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Carine de Freitas Souza

IMPLICAÇÕES FISIOLÓGICAS DO USO DO ÓLEO ESSENCIAL DE *Lippia alba*, **QUIMIOTIPOS LINALOL E CITRAL, NA ANESTESIA DE** *Rhamdia quelen*

Santa Maria, RS
2018

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Tese apresentada ao Curso de Pós-Graduação em Biodiversidade Animal, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para obtenção do título de **Doutor em Ciências Biológicas: Biodiversidade Animal**

Orientador: Prof.º Dr. Bernardo Baldisserotto

Co-orientadora: Prof.° Drª Berta Maria Heinzmann

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DEDICATÓRIA

A Deus, esta força incrível, magnânima e oniponte que move minha vida.

Aos meus pais, Volmar e Roselei, que muitas vezes se doaram e renunciaram aos seus sonhos, para que eu pudesse realizar os meus. Quero dizer que essa conquista não é só minha, mas nossa. Tudo que consegui só foi possível graças ao amor, apoio e dedicação que vocês sempre tiveram por mim. Sempre me ensinaram agir com respeito, simplicidade, dignidade, honestidade e amor ao próximo. E graças à união de todos, os obstáculos foram ultrapassados, vitórias foram conquistadas e alegrias divididas. Agradeço pela paciência e compreensão com minha ausência durante essa longa jornada. Muitíssimo obrigado.

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“Os sonhos não determinam o lugar onde vocês vão chegar, mas produzem a força necessária para tirá-los do lugar em que vocês estão. Sonhem com as estrelas para que vocês possam pisar pelo menos na Lua. Sonhem com a Lua para que vocês possam pisar pelo menos nos altos montes. Sonhem com os altos montes para que vocês possam ter dignidade quando atravessarem os vales das perdas e das frustrações. Bons alunos aprendem a matemática numérica, alunos fascinantes vão além, aprendem a matemática da emoção, que não tem conta exata e que rompe a regra da lógica. Nessa matemática você só aprende a multiplicar quando aprende a dividir, só consegue ganhar quando aprende a perder, só consegue receber, quando aprende a se do ¨

(Augusto Cury)

RESUMO

IMPLICAÇÕES FISIOLÓGICAS DO USO DO ÓLEO ESSENCIAL DE *Lippia alba*, QUIMIOTIPOS LINALOL E CITRAL, NA ANESTESIA DE *Rhamdia quelen*

AUTORA: Carine de Freitas Souza

ORIENTADOR: Bernardo Baldisserotto

Recentemente, foi reportado que o óleo essencial (OE) de *Lippia alba*, quimiotipo linalol, causa anestesia em peixes da espécie *Rhamdia quelen*. No entanto, sabe-se que uma mesma espécie vegetal pode apresentar diferenças em sua composição química, que varia conforme o quimiotipo, local de coleta, época do ano e tipo de solo. Deste modo, faz-se necessário a investigação cautelosa, a fim de averiguar se os efeitos anestésicos e sedativos do OE de *L. alba* são mantidos frente a diferentes quimiotipos, bem como analisar seus efeitos na fisiologia dos peixes, antes de recomendá-lo como anestésico seguro. O presente estudo avaliou os efeitos sanguíneos, plasmáticos e bioquímicos decorrentes da anestesia com OE de *L. alba* de dois diferentes quimiotipos: linalol (OE-L) e citral (OE-C) em *R. quelen*. No primeiro experimento foram utilizadas as concentrações de 100 e 300 µL/L para anestesiar os peixes. Durante a anestesia e recuperação, foi coletado sangue dos animais para averiguar parâmetros iônicos, sanguíneos e bioquímicos. Os níveis plasmáticos de Na^+, K^+ e Ca^{2+} não apresentaram nenhuma alteração durante a indução e recuperação anestésica em todos os tratamentos. No entanto, a glicemia aumentou durante a anestesia com ambos anestésicos. Os níveis de creatinina apresentaram um aumento ao final da recuperação anestésica em peixes anestesiados com OE-C. Deste modo, um segundo estudo foi realizado a fim de verificar se as alterações obtidas no primeiro experimento poderiam ser indicativas de algum tipo de dano tecidual. Dessa forma, num segundo experimento, objetivamos averiguar os efeitos desta anestesia, sob parâmetros bioquímicos, antioxidantes e de dano tecidual, repetindo o mesmo desenho experimental anteriormente utilizado. Como resultado, foi observado que embora a anestesia com OE-C e OE-L evite aumento dos níveis de cortisol plasmático, OE-C aumentou os níveis de substâncias reativas ao ácido tiobarbitúrico, bem como a carbonilação das proteínas no fígado e no rim dos peixes. Por outro lado, OE-L não causa nenhum tipo de dano tecidual. Em suma, este estudo comprova que diferentes quimiotipos de uma mesma espécie vegetal podem causar diferentes efeitos fisiológicos. Por fim, recomendamos o uso do OE-L como anestésico eficaz e seguro para *R. quelen*.

Palavras-chave: cortisol, estresse oxidativo, gases sanguíneos, íons, peixes

ABSTRACT

PHYSIOLOGICAL IMPLICATIONS OF THE USE OF THE ESSENTIAL OIL OF *Lippia alba*, chemotypes LINALOOL AND CITRAL, IN ANESTHESIA OF *Rhamdia quelen*

AUTHOR: Carine de Freitas Souza

ADVISOR: Bernardo Baldisserotto

Recently, it was reported that the *Lippia alba* essential oil (EO) linalol chemotype causes anesthesia in fish of *Rhamdia quelen* species. However, it is known that a given vegetal species can present differences in its chemical composition, which varies according to the chemotype, local of sampling, time of year and type of soil. In this way, it is necessary a cautious investigation to determine whether anesthetic and sedative effects of *L. alba* EO are maintained at different chemotypes, as well as to analyze their effects on fish physiology before recommending it as a safe anesthetic. The present study evaluated the blood, plasmatic and biochemical effects caused from anesthesia with two different chemotypes of *L. alba* EO: linalol (EO-L) and citral (EO-C) in *R. quelen.* In the first experiment the concentrations of 100 and 300 µL/L were used to anesthetize the fish. Blood was collected through anesthesia and recovery in order to investigate the ionic, blood and biochemical parameters. The plasma Na^+, K^+ and Ca^{2+} levels did not show alterations during anesthesia induction and recovery with both treatments. However, blood glucose increased during anesthesia with both anesthetics. Plasmatic creatinine levels increased at the end of recovery in fish anesthetized with EO-C. In this sense, a second experiment was carried out in order to verify whether the alterations observed in the first experiment could be indicative of tissue damage. Thus, the aim of the second experiment was to investigate the effects of anesthesia on biochemical, antioxidant and tissue damage parameters, replicating the same experimental model used previously. As result, it was observed that although anesthesia with EO-C and EO-l avoided the increase on plasma cortisol levels, EO-C increased thiobarbituric reactive acid substances, as well as protein carbonylation in liver and kidney of fish. On the other hand, EO-L did not cause any type of tissue damage. Thus, this study proves that different chemotypes of a given vegetal species can provoke different physiological effects. In summary, we recommended the use of EO-L as an effective and safe anesthetic to *R. quelen.*

