UNIVERSIDADE FEDERAL DE MINAS GERAIS – UFMG
ESCOLA DE VETERINÁRIA
PÓS-GRADUAÇÃO EM ZOOTECNIA

USO DO ÓLEO ESSENCIAL DE *Ocimum gratissimum* L **DURANTE ANESTESIA E TRANSPORTE DO PACAMÃ** *Lophiosilurus alexandri***: HEMATOLOGIA, BIOQUÍMICA E ESTRESSE OXIDATIVO**

TÚLIO PACHECO BOAVENTURA

Belo Horizonte
Escola de Veterinária – UFMG
2022

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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 título de Doutor em Zootecnia.

Área de concentração: Produção Animal/Aquacultura.

Orientador: Prof. Dr. Ronald Kennedy Luz

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Às 09:00 horas do dia 17 de fevereiro de 2022, reuniu-se, remotamente, a Comissão Examinadora de Tese, indicada pelo colegiado no dia 28/01/2022, para julgar, em exame final, a defesa da tese intitulada: **USO DO ÓLEO ESSENCIAL DE** *Ocimum gratissimum* L **DURANTE ANESTESIA E TRANSPORTE DO PACAMÃ** *Lophiosilurus alexandri*: **HEMATOLOGIA, BIOQUÍMICA E ESTRESSE OXIDATIVO**, como requisito final para a obtenção do Grau de **Doutor em Zootecnia, área de concentração Produção Animal - Aquacultura**
Abrindo a sessão, o Presidente da Comissão, Prof. Ronald Kennedy Luz, após dar a conhecer aos presentes o teor das Normas Regulamentares da Defesa de Tese, passou a palavra ao (a) candidato (a), para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa do candidato (a). Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento da tese, tendo sido atribuídas as seguintes indicações:

	Aprovada	Reprovada
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Prof.(a)/Dr.(a) Rodrigo Takata	x	
Prof.(a)/Dr.(a) Glauber David Almeida Palheta	x	
Prof.(a)/Dr.(a) Ronald Kennedy Luz	x	

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Belo Horizonte, 17 de fevereiro de 2022.

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“Impossível é uma palavra muito grande que
gente pequena usa pra tentar nos oprimir”
Pregador Luo e Chorão (Charlie Brown Jr)

RESUMO

Anestésicos vêm sendo amplamente utilizados na aquacultura. Entretanto, seu uso também pode induzir algumas respostas indesejáveis que devem ser verificadas antes de ser utilizado em determinada espécie. Nesse estudo foi verificado o uso do óleo essencial de *Ocimum gratissimum* (OEOG) como anestésico durante o manejo e transporte para juvenis de *Lophiosilurus alexandri* durante o manejo e transporte. Para isso, foram realizados três experimentos. No primeiro foi avaliado os intervalos de indução e recuperação do *L. alexandri* submetidos a diferentes concentrações do OEOG. No experimento 2, foi verificado o efeito das concentrações de: 0, 10 e 90 mg/L do OEOG sobre os parâmetros sanguíneos e os danos teciduais durante a indução e 1 hora após a recuperação. Já, no experimento 3, foram utilizadas as concentrações de 0, 5 e 10 mg/L do OEOG durante o transporte do *L. alexandri* e avaliados os parâmetros de qualidade da água, sanguíneos e danos teciduais. No experimento 1, o aumento da concentração do OEOG proporcionou menor tempo para indução e maior para a recuperação. Os animais anestesiados com 90 e 150 mg/L do OEOG apresentaram tempo de indução e recuperação dentro dos intervalos recomendados pela literatura. No experimento 2, foi observado que o uso de 90 mg/L do OEOG evitou níveis mais elevados de glicose e cortisol plasmático 1 hora após o manejo. Entretanto, após a indução e uma hora após a recuperação, os animais anestesiados com 90 mg/L do OEOG apresentaram concentrações mais elevadas de espécies reativas de oxigênio (EROS) no fígado e no cérebro. Os juvenis anestesiados com 10 mg/L do OEOG só apresentaram o aumento das EROS hepática 1 hora após a recuperação da anestesia. Entretanto, houve resposta do sistema de defesa antioxidante, sendo observado aumento da enzima glutatioana S-transferase cerebral e hepática. No experimento 3 foi verificado que a adição do OEOG na água reduziu o metabolismo dos juvenis, garantindo maiores concentrações de oxigênio dissolvido e menores níveis de amônia na água após o transporte. Os juvenis transportados com 10 mg/L de OEOG, também apresentaram batimentos

operculares reduzidos, menores níveis de hemoglobina, aspartato aminotransferase e glicose. Em relação aos parâmetros de estresse oxidativo, o uso de 10 mg/L de OEOG reduziu os níveis de oxidação lipídica e proteica nos tecidos, evitando a formação de hidroperóxido lipídico e proteína carbonila. Conclui-se que OEOG é um bom anestésico para ser utilizado durante o manejo e o transporte do *L. alexandri*. O uso do OEOG durante o manejo e o transporte do *L. alexandri* evita o aumento dos níveis de glicose e cortisol plasmático sem causar danos teciduais e grandes influencias nos demais parâmetros sanguíneos. Portanto, recomendamos o uso de 90 mg/L do OEOG para o manejo de biometria e de 10 mg/L do OEOG para o transporte de juvenis de *L. alexandri*.

Palavras chaves: Bem-estar, anestesia, peixes, manejo, transporte

ABSTRACT

Anesthetics have been widely used in aquaculture. However, its use can also induce some undesirable responses that must be verified before being used in a specific species. In this study, the use of essential oil *Ocimum gratissimum* (EOOG) was verified as an anesthetic for juveniles of *Lophiosilurus alexandri* during handling and transport. Three experiments were carried out. In the first, the induction and recovery intervals of *L. alexandri* submitted to different EOOG concentrations were evaluated. In experiment 2, the effect of EOOG concentrations of: 0, 10 and 90 mg/L on blood parameters and tissue damage during induction and 1 hour after recovery was verified. In experiment 3, concentrations of 0, 5 and 10 mg/L of EOOG were used during the transport of *L. alexandri*, and the parameters of water quality, blood and tissue damage were evaluated. In experiment 1, the increase in EOOG concentration provided less time for induction and more time for recovery. Animals anesthetized with 90 and 150 mg/L of the EOOG showed induction and recovery times within the intervals recommended by the literature. In experiment 2, it was observed that the use of 90 mg/L of EOOG prevented the highest levels of glucose and plasma cortisol 1 hour after handling. However, after induction and one hour after recovery, animals anesthetized with 90 mg/L of EOOG showed higher concentrations of reactive oxygen species (ROS) in the liver and brain. Juveniles anesthetized with 10 mg/L of EOOG only presented an increase in hepatic ROS 1 hour after recovery from anesthesia. However, there was a response from the antioxidant defense system, with an increase in the cerebral and hepatic glutathione S-transferase enzyme. In the third experiment, it was verified that the addition of EOOG in the water reduced the metabolism of the juveniles, guaranteeing higher concentrations of dissolved oxygen and lower levels of ammonia in the water after transport. Juveniles transported with 10 mg/L of EOOG, also presented reduced opercular beats, lower hemoglobin levels, aspartate aminotransferase and glucose. Regarding the oxidative stress parameters, the use of 10 mg/L of EOOG reduced the levels of lipid and protein oxidation in the tissues, preventing the formation of lipid hydroperoxide and carbonyl

protein. It is concluded that EOOG is a good anesthetic to be used during the handling and transport of *L. alexandri*. The use of EOOG during the handling and transport of *L. alexandri* prevents the increase in the levels of glucose and plasma cortisol without causing tissue damage and major influences on the other blood parameters. Therefore, we recommend the use of 90 mg/L of EOOG for the management and 10 mg/L of EOOG for the transport of juveniles of *L. alexandri*.

Key words: Welfare, anesthesia, fish, handling, transport.

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LISTA DE SIGLAS, ABREVIATURAS E SÍMBOLOS

°C	Graus Celsius
%	Porcentagem
ANOVA	Análise de variância
ALT	Alanina aminotrferaseans
AST	Aspartato aminotransferase
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CAT	Catalase
CEUA	Comissão de Ética no Uso de Animais
Cm	Centímetros
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
EROS	Espécies reativas de oxigênio
G	Gramas
GPx	Glutationa peroxidase
GST	Glutationa S-transferase
L	Litros
Mg	Miligramas
MS-222	Tricaína metano sulfonato
PPT	Proteína plasmática total
OEOG	Óleo essencial de *Ocimum gratissimum*
O_2	Oxigênio
O_2^-	Radical ânion superóxido ($O_2^{-\cdot}$)
·OH	Radical hidroxila
H_2O	Água

H_2O_2	Peroxido de hidrogênio
Ph	Potencial hidrogeniônico
SOD	Superóxido dismutase

SUMÁRIO

1. INTRODUÇÃO

Durante o ciclo de produção na piscicultura, os peixes estão acondicionados a diversas situações de manejo que causam o estresse, tais como: biometria, captura e transporte (Bacchetta et al., 2020; Kanani et al., 2013; Montero et al., 1999). O estresse gerado por essas situações pode impactar diretamente no desempenho produtivo, causando redução do crescimento, resposta imunológica e conversão alimentar (Barton, 2002). Além disso, esses manejos também podem causar danos epiteliais, perda de muco e escamas que podem contribuir para proliferação de bactérias, fungos e parasitas (Ross et al., 2008).

Nesse contexto, os anestésicos, sejam naturais ou sintéticos, vem sendo amplamente utilizados na piscicultura para promover o bem-estar dos animais, facilitar o manejo e preservar a integridade física dos trabalhadores (Ross et al., 2008). Além disso, essas substâncias também são utilizadas para reduzir a deterioração da água durante o transporte (Parodi et al., 2014). Apesar dessas vantagens o uso dos anestésicos também pode induzir algumas respostas indesejáveis (Kanani et al., 2013).

Recentemente, o *Ocimum gratissimum* (OEOG) mostrou potencial para ser utilizado como anestésico em peixes (Benovit et al., 2012; Boijink et al., 2016; Ribeiro et al., 2016; Silva et al., 2012; Silva et al., 2015). No entando, estudos sobre o uso do OEOG durante o transporte de peixes e suas influencias sobre os danos aos tecidos são raros, e mais estudos devem ser realizados.

Neste sentido, o objetivo desse trabalho foi verificar o uso do OEOG como anestésico durante o manejo e o transporte de juvenis *L. alexandri* e suas influências sobre os parâmetros sanguíneos e de estresse oxidativo.

2. REVISÃO DE LITERATURA

2.1. Estresse em peixes

Segundo Barton (2002), as respostas ao estresse em peixes são um evento integrado, mas podem ser classificadas como respostas primárias, secundárias e terciárias. A resposta primária, também conhecida como neuroendócrina, corresponde à ativação dos centros cerebrais e a liberação de catecolaminas (adrenalina e noradrenalina) e corticosteroides (cortisol) na corrente sanguínea. A resposta secundária é caracterizada pelos efeitos causados pela ação desses hormônios que podem levar ao aumento dos batimentos cardíacos, consumo de oxigênio, distúrbios osmorregulatórios e mobilização de substratos de energia. Já, a resposta terciaria é baseada nas alterações decorrentes das respostas primárias e secundárias, podendo afetar toda uma população, causando redução do crescimento, reprodução e resposta do sistema imunológico (Barton, 2002; Pickering e Pottinger, 1989). Dessa forma, o monitoramento dessas respostas vem sendo frequentemente utilizado como indicativo de estresse em peixes.

2.1.2. Marcadores de estresses

Devido às alterações causas pelo estresse, a hematologia e bioquímica vem sendo frequentemente utilizadas como indicadores de estresses em peixes (Célia et al., 2012; Lusková et al., 2002; Pickering e Pottinger, 1989; Silva et al., 2012).

2.1.3. Marcadores hematológicos

O sangue é responsável por distribuir calor, auxiliar na regulação osmótica (Russo et al., 2008), transportar gases respiratórios, nutrientes, produtos de excreção e anticorpos (Junior et al., 2010). Os eritrócitos são as células mais abundantes da corrente sanguínea dos peixes e são responsáveis por transportar oxigênio e gás carbônico através da hemoglobina (Faenge, 1994). A porcentagem de volume ocupado na corrente sanguínea pelos eritrócitos pode ser dada através da mensuração do hematócrito (HTC) (Goldenfarb et al., 1971). Em condições de estresse, hipóxia (Moraes et al., 2002) e esforço físico aumentado (Acerete et al., 2004), os peixes podem aumentar a concentração de eritrócitos, hematócrito e hemoglobina como uma resposta fisiológica para melhorar a oxigenação do sangue (Montero et al., 1999).

2.1.4 Marcadores bioquímicos

O cortisol é o principal corticosteroide liberado na corrente sanguínea dos peixes durante o estresse primário e vem sendo frequentemente utilizado como indicador de estresse em diversas pesquisas (Barton, 2002; Pickering e Pottinger, 1989; Silva et al., 2015; Wendelaar. 1997). A liberação das catecolaminas e do cortisol na corrente sanguínea causam diversas alterações hematológicas, metabólicas e hidrominerais no organismo do animal, que também podem ser utilizadas como indicadores de estresse (Parodi et al., 2014).

Devido ao aumento dos batimentos cardíacos e consumo de oxigênio durante o estresse, o organismo do animal sente a necessidade de aumentar a produção de energia para suprir essa nova demanda energética (Velisek et al., 2011). Isso faz com que ocorra uma grande mobilização de reservas energéticas e, consequentemente, o aumento dos níveis de glicoses na corrente sanguínea (Wells e Pankhurst, 1999).

As enzimas aspartato aminotransferase (AST) e alanina aminotransferase (ALT) são indicadoras do estado de saúde dos tecidos. A ALT está diretamente relacionada às funções hepáticas e a AST a diversos tipos de tecidos (Taheri et al., 2018). Além disso, essas enzimas também podem ser utilizadas como indicadores de estresses, pois estão diretamente envolvidas na quebra de proteínas para a mobilização de aminoácidos via gliconeogênese para a produção de glicose (Chatterjee et al., 2006).

