880
Beecf (mmol L^{-1})	-22.75 ± 0.96	-23.40 ± 1.67	-24.00 ± 1.58	-23.43 ± 1.45	6.30	0.4743
Hb (g dL^{-1})	5.53 ± 0.23	6.70 ± 0.42	7.15 ± 1.40	6.55 ± 1.05	13.77	0.1162

*The *P-value* refers to test F (P>0.05); CV (%) = Coefficient of variation.

4. Discussion

This study evaluated for the first time the anesthetic effects of chitosan-coated zein nanoparticles containing eugenol on juvenile *O. niloticus* and obtained positive results. The produced zein nanoparticles coated with chitosan had the ability to adhere to the mucus of fish and had physico-chemical characteristics that differed from those published thus far, thus demonstrating the originality and relevance of this work.

The adjustment made to the pH of the solution to form zein nanoparticles coated with chitosan containing eugenol was expected to affect water quality. This indeed was the case, however, the exposure time for anesthesia was short and the pH was within acceptable limits for the cultivation of *O. niloticus* during the entire 60-minute evaluation period (Shoko et al., 2014).

The results presented here show that the zein nanoparticles coated with chitosan containing eugenol remained stable, well dispersed and had positive loads and particle size distribution for a long period of time (up to 60 days). Yostawonkul et al. (2019) also produced

nanoparticles containing eugenol, however, their zeta potential was negative, which presents other physiological functions, such as improved tissue penetration. The nanoparticles of the resent study had positive zeta potential, which improves adherence to fish mucus, as verified by Charlie-Silva et al. (2018). Recent studies have evaluated the interaction between nanoparticles with positive loads and adherence to mucus membranes and found that this interaction can help to decrease the amount of drug being absorbed and enhance anesthetic effects, as observed in the present study (Costa et al., 2015; Charlie-Silva et al., 2018).

The anesthetic induction time for fish of the control group (eugenol-80 mg L^{-1}) was similar to that for NPZMA-80 mg L^{-1} and NPZMA-40 mg L^{-1} (116 s), which was less than three minutes to complete anesthesia and less than five minutes for recovery, as recommended by Kenne et al. (1998) and Ross and Ross (2008). This demonstrates the efficiency of using eugenol in both forms of use. Eugenol has a proven effect on the anesthesia of fish (Cunha et al., 2010; Ribeiro et al., 2015; He et al., 2020). In addition, the present study found that using mucoadhesive nanoparticles reduced the use of eugenol by 50%, as observed for the treatment of NPZMA-40 mg L^{-1}, which showed the same anesthetic induction time as eugenol-80 mg L^{-1}. A recent study also demonstrated the effects of lipid nanoparticles containing eugenol on anesthesia of *O. niloticus* (mean biomass 10 g), with a concentration of 40 mg L^{-1} having an induction time of 40 s and a recovery time of 193 s (Yostawonkul et al., 2019), but with 25% yield in relation to the use of eugenol.

The present study found variation in fish behavior, with fish submitted to the test with mucoadhesive nanoparticles being less responsive and without movement, in contrast to other treatments. As far as is known, this is the first report of the effects of nanoparticles on fish behavior. On the other hand, using a mammal animal model, Lima et al. (2019) reported depression, anxiety-like behavior and memory impairment in mice exposed to mucoadhesive zein nanoparticles.

Fish submitted to control, eugenol-80 mg L^{-1} and NPZMA-40 mg L^{-1} did not exhibit changes in hemogasometric variables, indicating that nanoparticles can be used to help reduce the concentration of drug used. Clove oil has been shown to have limited solubility in water and is generally used in higher concentrations for the induction and/or sedation of fish (Yostawonkul et al. 2019) and is thus able to modulate physiological and hemogasometric variables. In addition, alcohols or detergents are used as solvents to dissolve clove oil (Yostawonkul et al. (2019). In this sense, Luis et al. (2019) reviewed the possible uses of

different nanoparticle systems, highlighting essential oils and new nanotechnology strategies to improve the solubility and efficacy of drugs in aquaculture, and the use of eugenol in immersion baths for juvenile matrinxã, *Brycon amazonicus*, also without changes in the hydroelectric balance of the animals (Barbosa et al., 2007). Honorato et al. (2014) evaluated different concentrations of eugenol for juvenile Amazonian jundiá, *Leiarius marmoratus*, and also did not observe such changes in variables including pH, Glu, PCO_2, Na^+ and K^+, which corroborates the results of the present study. Thus, the use of eugenol directly in water (eugenol-80 mg L^{-1}) or through zein nanoparticles coated with chitosan containing eugenol (NPZMA-40 mg L^{-1}) proved to be safe ways of maintaining animal welfare while being efficient at inducing anesthesia.

5. Conclusion

Chitosan-coated zein nanoparticles containing eugenol at a concentration of 40 mg L^{-1} enhance the use of eugenol as an anesthetic by reducing the required concentration without affecting hemogasometric variables of juvenile *O. niloticus*.

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ARTIGO 4

Essential oil of *Ocimum gratissimum* (Linnaeus, 1753): efficacy for anesthesia and transport of *Oreochromis niloticus*

Running Head

O. gratissimum **for anesthesia and transport of tilapia**

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Abstract

This study aimed to evaluate the essential oil of *Ocimum gratissimum* L. (EOOG) for anesthesia and in the transport of *Oreochromis niloticus*. Experiment I determined the time of anesthesia induction and recovery during anesthesia of *O. niloticus* exposed to different concentrations of EOOG (0, 30, 90, 150 and 300 mg L^{-1}). Based on data from Experiment I, Experiment II evaluated the effect of 0, 30 and 90 mg L^{-1} EOOG on blood parameters and oxidative stress immediately after anesthesia induction and one hour after recovery. Experiment III evaluated the effect of 0, 5 and 10 mg L^{-1} EOOG on blood variables immediately after 4.5 hours of transport of juveniles. Concentrations between 90 and 150 mg L^{-1} EOOG were efficient for anesthesia and recovery. The use of 90 mg L^{-1} of EOOG prevented an increase in plasma glucose. Others changes in blood parameters and oxidative stress are discussed. The use of 10 mg L^{-1} EOOG in transport increased plasma glucose and decreased hematocrit values immediately after transport. It is concluded that the use of 90 and 150 mg L^{-1} EOOG causes anesthesia and recovery in *O. niloticus* within the time intervals considered ideal. The use of 90 mg L^{-1} EOOG favored stable plasma glucose soon after anesthesia induction and one hour after recovery, but caused changes in the antioxidant defense system by increasing hepatic and kidney ROS. The transport of 12 g *O. niloticus* for 4.5 hours can be performed with concentration of 5 mg L^{-1} of EOOG.

Key words: Anesthesia, Handling stress, Metabolism, Nile tilapia, Tissue damage

1. Introduction

The usual handling procedures employed in fish farms trigger stress to animals, which can compromise their productive and reproductive performances (Barton, 2002; Gressler et al., 2012). Among the adopted handling procedures, biometry and transport of fish are routine in fish farms. These practices can harm fish, increasing their susceptibility to infectious and pathogenic diseases, besides demanding a greater amount of energy (Gimbo et al., 2008; Goes et al., 2017), which is provided through gluconeogenesis, glycogenolysis and increased protein turnover (Barton and Iwama, 1991; Wendellar Bonga, 1997; Mommsen et al., 1999; Ribeiro et al., 2019). In addition, these handling practices can cause changes in fish metabolism, with oxidative damage through the production of reactive oxygen species (ROS), which often leads to lipid peroxidation (Matés, 2000; Veliske et al., 2011; Souza et al., 2018; Baldissera et al., 2020).

In this sense, the use of anesthetics has provided better control for the handling procedures used in fish production (Cunha et al., 2010; Becker et al., 2012; Becker et al., 2017; Rotili et al., 2012; Ribeiro et al., 2013; Ribeiro et al., 2015), by reducing swimming activity and stress of the animals (Ross and Ross, 2008; Zahl et al., 2010; Salbego et al., 2017). Anesthetics (at sedative concentrations) also showed positive effects during transport, including reduced waterborne ammonia and greater survival (Becker et al., 2012; Gil et al., 2016; Navarro et al., 2016; Boaventura et al., 2021a). However, responses to a given anesthetic may differ among species, and so prior characterization of efficacy is necessary (King et al., 2009).

The plant *Ocimum gratissimum* L. (Lamiaceae), popularly known as "alfavaca" or basil, is a native species of Africa that is being used in different countries as a condiment, sedative and stress reducer and to treat headaches in humans (Albuquerque et al., 2007). The essential oil of the leaves of this plant showed safety and efficacy as anesthesia for some species of fish, such as the Brazilian flounder *Paralichthys orbignyanus* (Benovit et al., 2012), silver catfish *Rhamdia quelen* (Silva et al., 2012; Silva et al., 2015; Bandeira et al., 2017), matrinxã *Brycon amazonicus* (Ribeiro et al., 2016), Nile tilapia *Oreochromis niloticus* (Adewale et al., 2017), cachara *Pseudoplatystoma reticulatum* (Silva et al., 2020) and pacamã *Lophiosilurus alexandri* (Boaventura et al., 2020).

Oreochromis niloticus is the fourth most produced fish species worldwide, contributing approximately 4.5 million tons/year (FAO, 2018). This high production is attributed to its excellent characteristics that make it favorable to commercial cultivation, as well as its white meat with good organoleptic characteristics and lack of "Y" shaped spines, which facilitates fillet processing (Simões et al., 2007). Therefore, the evaluation of the anesthetic and sedative effects of this essential oil and its benefits in the transport of juveniles of this species has become important.

Thus, the objective of this study was to evaluate different concentrations of the essential oil of *O. gratissimum* L. (EOOG) for induction and recovery times of anesthesia, blood and oxidative stress parameters and its use in the transport of *O. niloticus* juveniles. Based on previous studies with the extract of this plant in *O. niloticus* (Adewale et al., 2017) and of the EOOG in other species (Silva et al., 2012; Silva et al., 2015; Ribeiro et al., 2016; Silva et al., 2020; Boaventura et al., 2020; Boaventura et al., 2021a), we suppose that this essential oil is effective for anesthesia, reduces the stress of transport and improves the oxidative status of *O. niloticus*.

2. Materials and Methods

2.1 Compliance with Ethical Standards

Three experiments were carried out at the Laboratório de Aquacultura (LAQUA) of the Escola de Veterinária (EV) of the Universidade Federal de Minas Gerais (UFMG), with approval of the Comissão de Ética no Uso de Animais (CEUA) of this institution (registration numbers 324/2018 and 42/2020).

2.2 Animals and environmental acclimation

Juveniles of *O. niloticus* were grown in rectangular tanks, with a useful volume of 100 L, in a water recirculation system with mechanical and biological filtration. Temperature was maintained at 27.69 ± 0.35 ºC and pH at 7.09 ± 0.14 (both measured with a Hanna HI98130 multiparameter probe), dissolved oxygen levels at 6.65 ± 0.28 mg L^{-1} (measured with an EcoSense® DO200A oximeter) and total ammonia at 0.59 ± 0.03 mg L^{-1} (determined with a Alfakit Labcon Test colorimetric kit). The photoperiod was 12L:12D (controlled by Loud Advanced Technology TL63A digital timer). During the lights-on period, the average luminous flux incident on the water surface was 140 lux (measured with Instrutemp ITLD260

luximeter) and 0 lux after lights were turned off. The fish were fed with commercial feed (5 mm in diameter) containing 320 g kg^{-1} of crude protein, 3200 kcal kg^{-1} of digestible energy, 70 g kg^{-1} of ether extract, 130 g kg^{-1} of mineral matter, 25 g kg^{-1} of calcium and 10 g kg^{-1} of phosphorus, twice a day (8:00 am and 4:00 pm) until apparent satiety. The animals were fasted for 24 hours before use; thus, the food was provided until the first meal of the day before each test. A water renewal of 50% of the system volume was performed twice a week.

The essential oil of the leaves of *O. gratissimum* (EOOG) (73.6% eugenol) was obtained according to Silva et al. (2012). Ethyl alcohol (98.1%) was used in a 1:10 (V / V) proportion in all concentrations to homogenize the EOOG in water. The volume of ethyl alcohol equivalent to the highest concentration of the EOOG was used for the ethanol treatment (0 mg L^{-1}).

Experiment I – Anesthetic effect of EOOG for juvenile *O. niloticus*

Seventy-two *O. niloticus* juveniles (weight 45.36 ± 10.70 g; total length 13.16 ± 1.12 cm) were fasted for 24 hours prior to use. The animals were distributed in a completely randomized design (n = 12 for each concentration) and exposed to the following EOOG concentrations: 0 (ethanol), 10, 30, 90, 150 or 300 mg L^{-1}. The fish were transferred individually to a 1 L aquarium and exposed to the different concentrations (each fish was used only once). The ethanol group was observed for 10 min (Ferreira et al., 2020), while a digital chronometer (Taksun Ts1809) was used to assess the time of anesthesia induction and recovery for the other concentrations. Fish were considered to have reached deep anesthesia when they completely lost swimming balance and did not respond to external stimuli (Keene et al., 1998; Ross and Ross, 2008), at which point they were weighed on an analytical balance (Mars AD5002) and measured with the aid of a ruler. After biometrics, the fish were transferred to 1 L aquaria with clean water (from the culture system itself), for anesthesia recovery. The ventilatory frequency (VF) of the fish was evaluated during anesthesia induction and recovery by counting opercular movements per minute (adapted from Alvarenga and Volpato, 1995). The animals were considered recovered when they responded to external stimuli and showed normal swimming balance (Keene et al., 1998; Ross and Ross, 2008).