Keywords: blood gases, plasma, ion, cortisol, fish

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Manuscrito I

LISTA DE ABREVIATURAS E SIGLAS

ACTH – Hormônio adrenocorticotrófico

ANOVA – Análise de variância

CAT – Catalase

CRH – Hormônio Liberador de Corticotrofina

EROs – Espécies reativas de oxigênio

GPx – Glutationa Peroxidase

GSH – Glutationa Reduzida

GSSG – Glutationa dissulfeto

GST – Glutationa–S–Transferase

H_2O_2 – Peróxido de Hidrogênio

HCO_3^- – Bicarbonato

$HO^\bullet$ - Radical hidroxila

HPI – Hipotálamo, pituitária, interrrenal

MDA – Malondialdeído

MS222 – Tricaína metanosulfato

$NADP^+$ – Nicotinamida adenina dinucleótido fosfato

NADPH – Nicotinamida adenina dinucleótido fosfato reduzida

NPSH - tióis não proteicos

$O2^{\bullet -}$ - Radical superóxido

OE – óleo essencial

OEC - óleo essencial quimiotipo citral

OEL – óleo essencial quimiotipo linalol

PC – Proteína Carbonil

***p*CO_2** – Pressão parcial de dióxido de carbono

pH – Potencial Hidrogeniônico

***p*O_2** – Pressão parcial de oxigênio

SNC – Sistema nervoso central

SOD – Superóxido dismutase

TBARS – Substâncias reativas ao ácido tiobarbitúrico

α-MSH – Hormônio estimulante de melanócitos

SUMÁRIO

1 INTRODUÇÃO

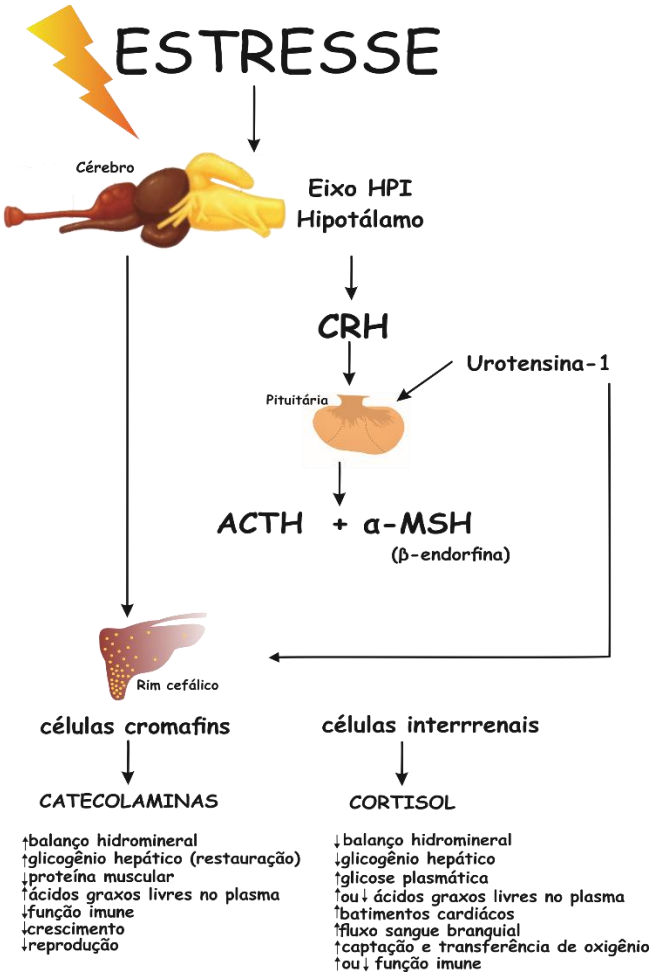
1.1 ESTRESSE EM PEIXES

O estresse está presente na piscicultura como consequência do manejo e pode ser definido como uma condição em que a homeostase é ameaçada ou perturbada em decorrência da ação de estímulos denominados estressores. Se um animal está submetido a estresse intenso e constante, a resposta fisiológica pode perder seu poder de se ajustar e tornar-se disfuncional, acarretando danos permanentes a sua saúde e bem-estar (WENDEELAR BONGA, 1997).

De acordo com Barton (2002), a resposta ao estresse em peixes é um evento integrado, mas é didaticamente separado entre resposta primária, secundária e terciária. A resposta primária compreende a ativação dos centros cerebrais, resultando em massiva liberação de catecolaminas (adrenalina e noradrenalina) e corticosteroides (cortisol), enquanto a resposta secundária é usualmente definida como a canalização das ações e dos efeitos imediatos desses hormônios em nível sanguíneo e tecidos, incluindo o aumento dos batimentos cardíacos e da absorção de oxigênio, a mobilização de substratos de energia e, ainda, a perturbação da osmorregulação. A limitação da capacidade do animal em tolerar estressores subsequentes ou adicionais também é atribuída a uma manifestação da resposta terciária (LIMA et al., 2006). A resposta terciária manifesta-se na população, traduzindo-se na inibição da reprodução (SMALL, 2004), crescimento e resposta imune (Figura 1) (WEENDELAR BONGA, 1997; TORT, 2011).

Antes de mais nada, é importante ressaltar que peixes são animais que ao longo da escala evolutiva enfrentaram diferentes adversidades, tanto bióticas quanto abióticas, e obtiveram sucesso na sobrevivência em variados habitats devido ao desenvolvimento de diferentes estratégias que envolveram ajustes fisiológicos e moleculares, refletindo a versatilidade destes animais frente às oscilações ambientais (FERNANDES et al., 1999; VAL, et al., 2004).

Figura 1 – Respostas ao estresse em peixes. A ativação dos eixos simpático-cromafim e hipotálamo-hipófise-interrenais culminam na liberação de catecolaminas e cortisol respectivamente.



Fonte: Adaptado de Wendelaar Bonga, 1997. Ilustração da autora.

Na aquicultura, procedimentos de tratamento são comuns durante o trabalho de rotina ou pesquisa. Atividades como marcação, triagem, vacinação, biometria, transporte e procedimentos cirúrgicos estão associados com estresse agudo de animais e até mesmo a mortalidade (PÁLIC et al., 2006; VIDAL et al., 2008).

1.2 MARCADORES DE ESTRESSE EM PEIXES

Muitos dos ajustes fisiológicos apresentados pelos peixes frente a condições de perturbação ou estresse, como por exemplo durante a manipulação e transporte, podem ser detectados por vários parâmetros, tais como a concentração plasmática

de cortisol, catecolaminas, glicose, lactato, lipídios, eletrólitos, proteínas, creatinina, ureia e estudos hematológicos (DAVIS; PARKER, 1986, DAVIS; SCHRECK, 1997; WENDELAAR BONGA ,1997), além de marcadores do estresse oxidativo.

1.2.1 Parâmetros Bioquímicos

No estresse secundário, a liberação de catecolaminas e/ou cortisol tem como consequência alterações bioquímicas no sangue e nos tecidos dos peixes, incluindo desequilíbrio eletrolítico, hiperglicemia, hiperlactatemia, depleção das reservas glicogênicas, lipólise e inibição da síntese proteica, alterações nos níveis plasmáticos de aminoácidos, ácidos graxos livres, colesterol (MCDONALD; MILLIGAN, 1997; WENDELAAR BONGA, 1997; MILLIGAN, 2003).

Em peixes, a glicose é a fonte primária de energia, atendendo principalmente à demanda do cérebro e dos músculos. Numa situação de estresse, o aumento de seus níveis no sangue é relatado para vários teleósteos e se deve à ação das catecolaminas na quebra de glicogênio hepático (Wendelaar Bonga, 2011). Nesta situação, o lactato também pode ser utilizado como fonte glicogênica, em circunstância de maior disponibilidade (Mommsen et al., 1999). Sob situação de estresse, o organismo também pode apresentar um aumento na mobilização de aminoácidos, proveniente de quebras de proteínas corporais, além de alterações no metabolismo de lipídeos (Wendelaar Bonga, 2011).

Com relação às respostas ionorregulatórias, as evidências mostram que o cortisol é um importante hormônio na aclimatação dos peixes na água doce, tendo tanto ação glicorticoide, quanto mineralocorticoide. Pelo menos dois mecanismos ionorregulatórios estão envolvidos durante a recuperação metabólica após estresse agudo: ativação dos transportadores H^+/Na^+ e HCO_3^-/Cl^- e mudanças nos fluxos difusionais de Na^+ e Cl^-. Outrossim, o estresse também é capaz de causar alterações sobre outros parâmetros bioquímicos sanguíneos, como níveis de enzimas e substâncias com importantes funções metabólicas, como ureia e creatinina, que indicam a saúde geral dos peixes (Cnaani et al., 2004).

Desta forma, faz-se categórico o uso de parâmetros bioquímicos, tais como os níveis séricos e/ou teciduais de glicose, lactato, glicogênio, proteínas e íons para avaliar o estresse em peixes (Barton, 2002).

1.2.2 Parâmetros hematológicos: Gasometria

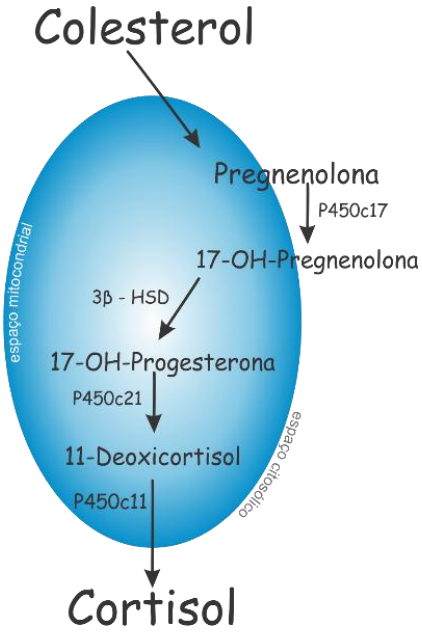
Da mesma maneira que as análises bioquímicas, os parâmetros de gasometria sanguínea são marcadores valiosos para o acompanhamento da saúde dos peixes. Neste sentido, a gasometria ou análise dos gases sanguíneos é uma ferramenta útil para o acompanhamento do processo de indução e recuperação anestésica, uma vez que estas análises detectam a eficácia das trocas gasosas respiratórias (oxigenação) e parâmetros metabólicos (equilíbrio ácido-base), usualmente como mensurações dos gases sanguíneos: pressão parcial de oxigênio (pO_2), pressão parcial de dióxido de carbono (pCO_2), pH sanguíneo e íon bicarbonato (HCO_3^-) através do cálculo de Siggard-Andersen (KING, 2000).

Além disto, a combinação destas duas análises configura uma excelente ferramenta para o acompanhamento das condições fisiológicas dos peixes, seja no controle de doenças, seja no estresse causado pela manipulação, seja na resposta aos anestésicos (ALDRIN et al., 1982; TAVARES DIAS et al., 2008; ARAÚJO et al., 2009; BARBAS et al., 2016).