A concentração de proteína plasmática total (PPT) exerce um papel importante na regulação osmótica do sangue e pode ser utilizada como um indicativo de distúrbios osmorregulatórios (Verdegem et al., 1997) e estresse físico e nutricional (Wells e Pankhurst, 1999; Wood et al., 1983). Quando submetido ao estresse, a liberação de adrenalina na corrente sanguínea causa o aumento da permeabilidade do epitélio, gerando um desequilíbrio osmorregulatório dos animais (Mazeaud et al., 1977). Esse desequilíbrio pode desencadear alteração no volume do plasma no sangue, que pode levar ao aumento da concentração ou diluição das PPT (Mazeaud et al., 1977).

2.2. Estresse oxidativo

O oxigênio é vital para manutenção da vida terrestre e aquática. Grande parte do oxigênio consumido é direcionado para a oxidação de compostos orgânicos e produção de energia. No entanto, uma parte do oxigênio consumido é reduzido e transformado em uma série de compostos oxidativos, denominados espécies reativas de oxigênio (EROS) (Comhair e Erzurum, 2002).

Em condições normais, as células dos tecidos mantém um equilíbrio entre os agentes oxidantes e antioxidantes (Poli et al., 2012). Entretanto, diversas situações podem causar o desequilíbrio desse sistema, aumentando a concentração de EROS nas células causando uma situação denominada de estresse oxidativo (Lackner, 1998; Barbas et al., 2017).

2.2.1. Espécies reativas de oxigênio (EROS)

Os EROS são moléculas de vida curta, altamente reativas e são formadas por sucessivas reduções do oxigênio (O_2), que inclui a formação de radicais livres, como: radical ânion superóxido ($O_2^{-\cdot}$), radical hidroxila ($^{\cdot}OH^-$) e não radicais, como o peróxido de hidrogênio (H_2O_2) (Halliwell, 1989). A presença de elevadas concentrações das EROS nas células, podem causar diversos danos teciduais como a oxidação lipídica e protéica (Stadtman e Levine, 2003). A proteína carbonila (Stadtman e Levine, 2003) e o hidroperóxidos de lipídio (Esterbauer, 1996) são produtos originados da degradação protéica e lipídica, respectivamente, sendo frequentemente utilizadas como marcadores de estresse oxidativos (Esterbauer, 1996; Stadtman e Levine, 2003).

2.2.2. Sistema de defesa antioxidante

O sistema de defesa antioxidante é composto por substâncias enzimáticas e não enzimáticas, que são capazes de prevenir ou reduzir os danos oxidativos causados pelas EROS (Halliwell, 1989; Hidalgo et al., 2002; Khalil et al., 2017). A enzima superóxido dismutase (SOD) é uma das principais enzimas responsáveis pela eliminação das EROS produzidas nas células (Cheeseman e Slater, 1994). Essa enzima transforma o radical superóxido ($O_2^{-\cdot}$) em peroxido de hidrogênio (H_2O_2) e água (H_2O), com posterior conversão de H_2O_2 em H_2O e O_2 pela ação das enzimas catalase (CAT) e glutationa peroxidase (GPx) (Li et al., 2009; Velisek et al., 2011). A enzima glutationa S-transferase (GST) é uma enzima envolvida no processo de defesa antioxidante e desintoxicação do organismo de compostos endógenos e exógenos (xenobióticos) (Frova, 2006).

2.3. Anestesia em peixes

Os peixes podem ser anestesiados de três formas: inalação, injeção e aspersão branquial. A anestesia por inalação é o método mais simples e utilizado nas atividades rotineiras da aquacultura (Ross et al., 2008). Portanto, será o método adotado nessa pesquisa e abordado nessa revisão.

Nesse método, a substância anestésica é adicionada na água, sendo facilmente absorvida pelas brânquias, difundida para o sangue e transportada até os neurônios do sistema nervoso central (Silva et al., 2012). Como a maioria dos organismos aquáticos são sensíveis a mudanças bruscas de temperatura e pH, é aconselhável realizar a aclimatação dos peixes na água que será utilizada para o preparo da solução anestésica (Zahl et al., 2012). Outro ponto importante que também deve ser levado em consideração é a utilização de aeradores na água durante a indução, uma vez que a maioria das espécies de peixes utilizam o oxigênio dissolvido na água para a respiração (Flecknell, 2009).

Durante o procedimento de indução e recuperação, os peixes podem apresentar diferentes estágios de sedação, que são estabelecidos através de respostas comportamentais. Geralmente essas respostas são classificadas de I a IV. No estágio I ocorre a sedação leve e redução da reação a estímulos externos; no estágio II (excitatório) ocorre a perda parcial de equilíbrio e natação errática; no estágio III (anestesia) ocorre a perda total de equilíbrio e ausência de locomoção; enquanto no estágio IV (overdose) ocorre o colapso medular e morte por overdose (Small, 2003) (Tabela 1). Em um trabalho de revisão, Zahl et al. (2012) descreveram mais três subdivisões durante a anestesia (estagio III), sendo anestesia leve, anestesia cirúrgica e narcose profunda (Tabela 2).

Entretanto, esses indicadores são difíceis de serem avaliados, principalmente quando o anestésico é utilizado em concentrações mais elevadas. Devido a essa dificuldade, existem diversos autores que trabalharam com critérios mais simples para a avaliação do agente

anestésico, utilizando apenas o tempo necessário que o animal gasta para ser anestesiado (estágio III) (Tort et al., 2002; Bittencourt et al., 2012; Ostrensky et al., 2016).

Tabela 1- Respostas observadas durante os diferentes estágios de anestesia em peixes.

Estágio	Descrição	Aparência	Natação	Resposta	Respiração
I	Sedação	Desorientado Redução de reação a estímulos externos	Reduzida	Reduzida	Normal
II	Excitatória	Animado Perda parcial de equilíbrio	Aumentada e errática	Exagerada	Irregular ou aumentada
III	Anestesia	Anestesiado Perda de equilíbrio	Parada	Nenhuma	Rara ou ausente
IV	Morte	Moribundo	Parada	Nenhuma	Ausente

Fonte: adaptado de Small (2003).

Tabela 2- Subdivisões observadas durante o estágio III (sedação) da anestesia em peixes.

Estágio	Sub	Descrição	Aparência	Natação	Resposta	Respiração
III	**I**	Anestesia leve	Anestesiado	Parada	Reage a forte estímulos externos	Normal
	II	Anestesia cirúrgica	Anestesiado	Parada	Nenhuma	Rara
	III	Narcose profunda	Anestesiado	Parada	Nenhuma	Quase ausente

Adaptado de Zahl et al. (2012)

Geralmente, quando os peixes estão anestesiados (estágio III), ocorre a redução ou interrupção da ventilação das brânquias, com posterior redução dos níveis de oxigênio no sangue (Ross al et., 2008). Contudo, a anestesia nessa fase por um tempo prolongado e sem irrigação das brânquias, pode causar a morte dos animais por hipóxia (Jerez-Cepa et al., 2019). Por outro lado, durante a recuperação dos animais ocorre a reoxigenação dos tecidos, que podem aumentar a concentração de EROS nas células e provocar o estresse oxidativo (Gressler et al., 2014; Velisek et al., 2011).

A concentração e o tempo necessários para indução e recuperação dos animais podem variar em função do anestésico (Ostrensky et al., 2016), espécie (Fernandes et al., 2016) e tamanho dos animais (Ribeiro et al., 2013; Ribeiro et al., 2019; Tarkhani et al., 2017). Portanto, é necessário a sua validação antes de seu uso (King, 2009).

O anestésico ideal deve apresentar baixo custo de aquisição, não deixar resíduos na carne (Anderson et al., 1997), ser eficaz em baixas concentrações, apresentar toxicidade em dose muito superior a efetiva e não perturbar a homeostase dos animais (Keene et al., 1998). Os mesmos também devem ser capazes de induzir a anestesia (estagio III) em menos de três minutos, com um tempo de recuperação inferior a cinco minutos (Marking e Meyer, 1985).

2.3.1 Uso de anestésicos durante o manejo

Durante o manejo, os agentes anestésicos podem ser utilizados em diferentes concentrações para induzir a anestesia ou sedação dos animais. Geralmente, a sedação leve é utilizada durante os procedimentos mais simples e com baixo grau invasivo, como por exemplo, biometria, vacinação, manejo reprodutivo (Velisek et al., 2011) e transporte (King, 2009). Já, a anestesia é utilizada para procedimentos mais invasivos e dolorosos (Lepic et al., 2014), como implante de marcadores, rastreadores e cirurgias (Luo et al., 2015).

Apesar de ser utilizado para mitigar os efeitos do estresse durante o manejo, o uso dos anestésicos também pode induzir algumas respostas fisiológicas indesejáveis (Kanani et al., 2013), como alterações bioquímicas (Parodi et al., 2014), hematológicas (Abdolazizi et al., 2011; Hashimoto et al., 2016), aumento nas concentrações de glicose plasmática, cortisol (Park et al., 2008), enzimas aspartato aminotransferese (AST) (Feng et al., 2011), alanine transaminase (ALT) (Akinrotimi et al, 2018) e alterações no hematócrito (Ribeiro et al., 2019). Além disso, foram observados também danos teciduais e oxidativos em trutas-arco-íris (*Oncorhynchus mykiss*) (Velisek et al., 2011). Portanto, os efeitos do anestésico como promotor de bem estar durante o manejo é especifico para cada espécie, reforçando a necessidade de sua validação antes de seu uso.

2.3.2. Uso do anestésico durante o transporte

O transporte de peixe vivo é uma atividade rotineira em sistemas de produção aquícola. Geralmente, ele é realizado em pequenos sacos plásticos preenchidos com água e oxigênio ou em caixas de transporte contendo aeração ou injeção de oxigênio comprimido. Durante o transporte pode ocorrer a deterioração da água, devido a respiração e excreção de amônia pelos animais.

Os peixes sedados apresentam metabolismo reduzido e, consequentemente, baixo consumo de oxigênio e excreção de amônia, assim os anestésicos podem auxiliar na manutenção da qualidade da água durante o transporte (Parodi et al., 2014; Becker et al., 2017; Jerez-cepa et al., 2019). Além disso, o uso do anestésico durante o transporte pode reduzir o estresse e a agitação dos animais, evitando o choque mecânico e permitindo o transporte em densidades mais elevadas (Purbosari et al., 2019).

2.3.3 Anestésicos sintéticos x naturais

Os anéstesicos utilizados na aquacultura podem ser classificados como naturais ou sintéticos. Existem diversos trabalhos utilizando-se anestésicos sintéticos na aquacultura (Coyle et al., 2004), como 2-phenoxyethanol para dourada (*Sparus aurata)* e truta-arco-íris (*Oncorhynchus mykiss)* (Tort et al., 2002), quinaldina e tricaína metano sulfonato (MS-222) para o bagre americano (*Ictalurus punctatus)* (Small, 2003), tricaípara pacamã (*L. alexandri*) (Ribeiro et al., 2015) e benzocaina para tilápia (*Oreochromis niloticus)* (Rucinque et al., 2017) e pacamã (*L. alexandri*) (Ribeiro et al., 2019). Apesar de sua eficiência, os anestésicos sintéticos apresentam custo elevado e podem desencadear alguns problemas relacionados ao descarte e acúmulo de resíduos (Purbosari et al., 2019). Devido a esses problemas, diversos estudos vêm

sendo realizados em busca de alternativas naturais para substituir o uso dos anestésicos sintéticos.

Os compostos naturais, eugenol e mentol (Cunha et al., 2010; Bodur et al., 2018), presentes em óleos essenciais isolados de plantas (Saydmohammed e Pal, 2009), têm se mostrado eficientes para utilização como anestésicos em várias espécies de interesse para aquacultura (Ribeiro et al., 2019), como eugenol e mentol para o camarão de água doce (*Macrobrachium rosenbergii)* (Saydmohammed e Pal, 2009), óleo essencial de *Aloysia triphylla* para jundiá (*Rhamdia quelen) (*Parodi et al., 2014) e pacamã (*L. alexandri*) (Becker et al., 2017) e óleo de cravo da índia para truta-arco-íris *Oncorhynchus mykiss* (Keene et al., 1998), pacamã (*L. alexandri*) (Ribeiro et al., 2019) e bagre americano *(Ictalurus punctatus)* (Small, 2003).

Geralmente, esses compostos são comercializados na forma de óleo, sendo insolúvel em água. Portanto, antes do preparo da solução anestésica, esses compostos devem ser dissolvido em uma substância anfifílica, como por exemplo, álcool etílico (Simões et al., 2010).

2.3.4. Óleo essencial de Ocimum gratissimum (OEOG)

O *Ocimum gratissimum* L, popularmente conhecido como alfavaca ou manjericão, é uma espécie de planta herbácea da familia Lamiaceae e encontrada em todas as regiões tropicais do mundo (Freire et al., 2006). O *O. Gratissimum* vem sendo utilizado no mundo inteiro para diversas finalidades, tais como: condimento culinário, repelente contra insetos, sedativos, anticonvulsivante e como medicamento na medicina popular para tratar diversos tipos de doenças (Prabuh et al., 2009). Além disso, os habitantes das florestas tropicais brasileiras usam a sua raiz como elemento decorativo e sedativo para as crianças (Stasi et al., 2002; Costa. 2016).

O OEOG pode ser extraído por hidrodestilação e o rendimento da extração pode variar em função da época de colheita e da parte da planta utilizada para extração (Kpoviessi et al.,

2014). Os mesmos autores estudaram o rendimento do extrato etanólico do *O. gratissimum* extraído de diversas partes da planta em diferentes épocas de colheita (pré- floração e floração plena) e observaram uma variação no rendimento de 1,97 a 4,81 % (m/m). Do ponto de vista comercial, o OEOG pode ser encontrado por R$ 45,00 o frasco de 10 ml (cotação realizada pelo site: www.chadao.com.br no dia 14-01-22. O OEOG pode apresentar diferentes quimiotipos (eugenol, timol e geraniol) (Vieira et al., 2001) e apresenta potencial para ser utilizado como anestésico em peixes (Benovit et al., 2012; Boijink et al., 2016; Ribeiro et al., 2016; Silva et al., 2012; Silva et al., 2015). No entando, estudos sobre a influencia do OEOG sobre os danos teciduais são raros e mais estudos devem ser realizados.