After the experiment, fish of each anesthetic concentration were pooled and kept in 100 L tanks in a recirculating aquaculture system to observe the return to appetite and record their survival after 24 h. During this period the fish were fed two times a day until apparent satiety.

Experiment II – Hematological, biochemical and oxidative stress responses of juvenile *O. niloticus* submitted to different EOOG concentrations

Based on the results of Experiment I, a subsequent test was performed using the concentrations of 0 (ethanol), 30 and 90 mg L^{-1} EOOG, considering a control and concentrations below the ideal and at the ideal, respectively, for fish anesthesia according to the criteria of Keene et al. (1998) and Ross and Ross (2008). For this test, 72 *O. niloticus* juveniles (weight 41.36 ± 9.80 g; total length 12.70 ± 1.15 cm, n = 24 per treatment) were fasted for 24 h and then distributed in a completely randomized factorial design. The same procedures as described in Experiment I were adopted. However, in this experiment blood samples were collected immediately after anesthesia induction and one hour after anesthesia recovery (n= 12 fish for each concentration after anesthesia and 12 fish for each concentration after recovery). Fish were restrained with a damp cloth and blood (500 µL) was collected by caudal puncture using heparinized syringes (0.2% mg mL^{-1}). The fish were then euthanized through immersion in ice slurry and spinal cord sectioning so that gills, liver, kidney and brain could be removed and immediately stored in a -80ºC freezer for further analysis.

Hematological and biochemical analysis

Blood aliquots were used to quantify hematocrit. Capillary tubes were centrifuged at 10,000-rpm (Microline-Laborline®) for 10 minutes as described by Goldenfarb et al. (1971). Plasma protein was determined using a Goldberg manual refractometer. Blood was centrifuged at 4000 rpm for 10 minutes for biochemical analysis. Plasma glucose was determined using a monoreagent glucose commercial kit (K082 Bioclin), followed by reading with a spectrophotometer (Biochrom Libra S21 – S22).

Oxidative stress analysis

Liver, gills, kidney and brain were sampled for evaluation of levels of reactive oxygen species (ROS), superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), non-protein thiols (NPSH) and protein.

Levels of ROS were determined by the DCFH oxidation method described by LeBel et al. (1992). Fluorescence was measured using excitation and emission wavelengths of 485 and 538 nm, respectively. A calibration curve was established with 2′,7′-dichlorofluorescein (DCF) (0.1 nM-1 μM) as the standard, and results were expressed as U DFC per mg of protein.

The activity of SOD was determined according to the auto-oxidation principle of pyrogallol, inhibited in the presence of SOD. The optical density change was determined kinetically for two minutes at ten second intervals at 420 nm, according to methodology described by Beutler (1984). Activity was expressed as U mg per of protein.

As an index of lipid peroxidation, TBARS formation during an acid-heating reaction was determined as previously described by Ohkawa et al. (1979). A malondialdehyde (MDA) solution was used as a reference standard. TBARS levels were determined by absorbance at 532 nm and were expressed as MDA equivalent nmol MDA per g of tissue.

NPSH levels were determined colorimetrically at 412 nm as previously described by Ellman (1959) and published in detail by Souza et al. (2018). A cysteine solution was used as a reference standard. NPSH levels were expressed as μmol SH per g of tissue.

Protein concentration was determined by the Coomassie Blue method following the methodology described by Read and Northcote (1981) using bovine serum albumin as a standard.

Experiment III – Effect of EOOG on survival rate, feed return, water quality and blood parameters after transport of juvenile *O. niloticus*

One hundred and sixty *O. niloticus* juveniles (weight 12.2 ± 3.4 g; total length 9.5 ± 2.1 cm) were used for Experiment III. The water in the culture system had a temperature of 27.49 ± 0.35 ºC, pH of 7.65 ± 1.28 (Hanna HI98130 multiparameter probe), and dissolved oxygen levels of 7.09 ± 1.14 mg L^{-1} (measured by the EcoSense® DO200A oximeter). Animals were fasted for 24 h before transport. After fasting, blood was collected as previously described from a group of 10 fish (basal group). The fish were distributed in a completely random design in 15 plastic bags of 59.5 x 80.5 cm, with 8 L water, 2/3 dissolved oxygen and 10 fish

bag^{-1}. Three concentrations of EOOG were tested: 0 (ethanol 90 µL L^{-1}), 5 and 10 mg L^{-1}. The animals were transported in a car for 4.5 h and this time was chosen based in an average duration of *O. niloticus* transport from other studies (Navarro et al., 2016, Hohlenwerger et al., 2017; Ventura et al., 2020); after which the bags were opened and temperature, dissolved oxygen, pH and total ammonia were measured and two fish from each bag (n = 10 fish per treatment) were selected for blood collection for the analysis of plasma glucose, hematocrit and plasma protein, as described in the Experiment II. The remaining fish of each treatment were then placed in three rectangular tanks, with a useful volume of 100 L, in a water recirculation system under a controlled environment, as previously described, to evaluate survival rate and return to feeding up to 96 h after transport.

2.3 Statistical analysis

Homogeneity of variances and normality of the data were tested with the Brown-Forsythe and Shapiro-Wilk tests, respectively. The results of anesthesia induction and recovery times and ventilatory frequencies were submitted to one-way ANOVA, followed by regression analyses to better adjust the model ($P < 0.05$). Two-way ANOVA followed by Tukey's post-test was used for biochemical, hematological and oxidative stress variables ($P < 0.05$). The results of water quality variables were analyzed using the Kruskal-Wallis test ($P < 0.05$). All analyses were performed using the SigmaPlot program version 12.0.

3. Results

Experiment I

No mortality was observed after 24 hours of testing and all fish resumed feeding. Ethanol and 10 mg L^{-1} EOOG did not induce deep anesthesia. Anesthesia induction time had a quadratic effect ($P < 0.05$), with a minimum value at 215 mg L^{-1} (38.79 s) (Fig. 1A). Anesthesia recovery time was directly related to EOOG concentration ($P < 0.05$) and varied from 149.55 to 568.1 s (Fig. 1B).

The VF showed a quadratic effect during anesthesia induction ($P < 0.05$), with a minimum value at 208.33 mg L^{-1} (11.27 beats $minute^{-1}$) (Fig. 1C). During anesthesia recovery, VF showed an inverse relationship with EOOG concentration ($P < 0.05$) (Fig. 1D).

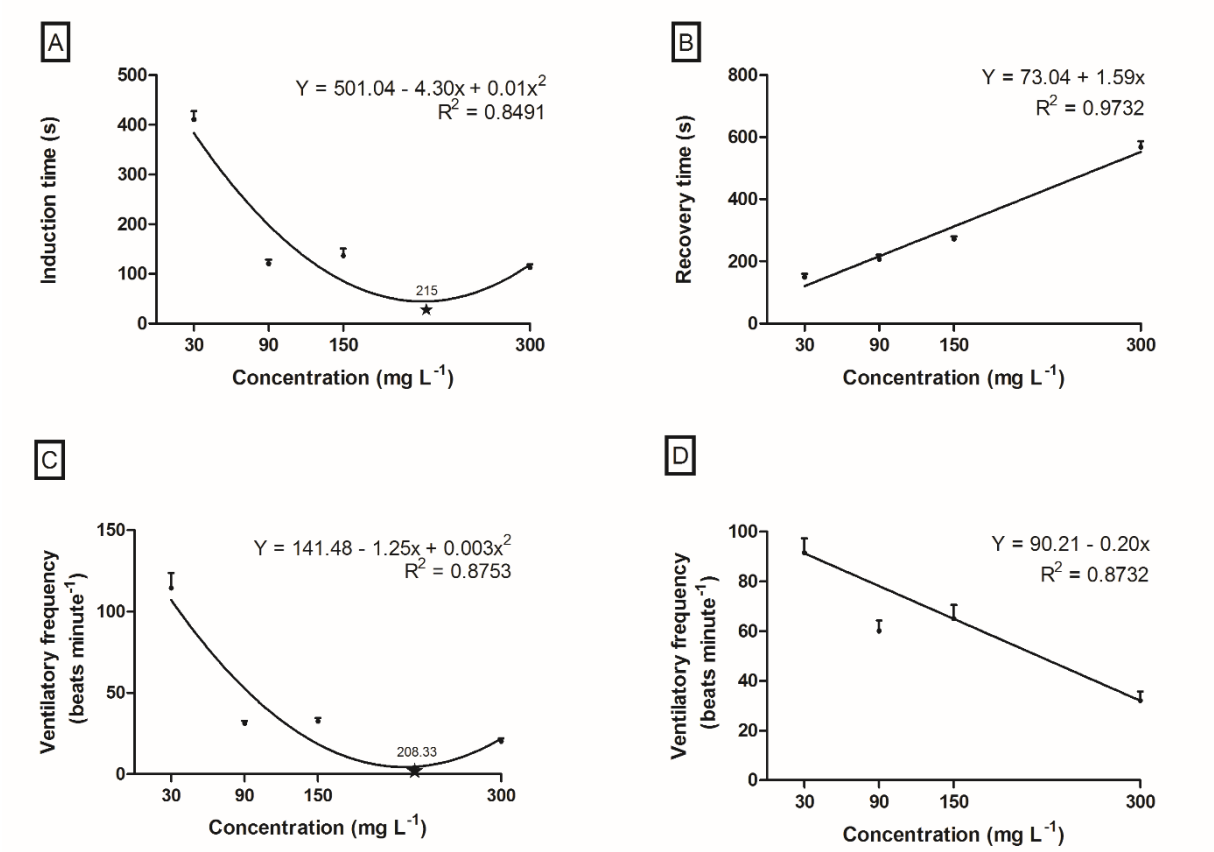

Fig 1 - Anesthesia induction time (A), anesthesia recovery time (B), ventilatory frequency during induction (C) and ventilatory frequency during recovery (D) (mean ± standard deviations) of *Oreochromis niloticus* subjected to different concentrations of the essential oil of *Ocimum gratissimum* (stars represent the minimum value of the equations).

Experiment II

Biochemistry and blood hematology

After anesthesia induction, *O. niloticus* juveniles submitted to 30 mg L^{-1} EOOG had higher plasma glucose values ($P < 0.05$) compared to those exposed to 0 mg L^{-1} (Fig. 2A). One hour after recovery, plasma glucose values were equal among the concentrations evaluated ($P > 0.05$). However, there was a reduction in plasma glucose levels for the

concentration of 30 mg L^{-1} EOOG after 1 h recovery compared to after anesthesia induction ($P < 0.05$).

O. niloticus juveniles exposed to 90 mg L^{-1} EOOG had higher hematocrit values after anesthesia induction compared to treatments of 0 and 30 mg L^{-1} ($P < 0.05$), which were similar to each other ($P > 0.05$) (Fig 2B). One hour after recovery, hematocrit was similar between all concentrations evaluated ($P > 0.05$). There was no significant difference in plasma protein for the tested concentrations between the two evaluated times ($P > 0.05$). There was also no significant difference among the concentrations assessed during induction and one hour after recovery, nor between times (Fig. 2C).