1.2.3 Cortisol

O cortisol plasmático é um hormônio corticosteroide sintetizado a partir do colesterol (Figura 2), sendo o principal marcador para avaliação de estresse, largamente utilizado em peixes de qualquer estágio de desenvolvimento (WEENDELAR BONGA, 1997; MOMMSEN et al., 1999). O estresse pode induzir a liberação de adrenalina e noradrenalina pelas células cromafins em resposta à estimulação do sistema nervoso simpático, o que pode aumentar os níveis de glicose no sangue (MOMMSEN et al., 1999). Desta forma, os níveis de glicose também podem fornecer informações importantes sobre o estado dos peixes.

Figura 2 - Biossíntese de cortisol em peixes teleósteos.

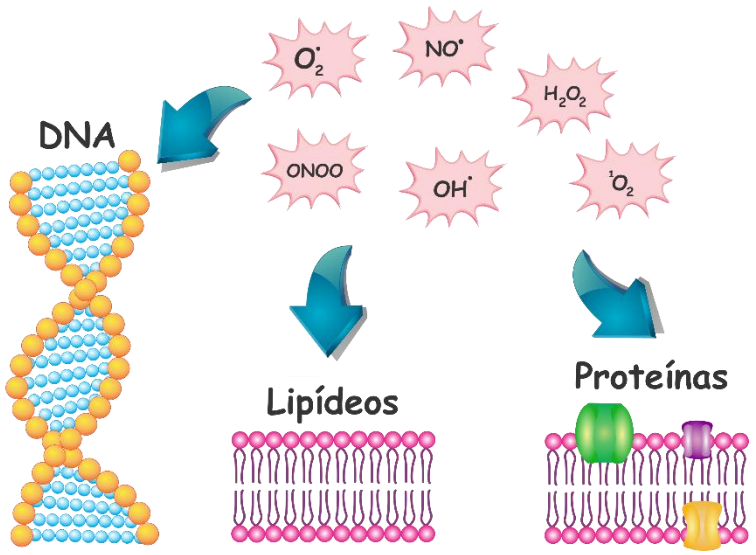


Fonte: Adaptado de Mommsen, Vijayan, Moon, 1999. Ilustração da autora.

1.2.4 Marcadores de estresse oxidativo

O oxigênio é essencial para a maioria das formas de vida, incluindo os peixes, mas também é inerentemente tóxico, devido à sua biotransformação em espécies reativas ao oxigênio (EROs) (HERMES-LIMA; ZENTENO-SAVIN, 2002). Algumas EROs são radicais livres, tais como os radicais superóxido (O_2•-) e hidroxila (•OH), e outras são agentes oxidantes não radicais, como o peróxido de hidrogênio (H_2O_2) (BARBOSA et al., 2010). Normalmente, cada célula animal mantém um estado de homeostase entre os oxidantes e agentes antioxidantes (POLI et al., 2004). Caso o sistema de defesa antioxidante esteja comprometido, pode ser estabelecido um desequilíbrio pró-oxidante celular, denominado estresse oxidativo, podendo acarretar em danos em lipídeos (ESTERBAUER; CHEESEMAN, 1990), proteínas celulares (HALLIWELL; CHIRICO, 1993) e DNA (MARNETT, 1999), comprometer a sobrevivência dos peixes (Figura 3). O termo antioxidante pode ser considerado como qualquer substância que atrase, previna ou remova o dano oxidativo de uma molécula-alvo (HALLIWELL; GUTTERIDGE, 2007).

Figura 3 - Dano oxidativo a macromoléculas biológicas



Fonte: Adaptado de Torres, 2003. Ilustração da autora.

Para combater estas espécies reativas de oxigênio existe um sistema de defesa antioxidante, composto por mecanismos enzimáticos e não enzimáticos. As principais enzimas antioxidantes são a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa-S-transferase (GST), e os antioxidantes não-enzimáticos, como a glutationa reduzida (GSH) (Figura 4), todas elas abundantes nos tecidos de peixes (STOREY, 1996; BIANCHI; ANTUNES,1999; VAN DER OOST et al., 2003; WENDELAAR BONGA, 2011).

O radical superóxido é a primeira ERO formada, podendo agir como oxidante ou como redutor, dando origem a outras espécies reativas (FRIDOVICH, 1989; WANG et al., 2015), e é formado através de diversas vias metabólicas, como por exemplo, a cadeia respiratória na mitocôndria, a via síntese de prostaglandinas no retículo endoplasmático liso e no sistema P-450 e o sistema xantina/oxidase, através da NADPH oxidase em células fagocíticas (RIBEIRO et al., 2005). Diante desta situação, a SOD é uma metaloenzima essencial ao sistema de defesa antioxidante, pois catalisa a dismutação do radical superóxido, formando peróxido de hidrogênio. O peróxido de hidrogênio (H_2O_2), por sua vez, será degradado pela CAT ou pela GPx (RIBEIRO et al., 2005).

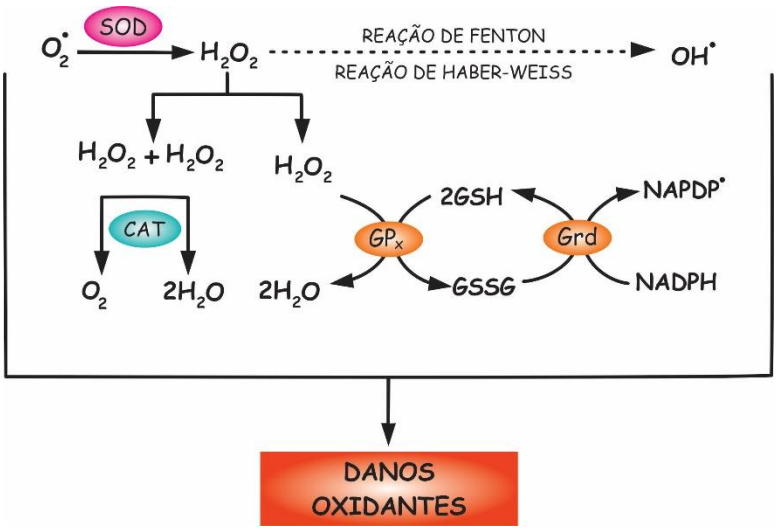
A CAT é uma peroxidase que degrada o peróxido de hidrogênio em oxigênio e água. Esta enzima é encontrada no citoplasma, em grande concentração nos peroxissomos de todos os tecidos animais, porém é mais eficiente quando as concentrações intracelulares de H_2O_2 são mais elevadas. Pequenos aumentos no H_2O_2 parecem ser mais bem controlados pela GPx (HERMES-LIMA, 2004), sendo a principal peroxidase em peixes.

A GPx converte a GSH à glutationa oxidada (GSSG), removendo H_2O_2 e formando água. Dessa forma, tanto, CAT quanto GPx evitam o acúmulo de radical superóxido e peróxido de hidrogênio para que não haja produção de radical hidroxil, contra o qual não existe sistema enzimático de defesa (YU, 1994). Dentro deste contexto, o perfeito equilíbrio entre as enzimas antioxidantes (SOD, CAT, GPx) é importante para a manutenção da integridade celular, mantendo o equilíbrio homeostático.

A GST é a principal enzima de desintoxicação da segunda fase da defesa antioxidante. Ela também envolve reações de conjugação na presença da GSH, no entanto desempenha um papel importante na detoxificação e eliminação de compostos eletrofílicos. Esta desintoxicação ocorre através de conjugados de glutationa com xenobióticos e produtos aldeídos produzidos na peroxidação lipídica (hidroperóxidos), tornando os produtos mais solúveis em água (HERMES-LIMA, 2004; LIMÓN-PACHECO; GONSEBATT, 2009).

Além de atuar como coenzima em reações enzimáticas, a GSH também está envolvida em vários processos fisiológicos como a degradação de peróxidos endógenos, formação de moléculas bioativas, detoxificação de toxinas e participação no transporte de aminoácidos (STAMLER; SLIVKA, 1996). A GSH participa ainda a decomposição do H_2O_2, potencialmente tóxico, que é convertido em H_2O em reação catalisada pela GPx, oxidando a GSH; a glutationa oxidada resultante é reciclada à forma reduzida pela glutationa redutase e NADPH (BOURAOUI, 2008).

Figura 4 - Integração dos sistemas de defesa enzimático.



Fonte: Adaptado de Barbosa et al., 2010. Ilustração da autora.

Quando o sistema de defesa antioxidante é insuficiente ou inativado, podem ocorrer danos aos lipídeos. Para mensurar o dano tecidual a peroxidação lipídica é quantificada pela formação de malondialdeído (MDA) (ESTERBAUER; SCHAUR; ZOLLNER, 1991) e o dano proteico pela carbonilação de proteínas (STADTMAN; LEVINE, 2003).

A peroxidação lipídica é uma das mais importantes causas de lesão e morte celular decorrente do estresse oxidativo. Essencialmente, a peroxidação lipídica consiste em uma reação em cadeia, na maioria dos casos catalisada por metais de transição, na qual oxidantes fortes causam a quebra dos fosfolipídios de membrana que contém ácidos graxos polinsaturados. Os danos da peroxidação lipídica para as membranas biológicas podem ser de vários níveis de severidade, dependendo da natureza e concentração do oxidante, variando desde reduções localizadas na fluidez da membrana até a ruptura total da integridade da bicamada (HALLIWELL; GUTTERIDGE, 2007).