2.4. Lophiosilurus alexandri (pacamã)

O pacamã *L. alexandri* é um peixe nativo e endêmico da bacia do rio São Francisco (López et al., 2000). Essa espécie, apresenta potencial para aquicultura (Becker et al., 2017; Costa et al., 2015; Kitagawa et al., 2015; Ribeiro et al., 2019) e vem sendo utilizada em programas de repovoamento (Sato, 2014) e estudos de marcação (Boaventura et al., 2019). Contudo, poucos estudos foram realizados para investigar os efeitos dos anestésicos durante o manejo e o transporte do *L. alexandri.*

Ribeiro et al. (2013) esturaram o efeito anéstesico do eugenol em diferentes tamanhos de juvenis de pacamã e observaram que o aumento da concentração de eugenol proporcionou menor tempo de indução a anestesia, e que os juvenis maiores necessitam de um maior tempo para serem anestesiados. Ribeiro et al. (2015) estudaram a eficácia de diferentes concentrações da tricaina (100, 160, 220, 280 e 340 mg/L) em diferentes fases de vida do pacamã (15, 21 e 60 dias após a eclosão) e observaram que concentrações acima de 280 mg/L podem causar mortalidade para as larvas com 15 dias de vida, enquanto todas as outras concentrações testdas

podem ser utilizadas com segurança. Becker et al. (2017) estudaram o uso do óleo essencial de *Aloysia triphylla* durante a anestesia e transporte do pacamã e observaram que o seu uso reduziu o metabolismo do animal durante o transporte e evitou o aumento da concentração de amonia na água. Favero et al. (2019) compararam o uso de diferentes concentrações de eugenol (0, 2,4 e 6 mg/L) durante o transporte do pacamã. Esses autores observaram que após o transporte, todos os animais apresentaram níveis de glicose acima do nível basal e que o uso de 4 e 6 mg/L de eugenol contribuiu com a redução dos níveis de glicose no sangue após 24 h do transporte. Ribeiro et al. (2019) avaliaram os parâmetros sanguíneos do pacamã submetidos a diferentes concentrações de benzocaina (60, 120, 240 e 480 mg/L) e eugenol (80, 160,329 e 640 mg/L) e observaram que todas as concentrações testadas de ambos os anestésicos provocaram o aumento da concentração de glicose. Contudo até o momento não há relatos para o uso de OEOG nesta espécie. Portanto, estudos adicionais sobre o uso de anestésicos e protocolos de anestesia seguros e eficientes para o manejo e transporte dessa especie devem ser realizados.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar o uso do óleo essencial de *Ocimum gratissimum* (OEOG) como anéstesico e sedativo durante o manejo e tranporte de juvenis de *L. alexandri.*

3.2. Objetivos específicos

Determinar os intervalos de indução e recuperação à anestesia dos juvenis submetidos às diferentes concentrações do OEOG.

Avaliar os efeitos de diferentes concentrações do OEOG durante o manejo do *L. alexandri.*

Avaliar os efeitos de diferentes concentrações do OEOG sobre os parâmetros sanguíneos e de estresse oxidativo de juvenis de *L. alexandri* durante o manejo.

Avaliar os efeitos de diferentes concentrações do OEOG durante o transporte do *L. alexandri.*

Avaliar os efeitos de diferentes concentrações do OEOG sobre os parâmetros sanguíneos e de estresse oxidativo do *L. alexandri* após o transporte.

Avaliar a influência de diferentes concentrações do OEOG sobre os parâmetros de qualidade da água durante o transporte de juvenis de *L. alexandri.*

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5. MANUSCRITO I

Essential oil of *Ocimum gratissimum* (Linnaeus, 1753) as anesthetic for *Lophiosilurus alexandri*: induction, recovery, hematology, biochemistry and oxidative stress

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Abstract

This study tested the use of essential oil of *Ocimum gratissimum* (EOOG) as an anesthetic for juvenile *Lophiosilurus alexandri* and its efficiency in mitigating the effects of handling stress. Two experiments were performed. Experiment 1 determined the time for anesthesia induction and recovery and the ventilatory frequency of juveniles exposed to different concentrations of EOOG (0, 10, 30, 90, 150 and 300 mg L^{-1}). Based on the results of Experiment 1, Experiment 2 evaluated the effects of 0, 10 and 90 mg L^{-1} of EOOG on blood parameters and oxidative stress in liver and brain immediately after induction and one hour after recovery. No mortality was observed in any experiment. In Experiment 1, times for anesthesia induction were shorter, and recovery times longer, with increased EOOG concentrations. Compared to the animals anesthetized with 0, 10 and 30 mg L^{-1} of EOOG, higher ventilatory frequencies were observed during induction for animals anesthetized with 90, 150 and 300 mg L^{-1}. In experiment 2, variations on hematological variables derived from EOOG exposition were determined, but parameters returned to baseline levels one hour after recovery. The use of 90 mg L^{-1} EOOG prevented increased plasma glucose and cortisol levels one hour after handling. However, animals anesthetized with 90 mg L^{-1} EOOG had higher concentrations of ROS (hepatic and brain) after induction and one hour after recovery. The increase in ROS for animals anesthetized with 90 mg L^{-1} was not matched by the antioxidant defense system, which showed a reduction in brain GST activity one hour after recovery. Juveniles anesthetized with 10 mg L^{-1} EOOG only presented an increase in hepatic ROS one hour after recovery from anesthesia, however, there was a response from the antioxidant defense system, with an increase in brain and hepatic GST. The increase in ROS in treatments submitted to handling under the effect of anesthesia did not cause tissue damage. It is concluded that the use of 90 and 150 mg L^{-1} EOOG cause anesthesia in *L. alexandri* within the ideal intervals of induction and recovery. The use of 90

mg L^{-1} EOOG prevented higher levels of plasma glucose and cortisol one hour after handling, and induced changes to the antioxidant defense system, increasing the concentration of liver and brain ROS, and reducing the activity of brain GST one hour after recovery.

Keywords: handling, welfare, carnivorous fish, eugenol

1. Introduction

Natural or synthetic anesthetics have been used to minimize stress to animals during handling in aquaculture production (Tarkhani et al., 2016; Souza et al., 2019a; Velisek et al., 2011). Several studies have investigated using synthetic anesthetics in aquaculture (Coyle et al., 2004), such as benzocaine for Nile tilapia, *Oreochromis niloticus* (Rucinque et al., 2017), and pacamã, *Lophiosilurus alexandri* (Ribeiro et al., 2019); quinaldine and MS-222 for channel catfish, *Ictalurus punctatus* (Small, 2003); and 2-phenoxyethanol for dorada, *Sparus aurata*, and rainbow trout, *Oncorhynchus mykiss* (Tort et al., 2002). MS-222 is the synthetic anesthetic most used in aquaculture (Popovic et al., 2012), but has a high cost in many countries and low efficacy in the control of plasma cortisol (Tarkhani et al., 2017). In addition, MS-222 is considered carcinogenic (Pirhonen and Schreck, 2003) and can cause cardiovascular and respiratory damage (Popovic et al., 2012).

Among natural alternatives to synthetic anesthetics, eugenol has been shown to be efficient as an anesthetic for several species of interest for aquaculture (Bodur et al., 2018; Hoseini et al., 2019), and is present in the composition of several essential oils extracted from plants and tested in fish (Saydmohammed and Pal, 2009; Aydin and Barbas, 2020), such as clove oil for rainbow trout (Keene et al., 1998), channel catfish (Small, 2003), pacamã (Ribeiro et al., 2013) and Nile tilapia (Kheawfu et al., 2017). However, the concentration and time required for induction and recovery vary according to each essential oil (Ostrensky et al., 2016), species (Fernandes et al., 2016) and size of the animals (Gomes et al., 2011; Tarkhani et al., 2017), therefore, it is necessary to evaluate each anesthetic before using in a given species (King, 2009).

Despite being used to mitigate the effects of stress after handling, natural anesthetics can also induce some undesirable responses (Kanani et al., 2013), such as biochemical (Parodi

et al., 2014; Souza et al., 2018) and hematological changes (Abdolazizi et al., 2011; Hashimoto et al., 2016; Ribeiro et al., 2019), such as increased concentrations of glucose , cortisol (Park et al., 2008), aspartate aminotransferase (AST) (Feng et al., 2011), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) enzymes (Akinrotimi et al., 2018). In addition, tissue and oxidative damage has also been observed in rainbow trout (Velisek et al., 2011) and silver catfish, *Rhamdia quelen* (Souza et al., 2018).

Basil (*Ocimum gratissimum* L.) is a species of herbaceous plant that is found in all tropical regions of the world (Freire et al., 2006). The essential oil extracted from this plant can present different chemotypes (eugenol, thymol and geraniol) (Vieira et al., 2001), and has the potential to be used as an anesthetic in fish (Benovit et al., 2012; Boijink et al., 2016; Ribeiro et al., 2016; Silva et al., 2012; Silva et al., 2015).

Pacamã (*L. alexandri*) is a native and endemic catfish of the São Francisco River basin in the state of Minas Gerais, Brazil (López et al., 2000). The species has a carnivorous feeding habit (Cardoso et al., 1996) and has been used in restocking programs (Sato, 2014) and marking studies (Boaventura et al., 2019). The meat of *L. alexandri* is white and tasty (Salaro et al., 2015; Sant´Ana et al., 2016), which makes it a promising species for aquaculture (Becker et al., 2017; Costa et al., 2015; Kitagawa et al., 2015; Ribeiro et al., 2019), and so the use of anesthetics and efficient anesthesia protocols for its handling need to be investigated.

Despite its importance for aquaculture and restocking programs, few studies have been conducted to investigate the effects of anesthetics on L. alexandri (Becker et al 2017; Favero et al., 2019; Ribeiro et al., 2013; Ribeiro et al 2015; Ribeiro et al 2019). In addition, studies on the effects of anesthetics on oxidative stress parameters are scarce for this species and should be verified. Therefore, the aim of this study was to test different concentrations of the essential oil of *O. gratissimum* L. (EOOG) for induction and recovery of anesthesia in *L. alexandri* and to verify its effects on biochemical, hematological and oxidative stress parameters.

2. Material and Methods

1. Animals and laboratory acclimation

One hundred and twenty juveniles of *L. alexandri* (94.30 ± 19.15 g and 18.12 ± 1.65 cm) were distributed among 12 tanks (10 fish/tank) each with 100 L of constantly-aerated water. The animals were acclimated for two weeks and fed with a commercial diet (32 % crude protein) twice a day (8:00 am and 4:00 pm) until apparent satiety. After feeding, the tanks were siphoned to remove feces and uneaten food. Water quality parameters were measured daily at 09:00 h. Temperature (26.9 ± 0.4 °C) and pH (7.73 ± 0.11) were measured throughout the acclimatization period using a portable COMBO pH meter (HANNA-HI 98107) while dissolved oxygen (6.58 ± 0.47 mg L^{-1}) was measured with a digital oximeter (HANNA-HI9146).

2.2. Animals and experimental design

Experimental tests were performed in the Aquaculture Laboratory at the Universidade Federal de Minas Gerais (UFMG). The methodology was approved by the Ethical and Animal Welfare Committee of the UFMG Process 407/2018 and 28/2019.

EOOG (73.6 % eugenol) was used as an anesthetic for both experiments and was obtained according to the methodology described by Silva et al. (2012). The EOOG was previously weighed and diluted 1:10 (V/V) in 95 % ethanol.

Experiment 1. Anesthesia induction and recovery of *Lophiosilurus alexandri* exposed to the essential oil of *O. gratissimum*

Sixty animals were fasted for 24 h prior to the experiment to assess induction and recovery times for the anesthesia. Six aquariums containing 2 L of water with different concentrations of the EOOG were used for anesthesia induction: 0, 10, 30, 90, 150 and 300 mg L^{-1} EOOG. Ten fish were used for each concentration and each fish was used only once. After anesthesia, animals were weighed on a precision scale (Mars AD2000 0.01 g precision) and measured for length with a digital caliper (Gamma - precision 0.01 mm). Recovery time was assessed by placing animals in 2 L aquariums containing only clean water. For the 0 mg L^{-1} EOOG, a concentration of ethanol equivalent to the dilution of 300 mg L^{-1} EOOG was added, and animals were kept for 90 seconds in each aquarium. Induction and recovery times were determined according to the criteria described by Schoettger and Julin (1967) with some adaptations. As *L. alexandri* is a benthic fish, deep anesthesia was considered when individuals turned with the abdomen facing upwards and were unable to return to the normal position, while recovery was considered when animals were able to return to and remain in the normal position (Ribeiro et al., 2015). After recovery, animals were transferred again to the 100 L tanks to determine the survival rate at 24 h after the procedure. Ventilatory frequency was also measured during anesthesia induction and recovery, according to Alvarenga and Volpato (1995).

Experiment 2. Physiological responses during anesthesia and one hour after recovery of *Lophiosilurus alexandri* exposed to different concentrations of EOOG

Previously, before any handling, blood and tissue sampling were collected from 10 animals as basal group. Experiment 2 was performed, based on the results of Experiment 1, using the following concentrations: 0; 10 and 90 mg L^{-1} EOOG. The 0 mg L^{-1} EOOG included a concentration of ethanol equivalent to the dilution of 90 mg L^{-1} EOOG. The concentrations of 10 and 90 mg L^{-1} EOOG were chosen because they are, respectively, outside and within the anesthesia induction times as indicated by Marking and Meyer (1985) and Keene et al. (1998). The same procedures described in Experiment 1 were adopted for Experiment 2, except that twenty fish were used for each concentration with ten being collected for blood and tissue sampling immediately after anesthesia and ten for sampling after one hour after recovery.

The fish were carefully contained with a damp cloth immediately after anesthetic induction for blood collection (approximately 1 mL) by caudal venipuncture using heparinized syringes. The animals were then euthanized by means of desensitization on ice and spinal cord sectioning for later removal of liver and brain, which were then stored in a freezer at -80 °C. Blood aliquots were used to determine hematocrit (Htc) and hemoglobin (Hg). Hematocrit was determined by the micro-hematocrit method (Goldenfarb et al., 1971) using capillary tubes. For hemoglobin analysis, 4 μL of blood was added and homogenized in 1 mL of Color Reagent (Bioclin®), followed by reading on a Term Plate Analyzer Basic® spectrophotometer (Tonks, 1983).