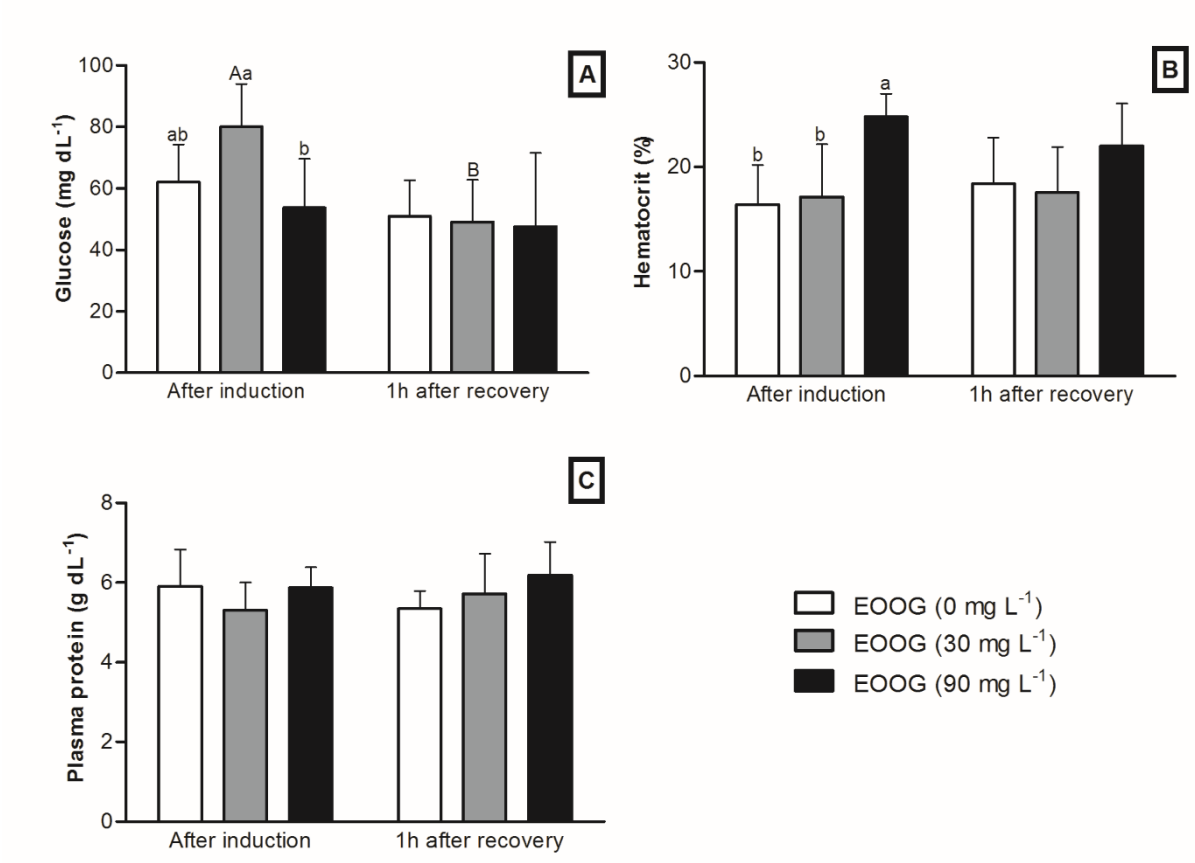

Fig 2 - Biochemical and hematological parameters of *Oreochromis niloticus* measured immediately after anesthesia induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after recovery. Ethanol group: 0 mg L^{-1} EOOG. (A) plasma glucose, (B) hematocrit and (C) plasma protein. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant differences between times for a given concentration.

Oxidative stress variables

After induction, higher levels of hepatic ROS were observed for fish anesthetized with 30 and 90 mg L^{-1} EOOG ($P < 0.05$) (Fig. 3A). These values remained higher than those for fish exposed to 0 mg L^{-1} EOOG one hour after recovery, when fish that were submitted to 30 mg L^{-1} EOOG presented higher levels of hepatic ROS than those recovering from 90 mg L^{-1} EOOG ($P < 0.05$). There were no differences in gill ROS between times ($P < 0.05$) for the three EOOG concentrations assessed. The EOOG concentrations evaluated did not influence the levels of ROS in the gills after anesthesia induction and 1 h after recovery ($P > 0.05$) (Fig. 3B).

There were no significant differences among the tested EOOG concentrations for ROS levels in the kidney after anesthesia induction ($P > 0.05$) (Fig. 3C). However, there was a decrease in the level of ROS in the kidney for fish of the 0 mg L^{-1} EOOG treatment 1 h after recovery and an increase in those of the 30 mg L^{-1} EOOG treatment ($P < 0.05$). The highest kidney ROS levels after recovery were for fish anesthetized with 30 mg L^{-1} EOOG, followed by those anesthetized with 90 mg L^{-1} EOOG ($P < 0.05$).

After anesthesia induction, the highest levels of brain ROS were observed in fish anesthetized with 30 and 90 mg L^{-1} EOOG ($P < 0.05$) (Fig. 3D). One hour after recovery, the highest level of brain ROS was found in fish exposed to 30 mg L^{-1} EOOG, with this value being higher than that observed immediately after anesthesia induction ($P < 0.05$). The other EOOG concentrations evaluated did not differ significantly between times ($P > 0.05$).

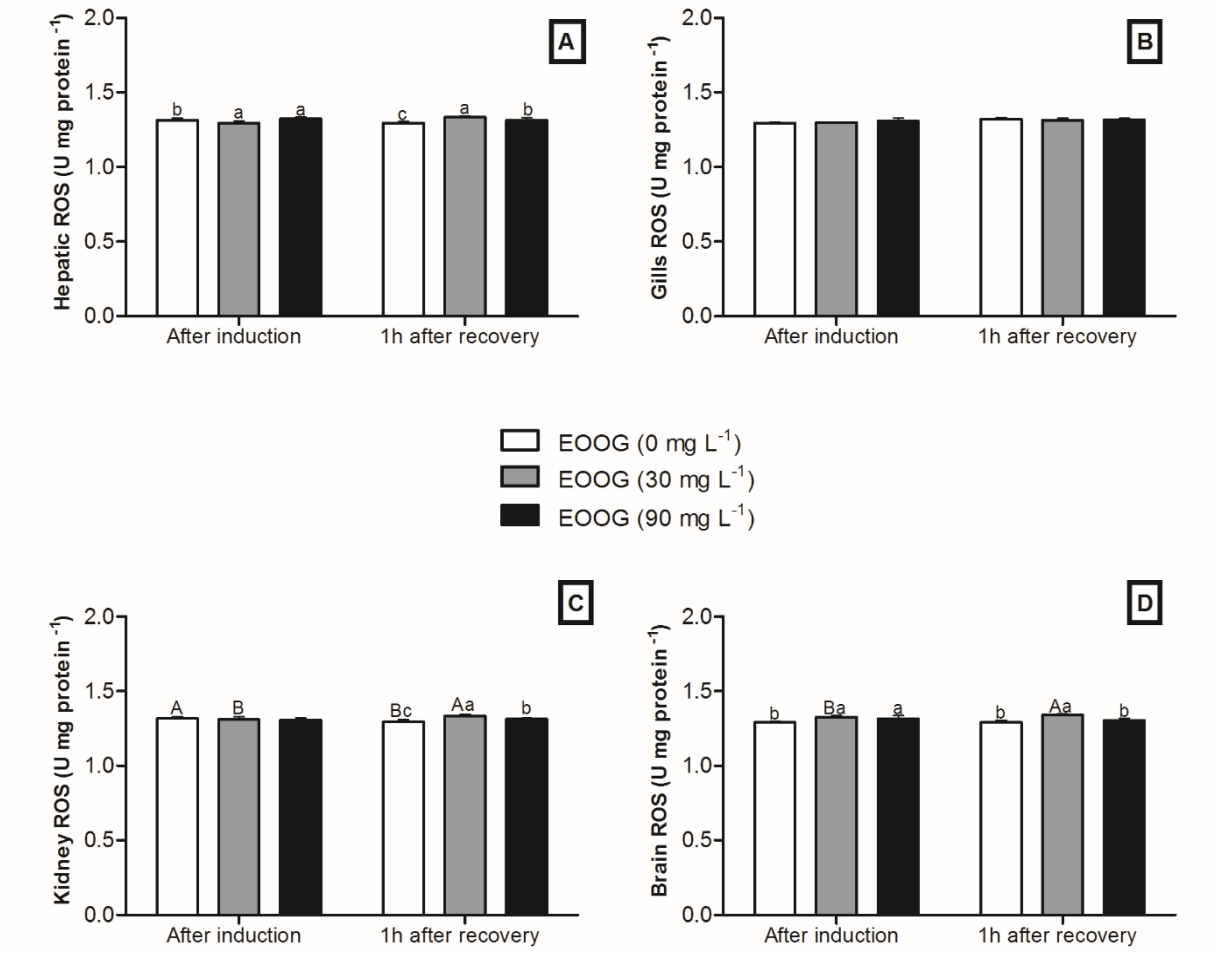

Fig 3 - Reactive oxygen species (ROS) of liver (A), gills (B), kidney (C) and brain (D) of *Oreochromis niloticus* measured immediately after anesthesia induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after recovery. Ethanol group: 0 mg L^{-1} EOOG. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration.

The highest hepatic SOD activity after anesthetic induction was for fish exposed to 30 mg L^{-1} EOOG ($P < 0.05$). One hour after recovery, this group showed the lowest hepatic SOD activity, followed by the group submitted to 90 mg L^{-1} EOOG ($P < 0.05$). There was an increase in hepatic SOD activity from induction to one hour after recovery for fish exposed to 0 mg L^{-1} EOOG and a decrease in those exposed to 30 mg L^{-1} EOOG ($P < 0.05$) (Fig. 4A).

The lowest SOD activity in the gills after anesthesia induction was for fish anesthetized with 90 mg L^{-1} EOOG ($P < 0.05$). One hour after recovery there were no

significant differences among the assessed EOOG concentrations ($P > 0.05$). However, there was a reduction in SOD activity in the gills in fish exposed to 0 and 30 mg L^{-1} EOOG one hour after recovery and an increase in those exposed to 90 mg L^{-1} EOOG ($P < 0.05$) (Fig. 4B).

No significant differences were observed for kidney SOD activity after anesthesia induction ($P > 0.05$). One hour after recovery, the lowest SOD activity was for fish exposed to 30 mg L^{-1} EOOG and the highest activity for those exposed to 0 mg L^{-1} EOOG ($P < 0.05$). There were no significant differences in kidney SOD activity between times for the tested concentrations ($P > 0.05$) (Fig. 4C).

After anesthesia induction, brain SOD activity was significantly lower for fish exposed to both 30 and 90 mg L^{-1} EOOG compared those exposed to ethanol (0 mg L^{-1} EOOG) ($P < 0.05$). One hour after recovery, the lowest SOD activity was observed in fish exposed to 30 mg L^{-1} EOOG ($P < 0.05$). There was a reduction in brain SOD activity between times in fish submitted to 30 mg L^{-1} EOOG and an increase in those exposed to 90 mg L^{-1} EOOG ($P < 0.05$) (Fig. 4D).

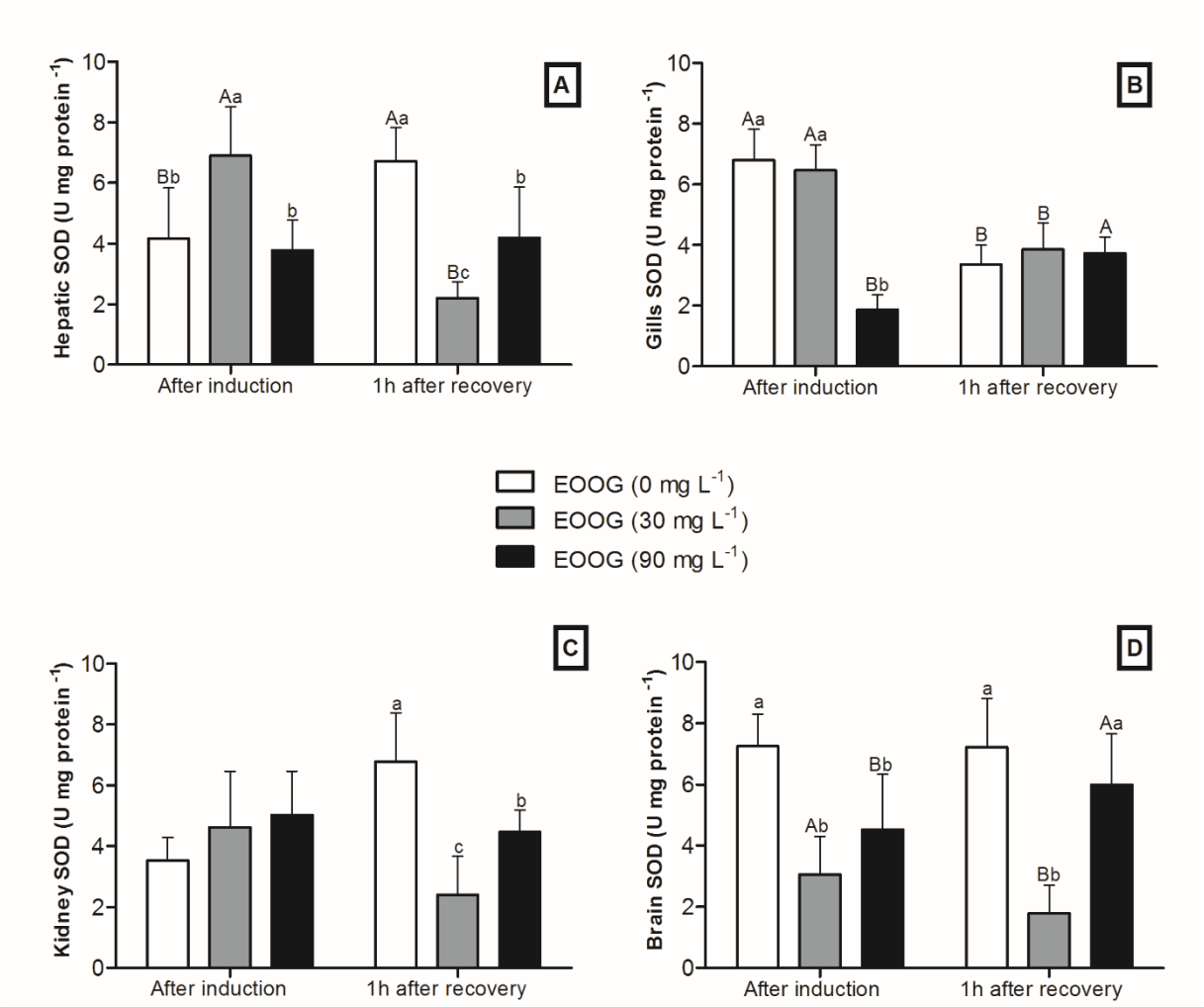

Fig 4 - Superoxide dismutase (SOD) of liver (A), gills (B), kidney (C) and brain (D) of *Oreochromis niloticus* measured immediately after anesthetic induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after recovery. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration.