Além de danos aos lipídios, o estresse oxidativo pode conferir danos às proteínas, que por sua vez, são alvo da oxidação celular nos locais onde sofrem modificações covalentes, possuindo poucos mecanismos celulares para reparo ou proteção. As proteínas carboniladas são produto da oxidação de proteínas, num processo que pode ser irreversível, sendo considerado o mais importante dos danos causados pelas EROs nas proteínas. O aumento da concentração destas proteínas

resulta em diversas doenças, tornando proteínas carboniladas um biomarcador de estresse oxidativo (SCHACTER, 2000; HERMES-LIMA, 2004)

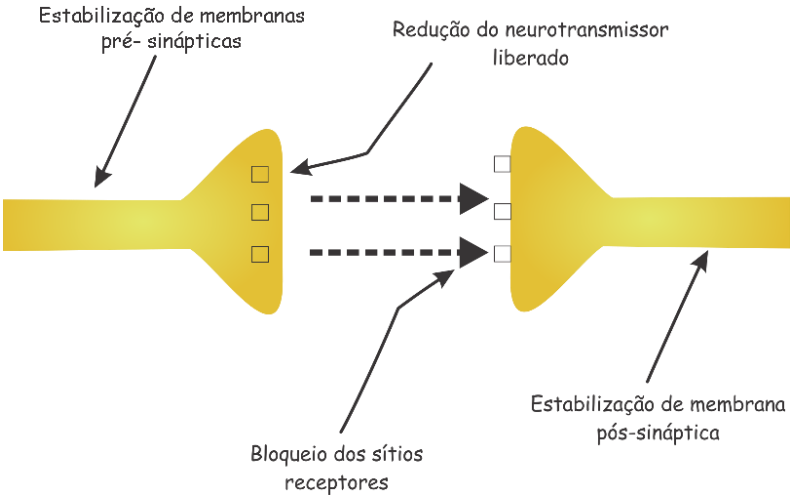
As espécies reativas ao oxigênio (EROs), além de causar danos aos lipídeos e proteínas, podem ocasionar também dano ao DNA (MARNETT, 1999), sendo considerado o mais importante dano oriundo do metabolismo celular.

1.3 ANESTESIA E SEDAÇÃO DE PEIXES

Os anestésicos têm sido estudados como uma importante ferramenta para minimizar os possíveis danos em peixes decorrentes das atividades de piscicultura (FAÇANHA; GOMES, 2005). O uso de anestésicos no manejo de peixes foi iniciado a partir de observações dos indígenas americanos, que colocavam timbó de mata (*Derris elliptica*), rica em rotenona, para sedar e capturar os peixes na natureza (HOMMA, 2014). Desde então, foram estudados diversos agentes químicos e diferentes procedimentos para insensibilização de peixes (HOSKONEN; PIRHONEN, 2004; PALIC et al., 2006; ROSS; ROSS, 2008; GRESSLER et al., 2012; TONI et al, 2015).

Em peixes, a indução à anestesia geralmente é feita através da imersão em água contendo algum anestésico, o qual é primeiramente captado pelas brânquias, difundido para o sangue e transportado até os neurônios do sistema nervoso central (SNC) (Figura 5), deprimindo-o (ROSS; ROSS, 2008). No entanto, pouco se sabe sobre o modo preciso de ação dos anestésicos em peixes. Acredita-se, porém, que este seja similar à anestesia inalatória utilizada em animais terrestres (UETA et al., 2007). Para a indução e a profundidade da anestesia em peixes são geralmente traçados padrões de monitoramento comportamental, como a observação da atividade natatória, comportamento, taxa de ventilação branquial, movimento dos olhos, respostas reflexas, e até mesmo observação de frequência cardíaca (SNEDDON, 2012). É fundamental que durante a indução se observe se o peixe possui alguma intolerância ao anestésico, através da observação do seu comportamento (como por exemplo “espasmos”), para evitar estresse adicional ao animal durante o processo de anestesia e até mesmo para mensurar a mortalidade que possa ocorrer posterior à recuperação (WALSH; PEASE, 2002; COYLE; DURBOROW; TIDWELL, 2004; HOSKONEN; PIRHONEN, 2004).

Figura 5 - Atuação de anestésicos nas sinapses do sistema nervoso central.



Fonte: Adaptado de ROSS; ROSS (2008)

Durante a anestesia, a observação de fatores biológicos e ambientais que podem influenciar a eficácia anestésica devem ser verificados. Entre os fatores biológicos encontramos: espécie, idade, sexo, condição e peso corporal, estado de desenvolvimento e taxa metabólica (SNEDDON, 2012). Quanto aos fatores ambientais, o de maior importância na anestesia de peixes parece ser a temperatura, que determina a taxa metabólica do peixe (MYLONAS et al, 2005; ZAHL et al 2011). O pH da água também possui bastante influência (ROUBACH; ROSS; ROSS, 2008) e está diretamente ligado à eficiência de certos anestésicos sintéticos, causando até mesmo reações estressantes, como por exemplo, a exposição de peixe a pHs ácidos, quando expostos a MS-222 e 2-fenoxietanol (SNEDDON, 2012). Para que esses fatores ambientais não sejam problema durante a anestesia, o ideal é anestesiar os peixes sempre na mesma temperatura e pH, para que possa ser utilizada sempre mesma concentração do anestésico. Além dos fatores abióticos citados anteriormente, os níveis de oxigênio dissolvido também podem afetar a eficácia anestésica (KING et al., 2005; PARK et al, 2008; ZAHL et al., 2009).

Um anestésico ideal deve possuir vários atributos, tais como viabilidade econômica, praticidade no uso, eficácia (CHO; HEATH, 2000) e não ser aversivo aos animais (READMAN et al., 2013). É desejável que os anestésicos apresentem ação rápida sobre o sistema nervoso, levando à indução, imobilização e recuperação

rapidamente, não ocasionando complicações posteriores para os animais (IWAMA; ACKERMAN, 1994; PIRHONEN; SCHRECK, 2002; GONÇALVES et al. 2008).

Nos Estados Unidos, o FDA (Food and Drug Administration – agência norte-americana reguladora de fármacos e alimentos) norteia as leis para o uso de anestésicos para uso em aquicultura, porém no Brasil não há nenhuma legislação definida a respeito da utilização de anestésicos para este fim (COSTA et al., 2013; CUNHA, 2017). Mundialmente, os anestésicos mais utilizados em animais aquáticos são a tricaína metano sulfonato (MS-222), a quinaldina, o 2-fenoxietanol, porém estes anestésicos sintéticos provocam efeitos adversos em peixes, como o aumento da sensibilidade e suscetibilidade ao estresse (KIESSLING et al., 2009). Desta forma, é necessário a busca de alternativas seguras para procedimentos de anestesia em animais aquáticos.

1.4 ANESTÉSICOS NATURAIS

Dentre as alternativas naturais, temos os óleos essenciais, que são compostos formados por misturas de metabólitos secundários voláteis e de odor forte, concentrados em diferentes órgãos da planta (BAKKALI et al., 2008; FRANZ; NOVAK, 2010).

Vários estudos farmacológicos têm evidenciado a atividade sedativa e anestésica de óleos essenciais em peixes e camarões (CUNHA et al., 2010; BECKER et al., 2012; PARODI et al., 2012; TONI et al., 2014, SALBEGO et al., 2014) para uso em procedimentos de rotina, como a manipulação e biometria e para o transporte de peixes. Por exemplo, jundiás (*Rhamdia quelen*) transportados com a adição de 10 µL/L de óleo essencial de *Lippia alba* na água do transporte apresentaram melhora no estado redox (AZAMBUJA et al., 2011). ZEPPENFELD et al. (2014) utilizando 30 e 40 µL/L de OE *Aloysia triphylla*, verificaram que além de melhorar o estado redox de *R. quelen*, este OE reduziu os níveis de cortisol plasmático, excreção de amônia e mudanças ionorregulatórias durante o transporte. Estudos mostram que a adição de agentes anestésicos na água para transporte de peixes auxilia nos processos osmorregulatórios, evitando o estresse. Becker et al. (2012;2013) e Parodi et al. (2014) relataram a adição do eugenol, OEs de *L. alba*, *A. triphylla* e extrato metanólico de *Condalia buxifolia*na água do transporte de *R. quelen* reduz a perda de íons e mortalidade.

O uso de anestésicos extraídos de plantas em concentrações baixas, ou seja, que causam apenas sedação leve, propicia uma condição ideal para o transporte de peixes que apresentam atividade reduzida, mas ainda são capazes de manter o equilíbrio parcial e a capacidade de nadar para evitar danos físicos resultantes da colisão com os sacos plásticos (COOKE et al., 2004), além de poder reduzir o estresse oxidativo (AZAMBUJA et al., 2011; ZEPPENFELD et al., 2014; SALBEGO et al., 2014; SOUZA et al., 2018 *in press*).