Blood was centrifuged at 1,792 × g for 10 minutes and the plasma was separated for biochemical and enzymatic analyzes. Cortisol (COR) was determined using a commercial ELISA kit (enzyme-linked immunosorbent assay kit - cortisol test, DRG International) which is an acceptable procedure for fish (Barcellos et al., 2010; Mattioli et al., 2017). Commercial

kits from Biotécnica® were used for glucose (GLU), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), followed by their reading in an automatic device (Cobas-Mira Plus®-Roche).

2.3. Hepatic and brain protein levels

The hepatic and brain protein concentrations were determined by the Coomassie Blue method following the methodology described by Read and Northcote (1981) using bovine serum albumin as a standard.

2.4. Hepatic and brain reactive oxygen species (ROS) levels

ROS levels 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 DCF per mg of protein.

2.5. Hepatic and brain lipid peroxidation (LOOH)

LOOH was determined using the FOX methodology (Hermes-Lima et al. 1995, with some modifications by the authors), which is based on the oxidation of Fe (II) under acidic conditions. The FOX method measures lipid peroxides, one of the main products of lipid peroxidation. LOOH was measured by sequentially adding $FeSO_4$ (1 mM), H_2SO_4 (0.25 M), xylenol orange (1 mM, Sigma) and MilliQ water. Samples or methanol (blanks) were added and incubated for 30 min, after which absorbance (550 nm) was determined with Cumene

hydroperoxide (CHP; Sigma) as a standard. LOOH was expressed in cumene hydroperoxide (CHP) equivalents per nmol per mL of serum.

2.6. Hepatic and brain protein carbonyl formation

Protein carbonyl formation was measured by the spectrophotometric assay described by Reznick and Packer (1994) with some modifications. One hundred microliters of supernatant containing approximately 0.15 mg of protein was treated with 200 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for one hour. The proteins present in the samples were then precipitated with 500 µL of 20 % TCA and centrifuged for 5 min at 10,000 × g. The pellet was suspended in 300 µL of 6 M guanidine prepared in 2.5 N HCl. The difference between the DNPH-treated and HCl-treated (blank) samples was used to calculate carbonyl content determined at 365 nm. The results were calculated as nmol carbonyl groups per mg protein, using the extinction coefficient of 22,000 M^{-1} cm^{-1} for aliphatic hydrazones.

2.7. Hepatic and brain superoxide dismutase (SOD)

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 420 nm at ten second intervals, according to methodology described by Beutler (1984). Activity was expressed as U mg $protein^{-1}$.

2.8. Hepatic and brain glutathione S-transferase (GST) activity

GST activity was measured according to Mannervik and Guthenberg (1981) with slight modifications. GST activity was measured by the rate of formation of dinitrophenyl-S-glutathione at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants (approximately 0.045 mg of protein). The results were calculated and expressed as U mg protein^{-1}.

2.9. Hepatic and brain glutathione peroxidase (GPx)

GPx activity was measured indirectly from monitoring the oxidation rate of NADPH at 340 nm using cumene hydroperoxide (CuOOH), according to Wendel (1981). The enzymatic activity was expressed as U mg protein^{-1}.

2.10. Statistical analysis

The anesthesia induction and recovery times obtained in Experiment 1 were subjected to exponential and linear regression analyses. The ventilatory frequency, and hematological and biochemical data were submitted to the Shapiro Wilk test for normality and the Levene test for homoscedasticity. Parametric results were analyzed by two-way ANOVA followed by Tukey's test, or when homogeneity of variance was not obtained, by the Scheirer-Ray-Hare extension of Kruskal Wallis tests and the Nemenyi test. Both at 95 % probability ($P<0.05$). SigmaPlot and Infostat software were used for data analysis.

3. Results

Experiment 1

3.1. Time for anesthesia induction and recovery

No mortality was observed 24 hours after the tests were performed. Animals exposed to only ethanol (0 mg L^{-1} EOOG) were not sedated or anesthetized. Increased concentrations of EOOG resulted in shorter times for anesthesia induction up to 90 mg L^{-1} EOOG (fig. 1A) and longer times for recovery (fig. 1B).

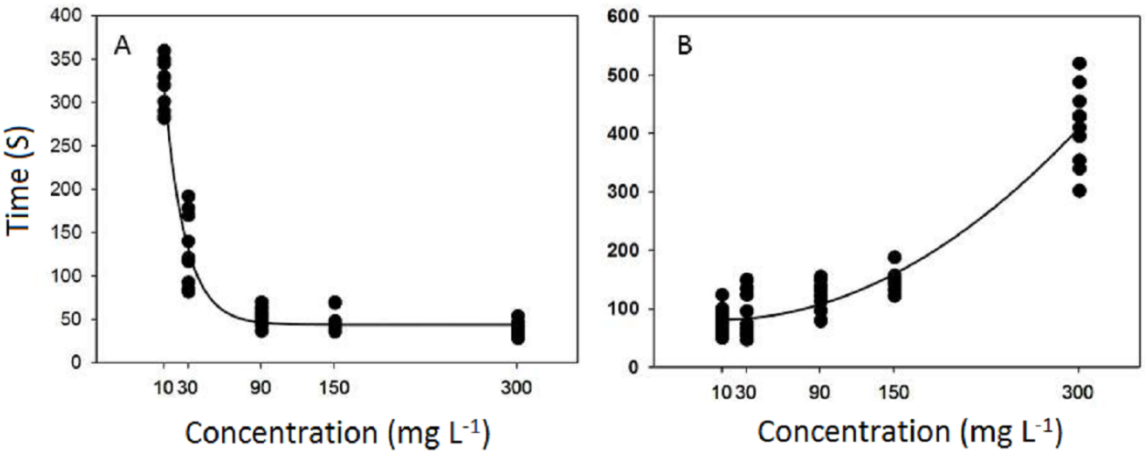

Figure 1- Time required for induction (A) and recovery (B) of Lophiosilurus alexandri from anesthesia with the essential oil of Ocimum gratissimum (EOOG). The equations were adjusted to the data: Induction Y= 43.8170 + 490.9770 e (-0,0579x), R2=0.95, recove y=81.4804 - 0.00486*x+0.0038x^2, R^2=0.9127.

0 mg L^{-1} EOOG and those exposed to 10 and 30 mg L^{-1} of EOOG had lower ventilatory frequencies during anesthesia induction compared to the other treatments (concentrations of 90,

150 and 300 mg L^{-1} EOOG) (Table 1). However, the ventilatory frequency of fish of the 150 mg L^{-1} EOOG treatment was statistically similar to fish of the 30 mg L^{-1} EOOG treatment. During recovery, the highest ventilatory frequencies were recorded for fish exposed to 90 mg L^{-1} EOOG, while the lowest frequency was observed for the those submitted to 300 mg L^{-1} EOOG. Animals submitted to 90, 150 and 300 mg L^{-1} EOOG had reduced ventilatory frequencies during recovery compared to their respective ventilatory frequencies through anesthesia induction.

Table 1- Effects of the essential oil of *Ocimum gratissimum* on the ventilatory frequency (opercular or buccal movements per minute) of *Lophiosilurus alexandri*

Time	Treatment					
	0 mg L^{-1}	10 mg L^{-1}	30 mg L^{-1}	90 mg L^{-1}	150 mg L^{-1}	300 mg L^{-1}
During anesthesia	33.47±5.16a	35.85±6.55a	38.24±7.28ab	57.76±15.70c*	46.17±5.05bc*	55.55±15.45c*
During recovery	33.11±8.96a	32.67±10.90a	36.94±10.54ab	43.64±6.79b	31.65±6.48ac	21.59±9.76c

Values expressed as mean ± standard deviation. The data were analyzed by the Kruskal-Wallis at 5% probability. Different lowercase letters indicate differences between concentrations at the same time. Asterisks indicate significant differences between times at the same concentration.

Experiment 2

3.2. Hematology and blood biochemistry

3.2.1. Hematology

Juveniles anesthetized with 10 and 90 mg L^{-1} EOOG showed hematocrit values above the baseline level ($P<0.05$) (Fig 2.A), but there was no significant difference between treatments. The highest hematocrit one hour after recovery was that for animals exposed to 90 mg L^{-1} EOOG. Anesthesia with EOOG and exposure to 0 mg L^{-1} EOOG did not change hemoglobin concentration ($P>0.05$), but one hour after recovery fish exposed to 0 mg L^{-1} EOOG showed higher levels than in the anesthesia induction period (Fig 2.B).

3.2.2. Plasma biochemical parameters

Juveniles anesthetized with 90 mg L^{-1} EOOG showed ALT and AST values above baseline level ($P<0.05$) (Figs. 2C and 2D). The concentrations of both enzymes for the 90 mg L^{-1} EOOG treatment had returned to baseline one hour after recovery. The ALT enzyme in the 0 mg L^{-1} EOOG was higher ($P<0.05$) in the recovery period than the baseline (Fig. 2C).

Plasma cortisol levels were not affected by the treatments ($P>0.05$) (Fig. 2E). However, fish treated with 90 mg L^{-1} EOOG had higher values one hour after recovery than when anesthetized and the 0 mg L^{-1} EOOG showed values above baseline level (Fig. 2E). Anesthesia with 90 mg L^{-1} EOOG increased plasma glucose levels compared to the baseline and the other treatments ($P<0.05$); however, plasma glucose levels of fish from this treatment returned to

baseline levels by one hour after recovery while these levels remained higher than baseline for fish from the 0 and 10 mg L^{-1} EOOG (fig. 2F).

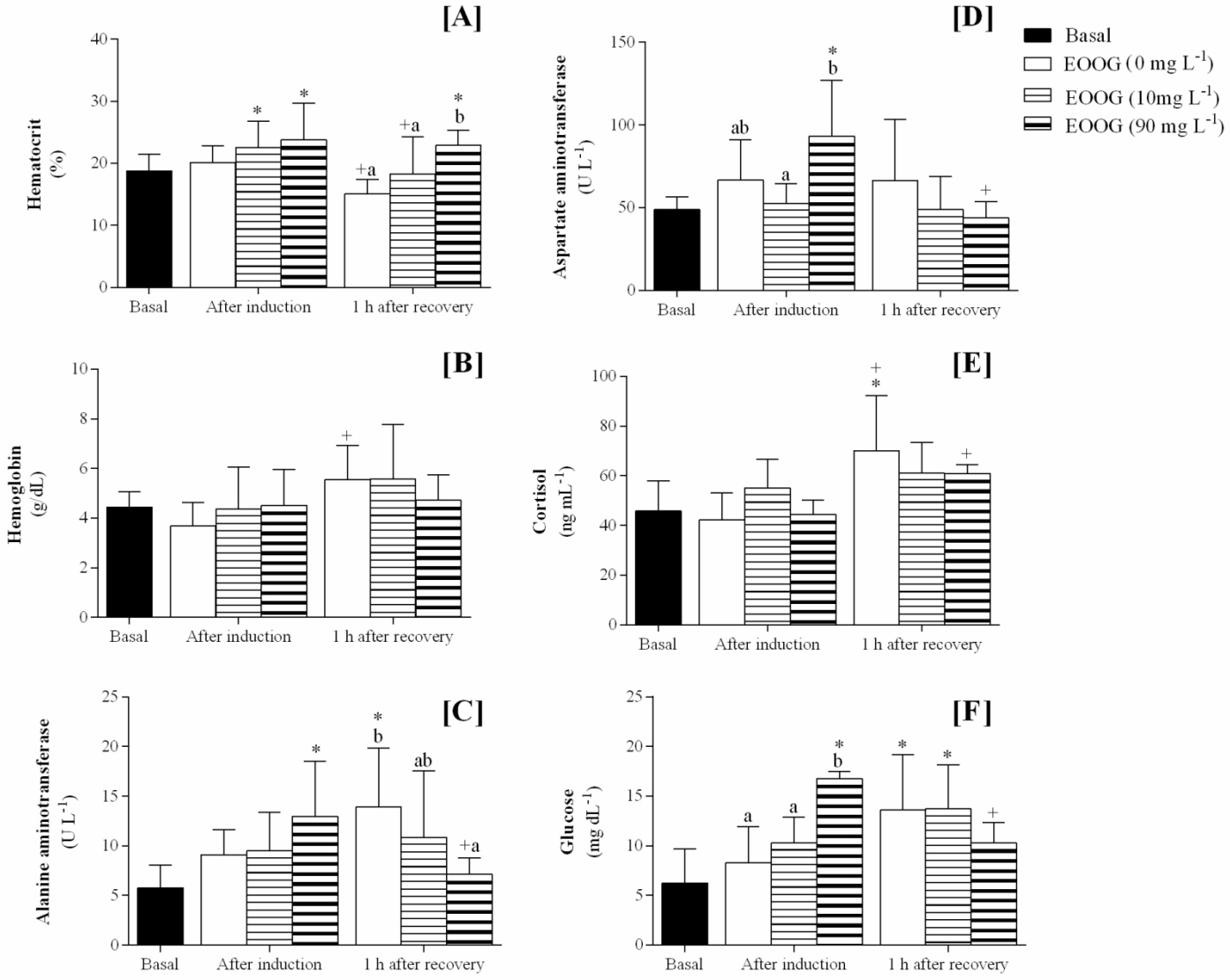

Figure 2- Biochemical and hematological parameters of *Lophiosilurus alexandri* measured immediately after anesthesia with different concentrations of essential oil of *Ocimum gratissimum* (EOOG) and one hour after recovery. Hematocrit (A), hemoglobin (B), alanine aminotransferase (C), aspartate aminotransferase (D), cortisol (E) and glucose (F). Values are expressed as the mean ± standard deviation (N=10). Asterisk represents a significant difference between treatments and baseline. Lowercase letters indicate significant differences between treatments at the same point in time ($p<0.05$). + indicate significant differences between points in time within the same treatment ($p<0.05$).