After anesthesia induction and 1 h after recovery from EOOG exposure there were no significant differences in hepatic TBARS levels among the assessed concentrations ($P > 0.05$). Between times, there was an increase in hepatic TBARS levels in fish exposed to 30 mg L^{-1} EOOG ($P < 0.05$) (Fig. 5A).

After anesthesia induction, fish exposed to 30 mg L^{-1} EOOG had significantly lower TBARS levels in the gills than those exposed to 0 mg L^{-1} EOOG ($P < 0.05$). One hour after

recovery there were no significant differences in gill TBARS levels among EOOG concentrations ($P > 0.05$). Between times there was a reduction in gill TBARS levels for fish submitted to 0 mg L^{-1} EOOG and an increase in those exposed to 30 mg L^{-1} f EOOG ($P < 0.05$) (Fig. 5B).

After anesthetic induction, no significant differences were observed among the assessed concentrations of EOOG for kidney TBARS levels ($P > 0.05$). One hour after recovery, higher kidney TBARS levels were observed for fish exposed to 30 and 90 mg L^{-1} EOOG compared to those exposed to 0 mg L^{-1} EOOG ($P < 0.05$). There was an increase in kidney TBARS levels for fish exposed to 30 and 90 mg L^{-1} EOOG one hour after recovery compared to after induction ($P < 0.05$) (Fig. 5C).

Fish exposed to 30 mg L^{-1} of EOOG had higher brain TBARS levels both after anesthesia induction and one hour after recovery than those submitted to 0 mg L^{-1} EOOG ($P < 0.05$). There were no differences in brain TBARS levels between times for all concentrations of EOOG ($P > 0.05$) (Fig. 5D).

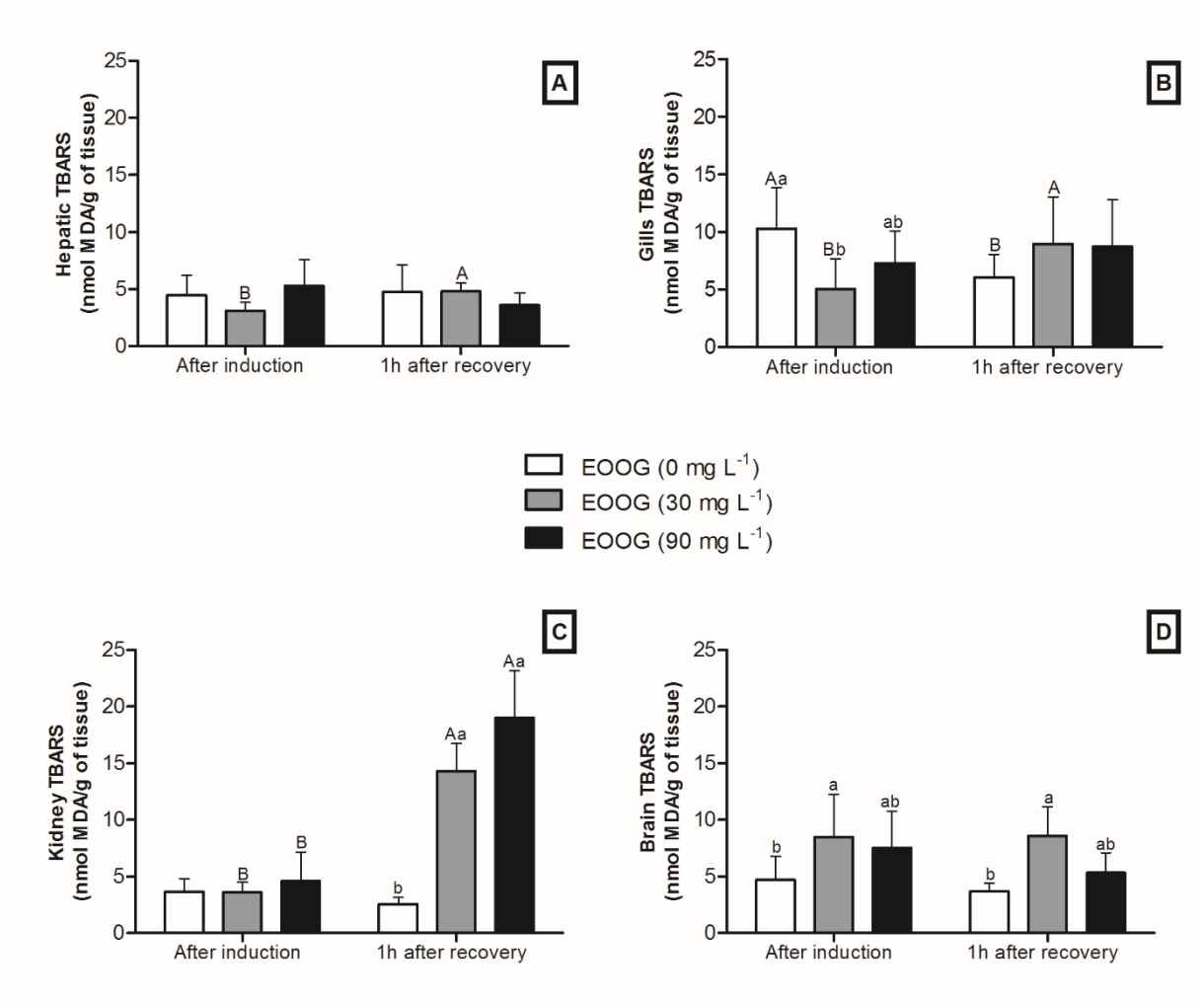

Fig 5 - Thiobarbituric acid reactive substances (TBARS) of liver (A), gills (B), kidney (C) and brain (D) of *Oreochromis niloticus* measured immediately after anesthetic induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after recovery. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by the Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration.

There were no significant differences in liver NPSH activity among EOOG concentrations after anesthesia induction and one hour after recovery, and no differences between times for any of the concentrations ($P > 0.05$) (Fig. 6A). Fish exposed to 30 and 90 mg L^{-1} of EOOG had significantly lower gill NPSH activity after anesthetic induction compared to in those exposed to 0 mg L^{-1} EOOG ($P < 0.05$). However, 1 h after recovery, fish exposed to 30 mg L^{-1} EOOG had significantly lower gill NPSH activity than did fish exposed

to 0 mg $^{-1}$ EOOG ($P < 0.05$). Between times, gill NPSH activity increased for fish exposed to 30 mg L^{-1} of EOOG and decreased for those exposed to 0 mg L^{-1} EOOG (Fig. 6B).

No significant differences were observed in kidney NPSH activity after anesthesia induction for all concentrations tested ($P > 0.05$). However, 1 h after recovery, fish exposed to 30 mg L^{-1} EOOG had the highest kidney NPSH activity, followed by those exposed to 90 mg L^{-1} EOOG. Between times there was only a significant increase in kidney NPSH activity for fish exposed to 30 mg L^{-1} EOOG ($P < 0.05$) (Fig. 6C). There were no significant differences in brain NPSH activity among EOOG concentrations assessed after anesthesia induction or between times for any of the concentrations ($P > 0.05$). However, 1 h after recovery, the highest brain NPSH activity was for fish exposed to 30 mg L^{-1} EOOG ($P < 0.05$) (Fig. 6D).

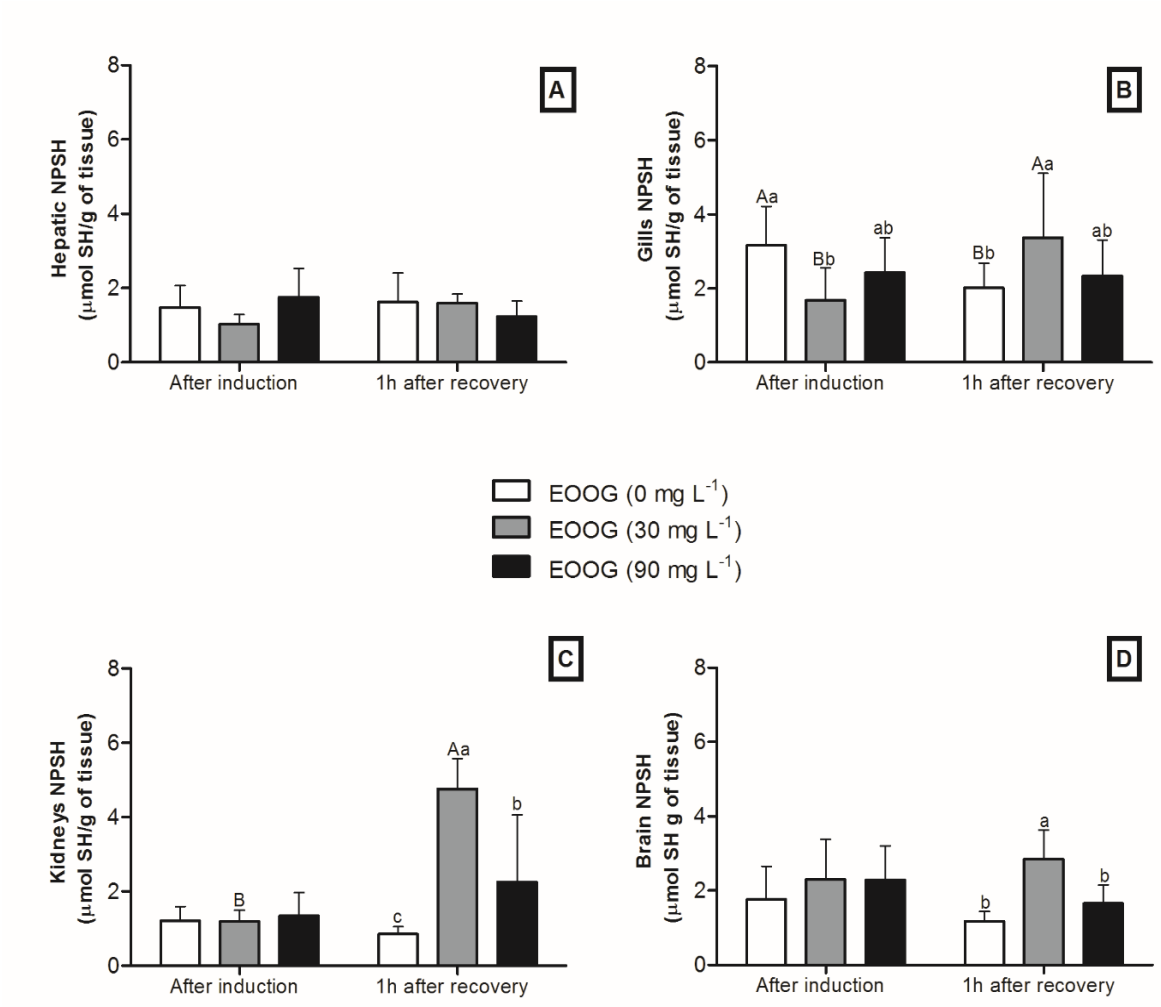

Fig 6 - Non-protein thiols (NPSH) of liver (A), gills (B), kidney (C) and brain (D) of *Oreochromis niloticus* measured immediately after anesthetic induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after

recovery. Ethanol group: 0 mg L^{-1} EOOG. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration.

After anesthesia induction, significantly lower and higher hepatic protein levels were observed in fish exposed to 30 and 90 mg L^{-1} EOOG, respectively, compared to those exposed to 0 mg L^{-1} EOOG ($P < 0.05$). One hour after recovery, the highest and lowest hepatic protein levels were for fish exposed to 30 and 0 mg L^{-1} EOOG, respectively ($P < 0.05$). Between times, there was an increase in hepatic protein levels for fish exposed to 30 mg L^{-1} EOOG and a decrease for those exposed to 0 mg L^{-1} EOOG ($P < 0.05$) (Fig. 7A).

The highest gill protein levels after anesthesia induction was for in fish exposed to 90 mg L^{-1} EOOG ($P < 0.05$). One hour after recovery, there were no significant differences in gill protein levels among EOOG concentrations ($P > 0.05$). Between the times, there was an increase in gill protein levels for fish exposed to 0 and 30 mg L^{-1} EOOG ($P < 0.05$) (Fig. 7B).

There were no significant differences among the three EOOG concentrations for kidney protein levels after anesthetic induction ($P > 0.05$). However, 1 h after recovery, fish exposed to 30 mg L^{-1} EOOG had the highest kidney protein levels ($P < 0.05$). Between the times there was an increase and a decrease in kidney protein levels for 30 and 0 mg L^{-1} of EOOG, respectively ($P < 0.05$) (Fig. 7C). Fish exposed to 30 mg L^{-1} EOOG had the highest brain protein levels, both after anesthesia induction and 1 h after recovery ($P < 0.05$). Between the times, there was an increase in brain protein level for fish exposed to 30 mg L^{-1} EOOG ($P < 0.05$) (Fig. 7D).