1.4.1 Óleo essencial de *Lippia alba* como anestésico

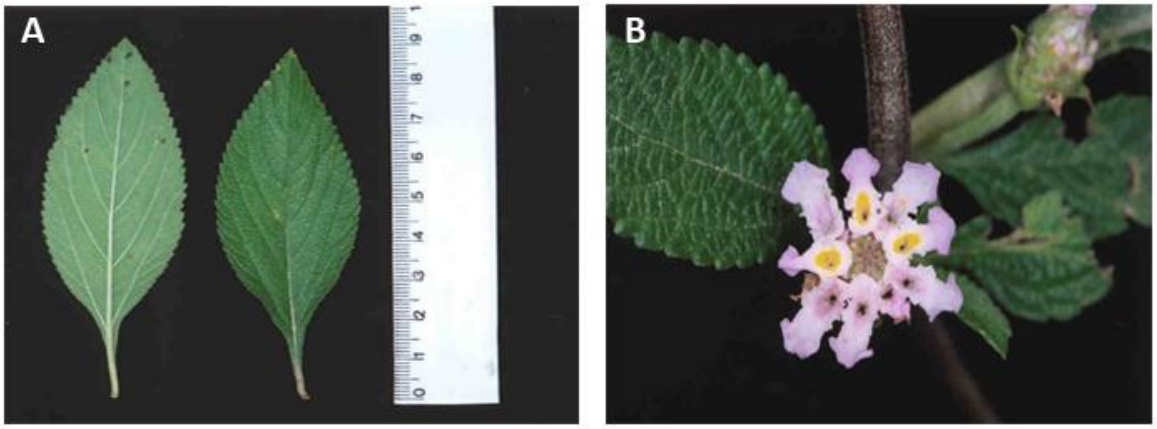
O gênero *Lippia* (Verbenaceae) possui cerca de 175 gêneros e 2.800 espécies difundidas nos trópicos e subtrópicos em regiões temperadas do hemisfério Sul e poucas espécies no hemisfério Norte (COSTA, 2003). Dentro deste gênero podemos destacar a *Lippia alba* (Mill.) N.E. Brown (Figuras 6 e 7), vulgarmente conhecida como erva-cidreira brasileira, sendo amplamente utilizada pela população devido ao seu efeito tranquilizante, antiespasmódico, analgésico, ansiolítico e expectorante (SETTE-DE-SOUZA et al., 2014).

O OE de *L. alba* foi primeiramente descrito por Cunha et al. (2010) como anestésico para peixes, sendo este trabalho pioneiro para diversas investigações futuras. Estudos posteriores confirmaram o efeito anestésico e sedativo deste OE para cavalo-marinho (*Hippocampus reidi*) (CUNHA et al., 2011), camarão branco *(Litopenaeus vannamei)* (PARODI et al., 2012), tilápia nilótica (*Oreochromis niloticus*) (HOHLENWERGER et al., 2016) e sedativo para dorada (*Sparus aurata*) (TONI et al., 2015). Além disto, o OE de *L. alba* ameniza o estresse oxidativo em jundiás transportados por até 7 h (AZAMBUJA et al., 2011). Por fim, estudo conduzido por Heldwein et al. (2012) demonstrou que o quimiotipo linalol possui ação gabaérgica.

Figura 6 –Exemplar de *Lippia alba*

Fonte: Universidade Federal do Ceará - Campus do PICI

Figura 7 - Detalhes da *Lippia alba*. Folha (A) e Inflorescência (B).



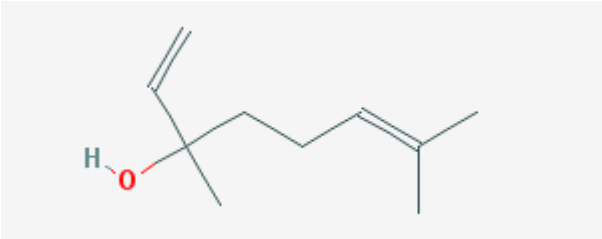
Fonte: APNE/CNIP

1.4.2 Óleo essencial de *Lippia alba* e seus diferentes quimiotipos

Óleos essenciais podem apresentar variação quantitativa e qualitativa em sua constituição (MATOS,1996; ZOGHBI et al.,1998). A composição do OE de *L. alba* pode variar com o local de coleta, tipos de cultivo, clima e época do ano (PASCUAL et al., 2001) e a espécie pode ser diferenciada em quimiotipos de acordo com a predominância de componentes químicos específicos, tais como carvona, citral, linalol, ß-cariofileno, tagetenone, limoneno, mirceno, γ-terpinen, cânfora-1,8-cineol ou estragol (HENNEBELLE et al., 2006; 2008; JANNUZZI et al., 2010; VALE et al. 2012; VICCINI et al., 2014). Estudos demonstraram que diferentes quimiotipos de uma mesma espécie vegetal podem apresentar atividades farmacológicas distintas

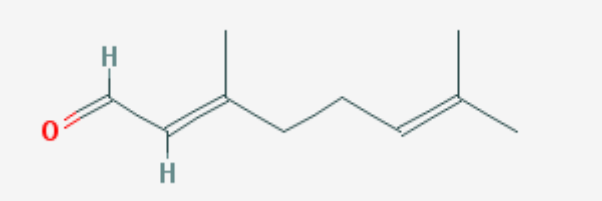
(CORRÊA, 1992), por isso, neste estudo, trabalharemos com os quimiotipos linalol (Figura 8) e citral (Figura 9).

Figura 8 – Estrutura química do linalol



Fonte: Pubchem. https://pubchem.ncbi.nlm.nih.gov/compound/6549#section=Top

Figura 9 – Estrutura química do citral



Fonte: Pubchem. https://pubchem.ncbi.nlm.nih.gov/compound/638011#section=Top

1.5 MODELO EXPERIMENTAL

Rhamdia quelen (QUOY; GAIMARD, 1824) é um peixe da família Heptapteridae, com distribuição neotropical, do sudeste do México ao norte, e centro da Argentina ao sul, sendo conhecida popularmente no Brasil como jundiá (GOMES et al., 2000) (Figura 10). Esta espécie nativa tem despertado grande interesse no sul do Brasil devido à resistência ao manejo, crescimento rápido, mesmo nos meses de inverno, boa eficiência alimentar, carne saborosa e sem espinhos intramusculares (BOCHI et al., 2008). Além disto, recentemente, o jundiá tem sido utilizado como modelo experimental de avaliação de eficácia anestésica de vários extrativos vegetais (CUNHA et al., 2010, SILVA et al., 2012, 2013, GRESSLER et al., 2014, PARODI et al., 2014, TONI et al., 2014).

Figura 10. Exemplar de jundiá, *Rhamdia quelen.*

Fonte: Carine de Freitas Souza

1.6 ESTRUTURA DA TESE

Em um primeiro momento esta tese aborda a análise do efeito anestésico de dois quimiotipos do OE de *Lippia alba*, bem como seu efeito sob a fisiologia de *R. quelen*, utilizando-se de ferramentas de gasometria e bioquímica plasmática. Após esta primeira análise, foi possível verificar que diferentes quimiotipos de uma mesma espécie vegetal poderiam causar efeitos distintos sob a fisiologia do peixe, embora ambos apresentassem efeito anestésico. Posteriormente, foram realizados novos estudos com o propósito de investigar detalhadamente se o óleo essencial quimiotipo citral (OE-C) poderia causar algum tipo de dano tecidual com base nos resultados obtidos previamente no primeiro estudo. A presente tese está organizada na forma de manuscritos, os quais estão estruturados de acordo com as normas das revistas as quais foram publicados/aceitos e está dividida em dois manuscritos, a saber:

Manuscrito I. *Respostas fisiológicas de Rhamdia quelen (Siluriformes: Heptapteridae) na anestesia com óleos essenciais de dois quimiotipos diferentes de Lippia alba.* Neste capítulo analisamos o efeito anestésico dos diferentes quimiotipos do óleo essencial de *L. alba* (citral e linalol), bem como seus efeitos sob parâmetros sanguíneos e plasmáticos dos peixes. Para isso foram realizadas análises de gasometria e bioquímica.

Manuscrito II. *Quimiotipos de citral e linalol de óleo essencial de Lippia alba como anestésicos para peixes: uma análise fisiológica detalhada de efeitos colaterais durante a recuperação anestésica em jundiás (Rhamdia quelen)***.** Este capítulo trata diretamente dos efeitos da anestesia sob parâmetros bioquímicos, antioxidantes e dano aos lipídeos e proteína em fígado e rim, tecidos responsáveis pela metabolização e excreção, respectivamente. Desta forma, identificamos se os óleos essenciais utilizados como anestésicos apresentam algum dano tecidual, e se estes podem ou não ser utilizados com segurança sem que provoquem distúrbios fisiológicos em *R. quelen*.

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2 OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar o uso dos OEs de *Lippia alba* quimiotipos citral e linalol como anestésicos e sedativos para peixes e suas implicações sobre a fisiologia dos mesmos.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a eficácia anestésica na indução e recuperação dos OE de *Lippia alba* quimiotipos citral e linalol em jundiás (*Rhamdia quelen*) através de parâmetros sanguíneos.