3.3. Enzymatic oxidative and prooxidant stress variables

3.3.1. Hepatic and brain reactive oxygen species (ROS) levels

Levels of hepatic ROS for the 90 mg L^{-1} EOOG treatment after induction were higher than baseline ($P< 0.05$) (fig. 3A). Hepatic ROS levels for the 10 and 90 mg L^{-1} EOOG treatments remained high relative to baseline and the 0 mg L^{-1} EOOG at one hour after recovery. Fish anesthetized with 90 mg L^{-1} EOOG had the highest brain ROS after induction and one hour after recovery ($P< 0.05$) (fig 3D).

3.3.2. Hepatic and brain lipid peroxidation (LOOH)

No differences were observed for hepatic and brain LOOH compared to the 0 mg L^{-1} EOOG and baseline (fig 3 B, E). However, brain LOOH for the 10 mg L^{-1} EOOG treatment was higher after induction compared to one hour after recovery (fig 3E).

3.3.3. Hepatic and brain protein carbonyl formation

There was no significant difference in hepatic protein carbonyl among treatments and baseline ($P>0.05$). Higher values were found for the 10 and 90 L^{-1} EOOG treatments compared to the 0 mg L^{-1} EOOG (fig. 3C) at one hour after recovery. Values for brain protein carbonyl were lower after anesthesia induction for fish exposed to 10 and 90 L^{-1} EOOG concentrations compared to the 0 mg L^{-1} EOOG ($P<0.05$). These values returned to baseline one hour after recovery (Fig. 3F).

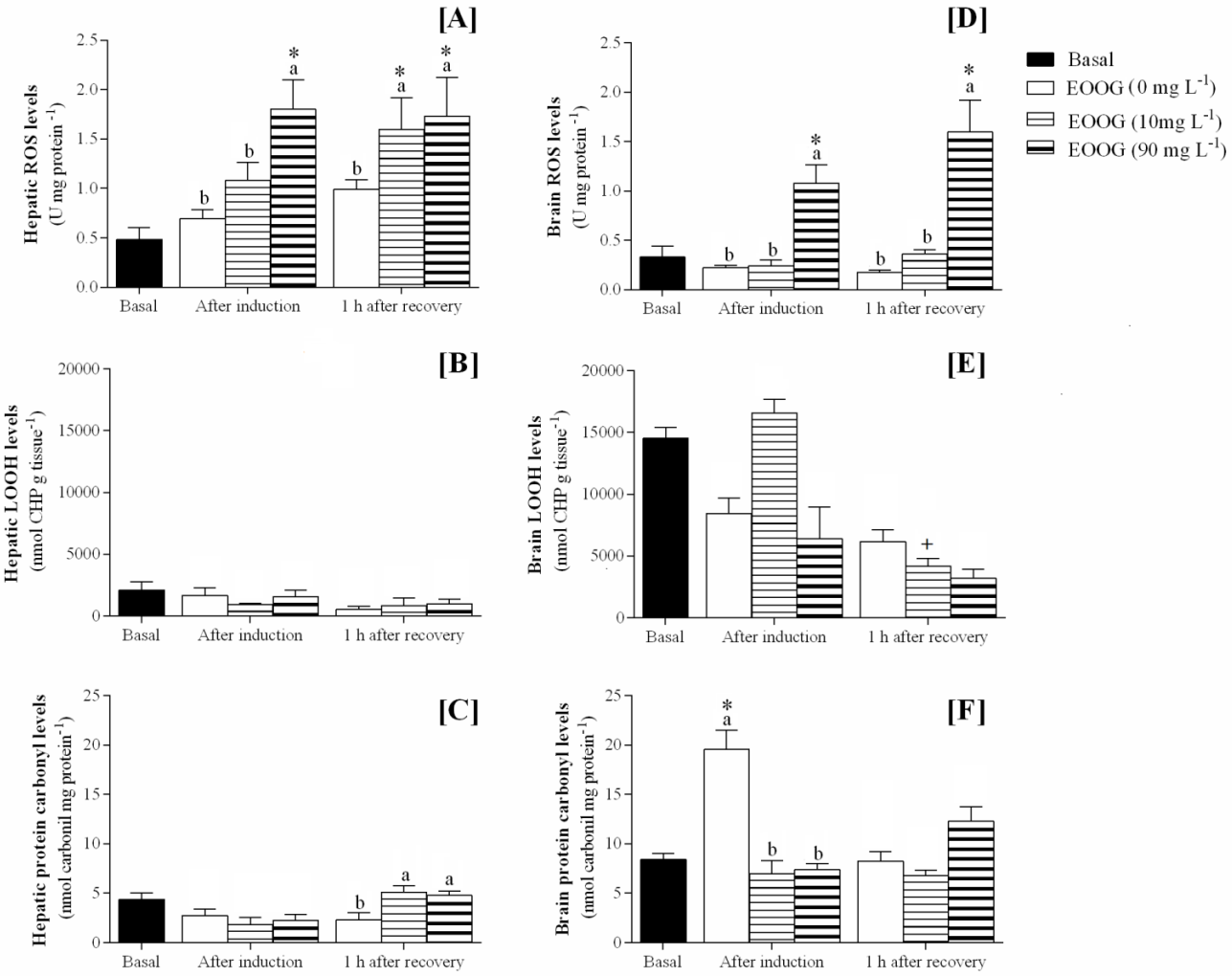

Figure 3- Prooxidant variables: (A,D) Reactive oxygen species (ROS), (B,E) lipid hydroperoxides (LOOH) and (C,F) Protein carbonylation (PC) levels in the liver and brain of *Lophiosilurus alexandri* (n = 8) measured immediately after anesthesia with different concentrations of essential oil of *Ocimum gratissimum* (EOOG) and one hour after recovery. Values are means ± standard error. An asterisk indicates a significant difference compared to baseline. Lowercase letters indicate significant differences between treatments at the same point in time ($p<0.05$). + indicate significant differences between points in time within the same treatment ($p<0.05$).

3.3.4. Hepatic and brain superoxide dismutase (SOD)

Hepatic SOD activity was below baseline for fish exposed to 10 mg L^{-1} EOOG after induction, and for the 0 mg L^{-1} EOOG one hour after recovery (Fig. 4A). There was no difference among treatments ($P>0.05$) for brain SOD activity after induction. Fish exposed to 90 mg L^{-1} EOOG had SOD values above baseline and the 0 mg L^{-1} EOOG one hour after recovery (Fig. 4D).

3.3.5. Hepatic and brain glutathione S-transferase (GST)

Fish exposed to 10 mg L^{-1} EOOG had higher levels of hepatic GST activity than baseline and the 0 mg L^{-1} EOOG ($P<0.05$) after induction and one hour after recovery (Fig. 4B). Brain GST activity after induction in fish exposed to 10 mg L^{-1} EOOG was higher than baseline and other treatments ($P<0.05$), while the lowest value was observed for the 90 mg L^{-1} EOOG treatment. All treatments showed a reduction in brain GST activity one hour after recovery compared to after induction, while the 90 mg L^{-1} EOOG treatment had lower activity than baseline and the lowest activity ($P<0.05$) (Fig. 4E).

3.3.6. Hepatic and brain glutathione peroxidase (GPx)

No significant differences among treatments and baseline were recorded for hepatic and brain GPx activity after anesthesia induction and one hour after recovery ($P>0.05$) (Figs. 4C, F).

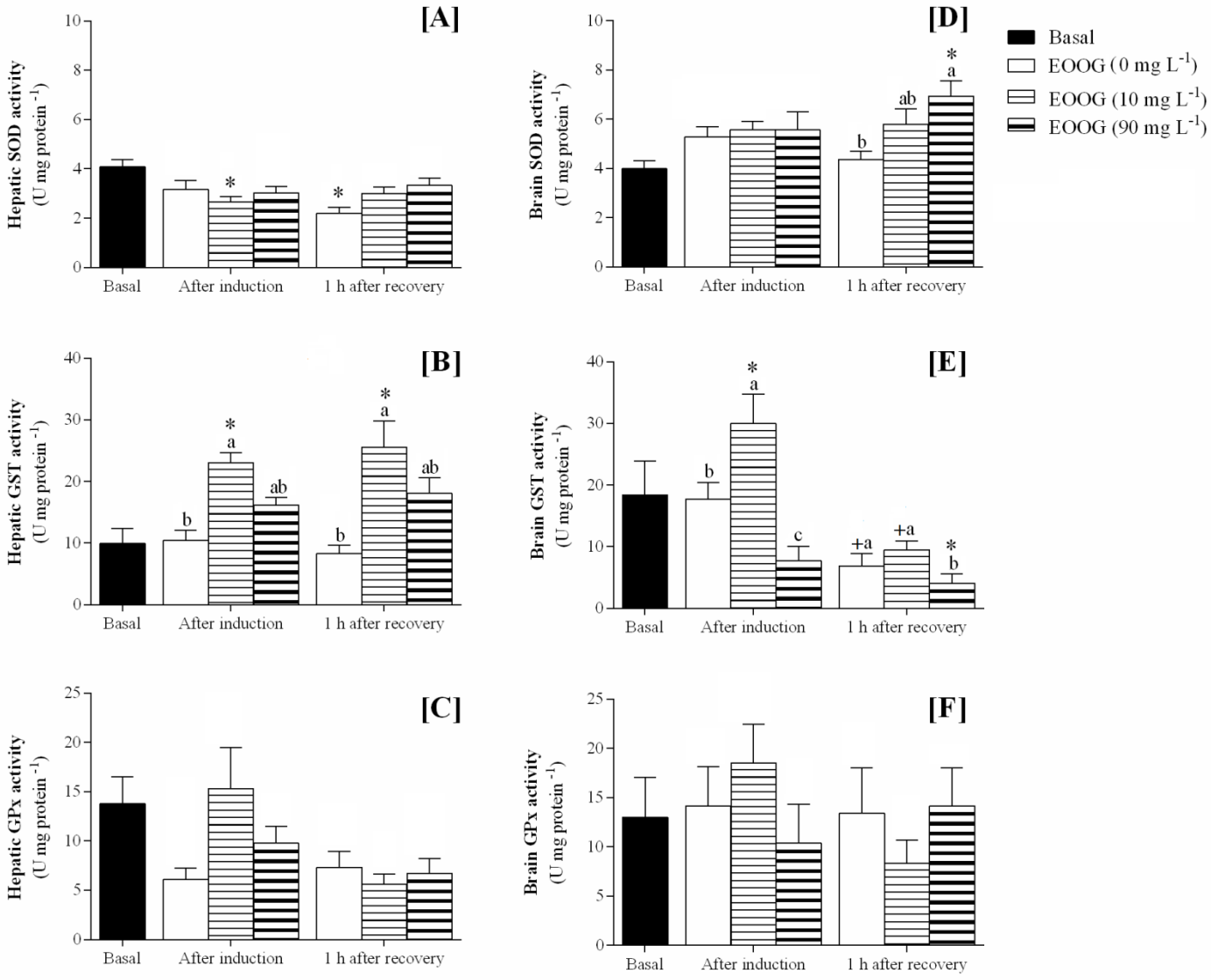

Figure 4- Enzymatic oxidative stress variables: (A,D) superoxide dismutase (SOD), (B,E) glutathione-s-transferase (GST) and (C,F) glutathione-peroxidase (GPx) activities in the liver and brain of *Lophiosilurus alexandri* (n = 8) measured immediately after anesthesia with different concentration of essential oil of *Ocimum gratissimum* (EOOG) and one hour after recovery. Values are means ± standard error. An asterisk indicates a significant difference compared to baseline. Lowercase letters indicate significant differences between treatments at the same point in time ($p<0.05$). + indicate significant differences between points in time within the same treatment ($p<0.05$).

4. Discussion

The findings indicate that the EOOG is a safe and efficient natural anesthetic to be used for tropical fish farming. Becker et al. (2017) also did not observe mortality for juvenile *L. alexandri* submitted to different concentrations of the essential oil of *Aloysia triphylla*. According to Marking and Meyer (1985) and Keene et al. (1998), an ideal anesthetic should induce anesthesia in less than 3 min and have a recovery time of less than 5 min. Based on these data, concentrations of 90 mg L^{-1} (induction < 2 min and recovery < 2 min) and 150 mg L^{-1} (induction < 1 min and recovery < 3 min) EOOG would be the most indicated for *L. alexandri* juveniles with an average weight of 100 g. Boijink et al. (2016) studied the use of EOOG in *Colossoma macropomum* and indicated the use of concentrations between 50 and 100 mg L^{-1} because of the rapid anesthesia induction (<4 min).

In the present study, juveniles exposed to concentrations above 90 mg L^{-1} EOOG showed an increase in ventilatory frequency during anesthesia induction, followed by a reduction during recovery. Similar behavior was observed by Roohi and Imanpoor (2015) for common carp (*Cyprinus carpio*) anesthetized with methyl salicylate. Increased opercular beating during induction may be related to agitation from a stressor in water (anesthetic) (Matthews and Varga, 2012).

The increased hematocrit in *L. alexandri* after anesthesia induction may be related to the reduced ventilatory frequency and hypoxia caused by anesthesia (Hill and Forster, 2004). In addition, increased hematocrit during anesthesia of fish with essential oils containing eugenol has also been reported for other species (Hill and Forster, 2004; Pádua et al., 2012; Tort et al., 2002).

When fish are exposed to handling stress, catecholamines and corticosteroids are released and, consequently, blood glucose increases to produce energy that allows an animal to

adapt to stress (Velisek et al., 2011). One hour after recovery, plasma cortisol and glucose of *L. alexandri* anesthetized with 90 mg L^{-1} returned to baseline values, showing that the use of this concentration contributes to reducing handling stress. In contrast, one hour after recovery, plasma glucose levels of animals of the exposed to 0 mg L^{-1} and 10 mg L^{-1} EOOG were still above baseline levels, showing that handling of fish in water (0 mg L^{-1} EOOG) and anesthesia with a prolonged induction time also causes increased plasma glucose for this species. Reduction of cortisol by the use of EOOG may be related to alterations to the expression of genes involved in the hypothalamic-pituitary-interrenal axis that mediate responses to stress (Souza et al., 2019a; Souza et al., 2019b). Iversen et al. (2003) evaluated the efficiency of four anesthetics (metomidate, clove oil, Aqui-S and benzocaine) during the management of Atlantic salmon (*Salmo salar*) and observed that metomidate (≥ 2 mg L^{-1}), clove oil and Aqui-S (≥ 20 mg L^{-1}) prevented increased plasma cortisol. Teixeira et al. (2017) evaluated the effectiveness of *A. triphylla* essential oil (EOAT) as an anesthetic in the management of Nile tilapia juveniles and observed a significant reduction in plasma cortisol levels one hour after handling in individuals anaesthetized with 300 μL L^{-1} EOAT compared to the control group (0 mg μL L^{-1} EOAT).