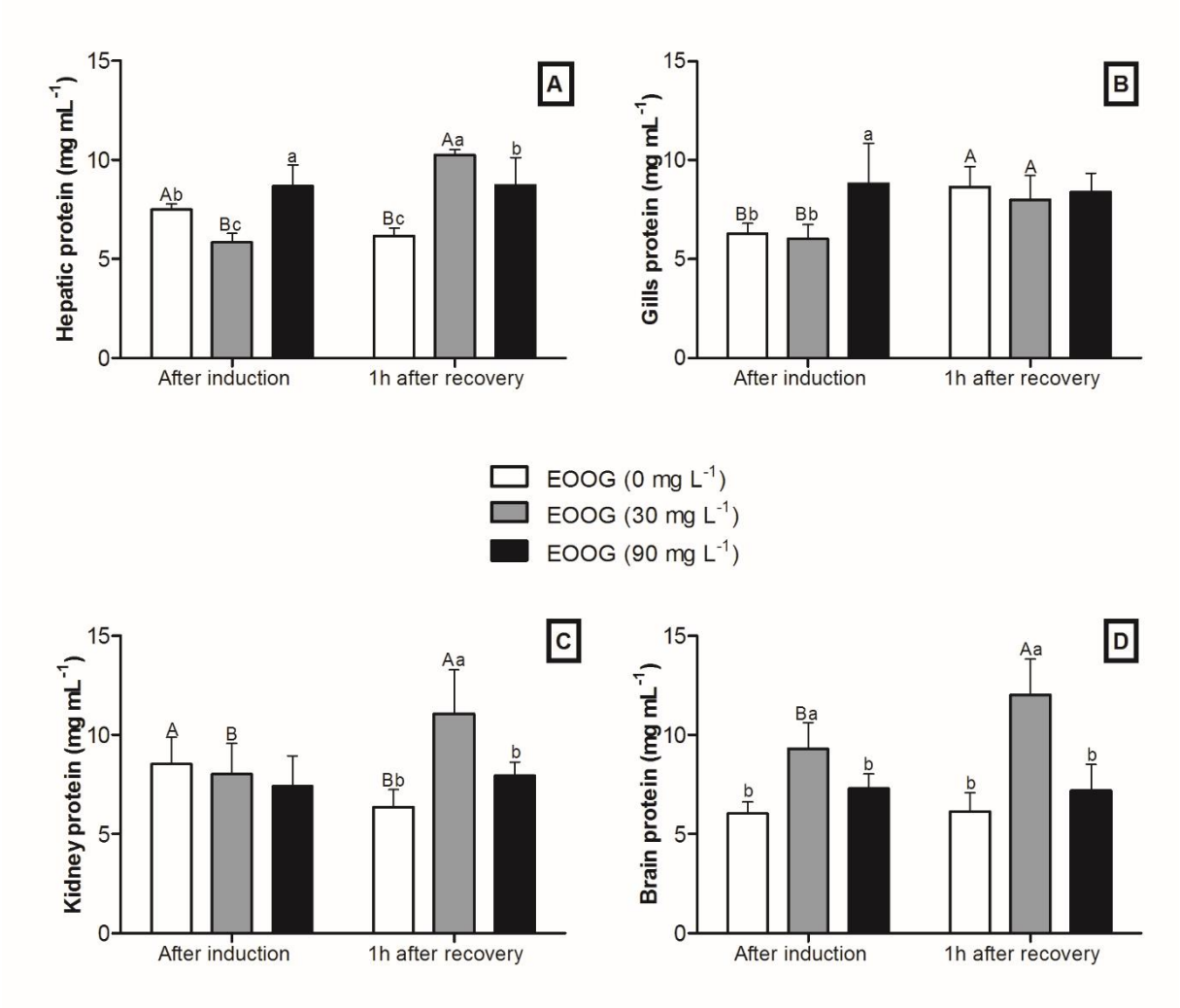

Fig 7 - Protein levels of liver (A), gills (B), kidney (C) and brain (D) of *Oreochromis niloticus* measured immediately after anesthetic induction with essential oil of *Ocimum gratissimum* (EOOG) and 1 h after recovery. Ethanol group: 0 mg L^{-1} EOOG. Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration.

Experiment III

Water quality

There were no significant differences ($P > 0.05$) in water quality variables immediately after the transport of *O. niloticus* juveniles exposed to different EOOG concentrations (Table 1).

Table 1. Water quality parameters (mean ± standard deviation) after 4.5 hours of transport of juvenile *O. niloticus* in plastic bags containing water with different concentrations of essential oil of *Ocimum gratissimum* L.

EOOG concentrations	Temperature (º C) [ns]	Dissolved oxygen (mg L^{-1}) [ns]	pH [ns]	Total ammonia (mg L^{-1}) [ns]
0 mg L^{-1} (ethanol)	25.74 ± 0.11	15.14 ± 1.58	6.49 ± 0.13	0.54 ± 0.41
5 mg L^{-1}	27.70 ± 0.21	14.10 ± 1.09	6.61 ± 0.13	0.59 ± 0.38
10 mg L^{-1}	25.68 ± 0.16	13.53 ± 1.38	6.64 ± 0.07	0.68 ± 0.43
P - values	0.7881	0.357	0.1506	0.9702

[ns] not significant

Blood biochemical and hematological parameters

Survival was 100% at 96 h post-transport and all animals resumed feeding within 24 h. After transport, fish transported with 10 mg L^{-1} EOOG had significantly higher plasma glucose levels and lower hematocrit than the basal group and the other treatments ($P < 0.05$) (Fig. 8A, B). Plasma protein was not affected by transport for any of the EOOG concentrations ($P > 0.05$) (Fig. 8C).

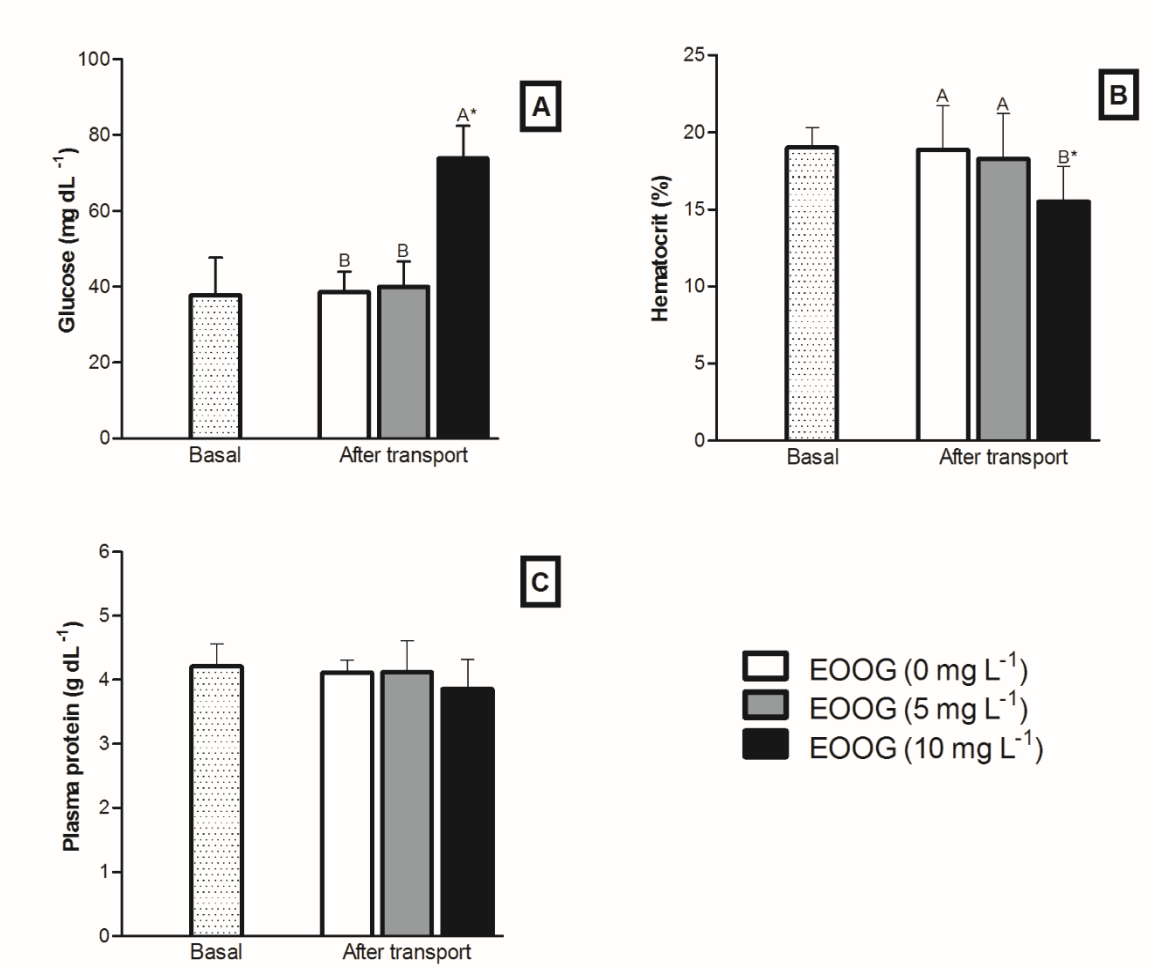

Fig 8. Biochemical and hematological parameters of *Oreochromis niloticus* measured after transport with essential oil of *Ocimum gratissimum* (EOOG). Basal group: fish not transported. Ethanol group: 0 mg L^{-1} EOOG. Plasma glucose (A), hematocrit (B) and plasma protein (C). Values (mean ± standard deviations) (N = 12 animals per treatment). The data were analyzed by two-way Anova, followed by Tukey post-test ($P < 0.05$). Different lowercase letters indicate a significant difference between concentrations at the same time (after induction or 1 h after recovery). Different capital letters indicate a significant difference between times for a given concentration. Asterisk indicates a significant difference from the basal group.

4. Discussion

EOOG proved to be efficient and safe as anesthesia and for transport of *O. niloticus*, with induction and recovery times within acceptable limits for fish and minimal influence on biochemical, hematological and water quality variables. However, the use of EOOG favored lipid peroxidation in the kidney, which indicates the possible development of oxidative stress.

No mortality was observed during anesthesia and sedation of juvenile *O. niloticus*. Netto et al. (2017) also did not observe mortality rates for this species after being subjected

different concentrations of the essential oils of *O. basilicum* and *Cymbopogum flexuosus* for anesthesia and sedation. EOOG also proved to be safe for *R. quelen* (Silva et al., 2015), *Colossoma macropomum* (Boijink et al., 2016), *B. amazonicus* (Ribeiro et al., 2016) and *O. niloticus* (Adewale et al., 2017). According to Keene et al. (1998) and Ross and Ross (2008), the ideal concentration of an anesthetic should induce anesthesia in fish in less than 180 seconds and have a recovery time of less than 300 seconds. Thus, 90 and 150 mg L^{-1} EOOG would be the most suitable concentrations for juvenile *O. niloticus* with an average weight of 45 g. Adewale et al. (2017) indicated EOOG concentrations between 60 to 100 mg L^{-1} for anesthesia of *O. niloticus* with an average weight of 20 g.

Ventilation frequency is a useful parameter for understanding the physiology of fish when using anesthetics (Alvarenga and Volpato, 1995). The juvenile of *O. niloticus* in the present study had a higher VF during anesthesia induction and recovery with 30 mg L^{-1} EOOG. Increased fish VF during induction may be related to the disturbance caused by a stressor in the water (anesthetic) (Matthews and Varga, 2012). However, after the first minutes of contact with an anesthetic, VF decreases considerably (Becker et al., 2012). Silva et al. (2019) did not establish a direct relationship between anesthetic concentration of the essential oils of *L. alba* and *L. origanoides* and VF for *C. macropomum*, with the exception of the anesthesia stage at 200 µL L^{-1} *L. origanoides* EO, which led to a lower VF than did 25 µL L^{-1}, suggesting that this relationship may be species-specific.

Plasma glucose was higher in fish anesthetized with 30 mg L^{-1} EOOG immediately after induction. Fish in stressful situations, such as biometry and transport, for example, release catecholamines and corticosteroids that activate the processes of gluconeogenesis and glycogenolysis, which mobilize and increase glucose in the bloodstream to deal with the increased energy demand (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Pankhust et al., 2011). However, the hyperglycemia observed with 30 mg L^{-1} EOOG after induction may be related to increased fish agitation and hypoxia caused during this step. A similar phenomenon was described by Hohlenwerger et al. (2016) during anesthesia of *O. niloticus* with the essential oil of *L. alba*, with the glycemia tending to gradually return to normal levels (Teixeira et al., 2017; Santos et al., 2020), as observed after 1 h of recovery in the present study.

The elevation of hematocrit in *O. niloticus* juveniles anesthetized with 90 mg L^{-1} EOOG characterizes hemoconcentration caused by osmoregulatory adjustments (Houston et

al., 1996; McDonald and Milligan et al., 1997); however, all treatments showed similar values at 1 h post-recovery. Increased hematocrit has also been observed for other species during anesthesia with eugenol (Hill and Foster, 2004; Pádua et al., 2012; Ribeiro et al., 2019).