- Verificar alterações fisiológicas e possíveis danos teciduais em jundiás anestesiados com os OEs de *Lippia* quimiotipos citral e linalol através de análises bioquímicas, histológicas e parâmetros de estresse oxidativo.

3 DESENVOLVIMENTO

3.1 MANUSCRITO I

PHYSIOLOGICAL RESPONSES OF *RHAMDIA QUELEN* (SILURIFORMES: HEPTAPTERIDAE) TO ANESTHESIA WITH ESSENTIAL OILS FROM TWO DIFFERENT CHEMOTYPES OF *LIPPIA ALBA*

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Physiological responses of *Rhamdia quelen* (Siluriformes: Heptapteridae) to anesthesia with essential oils from two different chemotypes of *Lippia alba*

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The aim of this study was to evaluate if *Lippia alba* has different chemotypes according to the chemical composition of the essential oil (EO) considering collection site, and if the EO may have different effects on blood and plasma parameters in silver catfish, *Rhamdia quelen,* during and immediately after anesthesia. The citral (EO-C) and linalool (EO-L) chemotypes were identified, and both presented similar anesthetic effects for silver catfish. Fish were exposed to two concentrations of each EO, which induced slow and fast anesthesia (100 and 300 μL L^{-1}, respectively). Blood ions did not change at any time of anesthesia induction and recovery and, therefore, the electrolyte balance was not altered. Blood gases oscillated through all exposure and recovery times, but there was an increase in pO_2 after 10 min recovery in fish anesthetized with EO-C. Glucose increased in fish exposed to both EOs when compared with the control group. Overall, exposure to both EOs (except 100 μL L^{-1} EO-L at most times) reduced plasma cortisol levels compared to the control and/or ethanol groups. However, as plasma creatinine levels in fish anesthetized with EO-C were higher than control fish, the use of EO-L is preferable.

Keywords: Blood gas, Cortisol, Glucose, Plasma ions, Silver Catfish.

O objetivo deste estudo foi avaliar se *Lippia alba* apresenta diferentes quimiotipos de acordo com a composição química do óleo essencial (OE), considerando local de coleta e se o OE causa diferentes efeitos nos parâmetros sanguíneos e plasmáticos em jundiá, *Rhamdia quelen,* durante e imediatamente após a anestesia. Os quimiotipos citral (OE-C) e linalol (OE-L) foram identificados e ambos apresentaram efeito anestésico semelhante para jundiá. Os peixes foram expostos a duas concentrações de cada OE, que induziram anestesia lenta e rápida (100 e 300 mL L^{-1}, respectivamente). Íons sanguíneos não se alteraram em nenhum tempo e consequentemente, o equilíbrio eletrolítico não foi alterado. Os gases sanguíneos oscilaram durante todo tempo de exposição e recuperação, mas houve aumento na pO_2 após 10 min de recuperação em peixes anestesiados com OE-C. Níveis sanguíneos de glicose aumentaram nos peixes expostos a ambos OEs quando comparados com o grupo controle. De um modo geral, a exposição a ambos OEs (exceto 100 μL L^{-1} OE-L na maioria dos tempos) reduziu o cortisol plasmático comparado aos grupos controle e etanol. No entanto, como os níveis de creatinina plasmática em peixes anestesiados com OE-C foram maiores que nos peixes controle, é preferível o uso do OE-L.

Palavras-chave: Cortisol, Gases sanguíneos, Glicose, Íons plasmáticos, Jundiá.

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Introduction

In aquaculture, anesthetics (synthetics or plant extratives) are widely employed: from light sedation, to reduce stress during handling and non-invasive procedures, to full anesthesia to avoid pain during surgery and larger interventions (Small, 2003; Roubach *et al*., 2005; Ross, Ross, 2008; Kiessling *et al*., 2009; Neiffer, Stamper, 2009; Silva *et al*., 2013a; Roohi, Imanpoor, 2015).

The anesthetic efficacy of several essential oils (EOs), such as *Hyptis mutabilis* (Silva *et al*., 2013a), *Ocotea acutifolia* (Silva *et al*., 2013b), *Hesperozygis ringens* (Silva *et al*., 2013b; Toni *et al*., 2014), *Aloysia triphylla* (Parodi *et al*., 2014) and *Ocimum gratissimum* (Silva *et al*., 2015), has been demonstrated in fish. The EO of *Lippia alba* has been highlighted in the last decade through studies reporting its potential as antioxidant, anesthetic and sedative for fish (Cunha *et al*., 2010; Becker *et al*., 2012; Heldwein *et al*., 2014; Toni *et al*., 2014; Hohlenwerger *et al*., 2016).

Lippia alba occurs in all regions of Brazil (Zétola *et al*., 2002; Oliveira *et al*., 2006; Neto *et al*., 2009; Cunha *et al*., 2010; Teles *et al*., 2012; Vale *et al*., 2012; Soares *et al*., 2016). Due to its genetic variation, wide geographical distribution and exposure to different soil and weather conditions, and distinct seasons of collection, *L. alba* can produce EOs with different chemical composition, which expresses the occurrence of distinct chemotypes (Pascual *et al*., 2001; Hennebelle *et al*., 2008; Maffei *et al*., 2011; Teles *et al*., 2012). There are numerous chemotypes of *L. alba* in Brazil, such as: citral, linalool, ß-caryophyllene, tagetenone, limonene, carvone, myrcene, γ-terpinene, camphor-1,8-cineole and estragole, which produce different pharmacological effects (Oliveira *et al*., 2006; Hennebelle *et al*., 2008; Vale *et al*. 2012; Viccini *et al*., 2014). Thus, the distinct composition of same EO may result in different physiological and pharmacological effects during anesthesia.

Hematological and biochemical parameters of fish are valuable markers, since they can be used as indicators of physiological conditions, as well as in the control of diseases and stress manipulation (Aldrin *et al*., 1982; Tavares-Dias *et al*., 2008; Araújo *et al*., 2009). Plasma cortisol is one of the most used indicators to evaluate stress in fish (Wendelaar Bonga, 1997) and the two major actions of this hormone are the control of the ionoregulatory balance and energy metabolism (Liew *et al*., 2015). The electrolytic imbalance can be observed by changes in plasma or blood ions (McDonald, Milligan, 1997; Wendelaar Bonga, 1997; Takahashi *et al*., 2006). Glucose levels are also widely used as indicator of stress, hyperglycaemia being reported for several teleosts in this situation (Barton, Iwama, 1991). Stress also has an effect on other blood biochemical parameters such as levels of enzymes and substances with important metabolic functions, such as urea and creatinine, which indicate the overall health of the fish (Cnaani *et al*., 2004).

Physiological effects of the EO of *L. alba* cultivated in southern Brazil as anesthetic and sedative for silver catfish, *Rhamdia quelen*, was verified by many authors (Cunha *et al*., 2010; Heldwein *et al*., 2014; Toni *et al*., 2014; Salbego *et al*., 2014), but only the linalool chemotype. Therefore, it is of interest to investigate the anesthetic and physiological effects of EO obtained from other chemotypes of *L. alba*. Considering that a different chemotype of *L. alba* (myrcene-citral) cultivated in northern Brazil was identified by Oliveira *et al*. (2006), the aim of this study was to investigate a possible geographic effect in the EO composition (*L. alba* cultivated in northern and southern Brazil) and, if these EOs have different compositions, to evaluate their sedative and anesthetic effects in silver catfish, as well as their physiological effects on blood and plasma parameters.

Materials and Methods

Animals. One hundred sixty-eight juveniles silver catfish (*Rhamdia quelen*; 51.17 ± 1.69 g and 20.21 ± 1.40 cm) were obtained from a local fish farm and brought to the Fish Physiology Laboratory at the Universidade Federal de Santa Maria (UFSM). The species was identified at the Ichthyology Laboratory (Universidade Federal do Rio Grande do Sul) and a voucher specimen was deposited in this laboratory at number UFRGS 19612. The fish were maintained for one week in 250 L tanks (50 fish/tank) with continuous aeration; temperature 21 ± 2 °C; pH 6.5-7.5 and dissolved oxygen above 5.5 mg L^{-1}. The animals were fed once a day with commercial feed and kept fasted for a period of 24 h prior to the experiments. The experimental protocol was approved by the Committee on Animal Experimentation - UFSM, under the registration number 074/2014.

Essential oils extraction and analysis. The specimens of *Lippia alba* linalool chemotype were cultivated at the Centro de Educação Superior do Norte (CESNORS-UFSM) - Frederico Westphalen, Rio Grande do Sul State, southern Brazil (27°23'48"S, 53°25'45"W), soil classified as Oxisol typical clayey. The climate is Cfa (humid subtropical) with average annual temperature of 19.1°C and rainfall of 1892 mm. Those from the citral chemotype were cultivated in Santarém, Pará state, northern Brazil (02°26'35"S, 054°54'54"W), soil classified as ultisol yellow Hapludox + yellow latosol Hapludox, but in the culture it was used black soil and cattle manure (3:1). The climate is Am (humid tropical) with average annual temperature of 25.9°C and rainfall of 2,150 mm.