Hyperglycemia in anesthetized animals can occur due to increased agitation and hypoxia during anesthesia. However, blood glucose tends to return to normal levels gradually (Teixeira et al., 2017), as observed in the present study for animals exposed 90 mg L^{-1} EOOG. The enzymes AST and ALT are both indicative of the tissue health, with ALT being directly related to liver functions and AST to various types of tissues (Taheri et al., 2018). In addition, these enzymes can also be used as stress indicators because they are involved in the mobilization of amino acids via gluconeogenesis for the production of glucose (Chatterjee et al., 2006). However, the higher levels of ALT and AST shortly after anesthesia induction in *L. alexandri* exposed to 90 mg L^{-1} EOOG may be related to the increase in glucose levels observed during

the same period, as may be the higher ALT levels for the 0 mg L^{-1} EOOG one hour after recovery.

Tissue cells maintain a balance between prooxidant and antioxidant agents in normal situations (Poli et al., 2012). When there is an imbalance between these agents, the oxidative stress process begins, characterized by the formation of ROS, which can cause lipid and protein oxidation and the formation of LOOH (Coyle, 2004) and protein carbonyl, respectively (Stadtman and Levine, 2003). During anesthesia induction, fish experience a situation similar to hypoxia due to decreased opercular beating. Tissue reoxygenation then occurs during recovery from anesthesia, which is usually accompanied by the formation of ROS in tissues (Gressler et al., 2014; Velisek et al., 2011). Baldissera et al. (2019) studied the branchial bioenergetic imbalance of *L. alexandri* submitted to hypoxia followed by reoxygenation and observed higher levels of ROS in the gills during reoxygenation. The handling of fish in water or in water containing low concentrations of ethanol may also influence the balance between prooxidant and antioxidant, with the possibility of an increase in thiobarbituric acid reactive substances and carbonil protein in the liver right after handling (Souza et al., 2017). In addition to the formation of ROS after recovery, animals exposed to 90 mg L^{-1} EOOG also showed an increase in hepatic and brain ROS immediately after induction. This increase may have occurred due to the organism's reaction to the EOOG, identifying it as aversive. The EOOG can present different chemotypes (eugenol, thymol and geraniol) (Vieira et al., 2001). Eugenol is the main constituent of the EOOG used in this work (Silva et al., 2012). However, despite its analgesic and antioxidant properties, depending on the type and concentration, eugenol can also have cytotoxic effects and cause an increase in ROS in tissues (Atsumi, 2005; Bezerra et al., 2017).

The presence of oxidative substances in cells stimulates the body's antioxidant defense system, which increases the concentration of antioxidant enzymes (Łuczaj et al., 2017; Yu,

1994). SOD is one of the main enzymes responsible for the elimination of ROS produced in cells (Cheeseman and Slater, 1994). This enzyme converts ROS to H_2O_2 and H_2O, with later conversion of H_2O_2 to H_2O and O_2 by the action of the enzymes catalase (CAT) and GPx (Li et al., 2009; Velisek et al., 2011). GST is an enzyme involved in the body's antioxidant defense and its process of detoxifying endogenous and exogenous compounds (xenobiotics) (Frova, 2006). Therefore, the highest concentrations of GST found for the treatment exposed to 10 mg L^{-1} EOOG may be related to the response of the body's antioxidant defense system against the anesthetic and ROS. The prooxidant agents of the treatment juveniles exposed 0 mg L^{-1} EOOG remained unchanged, which may be due to absence of the EOOG in water and low concentration of ROS found in the tissues. However, despite the increase in ROS, the treatment with the highest concentration of EOOG (90 mg L^{-1}) showed reduced brain GST activity, indicating that the antioxidant defense capacity was exceeded. Velisek et al. (2011) studied the effect of four anesthetics (clove oil, 2-phenoxyethanol, MS-222 and propiscin) on oxidative stress of rainbow trout. These authors observed that, at twenty-four hours after anesthesia, all four anesthetics caused tissue damage, higher levels of substances reactive to thiobarbituric acid and reduced activity of glutathione reductase (GR) in the brain. While higher activities of SOD, GR e GPx were found in the brain of the control treatment. In the present study, despite increased ROS in treatments submitted to handling under the effect of anesthesia, the higher levels of ROS did not contribute to lipid peroxidation and carbonylation. Souza et al. (2017) studied the use of two chemotypes (citral and linalool) of the essential oil of *Lippia alba* as an anesthetic in silver catfish, *Rhamdia quelen*. This study demonstrated that the use of both oils did not cause an increase in thiobarbituric acid reactive species in the kidney and liver during recovery, but found higher levels of carbonyl protein, and increases in SOD, CAT and GST activities for fish anesthetized with the citral chemotype.

Conclusion

In conclusion, the use of 90 and 150 mg L^{-1} of essential oil of *Ocimum gratissimum* (EOOG) causes anesthesia in *L. alexandri* within induction (< 3 minutes) and recovery (< 5 minutes) intervals considered ideal. The use of 90 mg L^{-1} EOOG prevented higher levels of plasma glucose and cortisol one hour after handling. On the other hand, although it did not cause tissue damage, the use of 90 mg L^{-1} EOOG induced changes to the antioxidant defense system, increasing the concentration of liver and brain ROS, and reducing the activity of brain GST one hour after recovery. Finally, future evaluations are needed over greater periods of time than one hour after recovery from anesthesia.

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6. MANUSCRITO II

The use of *Ocimum gratissimum* L. essential oil during the transport of *Lophiosilurus alexandri*: water quality, hematology, blood biochemistry and oxidative stress

(artigo publicado na Aquaculture)

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Abstract

This study evaluated the effects of the essential oil of *Ocimum gratissimum* L. (EOOG) on water quality, blood characteristics and tissue damage during transport of *Lophiosilurus alexandri*. One hundred and sixty juveniles of *L. alexandri* (123.44 ± 1.95 g and 23.71 ± 1.95 cm) were fasted for 24 h before transportation. Fish were then submitted to one of three treatments of transport water with five replicates each: 0 (control), 5 and 10 mg/L EOOG. The animals were transported for 4 h in plastic bags at a density of 1 juvenile/L and 10 animals per bag. The ventilatory frequency of one fish from each bag was determined after 3 h of transport by direct observation and counting of opercular beats. Blood, brain and liver samples were collected prior to (basal group) and at the end of transport from 10 animals each. Blood aliquots were used to determine hematocrit and hemoglobin, total plasma protein, glucose, aspartate aminotransferase and alanine aminotransferase levels. Samples of liver and brain were used to determine parameters and oxidative stress (reactive oxygen species, glutathione peroxidase, superoxide dismutase, glutathione S-transferase, lipid peroxidation and protein carbonyl formation). No mortality was observed 24 h after transport. The addition of EOOG to transport water appeared to reduce animal metabolism, thus ensuring higher dissolved oxygen and lower ammonia levels. Juveniles transported with 10 mg/L EOOG also had reduced opercular beats, lower levels of hemoglobin and blood aspartate aminotransferase and glucose. This concentration also reduced lipid and protein oxidation levels in tissues, thereby avoiding lipid peroxidation and the formation of carbonyl protein. The use of 10 mg/L EOOG during transport promotes a sedative effect in *L. alexandri*, with reduced oxygen consumption, ammonia excretion and biochemical changes and enhanced protection against oxidative damage.

Keywords: pacamã, welfare, anesthetic, carnivorous fish

1. Introduction

The transport of live fish is an important practice in aquaculture (Bui et al., 2013; Ribeiro et al., 2019; Shrivastava et al., 2017), and is often carried out in plastic bags inflated with pure oxygen or in containers with aeration or oxygen injection (Berka, 1986). The use of plastic bags with pure oxygen increases the concentration of dissolved oxygen in the water at the beginning of transport (Golombieski et al., 2003), which can increase the formation of reactive oxygen species (ROS) in cells (Barbas et al., 2017). Furthermore, respiration and ammonia excretion by animals can also deteriorate water quality during transport (Ross et al., 2009; Shrivastava et al., 2017). As sedated fish have reduced metabolism and, consequently, low oxygen consumption and ammonia excretion, anesthetics can be used to preserve water quality during transport and ensure animal welfare (Bucking, 2017; Cooke et al., 2004; Kaiser et al., 2006). However, the effects of a given anesthetic can be species specific, which makes evaluation necessary before use (Jerez-Cepa et al., 2019; Readman et al., 2017).

Ocimum gratissimum L., also known as basil, is a native species of Africa that can be found in all tropical regions of the world (Freire et al., 2006). The essential oil extracted from this plant can present different chemotypes that are classified according to their main component (eugenol, thymol and geraniol) (Vieira et al., 2001). The essential oil of *Ocimum gratissimum* (EOOG) has anti-inflammatory and anti-oxidant properties (Shittu et al., 2016; Alabi et al., 2018) and the eugenol chemotype has been shown to be an efficient anesthetic for *Rhamdia quelen* (Silva et al., 2015), *Colossoma macropomum* (Boijink et al., 2016), *Brycon amazonicus* (Ribeiro et al., 2016), *Astyanax bimaculatus* (Silva et al., 2019) and *Paralichthys orbignyanus* (Benovit et al., 2012). However, studies on the use of EOOG during fish transport and its influence on oxidative stress are rare and so further studies should be carried out.

Pacamã, *Lophiosilurus alexandri*, is a carnivorous fish (Cardoso et al., 1996), native and endemic to the São Francisco River basin in the state of Minas Gerais, Brazil (López et al.,

2000). The species has been used in restocking programs (Sato, 2014) and tagging studies (Boaventura et al., 2019). It possesses preferential nocturnal eating habits (Kitagawa et al., 2015) and has white tasty meat (Sant´Ana et al., 2016), which makes it promising for aquaculture (Becker et al., 2017; Costa et al., 2015; Kitagawa et al., 2015; Ribeiro et al., 2019).

The objective of this work was to test the effects of different concentrations of EOOG in transport water on water quality and post-transport biochemical, hematological and oxidative stress parameters of *L. alexandri*.

2. Materials and methods

The experiment was performed at the Aquaculture Laboratory of the Universidade Federal de Minas Gerais (UFMG) and the methodology was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Minas Gerais Process CEUA: 177/2019.

2.1. Animals and environmental acclimation

One hundred and sixty juveniles of *L. alexandri* (123.44 ± 1.95 g and 23.71 ± 1.95 cm) were acclimated for 15 days in a 7000 L circular tank with constant aeration. The juveniles were fed with commercial feed containing 32% crude protein supplied until apparent satiety twice a day (08:00 and 16:00 h). Ten percent of the water in the tank was changed 30 minutes after each feeding to remove feces and uneaten food. Water quality parameters were measured daily at 10:00 am. Dissolved oxygen was measured using an EcoSense DO200A oximeter and temperature and pH with a HANNA HI 98107 portable combo pH meter. Water temperature

remained at 27.2 ± 0.6 °C, dissolved oxygen levels at 7.45 ± 0.54 mg/L and pH at 7.57 ± 0.13 throughout acclimation. The animals were fasted for 24 h prior to the experiment.

2.2. Experimental design

Extraction of EOOG followed Silva et al. (2012). In brief, aerial parts of *O. gratissimum* L. were collected and dried for three days in a ventilated drying oven at 45 °C. The essential oil was then obtained by hydrodistillation for 3 h using a Clevenger-type apparatus (European Pharmacopoeia, 2007). The EOOG was weighed and diluted 1:10 (V/V) in 95% ethanol prior to the experiment. Fish were divided among three transport water treatments with five replicates each: 0 (control), 5 and 10 mg/L EOOG. The control treatment contained the volume of ethanol used to dilute the highest concentration of EOOG. The animals were transported for a period of 4 h in 25-L plastic bags containing 10 L of water supplemented with pure oxygen at a loading density of 1 juvenile/L (10 juveniles each bag). Ventilatory frequency was determined after 3 h of transport as beats per minute for the first 20 opercular beats (Alvarenga and Volpato, 1995), by observing one animal in each bag for a total of five animals per treatment. Blood, brain and liver samples were collected from 10 animals prior to transport for baseline measurements and from 10 animals (N = 2 from each transport bag) from each treatment immediately after transport. The remaining animals of each replicate were transferred to 100 L tanks to determine survival at 24 h post-transport.

2.3. Water quality parameters

Water quality parameters were measured before and after transport. Dissolved oxygen was measured using a Politerm model POL-69 oximeter; pH and temperature were measured with a Hanna Instruments Combo model pH & EC meter; and total ammonia was measured by colorimetric test (Labcom).

2.4. Blood and tissue collection

The animals were held with a damp cloth and 1 mL of blood was collected by caudal venipuncture using heparinized syringes with subsequent addition of 10% heparin to the collected blood. The animals were then euthanized by means of desensitization on ice and decapitation, and the liver and brain were removed and immediately frozen at -80 ° C.

2.5. Hematology and biochemistry

Blood aliquots were used to determine hematocrit (Htc) (Goldenfarb et al., 1971) and hemoglobin (Hg) (Tonks, 1983). Blood was centrifuged at 1792 x g for 5 min and the plasma separated and used to determine glucose (GLU), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels using Biotécnica® commercial kits. The samples were read in an automatic device (Cobas-Mira Plus®-Roche), which was also used by other authors (Mattiolli et al., 2017; Fortes-Silva et al., 2018; Favero et al., 2019; Boaventura et al., 2020).

2.6. Oxidative stress

The individual samples of liver and brain were defrosted, weighed, crushed and homogenized (Potter-Elvehjem glass homogenizer) (1:10 w v^{-1}) in a medium containing 120 mM potassium chloride and 30 mM phosphate buffer (pH 7.4). The samples were centrifuged for 5 min at 10,000 × g and the supernatant fraction obtained. Part of each supernatant was used to evaluate reactive oxygen species (ROS) and levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione S-transferase (GST), lipid peroxidation (LPO), and protein carbonyl formation (PC).