Tissue cells maintain a balance between prooxidant and antioxidant agents in situations that do not cause discomfort to animals (Poli et al., 2012). When an imbalance occurs between these agents, the oxidative stress process begins, characterized by the formation of ROS, which cause lipid and protein oxidation (Esterbauer, 1996; Baldissera et al., 2020). At the time of anesthesia induction, fish may experience a situation similar to hypoxia due to decreased VF. During recovery from anesthesia, tissue reoxygenation occurs and may affect the synthesis of ROS in tissues (Gressler et al., 2014; Velisek et al., 2011). The handling of fish in water and water with low concentrations of ethanol can affect the prooxidant and antioxidant balance and cause tissue damage (Souza et al., 2018). In the current study, ROS was produced in the liver and kidney 1 h after recovery in *O. niloticus* anesthetized with EOOG. This increase can be understood as an aversive response of the organism to EOOG. The main compound of EOOG used in this study was eugenol (Silva et al., 2012). Despite its analgesic and antioxidant properties, the concentration of eugenol used can cause cytotoxic effects and increase ROS in tissues (Atsumi, 2015; Bezerra et al., 2017).

The increase of antioxidant enzymes in tissues is related to oxidative substances that stimulate the antioxidant defense system (Yu, 1994; Luczaj et al., 2017). SOD is one of the main enzymes involved in the clearance of ROS produced in cells (Cheeseman and Slater, 1994). This enzyme functions by converting ROS to H_2O_2 and H_2O, and then convert H_2O_2 and O_2 by the enzymatic action of catalases (CAT) and glutathione peroxidase (GPx) (Li et al., 2009; Velisek et al., 2011). The highest values for hepatic, kidney and brain SOD were observed 1 h after recovery for *O. niloticus* juveniles exposed to 0 and 90 mg L^{-1} EOOG. This increase suggests an attempt by the organism to revert to the synthesis of ROS in tissues after anesthesia with EOOG.

TBARS analysis is commonly used to assess oxidative damage, with lipid peroxidation being responsible for the loss of cell function under oxidative stress (Huang et al., 2003). Higher levels of TBARS in the kidney were observed 1 h after recovery of fish exposed to EOOG. Increased levels of TBARS in the kidney characterize the synthesis of lipid peroxidation, which indicates the possible development of oxidative stress when using EOOG to anesthetize *O. niloticus*. Recently, Souza et al. (2018) observed higher levels of

TBARS in the kidney of *R. quelen* immediately after anesthesia with *L. alba* essential oil of the linalool chemotype. However, these authors found that these values were reduced after eight hours of recovery. In addition, the levels of gill, kidney and brain NPSH and hepatic, kidney and brain protein remained elevated 1 h after the recovery of *O. niloticus* exposed to EOOG. This observation demonstrates the protection of cellular proteins against oxidation through the NPSH redox cycle or by directly detoxifying ROS caused by exposure to stressors (Ruas et al., 2008). When evaluating the effects of different chemotypes of the essential oil of *L. alba* in *R. quelen*, Souza et al. (2018) observed that the linalool chemotype was able to reduce damage to lipids and proteins in the liver and kidney; however, the same did not occur after 8 h of recovery of fish anesthetized with the citral chemotype, demonstrating that oxidative defenses were not completely capable of reducing ROS synthesis or preventing tissue damage.

The EOOG concentrations used in the present study did not lead to changes in water quality or plasma protein levels of *O. niloticus* juveniles at the end of transport. Favero et al. (2019) also did not find any differences in water quality parameters and plasma protein values for juvenile *Lophiosilurus alexandri* transported in water containing eugenol. In addition, the water quality parameters were within acceptable levels for *O. niloticus* at the end of transport (Shoko et al., 2014).

There was an increase of plasma glucose of *O. niloticus* immediately after transport with 10 mg L^{-1} EOOG. This hyperglycemia may be due to the increased energy demand and metabolic responses of fish during stressful situations (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Velisek et al., 2011). Juveniles of *C. macropomum* transported with eugenol also showed an increase in plasma glucose shortly after transport and a reduction to basal levels after 96 h of recovery (Santos et al., 2020). The lower hematocrit of *O. niloticus* transported with 10 mg L^{-1} EOOG may also be associated with acute and induced stress (McDonald and Milligan, 1997; Navarro et al., 2016) or hemodilution through osmoregulatory mechanisms (Houston et al., 1996). The use of eugenol as a sedative and anesthetic for juvenile *L. alexandri* also decreased hematocrit values (Favero et al., 2019; Ribeiro et al., 2019). However, despite these differences in glucose and hematocrit, survival after 96 hours was 100%, and the animals started to feed again within 24 hours after the end of the test.

5. Conclusions

Concentrations of 90 and 150 mg L^{-1} of essential oil of *Ocimum gratissimum* (EOOG) are considered ideal for anesthetizing *O. niloticus* juveniles with an average weight of 41 g. In spite of the use of 90 mg L^{-1} EOOG prevented elevated plasma glucose, it may have caused lipid damage to the kidneys and changes to the antioxidant defense system by increasing hepatic and brain ROS concentration and reducing brain TBARS activity. Additional studies analyzing the effect of these concentrations on different times of anesthesia and recovery will allow a better understanding of the efficacy of EOOG for *O. niloticus*. The concentration of 5 mg L^{-1} EOOG can be indicated for the transport of *O. niloticus* with an average weight of 12 g for 4.5 hours.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics approval

Three experiments were carried out at the Laboratório de Aquacultura (LAQUA) of the Escola de Veterinária (EV) of the Universidade Federal de Minas Gerais (UFMG), with approval of the Comissão de Ética no Uso de Animais (CEUA) of this institution (registration numbers 324/2018 and 42/2020).

Consent to participle

All names in author list have been involved in various stages of experimentation or writing.

Consent to publication

All authors agree with submit the paper for publication in the Journal of Fish Physiology and Biochemistry.

Availability of data and material

The data and material that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

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ARTIGO 5

Efficacy of *Hesperozygis ringens* essential oil as an anesthetic and for sedation of juvenile tambaqui (*Colossoma macropomum*) during simulated transport

Running Head

H. ringens **for anesthesia/transport of tambaqui**

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Abstract

This study evaluated the anesthetic and sedative effects of the essential oil of *Hesperozygis ringens* (EOHR) on juveniles *Colossoma macropomum*. Experiment 1 exposed juveniles (3.35 ± 0.73 g) to 0 (control), 75, 150, 300 and 450 µL L^{-1} of EOHR, with 10 replications each. Induction time showed a quadratic effect (R^2 = 0.8628), while recovery time showed a direct linear effect (R^2 = 0.7527). Concentrations between 150 and 450 µL L^{-1} EOHR are therefore recommended for use as anesthetic for *C. macropomum*. Experiment 2 evaluated 0, 5, 15 and 30 µL L^{-1} of EOHR for two periods of transport (4 and 24 h) of juveniles (1.46 ± 0.58 g) in 32 plastic bags (30 x 40 cm) containing 1 L of water, 2/3 of pure oxygen with 10 fish per bag. All EOHR treatments had lower dissolved oxygen (DO) after 24 h, while 15 and 30 µL L^{-1} EOHR had lower non-ionized ammonia after 4 and 24 h. Experiment 3 used the same procedures as Experiment 2 for 24 h of transport with juveniles (1.77 ± 0.58 g) in 16 plastic bags at the same density (10 fish L^{-1}). Concentrations of 15 and 30 µL L^{-1} EOHR had lower pH after 24 h and all EOHR treatments had lower DO. No mortality was observed. Experiment 4 tested the same concentrations of EOHR evaluated in Experiment 2 but using 16 plastic bags without fish. The concentrations of 15 and 30 µL L^{-1} EOHR had lower DO and pH after 24 h.

Key words: Anesthetic and sedative, Dissolved oxygen, Handling stress, Tambaqui, Water quality

1. Introduction

Procedures adopted in aquaculture, such as confinement, stripping of eggs and milt from brood-stock, sampling and transport, can trigger acute or chronic stress in fish (Barton 2002).

Anesthesia promotes safety for fish and handlers during different stressful management practices adopted in fish farms (Ross and Ross 2008). The effective concentrations of an anesthetic are associated with fish species and size (Ross and Ross 2008), thus prior characterization of anesthetic effectiveness is necessary (King 2009). Anesthesia induction and recovery times considered ideal for fish are up to 180 s and 300 s, respectively (Keene et al. 1998; Ross and Ross 2008).

Some essential oils, and their isolated compounds, extracted from plants are advantageous as anesthetics and/or sedatives over synthetic compounds. This is because these natural substances can mitigate the effects of stress caused by biometric handling and transport of live fish (Gressler et al. al. 2014; Braz et al. 2018; Souza et al. 2019; Brandão et al. 2021; Sousa et al. 2021), yet have the potential to be safer and more economical and environmentally friendly (Mirghaed et al. 2018; Aydın et al. 2019; Aydın and Barbas 2020). Espanta pulga, *Hesperozygis ringens*, is a native plant of South Brazil (Dolwitsch et al. 2020) whose essential oil (EOHR; pulegone 95.18% and limonene 1.28 %) (Toni et al. 2014) demonstrated anesthetic properties for the silver catfish *Rhamdia quelen* (Silva et al. 2013; Toni et al. 2015).

Tambaqui *Colossoma macropomum* is a migratory fish species native to the Amazon and Orinoco river basins (Araújo-Lima and Goulding 1998; Reis 2003). Its consumption is appreciated in Brazil, Colombia, Venezuela and Peru (Woynárovich and Anrooy 2019), with production in Brazilian being second only to that of Nile tilapia, *O. niloticus* (Peixe BR 2020). *Colossoma macropomum* is considered a rustic animal with fast growth (Araújo-Dairiki et al. 2018) and easy management (Araújo-Lima and Goulding 1998), which has made it popular for aquaculture (Gomes and Baldisserotto 2019). Given that alternative natural anesthetics are needed to promote greater safety and welfare during the various handlings of fish of commercial interest, this work aimed to analyze the effects of EOHR on anesthesia induction and recovery times and ventilatory frequency of small juveniles of *C. macropomum* and its use as a sedative in their simulated transport.

2. Material and Methods

The experiments were approved by the Ethics Committee on the Use of Animals of the Federal University of Minas Gerais (registration number 35/2021).

Anesthetic

The anesthetic used was extracted from fresh *H. ringens* leaves through a 3 h hydrodistillation using a Clevenger-type apparatus, as described by Toni et al. (2014). According to the same authors, the main components of the essential oil of *H. ringens* (EOHR) are pulegone (95.18 %) and limonene (1.28 %).

Fish maintenance

Juveniles of *C. macropomum* (with homogeneous weights, weighed during each experiment) were kept in 42-L tanks, at stocking density of 10 juvenile per tank, in a recirculating aquaculture system (RAS) for two weeks prior to all experiments. The fish were fed commercial feed (Supra, Brazil; size 1.7 mm) with 460 g kg^{-1} crude protein, 140 g kg^{-1} mineral matter and 80 g kg^{-1} ether extract (according to the manufacturer), up to apparent satiety twice a day (08:00 and 16:00 h). Drilling water was used and maintained at a temperature of 28.53 ± 0.34 ºC, with a pH of 7.23 ± 0.22 (Hanna HI98130 multiparameter probe), dissolved oxygen level of 4.71 ± 0.15 mg L^{-1} (EcoSense® DO200A oximeter) and non-ionized ammonia of 0.0028 ± 0.0015 mg L^{-1} (Alfakit Labcon Test colorimetric kit). Two water changes (50% of the RAS useful volume) were made each week. The fish were fasted for 24 h prior the start of each of the three experiments.

Experiment 1 – Anesthetic effect of EOHR for *C. macropomum* juveniles

Fifty juveniles of *C. macropomum* (total length 5.83 ± 0.54 cm, weight 3.35 ± 0.73 g) allocated in five tanks at a density of 10 fish per tank were submitted to the following concentrations of EOHR: 0 (control - only alcohol at the concentration used as diluent), 75, 150, 300 and 450 µL L^{-1}, according to Toni et al. (2014). The EOHR was previously diluted in 5 mL of ethyl alcohol (99.8% purity) (Ferreira et al. 2021). The fish were distributed in a completely randomized design (n = 10 fish for each concentration), and the control group was exposed to the same volume of ethyl alcohol (Ribeiro et al. 2015; Ferreira et al., 2021). Each fish was considered a replicate (n = 10 for each concentration). For induction, each fish (one

at a time) was allocated to a continuously aerated 1-L beaker containing water from the RAS and pre-established EOHR concentrations. Control-treated fish were observed for 10 min during anesthesia induction and for another 5 min during recovery. Anesthesia induction and recovery times were measured with a digital chronometer (Taksun Ts1809). The behavioral characteristics of deep anesthesia used in this test consisted of total loss of consciousness and absence of swimming (Small 2003; Ross and Ross 2008). Ventilatory frequency (VF) was directly counted during anesthesia induction (starting at the first contact with the anesthetic solution) through visualization and use of a manual counter, following Alvarenga and Volpato (1995) with modifications. After deep anesthesia, the animals underwent biometry (duration of 30 s), during which weight was measured using a Mars AD5002 analytical balance and total length with a ruler. Each fish was then placed in another 1-L beaker, containing water from the RAS without anesthetic, to assess anesthesia recovery time and count VF during recovery. Fish behavior indicating recovery consisted of the return of complete swimming balance (Small 2003; Ross and Ross 2008). The VF of fish of the control group was also measured during the observation of anesthesia recovery.