Botanical identification of *L. alba* linalool chemotype was made by Gilberto Dolejal Zanetti (Department of Industrial Pharmacy, UFSM) and a voucher specimen (SMDB 10050) was deposited in the herbarium of the Department of Biology (UFSM). The *L. alba* citral chemotype was identified by Dr. Fatima Salimena (Universidade Federal de Juiz de Fora) and a voucher was registered in the herbarium of this institution under number CESJ 65276.

The essential oils were obtained by hydrodistillation of fresh leaves for 3h in a Clevenger apparatus (European Pharmacopoeia, 2007) and stored at -4°C until utilization. The analysis of the EOs was performed by gas chromatography-mass spectrometry-total ion chromatogram using an Agilent 6890 gas chromatograph coupled with an Agilent 5973 mass selective detector and employing a HP5-MS column (5% phenyl, 95% methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 mm) as described by Silva *et al.* (2012). The constituents were identified by comparison of the Kovats retention index and their mass spectra with data from the mass spectral library (NIST, 2002) and the literature (Adams, 2001).

Experiment 1. Anesthetic induction and recovery times. Anesthesia induction and recovery were tested at concentrations of 25, 50, 100, 200, 300 µL L^{-1} for both EOs. Eight fish were used for each concentration tested, and each juvenile was used only once. Sedation was characterized by the decreased reactivity to external stimuli, and anesthesia by total loss of equilibrium and cessation of locomotion, according to Small (2003). The EOs were previously diluted in 95% ethanol (1:10). Ethanol at the highest concentration used does not have any anesthetic effect in silver catfish (Cunha *et al.*, 2010). After induction, fish were transferred to anesthetic-free aquaria to measure anesthesia recovery time. The fish were considered to be recovered when they returned to normal swimming and reacted to external stimuli.

Experiment 2. Exposure to anesthetics for physiological evaluation. Silver catfish were individually placed in an 8 L aquarium containing one of the EOs at 100 µL L^{-1} for up to 5 min or 300 µL L^{-1} for up to 2 min. These concentrations led to sedation and deep anesthesia, respectively (Cunha *et al.*, 2010). Afterwards, fish from all groups were transferred individually to 8 L aquaria with anesthetic-free water for up to 10 min for recovery. There were also groups subjected to 26700 µL L^{-1} ethanol (the concentration used to dilute the highest EO concentration) and water only (control), which were handled as outlined above.

Blood analysis. Blood was collected from the caudal vein of silver catfish in less than 30 s with heparinized syringes at 1, 2 and 5 min of exposure (groups exposed to the concentration of 300 µL L^{-1} were not assessed at 5 min) and 5 and 10 min of recovery (total of 30 fish per treatment, n = 6 for each EO, concentration and collection time, each fish was sampled only once). Control and ethanol exposed fish were held tightly for blood collection. An aliquot of this blood was used to measure Na^+, K^+, Ca_2^+, glucose, pH, partial pressures of O_2 (pO_2) and CO_2 (pCO_2) using the i-STAT® portable clinical analyzer with CG8+ cartridges (Abbott Laboratories, Chicago, IL, USA). The sample temperature was corrected to match the experimental water temperature (Roth, Rotabakk, 2012). The use of i-STAT® and calculations for blood gases have been described for several fish species (Jacobs *et al.*, 1993; Pidetcha *et al.*, 2000; Harrenstien *et al.*, 2005; Kristensen *et al.*, 2010; Barbas *et al.*, 2016).

Plasma analysis. The remaining blood collected was centrifuged (800 *x g* for 10 min) and the plasma was used for analysis of creatinine and urea using an automated Vitros 250 (Ortho - Clinical Diagnostics) and Johnson & Johnson dry chemistry kits. All tests were carried out in duplicate.

Plasma cortisol was also determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada). This analysis was previously validated (Souza *et al.*, 2015). Absorbance was measured in a spectrophotometer at 450 nm, and intra- and inter-assay coefficients of variation were 6.3% and 5.2%, respectively.

Statistical analysis. Data are reported as mean ± SE. The homogeneity of variances among groups was determined with the Levene test. All treatment groups were compared by two-way analysis of variance (time x concentration) and Tukey's test; or, when homogeneity of variances was not obtained, by the Scheirer-Ray-Hare extension of the Kruskal-Wallis test and the Nemenyi test. Analyses were performed using the STATISTICA software package, version 5.1 (StatSoft, Tulsa, OK, USA), and the minimum significance level was set at $p < 0.05$.

Results

Chemical composition of the essential oils. A total of 65 compounds were identified in the EO of *L. alba* collected in southern Brazil (EO-L) and 67 compounds in the EO of *L. alba* collected in northern Brazil (EO-C) (Tab. 1). The major component in the EO-L was β-linalool (50.56%), while the major compounds in the EO-C were E-citral (29.84%) and Z-citral (24.41%).

Anesthetic induction and recovery times. Both EO-C concentrations induced sedation faster than EO-L, but anesthesia was faster only at the lowest EO-C concentration. Fish anesthetized with EO-C took longer to recover than those anesthetized with EO-L (Fig. 1). Ethanol did not show any sedative or anesthetic effect.

Blood analysis. Blood pH, Na^+, K^+ and Ca^{2+} did not differ between any of the treatments and overall means were: pH (7.32 ± 0.04), Na^+ (134.90 ± 0.89 mmol L^{-1}), K^- (2.89 ± 0.12 mmol L^{-1}), Ca^{2+}(1.18 ± 0.06 mmol L^{-1}).

Overall, blood pO_2 levels of silver catfish anesthetized with both EOs and exposed to ethanol were lower than control fish, increasing after 10 min recovery (Figs. 2a-b). In contrast, an increase in pCO_2 was observed for fish anesthetized with EO-C and no significant change was observed for silver catfish anesthetized with EO-L. At the end of 10 min of recovery fish anesthetized with EO-C still maintained blood PCO_2 levels higher than control fish (Figs. 2c-d).

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Tab. 1. Chemical composition of the essential oils of *Lippia alba* collected from southern (linalool chemotype - EO-L) and northern (citral chemotype - EO-C) Brazil. RI calc= calculated Kovats retention index; RI ref= reference Kovats retention index; (Adams, 2001; NIST, 2002).

RT (min)	Constituent	Relative percentage (%)		RI calc	RI ref
		EO-L	EO-C		
11.334	sabinene	1.05	0.47	972	968[1,2]
13.597	limonene	0.63	6.15	1026	1029[2]
13.698	1,8-cineole	7.01	-	1029	1031[1,2]
14.535	E-β-ocimene	1.10	0.35	1049	1050[1]
14.882	γ-terpinene	-	3.16	1058	1060[1]
16.623	β-linalool	50.56	0.73	1100	1099[2]
21.599	Z-geraniol	0.49	3.57	1229	1230[1]
22.037	Z-citral	-	24.41	1241	1238[1,2]
22.587	E-geraniol	0.17	5.32	1256	1253[1]
23.131	E-citral	1.51	29.84	1270	1267[1,2]
27.375	β-elemene	2.66	0.30	1391	1391[1,2]
28.291	E-caryophyllene	4.56	0.99	1418	1419[1]
28.758	γ-elemene	1.27	0.08	1433	1437[1]
30.174	γ-muurolene	5.23	2.46	1476	1480[1]
30.815	bicyclogermacrene	0.22	3.72	1495	1500[1]
32.47	elemol	0.13	3.15	1549	1550[1]
32.717	germacrene B	2.37	0.23	1557	1561[1]
33.532	caryophyllene oxide	1.12	0.80	1584	1583[2]
	% Identified	80.08	85.73		

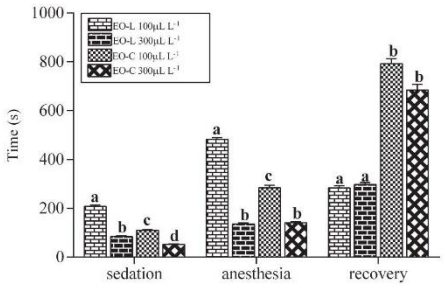

Fig. 1. Time required for silver catfish (*Rhamdia quelen*) anesthesia induction and recovery (n=8 for each concentration tested) using different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Stages are defined according to Small (2003). Values are mean ± SEM. Different letters indicate difference between concentrations and EOs in the same anesthetic stage. Based on two-way ANOVA followed by the Tukey *post hoc* test ($p < 0.05$).

Blood glucose levels increased in control and ethanol groups after 5 min, compared to initial values, returning to initial values at the end of the recovery period in the control group. Exposure to both EOs (except 300 µL L^{-1} EO-L) did not avoid this increase of blood glucose levels. At the end of the recovery period, the blood glucose levels of silver catfish exposed to ethanol and both EOs (except 300 µL L^{-1} EO-L) remained higher than the initial values and higher than those of the control group (Figs. 3a-b).

In the control group, plasma cortisol levels decreased after 2 min and remained low until the end of the recovery period. In fish exposed to ethanol, cortisol levels decreased up to 5 min after exposure and returned to the initial values after 5 min of recovery. Overall, exposure to both EOs (except 100 µL L^{-1} EO-L at most times) reduced plasma cortisol levels compared to the control and/or ethanol groups (Figs. 3c-d).