Hepatic and brain protein

Hepatic and brain protein concentrations were determined by the Coomassie Blue method following Read and Northcote (1981) using bovine serum albumin as a standard.

2.6.1 Enzymatic oxidative stress

Hepatic and brain superoxide dismutase (SOD)

The activity of superoxide dismutase was determined according to the pyrogallol auto-oxidation principle, 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 protein.

Hepatic and brain glutathione S-transferase (GST) activity

The activity of GST was measured, according to Mannervik and Guthenberg (1981) with slight modifications, as the rate of formation of dinitrophenyl-S-glutathione at 340 nm in a medium containing 50 mM potassium phosphate pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants (approximately 0.045 mg of protein). The results were calculated and expressed as U/mg protein.

Hepatic and brain glutathione peroxidase (GPx)

The activity of glutathione peroxidase (GPx) was measured indirectly by monitoring the oxidation rate of NADPH at 340 nm using cumene hydroperoxide (CuOOH), according to Wendel (1981). Enzymatic activity was expressed as U/mg protein.

2.6.2. Prooxidant variables

Hepatic and brain levels of reactive oxygen species (ROS)

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 the results expressed as U DCF/mg of protein

Hepatic and brain lipid peroxidation (LOOH)

LOOH was determined using the FOX methodology (Hermes-Lima et al. 1995, with some modifications by the authors), which is based on the oxidation of Fe (II) under acidic conditions. The FOX method measures lipid peroxides, one of the main products of lipid peroxidation. LOOH was measured by sequentially adding $FeSO_4$ (1 mM), H_2SO_4 (0.25 M), xylenol orange (1 mM, Sigma) and MilliQ water. Samples or methanol (blanks) were added and incubated for 30 min, after which absorbance (550 nm) was determined with cumene hydroperoxide (CHP; Sigma) as a standard. LOOH was expressed in cumene hydroperoxide (CHP) equivalents per nmol per mL of serum.

Hepatic and brain protein carbonyl formation

Protein carbonyl formation was measured by spectrophotometric assay following Reznick and Packer (1994) with some modifications. One hundred microliters of supernatant containing approximately 0.15 mg of protein was treated with 200 µL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl while another 100 µL of supernatant was treated with 2.5 N HCl (blank). Both were left in the dark for 1 h, after which protein was precipitated with 500 µL of 20% TCA and then centrifuged for 5 min at 10,000 × g. The pellet was suspended in 300 µL of 6 M guanidine prepared in 2.5 N HCl. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate carbonyl content determined at 365 nm. The results were calculated as nmol carbonyl groups/mg protein, using the extinction coefficient of 22,000/M cm for aliphatic hydrazones.

2.7. Statistical analysis

Data were submitted to the Shapiro-Wilk test for normality and the Levene test for homoscedasticity. Parametric results were analyzed by one-way ANOVA followed by Tukey's test, or when homogeneity of variance was not obtained, by the Scheirer-Ray-Hare extension of the Kruskal Wallis test and the Nemenyi test, both at 95 % probability ($P<0.05$). SigmaPlot and Infostat software were used for data analysis.

3. Results

3.1. Survival and water quality at the end of transport and ventilatory frequency during transport

No mortality was observed during and 24 h after transport. Water temperature and pH at the end of transport was similar among treatments and to the initial temperature and pH in the culture tank ($P > 0.05$). There was an increase in total ammonia after transport in all groups, with higher concentrations for the control and 5 mg/L EOOG ($P < 0.05$). All treatments with EOOG had higher dissolved oxygen levels than the control at the end of transport ($P < 0.05$). Fish transported with 10 mg/L EOOG had a lower ventilatory frequency than did control fish (Table 1).

Table 1- Water parameters before and after transport (4 h) and ventilatory frequency during transport (3 h) of pacamã (*Lophiosilurus alexandri*) in plastic bags containing different concentrations of the essential oil of *Ocimum gratissimum* L. (EOOG) in the water.

Parameter	Before transport	After transport (groups)					
	Basal	Control	EOOG (5 mg/L)	EOOG (10 mg/L)	*F-value*	d.f	*p-value*
Temperature (°C)	24.66±0.57	24.46±0.08	24.48±0.10	24.46±0.20	0.11	3	0.9527
pH	6.42±0.04	6.49±0.13	6.47±0.09	6.36±0.14	-	3	< 0.001
Total ammonia (mg/L)	0.5 ± 0	3.5 ± 0 a*	3.5±0 a*	2.3±0 b*	6.96	3	0.0027
Dissolved oxygen (mg/L)	7.2±0	3.67±0.79 b	9.73±3.13 a	9.13±0.72 a	10.74	3	> 0.001
Ventilatory frequency (beats/min)	23.2±1.03	27.46±3.67 a	18.0±3.2 c	15.6±0.54 c*	17.79	3	< 0.001

Values are expressed as mean ± standard deviation. Asterisk represents a significant difference between treatments and baseline. Different lowercase letters indicate significant differences between treatments ($p<0.05$). One-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance. F values for the normal distribution data. P values for normal and non-normal distribution data. *d.f.* = Degrees of freedom.

3.2. Blood parameters

No differences were observed for hematocrit (fig 1A), ALT (fig. 1D) and total plasma protein (fig. 1F) ($P > 0.05$) after 4 h of transport. Hemoglobin was lower in fish transported with 10 mg/L EOOG than basal and control fish ($P < 0.05$) (Fig. 1B). Fish transported with EOOG had lower AST than those of the control treatment (Fig. 1C) ($P < 0.05$). Plasma glucose levels were higher for control fish at the end of transport compared to basal fish, while EOOG reduced this increase (mainly with 10 mg/L EOOG; $P < 0.05$) (Fig. 1 E).

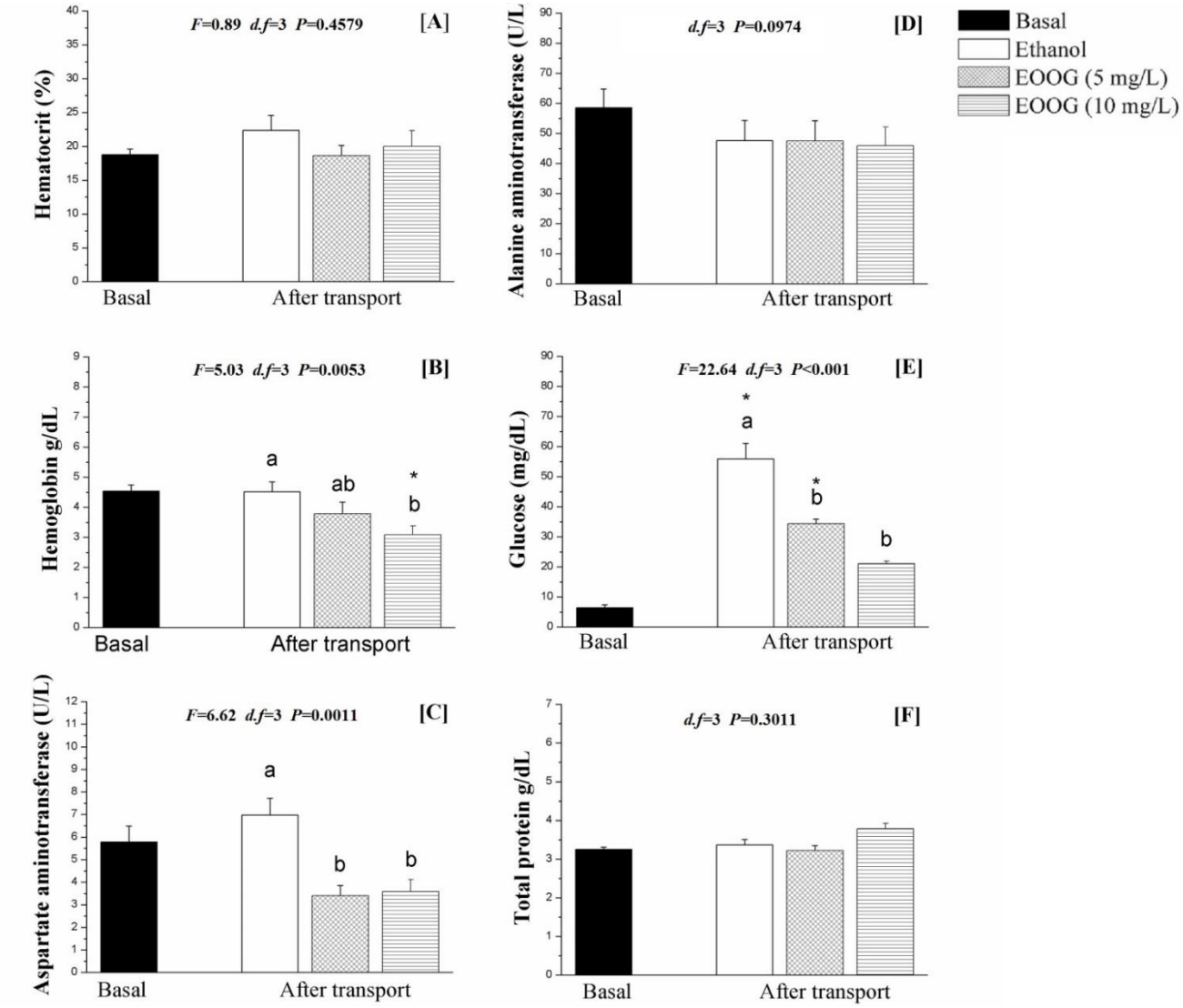

Figure 1. Biochemical and hematological parameters of *Lophiosilurus alexandri* measured immediately after transport (4 h) with essential oil of *Ocimum gratissimum* (EOOG). Hematocrit (A), hemoglobin (B), aspartate aminotransferase (C), alanine aminotransferase (D), glucose (E) and total plasma protein (F) N = 10. Values are means ± standard error. Asterisks represent significant differences between treatments and baseline. Different lowercase letters indicate significant differences between treatments ($p<0.05$). One-way ANOVA and Tukey's test or Scheirer-Ray-Hare extension of the Kruskal-Wallis and Nemenyi tests were used to determine statistical significance. F values for the normally distributed data; P values for normally and non-normally distributed data. *d.f.* = degrees of freedom.

3.4. Enzymatic oxidative and prooxidant stress variables

3.4.1. Enzymatic oxidative stress in liver and brain

There were no differences in hepatic SOD (fig. 2A), GST (fig. 2B) and GPx activities (fig. 2C) among treatments after transport ($P > 0.05$). However, hepatic SOD activity was lower than basal after transport for all treatments (Fig. 2A) ($P < 0.05$).

Brain SOD activity was not affected by transport (fig. 2D). Fish transported with 5 mg/L EOOG had higher brain GST activity than did those of control and 10 mg/L EOOG ($P < 0.05$) treatments, but not different from basal fish (fig 2E) ($P > 0.05$). Brain GST activity was lower than basal for control and 10 mg/L EOOG ($P < 0.05$) treatments. Fish transported with 10 mg/L EOOG had higher brain GPx activity than basal fish and fish of the other treatments (fig. 2F) ($P < 0.05$).

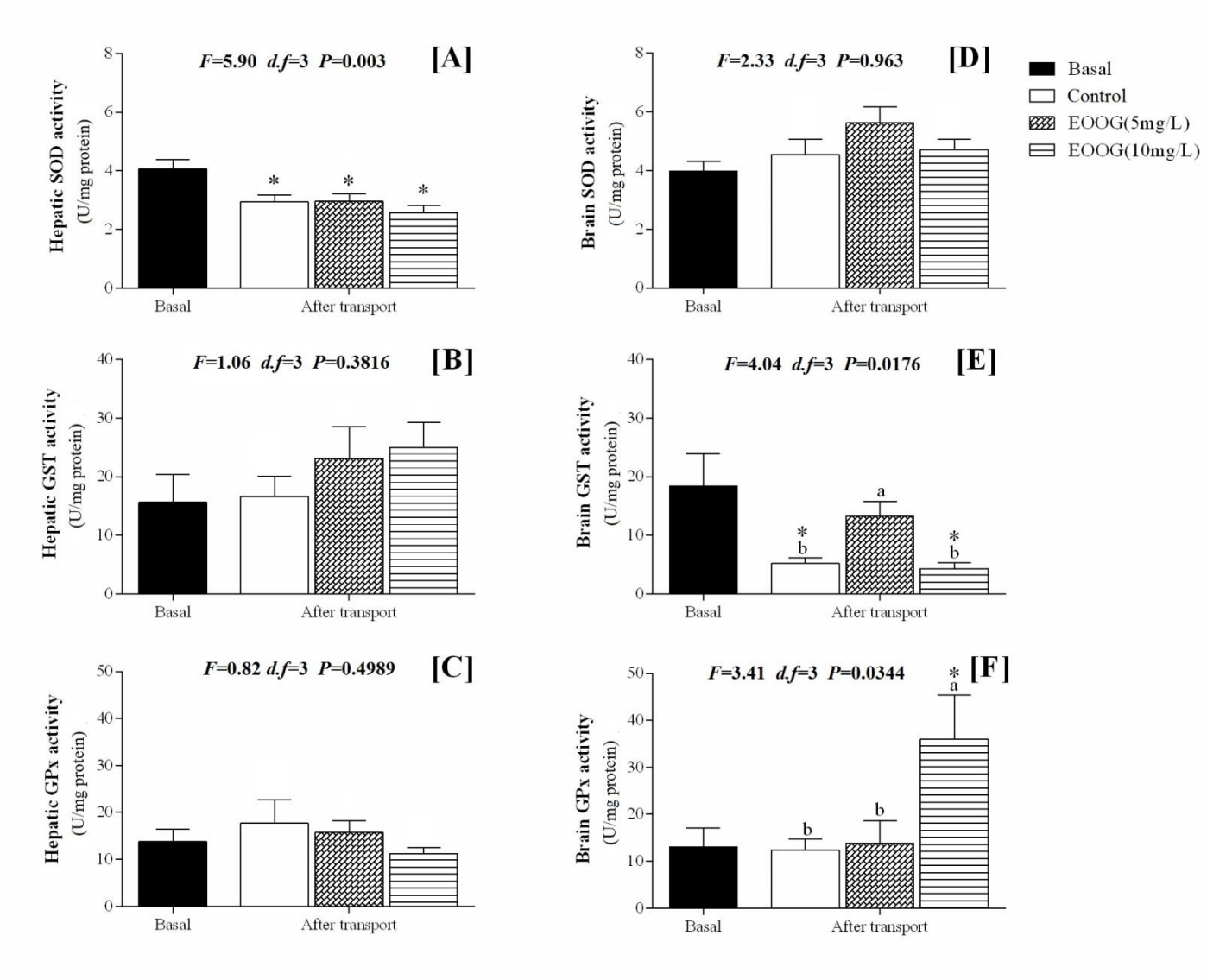

Figure 2. Enzymatic oxidative stress variables: [A, D] superoxide dismutase (SOD), [B, E] glutathione-s-transferase (GST) and [C, F] glutathione-peroxidase (GPx) activities in the liver and brain of *Lophiosilurus alexandri* (n = 8) immediately after transport (4 h) with the essential oil of *Ocimum gratissimum* (EOOG). Values are means ± standard error. Asterisks represent significant differences between treatments and baseline. Different lowercase letters indicate significant differences between treatments ($p<0.05$). One-way ANOVA and Tukey's test or Scheirer-Ray-Hare extension of the Kruskal-Wallis and Nemenyi tests were used to determine statistical significance. F values for the normally distributed data; P values for normally and non-normally distributed data. *d.f.* = degrees of freedom.