After the tests, fish of each anesthetic concentration were grouped and relocated in their respective original tanks (42-L) in the RAS to observe the return of feeding and record survival up to 96 h post-recovery. During this period, the animals were fed twice a day until apparent satiety and were observed for a period of 30 min (small amount of fish) to visualize the search and ingestion of food.

Experiment 2 – Effects of EOHR added to transport water for *C. macropomum* juveniles

Twenty-four hours before simulated transport, the temperature of the RAS (fish maintenance) was gradually reduced over four hours and then kept at 26.32 ± 0.07 ºC, with a pH of 7.47 ± 0.08, dissolved oxygen of 4.49 ± 0.32 mg L^{-1}, and non-ionized ammonia of 0.003 ± 0.0009 mg L^{-1}. Clean water, with a temperature of 26.47 ± 0.09 ºC, dissolved oxygen of 4.68 ± 0.24 mg L^{-1}, pH of 6.72 ± 0.12 and non-ionized ammonia of 0.0002 ± 0.0004 mg L^{-1}, was used to fill the plastic bags for transport.

A total of 320 juveniles of *C. macropomum* (total length 4.29 ± 0.44 cm, weight 1.46 ± 0.58 g) allocated in 32 tanks of the RAS at a density of 10 fish per tank were transferred to

32 plastic bags (30 x 40 cm) containing 1 L of water, 2/3 of pure oxygen and respective concentration of EOHR. The bags contained EOHR concentrations of 0 (control - only water), 5, 15 or 30 µL L^{-1}, and were subjected to simulated transport for two independent time periods (4 and 24 h) in a completely randomized design, with four replicates (bags) each. The EOHR was previously diluted in 5 mL of ethyl alcohol (99.8% purity). The plastic bags were inflated with pure oxygen and sealed with elastic material at 12:00 pm. For the transport simulation, the closed plastic bags were randomly allocated among polystyrene boxes and shaken every 30 min during the day (until 6 pm on the same day and from 8 am on the following day) to simulate the disturbance that occurs during the transport of live fish. After the simulated transport time periods, the respective plastic bags were opened and the water quality parameters (temperature, dissolved oxygen, pH and non-ionized ammonia) were immediately measured and animal survival determined. Ammonia excretion during transport was calculated following Garcia et al. (2015).

After the experiments, fish of each anesthetic concentration (EOHR) and time-period of simulated transport were grouped and relocated in their respective original tanks in the RAS (identified) to observe the return of feeding and record survival up to 96 h post-simulated transport. Assessment of appetite return and survival was performed as described in Experiment 1.

Experiment 3 – New simulated 24-hour transport of *C. macropomum* juveniles with different concentrations of EOHR

Based on the results of Experiment 2, in which low levels of dissolved oxygen were observed after 24 hours of transport with EOHR (see results), another 24-hour simulated transport was performed. Clean water with a temperature of 26.25 ± 0.07 ºC, dissolved oxygen of 4.68 ± 0.24 mg L^{-1}, pH of 6.84 ± 0.09 and non-ionized ammonia of 0.0002 ± 0.0008 mg L^{-1} was used to fill the plastic bags for transport. A total of 160 juveniles of *C. macropomum* (total length 4.67 ± 0.38 cm, weight 1.77 ± 0.58 g) distributed in 16 tanks at a density of 10 fish per tank were transferred to 16 plastic bags (30 x 40 cm), containing 1 L of water, 2/3 of pure oxygen and respective concentration of EOHR, at the same density (10 fish L^{-1}). The fish were randomly distributed among the plastic bags containing the EOHR concentrations of 0 (control - only water), 5, 15 and 30 µL L^{-1}, in a completely randomized

design with four replicates (bags). The same procedures described in Experiment 2 were adopted.

Experiment 4 – Effects of EOHR on water physicochemical parameters after 24 hours of simulated transport without fish

Based on the low dissolved oxygen levels observed in the treatments with EOHR at the end of experiments 2 and 3 (see results), a new test was performed without the use of fish to verify whether the EOHR itself has any effect on dissolved oxygen levels. This experiment evaluated the same EOHR concentrations: 0 (control - only water), 5, 15 and 30 µL L^{-1} in simulated transport water after 24 h. Clean water, with a temperature of 26.20 ± 0.08 ºC, dissolved oxygen of 4.21 ± 0.42 mg L^{-1}, pH of 7.04 ± 0.07 and non-ionized ammonia of 0.0001 ± 0.0006 mg L^{-1}, was used to fill the plastic bags for transport; 16 plastic bags (30 x 40 cm) containing 1 L of water and 2/3 of dissolved oxygen. Four concentrations of EOHR with four replicates (bags) each were distributed in a completely randomized design. The same procedures were adopted for the plastic bags as described in Experiment 2 (closing and opening, disturbance caused by simulated transport and analysis of water quality).

Statistical analysis

The data were subjected to the Shapiro-Wilk test for normality and Levene's test for homoscedasticity of variances. Parametric results (Experiment 1) were analyzed using one-way ANOVA and regression models ($p < 0.05$). Nonparametric results (Experiment 2, 3 and 4) were investigated by the Kruskal-Wallis test ($p < 0.05$). Infostat (version 2020) and R software (version 3.5.2) were used for data analysis.

3. Results

Experiment 1 – Anesthetic effect of EOHR for *C. macropomum* juveniles

No mortality was observed 96 h after the EOHR anesthesia test and all fish resumed feeding within 24 h.

Anesthesia induction time showed a quadratic effect ($R^2 = 0.8628$), with a minimum concentration at 333.0 µL L^{-1} (108 s) (Fig. 1a). Recovery time showed a direct linear effect

(R^2 = 0.7527) among EOHR concentrations and varied from 87 to 158 s (Fig. 1b). The different concentrations of EOHR did not affect VF (p > 0.05) during anesthesia induction (Fig. 1c). VF showed an inversely proportional relationship among EOHR concentrations throughout recovery (p < 0.05) and ranged from 81 to 173 opercular beats per minute (Fig. 1d).

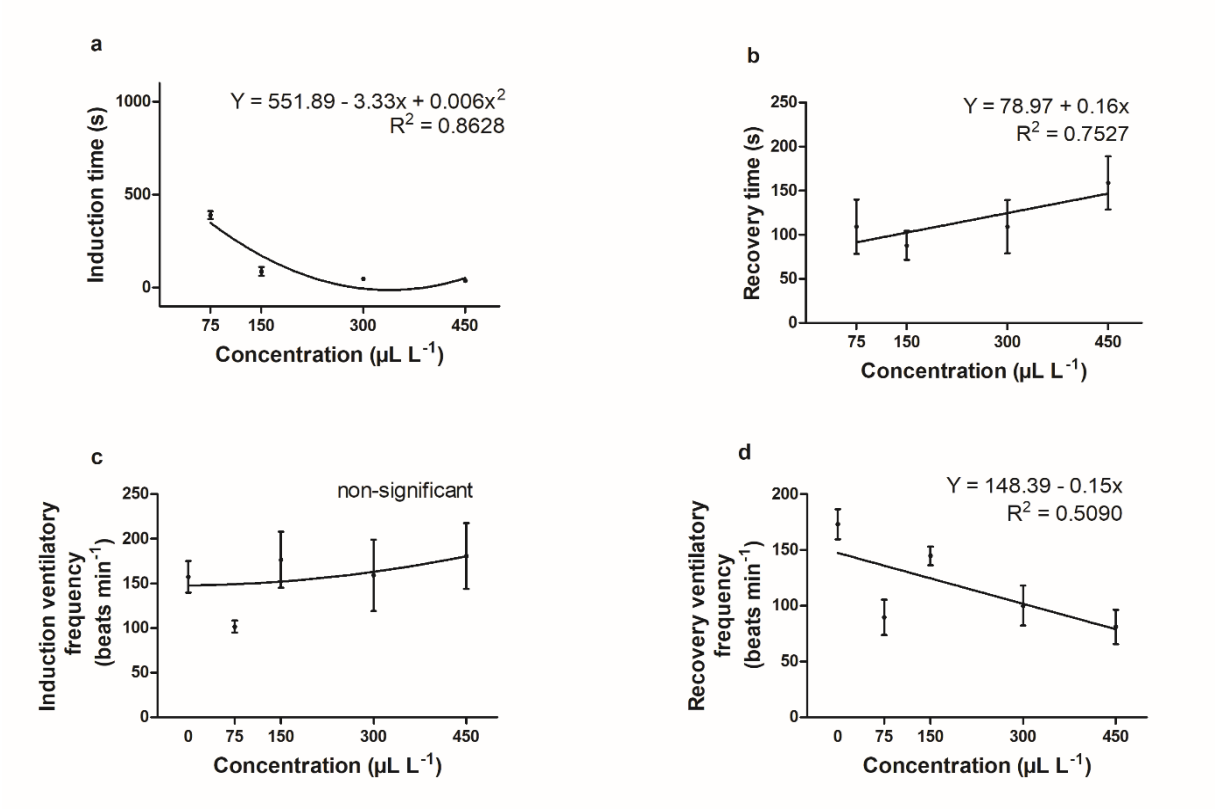

Fig 1. Anesthesia induction time (a) and recovery time (b) and ventilatory frequency during anesthesia induction (c) and recovery (d) (mean ± standard deviations) of juveniles of *Colossoma macropomum* subjected to different concentrations of essential oil of *Hesperozygis ringens* (star represents the minimum value of the equation). The equations were adjusted to the data: induction y = 551.89 - 3.33x + 0.006x^2, R^2 = 0.8628, recovery y = 8.97 + 0.16x, R^2 = 0.7527, induction ventilatory frequency (non significant), recovery ventilatory frequency y = 148.39 - 0.15x, R^2 = 0.5090.

Experiment 2 – Effects of EOHR added to transport water for *C. macropomum* juveniles

No mortality was observed 96 h after simulated transport with EOHR and all fish resumed feeding within 30 h.

After 4 h of simulated transport, the treatment with 30 µL L^{-1} EOHR had a lower water temperature than the treatments with 0 (control) and 5 µL L^{-1} EOHR (p < 0.05). Also

after 4 h of simulated transport, treatments with 15 and 30 µL L^{-1} EOHR had lower pH and non-ionized ammonia values than the control ($p < 0.05$). The treatments did not differ significantly for dissolved oxygen level after 4 h of simulated transport ($p > 0.05$) (Table 1), while all treatments with EOHR had lower ammonia excretion than the control (Fig. 2).

Table 1. Water quality parameters (mean ± standard deviation) at the end of 4 and 24 h of simulated transport of juveniles *Colossoma macropomum* in plastic bags containing only water (control) and different concentrations of EOHR.

	After 4 h of simulated transport			
Treatments	Temperature (ºC)	pH	Dissolved oxygen (mg L^{-1})	Non-ionized ammonia (mg L^{-1})
0 (control)	27.30 ± 0.08 a	7.39 ± 0.10 a	14.95 ± 1.37	0.010 ± 0.0033 a
5 µL L^{-1} EOHR	27.30 ± 0.14 a	7.21 ± 0.02 ab	12.12 ± 2.02	0.010 ± 0.0014 ab
15 µL L^{-1} EOHR	27.25 ± 0.17 ab	7.18 ± 0.03 bc	13.78 ± 1.44	0.004 ± 0.0010 b
30 µL L^{-1} EOHR	27.08 ± 0.05 b	7.15 ± 0.01 c	12.97 ± 1.62	0.002 ± 0.0006 b
P values	0.0348	0.0053	0.1127	0.0074
	After 24 h of simulated transport			
0 (control)	27.10 ± 0.08 ab	7.20 ± 0.08 a	14.15 ± 1.15 a	0.030 ± 0.0100 a
5 µL L^{-1} EOHR	27.15 ± 0.06 a	7.09 ± 0.09 ab	2.29 ± 1.46 b	0.001 ± 0.0009 ab
15 µL L^{-1} EOHR	26.95 ± 0.13 bc	6.87 ± 0.06 bc	2.18 ± 0.51 b	0.001 ± 0.000 b
30 µL L^{-1} EOHR	26.85 ± 0.17 c	6.81 ± 0.02 c	1.77 ± 0.41 b	0.001 ± 0.000 b
P values	0.0220	0.0048	0.0301	0.0055

Means followed by different letters in a column differ by Kruskal-Wallis test ($P < 0.05$).

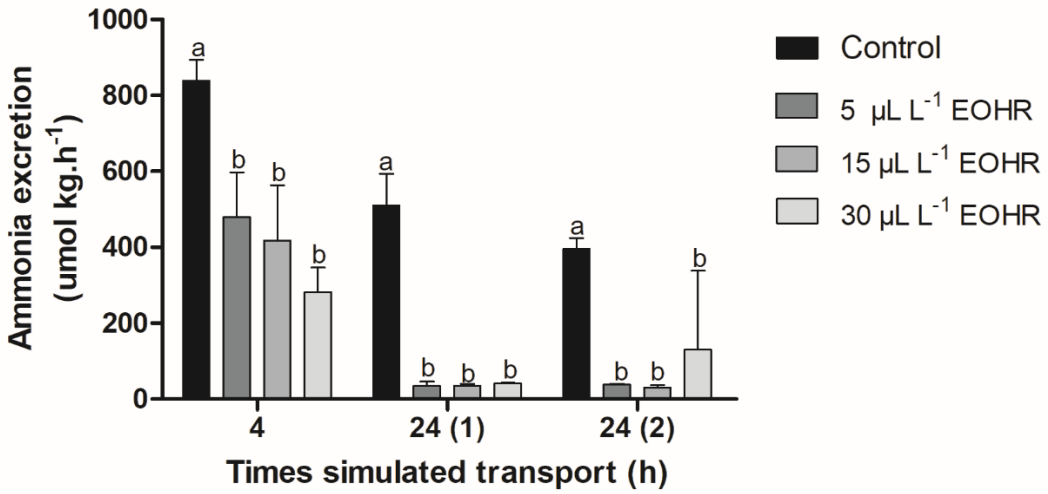

Fig 2. Ammonia excretion levels (mean ± standard deviation) for the different time periods of simulated transport of juveniles of *Colossoma macropomum* in plastic bags containing only water (control) and different concentrations of EOHR. (1) Experiment 2 and (2) Experiment 3. Means for the same time followed by different letters differ by the Kruskal-Wallis test ($p < 0.05$).

After 24 h of transport, the treatment with 30 µL L^{-1} EOHR had lower water temperature and pH values than the treatments with 0 (control) and 5 µL L^{-1} EOHR, and the treatment with 15 µL L^{-1} EOHR also had a lower pH than the control ($p < 0.05$). All treatments with EOHR had much lower dissolved oxygen levels than the control after 24 h of transport ($p < 0.05$). Also after 24 h, treatments with 15 and 30 µL L^{-1} EOHR had lower non-ionized ammonia levels than the control ($p < 0.05$) (Table 1), while all treatments with EOHR had lower ammonia excretion than the control (Fig. 2).

Experiment 3 – New simulated 24-hour transport of *C. macropomum* juveniles with different concentrations of EOHR

No mortality was observed 96 h after simulated transport with EOHR and all fish resumed feeding within 30 h.

After 24 h of transport, the treatment with 30 µL L^{-1} EOHR had higher water temperature than the treatments with 0 and 5 µL L^{-1} EOHR ($p < 0.05$), and treatments with 15 and 30 µL L^{-1} EOHR had lower pH than in the control ($p < 0.05$). As observed in Experiment

2, all treatments with EOHR had much lower dissolved oxygen levels than the control after 24 h of transport ($p < 0.05$). Also after 24 h, treatments with 15 and 30 µL L^{-1} EOHR had lower non-ionized ammonia levels than the control ($p < 0.05$) (Table 2), while treatments with EOHR had lower ammonia excretion than the control (Fig. 2).

Table 2. Water quality parameters (mean ± standard deviation) after the second simulated transport (24 h) of juveniles *Colossoma macropomum* in plastic bags containing only water (control) and different concentrations of EOHR

Treatments	Temperature (ºC)	pH	Dissolved oxygen (mg L^{-1})	Non-ionized ammonia (mg L^{-1})
0 (control)	27.28 ± 0.05 b	7.36 ± 0.15 a	14.38 ± 0.93 a	0.040 ± 0.0100 a
5 µL L^{-1} EOHR	27.33 ± 0.05 b	7.18 ± 0.08 ab	2.16 ± 0.12 b	0.003 ± 0.0005 ab
15 µL L^{-1} EOHR	27.38 ± 0.05 ab	7.08 ± 0.04 bc	2.23 ± 0.27 b	0.002 ± 0.0005 b
30 µL L^{-1} EOHR	27.58 ± 0.05 a	6.92 ± 0.05 c	2.16 ± 0.09 b	0.001 ± 0.0005 b
P values	0.0073	0.0049	0.0301	0.0051

Means followed by different letters in a column differ by Kruskal-Wallis test ($P < 0.05$).

Experiment 4 – Effects of EOHR on water physicochemical parameters after 24 hours of simulated transport without fish

After 24 h of transport, the bags without fish were opened, and the treatments with 15 and 30 µL L^{-1} EOHR had higher water temperatures than the control treatment ($p < 0.05$). As observed in Experiment 3, treatments with 15 and 30 µL L^{-1} EOHR had lower pH than did the control after 24 h of transport ($p < 0.05$), and all treatments with EOHR had lower dissolved oxygen levels than the control ($p < 0.05$). The treatments did not differ significantly for non-ionized ammonia after 24 h of transport ($p > 0.05$) (Table 3).

Table 3. Water quality parameters (mean ± standard deviation) after simulated transport (24 h) without fish containing water and different concentrations of EOHR

Treatments	Temperature (ºC)	pH	Dissolved oxygen (mg L^{-1})	Non-ionized ammonia (mg L^{-1})
0 (control)	26.47 ± 0.05 b	7.52 ± 0.09 a	14.41 ± 1.27 a	0.003 ± 0.0009
5 µL L^{-1} EOHR	26.57 ± 0.05 ab	7.23 ± 0.08 ab	7.82 ± 0.19 b	0.001 ± 0.0005
15 µL L^{-1} EOHR	26.63 ± 0.05 a	7.18 ± 0.01 b	7.67 ± 0.40 b	0.001 ± 0.0005
30 µL L^{-1} EOHR	26.63 ± 0.05 a	7.12 ± 0.04 b	7.85 ± 0.43 b	0.001 ± 0.0005
P values	0.0111	0.0076	0.0281	0.0587

Means followed by different letters in a column differ by Kruskal-Wallis test ($P < 0.05$).

4. Discussion

The EOHR promoted anesthesia and sedation of small juveniles of *C. macropomum*, with induction and recovery times considered ideal for fish. It also reduced ammonia excretion during transport, although it caused changes in water quality parameters.

No mortality was observed for *C. macropomum* juveniles during anesthesia and sedation with EOHR. Toni et al. (2014) and Toni et al. (2015) also did not observe mortality for *R. quelen* after being anesthetized and sedated with different concentrations of EOHR. Thus, EOHR concentrations between 150 and 450 μL L^{-1} can be recommended for individuals of *C. macropomum* with an average weight of 3 g; according to recommended induction and recovery times for fish anesthesia (Keene et al. 1998; Ross and Ross 2008). Toni et al. (2014) indicated 300 or 450 μL L^{-1} EOHR for *R. quelen* anesthesia with an average weight of 32 g, which indicates that the ideal concentration vary depending on the species and weight of the animals.

Evaluating the VF of fish is a non-invasive method that can be useful for understanding their physiology (Alvarenga and Volpato 1995; Becker et al. 2017; Aydin and Barbas 2020). Hyperventilation and increased oxygen consumption usually occur in fish at the beginning of anesthesia, and can be due to the detection of anesthetics in the water (Summerfelt and Smith 1990). Nevertheless, hypoventilation and, consequently, reduced oxygen consumption by fish may result from anesthesia (Houston et al. 1971). The VF of *C. macropomum* during recovery decreased with increasing EOHR concentration. Toni et al. (2014) also observed a reduction in VF for *R. quelen* when anesthetized with 300 or 450 μL L^{-1} EOHR. However, Hajek (2010) did not observe a difference in the VF of the common carp, *Cyprinus carpio* L., during anesthesia with 0.5 mL L^{-1} tea tree oil but found a decrease in VF during the first minutes of recovery. There are reports in the literature that different VF responses of fish in relation to the use of anesthetics may be related to the specific characteristics of the species (Becker et al. 2012; Cunha et al. 2017; Becker et al. 2018; Boaventura et al. 2020; Silva et al. 2019; Ferreira et al. 2020).

Fish survival was 100% during and 96 h after transport and the evaluated EOHR concentrations did not cause deep anesthesia in the fish, which is desirable during transport. When evaluating the effect of the EO of *O. gratissimum* (0, 5 and 10 mg L^{-1}) on *L. alexandri*, Boaventura et al. (2021) also did not observe mortality during and after 24 h of transport in

plastic bags. The use of the EOs of *Nectandra grandiflora* (30 µL L^{-1}) and *Protium heptaphylum* (50 mg L^{-1}) contributed to the well-being of *C. macropomum* during 6 and 22 h of transport, respectively, and did not cause mortality (Barbas et al. 2020; Silva et al. 2020), results that suggest the possibility of using different EOs for transporting fish.

Water quality parameters are extremely important for the successful transport of live fish (Sampaio and Freire 2016). The present study found changes in water temperature after both evaluated transport periods, but the changes were very small (1 ºC or less), and values were within the acceptable limits for the cultivation of *C. macropomum* (Silva and Fujimoto 2015). These changes were not related to the presence of EOHR in the water, as the results differed between experiments.

The reduced pH of the water at the end of transport with or without fish indicates that at least part of the variation in pH was due to the addition of EOHR to the water. However, the pH of the water only ranged from 6.8 to 7.3 at the end of the transport, which are appropriate levels for raising *C. macropomum* (Silva and Fujimoto 2015).

Dissolved oxygen levels of water containing EOHR after 24 h of simulated transport of *C. macropomum* demonstrated, in both experiments, higher oxygen consumption when using this EO. Furthermore, the simulated transport without fish showed that EOHR actually consumes oxygen, which was enhanced with the presence of fish (experiments 2 and 3). Oxygen consumption by fish during long periods of transport was already expected (Silva et al. 2020). However, what may have contributed to the oxygen consumption by EOHR could have been a reaction between the major compound of this EO (pulegone) and oxygen, resulting in the production of pulegone epoxide (Elgendy and Khayyat 2008). Nonetheless, despite the low dissolved oxygen levels in the water after 24 h of transport (approximately 2 mg L^{-1}), mortality was not observed during and after 96 h, and all animals resumed feeding after the end of the tests. *Colossoma macropomum* is known for its resistance to hypoxic conditions due to its region of origin (Saint Paul 1984; Weber 1996; Affonso et al. 2002). Neves et al. (2020) recently reported that *C. macropomum* was able to survive for three days at dissolved oxygen levels close to 0.92 mg L^{-1} and subsequently resume homeostasis. Thus, attention should be paid to long transports with the use of EOHR, as it can reduce the dissolved oxygen of water in plastic transport bags. The current study also indicated that, before using an EO for the transport of aquatic animals, it is important to determine whether the EO itself changes dissolved oxygen levels.

The transport of live fish in plastic bags tends to result in increased waterborne ammonia levels due to excretion by the fish (Sampaio and Freire 2016). In the present study, non-ionized ammonia levels were lower in the bags of fish transported for 4 and 24 h with 15 and 30 µL L^{-1} EOHR than in the control, and fish from all treatments with EOHR had lower ammonia excretion. This result indicates a possible reduction in animal metabolism. Likewise, a mixture of tea tree and clove EOs (10.4 mg L^{-1}) decreased ammonia levels in the water of *C. macropomum* transported for 36 h in plastic bags (Santos et al. 2020), while the use of 12.5 and 25 µL L^{-1} EO of *A. tryphilla* in the transport water of *L. alexandri* for 3 h also reduced ammonia excretion (Becker et al. 2017). These results indicate the efficiency of the use of these EOs to reduce animal metabolism during transport.

5. Conclusions

EOHR proved to be effective for anesthesia and sedation for short periods of transport (4 h) of juveniles of *C. macropomum*. Concentrations between 150 and 450 µL L^{-1} EOHR are recommended for *C. macropomum* at 3 g, as they promote anesthesia with induction and recovery times considered ideal for fish. Concentrations of 15 and 30 µL L^{-1} EOHR were able to reduce non-ionized ammonia levels during transport of *C. macropomum* at 2 g; however, its use in a closed system (i.e., plastic bag) for long periods should be avoided (24 h) as it reduces dissolved oxygen levels.

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Availability of data and material

The data and material that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

Declarations

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

Consent to participle

All names in the author list have been involved in various stages of experimentation and/or writing.

Consent for publication

All authors agree to submit the paper for publication in the journal Aquaculture International.

Conflict of interest

The authors declare no competing interests.

6. References

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CONSIDERAÇÕES FINAIS

Os resultados obtidos proporcionaram informações importantes sobre a concentração ideal de diferentes anestésicos para peixes de água doce em situações de manipulação, como biometria e transporte. Os anestésicos avaliados foram eficazes e seguros para as espécies estudadas de acordo com os parâmetros analisados. Contudo, para alguns dos anestésicos testados são necessárias análises eletroencefalográficas ou relacionadas à ativação/expressão de receptores de neurotransmissores para comprovação efetiva do seu efeito anestésico. Além disso, a utilização de novas tecnologias, como a nanotecnologia, é de suma importância para o desenvolvimento de uma piscicultura sustentável. Esta forma de aplicação de anestésico na aquicultura (nanoemulsão), pode contribuir na maior estabilidade dos compostos bioativos dos fármacos, melhorar sua solubilidade e disponibilidade, bem como aprimorar sua eficiência; garantindo assim maior segurança e bem-estar dos animais durante situações de manipulação.

Novos estudos de anestésicos para peixes devem investigar a morfologia e histologia de tecidos; e sobretudo avaliar o desempenho zootécnico dos animais após os testes de anestesia.