3.4.2. Prooxidant variables in liver and brain

Hepatic ROS levels were not affected by transport or treatments (fig. 3A) ($P > 0.05$). Levels of hepatic LOOH (fig. 3B) and protein carbonyl (fig. 3C) were lower in fish transported with 10 mg/L EOOG than basal fish and fish of the other treatments ($P < 0.05$). No differences were observed among treatments and basal for brain ROS (fig. 3D) and protein carbonyl (fig. 3F) ($P > 0.05$). Brain LOOH levels were lower in fish transported with 10 mg/L EOOG than basal fish and fish of the other treatments (fig. 3E) ($P < 0.05$).

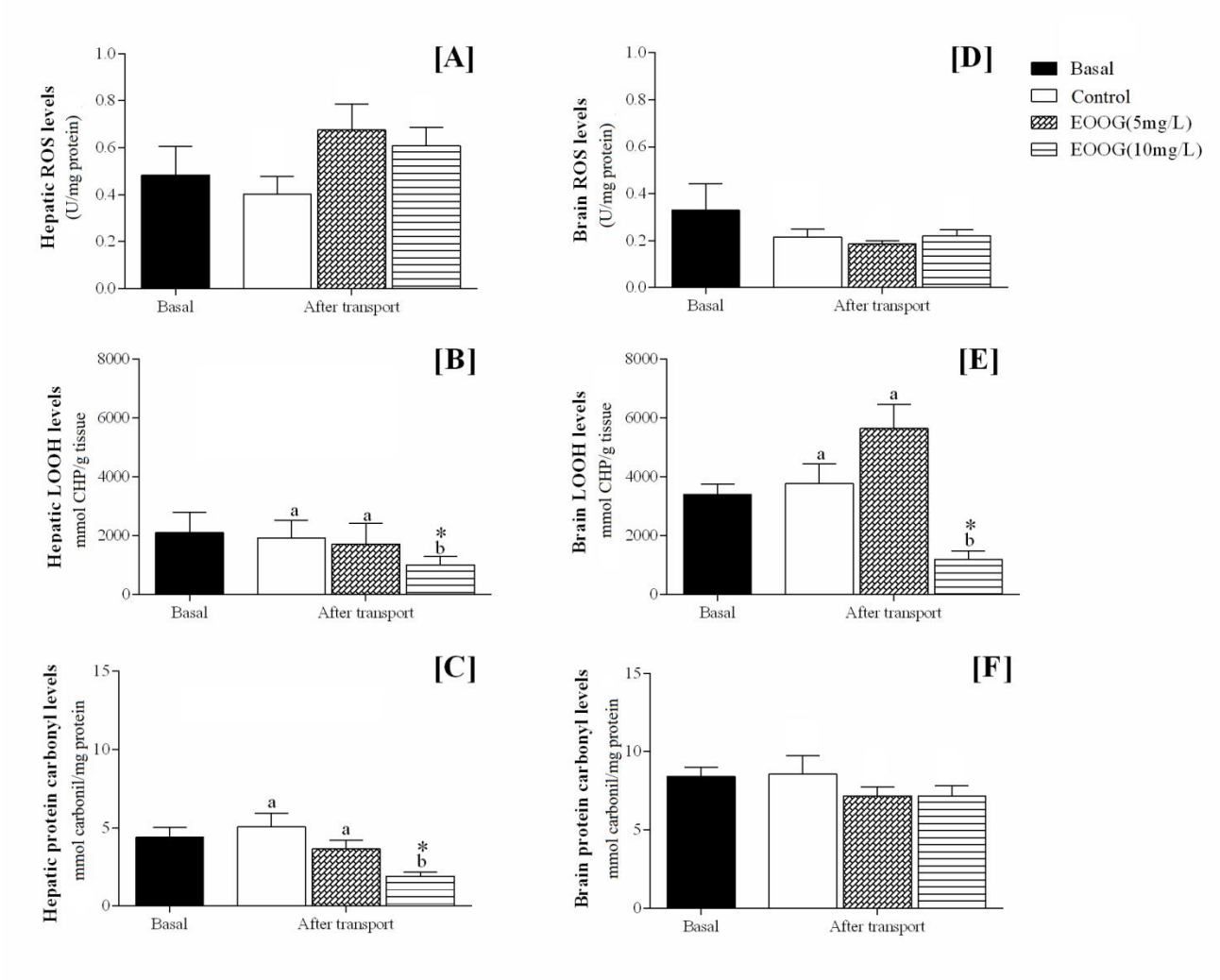

Figure 3. Prooxidant variables: [A, D] Reactive oxygen species (ROS), [B, E] lipid hydroperoxides (LOOH) and [C, F] protein carbonylation (PC) levels in the liver and brain of *Lophiosilurus alexandri* (n = 8) immediately after transport (4 h) with the essential oil of *Ocimum gratissimum* (EOOG). Values are means ± standard error. Asterisks represent significant differences between treatments and baseline. Different lowercase letters indicate significant differences between treatments ($p<0.05$). One-way ANOVA and Tukey's test or Scheirer-Ray-Hare extension of the Kruskal-Wallis and Nemenyi tests were used to determine statistical significance. F values for the normally distributed data; P values for normally and non-normally distributed data. *d.f.* = degrees of freedom.

Discussion

The addition of 5 and 10 mg/L EOOG to transport water did not induce deep anesthesia in juveniles of *L. alexandri* during transport, which is desirable. The consumption of oxygen and the accumulation of carbon dioxide and ammonia in water are significant problems during the transport of fish in plastic bags (Amend et al., 1982; Golombieski et al., 2003). In the present study, the water of animals transported with 10 mg/L of EOOG had a lower concentration of ammonia and a higher concentration of dissolved oxygen after transport. These results show that EOOG reduced animal metabolism and, consequently, ammonia excretion and oxygen consumption. Likewise, the addition of 12.5 and 25 μL/L of essential oil of *Aloysia triphylla* to water for 3 h of transport of *L. alexandri* juveniles also reduced metabolism and, consequently, the excretion of ammonia and oxygen consumption (Becker et al., 2017). Parodi et al. (2014) also observed lower ammonia excretion for *Rhamdia quelen* transported for 5 h with 40 μL/L of essential oil of *A. triphylla*.

The reduced metabolism of *L. alexandri* juveniles during transport with EOOG is further evidenced by their reduced ventilatory frequency compared to control fish. The use of 5 mg/L of clove oil for the transport of puffer fish (*Takifugu obscurus* and *Takifugu rubrips*) also reduced ventilatory frequency (Park, 2019). Becker et al. (2017) also observed a reduction in opercular beats of *L. alexandri* after 0.5 h of exposure to 25 μL/L of essential oil of *A. triphylla*.

Fish can increase blood hemoglobin concentration in response to stress and hypoxia to improve oxygen transport and supply the body's energy demand (Souza and Bonilla-Rodriguez, 2007). However, animals transported with 10 mg/L EOOG had hemoglobin values below basal, which may be associated with its sedative effect causing lower oxygen consumption during transport. Normally, during the transport of animals, there is an increase in carbon dioxide concentration of the water and, consequently, a reduction in pH (Simões et al., 2011); the present study, however, found no change in pH during transport.

When fish are subjected to the stress of transport, catecholamines and corticosteroids are released and, consequently, energetic reserves are mobilized to produce energy for the organism to adapt (Iversen et al., 2005; Iversen et al., 2009). Due to this mobilization, hyperglycemia has been used as an indicator of stress in fish (Gomes et al., 2006; Velisek et al., 2011). The juveniles of *L. alexandri* transported with EOOG had lower plasma glucose levels than did control individuals, indicating that the use of EOOG prevented stress and hyperglycemia during transport.

The enzymes AST and ALT are indicators of tissue damage (Mirghaed et al., 2018). They are also involved in the mobilization of amino acids via gluconeogenesis for the production of glucose (Chatterjee et al., 2006). Therefore, the finding that control fish had the highest level of AST may be related to the mobilization of aspartate for the production of glucose, since this treatment also had the highest levels of glucose.

Under normal conditions, cells maintain a balance between oxidizing and prooxidant agents (Poli et al., 2012). However, adverse situations, such as the presence of xenobiotics (Lackner, 1998) and hyperoxic conditions (Barbas et al., 2017), can cause imbalance to this system by increasing cellular production of ROS. The presence of high concentrations of ROS in cells can cause several types of tissue damage, such as lipid and protein oxidation (Stadtman and Levine, 2003). Carbonyl protein (Stadtman and Levine, 2003) and LOOH (Esterbauer, 1996) are frequently used as oxidative stress markers since they are products of protein and lipid degradation, respectively. (Esterbauer, 1996; Stadtman and Levine, 2003). In the present study, fish of all treatments had ROS levels similar to basal. However, animals transported with 10 mg/L EOOG had levels of hepatic protein carbonyl and hepatic and brain LOOH below basal, indicating that the use of 10 mg/L EOOG increased the protection of cells against oxidative damage, which may have been a result of the antioxidant property of EOOG (Souza et al., 2019). The use of essential oil of *Nectranda grandiflora* and hexanic extract of *Spilanthes*

acmella during the transport of *Colossoma macropomum* in hyperoxic conditions also increased protection against oxidative damage to tissues (Barbas et al., 2017).

The antioxidant defense system prevents cell damage when oxidative substances are present by increasing the concentration of antioxidant enzymes in the body (Poli et al., 2012). The enzyme SOD converts ROS into hydrogen peroxide and water and, thus, is one of the main enzymes responsible for the elimination of ROS (Cheeseman and Slater, 1994). The enzymes catalase (CAT) and GPx convert hydrogen peroxide into water and oxygen (Li et al., 2009; Velisek et al., 2011). The reduction found for hepatic SOD of *L. alexandri* after transport for all treatments may be related to the response of the body to oxidizing agents, which was enough to prevent an increase in ROS. The increase in brain GPx in fish transported with 10 mg/L of EOOG may have been a response by the organism to reduce the levels of oxidized substances in cells, since this was the only indicator of cellular damage analyzed that was not reduced with the use of 10 mg/L EOOG. In addition to its involvement in the antioxidant defense system, the enzyme GST also plays a very important role in detoxifying the body of xenobiotic agents (Frova, 2006). Therefore, the lowest concentrations of GST found in fish of the control and 10 mg/L EOOG treatment may be related to them having the highest concentrations of ammonia and anesthetic (EOOG), respectively.

Conclusion

It can be concluded that the use of 5 and 10 mg/L of EOOG has a sedative effect on juveniles of *L. alexandri*, which reduces oxygen consumption and ammonia excretion. The use of EOOG also prevented hyperglycemia and increased AST. In addition, 10 mg/L EOOG showed greater protection against oxidative damage than the other treatments. Thus, based on the results obtained here, 10 mg/L EOOG is recommended for *L. alexandri* transport. Although EOOG yielded good results, additional studies on its effects on the overall well-being of fish during and after transport and fillet quality are necessary.

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Considerações finais

O óleo essencial de *Ocimum gratissimum* L (OEOG) provou ser um anestésico eficiente para ser utilizado durante o manejo e o transporte do *L. alexandri*.

O uso de 90 e 150 mg/L do OEOG causa anestesia em *L. alexandri* em intervalos de indução (<3 minutos) e recuperação (<5 minutos) considerados ideais. O uso de 90 mg/L do OEOG evitou níveis mais elevados de glicose e cortisol plasmáticos uma hora após o manejo. Por outro lado, embora não tenha causado danos aos tecidos, o uso de 90 mg/ L OEOG proporcionou algumas alterações no sistema de defesa antioxidante, aumentando a concentração de ROS hepático e cerebral e reduzindo a atividade do GST cerebral em uma hora após a recuperação.

O uso de 5 e 10 mg/L de OEOG durante o transporte proporcionou efeito sedativo em juvenis de *L. alexandri*, reduzindo o consumo de oxigênio e a excreção de amônia. O uso dessas concentrações também evitou a hiperglicemia dos animais após o transporte. Além disso, os animais transportados com 10 mg / L do OEOG apresentaram maior proteção contra os danos oxidativos.

Com base nos resultados obtidos, recomenda-se o uso de 90 mg/L do OEOG para o manejo e de 10 mg / L do OEOG para o transporte de juvenis de *L. alexandri*.

Apesar de terem apresentado bons resultados, pesquisas futuras devem ser realizadas para verificar o tempo de retenção do OEOG na carne *L. alexandri,* sua influência sobre o consumo de ração e seus efeitos após um período maior de recuperação dos peixes após o manejo e transporte.

7. ANEXO

Figura 1. Fotografia do pacamã **(***Lophiosilurus alexandri*).

Fonte: acervo pessoal

Figura 2. Fotografia do alfavaca ou manjericão **(***Ocimum gratissimum*).

Fonte: adaptado de Costa. 2016.