Plasma creatinine values of control fish increased significantly after 5 min and remained high at the end of the recovery period. Fish exposed to ethanol and both EOs showed significantly higher creatinine levels 1 min after exposure compared to control fish and these levels returned to control values at the end of recovery only in those exposed to EO-L (Figs. 4a-b). Plasma urea levels in the control group remained constant at all evaluation times. Fish exposed to ethanol showed a significant increase in plasma urea levels when compared to control fish after 5 min of exposure and returned to control values at the end of the recovery period. Plasma urea was significantly higher with most anesthesia treatments and recovery times in fish exposed to both EOs when compared to the control and ethanol groups (Figs. 4c-d).

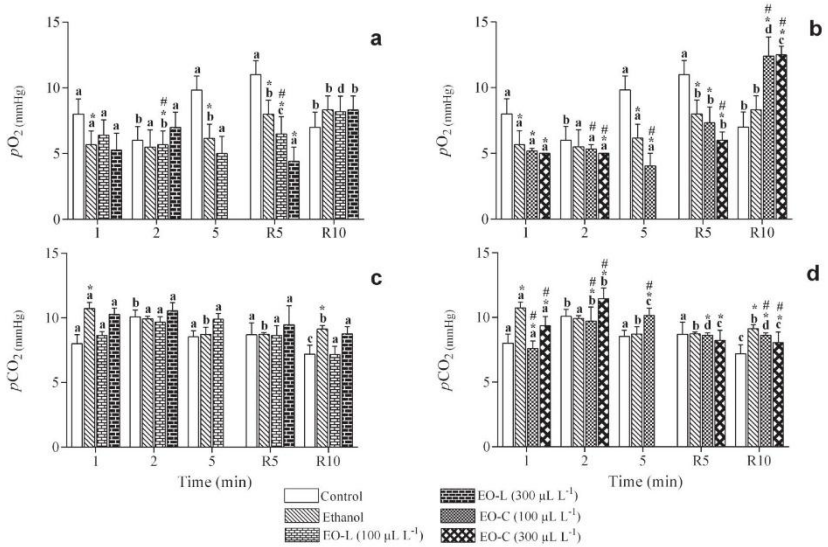

Fig. 2. Partial pressures of **a-b** oxygen (PO_2) and **c-d** carbon dioxide (PCO_2) in silver catfish (n = 6) (*Rhamdia quelen*) submitted to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are mean ± SEM. Different letters indicate significant difference between times within the same EO concentration. * indicate significant difference from control, # indicate significant difference from ethanol. Two-way ANOVA and Tukey's test or Scheirer-Ray-Hare extension of the Kruskal-Wallis test and Nemenyi test.

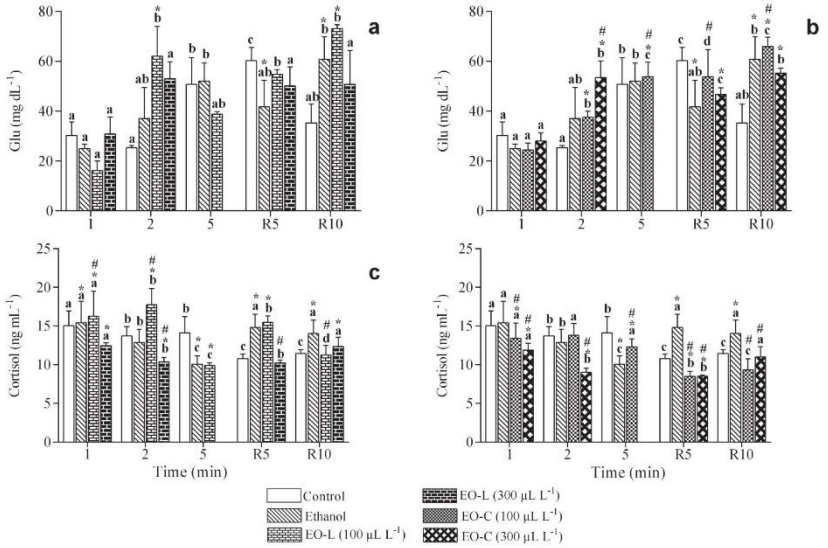

Fig. 3. a-b. Blood glucose (Glu) and **c-d.** plasma cortisol in silver catfish (*Rhamdia quelen*) (n = 6) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are mean ± SEM. Different letters indicate significant differences between times within the same treatment. * indicates significant difference from control, # indicates significant difference from ethanol. Two-way ANOVA and Tukey's test or Scheirer-Ray-Hare extension of the Kruskal-Wallis test and Nemenyi test were used to determine statistical significance.

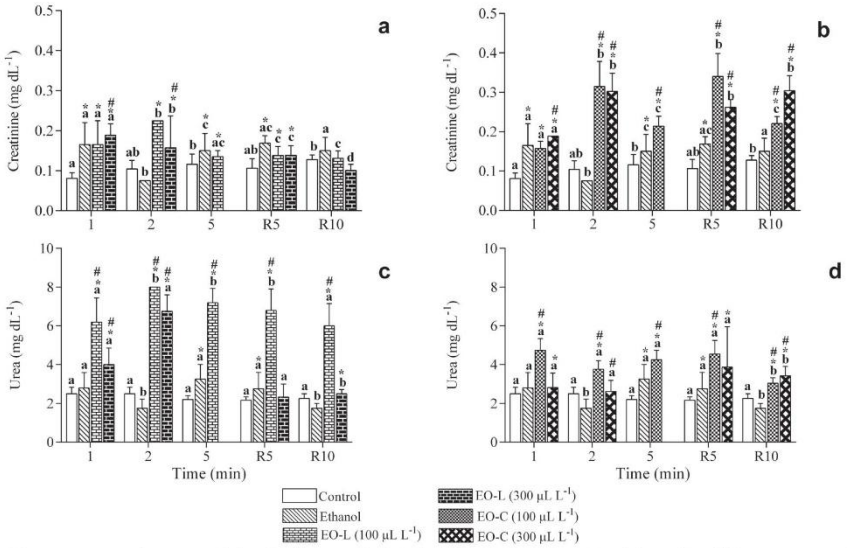

Fig. 4. a-b. Plasma urea and **c-d** creatinine in silver catfish (*Rhamdia quelen*) (n = 6) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are mean ± SEM. Different letters indicate significant differences between times within the same treatment. * indicates significant difference from control, # indicates significant difference from ethanol. Two-way ANOVA and Tukey's test or Scheirer-Ray-Hare extension of the Kruskal-Wallis test and Nemenyi test were used to determine statistical significance.

Discussion

Since EOs represent a chemical interface between plant and the surrounding environment, their syntheses are often affected by environmental conditions, thus expressing the occurrence of chemotypes or chemical races in the producing plant species (Gobbo-Neto, Lopes, 2007). Although there are many examples of the occurrence of geographic variations of EOs chemical composition in several plants (Figueiredo *et al*., 2008), the distribution of chemotypes is often not locally limited. In some species, different chemotypes can grow side by side (Schmidt *et al*., 2004).

The present study demonstrates that the EO from *L. alba* cultivated by our group in southern Brazil has linalool as its main compound (50.56%), and so it belongs to chemotype linalool. On the other hand, *L. alba* collected in northern Brazil can be classified in the chemotype citral, once this is the major compound of its EO (54.26%). Some authors indicated that geographical distribution and exposure to different soil and weather conditions, season of collection can affect the chemical composition of *L. alba* EO (Pascual *et al*., 2001; Hennebelle *et al*., 2008; Maffei *et al*., 2011; Teles *et al.*, 2012). However, specimens from the chemotypes citral, linalool and carvone, collected in different regions of Brazil, cultivated in similar conditions, maintained the same chemical composition, indicating that differences are due to genotypic variations (Tavares *et al.*, 2005).

The EO-C anesthetized silver catfish within approximately 2 min at 300 µL L^{-1}, inducing anesthesia faster than EO-L. Anesthesia recovery was slower with EO-C, but it can be considered and adequate anesthetic for silver catfish, because an ideal anesthetic must induce anesthesia up to 3 min and enable the recovery in about 10 min (Park *et al*., 2008). The anesthetic effect of EO-L in silver catfish involves the modulation of the benzodiazepine (BDZ) site of the GABAergic system (Heldwein *et al*., 2012). The EO-C blocks the excitability of rat sciatic nerves (Sousa *et al.*, 2015), but the anesthetic effect of the EO from *Aloysia triphylla* (which has citral as its major compound) in silver catfish is not related to a modulation of the BDZ site of the GABAA receptor (Santos *et al*., in press).

All parameters examined in this study are within the range previously observed for silver catfish (Barcellos *et al*., 2001; 2004). Blood Na^+, K^+ and Ca^{2+} of silver catfish were not affected by anesthesia with either EO tested. Similar results were found in the blood of *Amazon* catfish (*Leiarius marmoratus*) anesthetized with 10-200 µL L^{-1} eugenol (Honorato *et al*., 2014) and in the plasma of silver catfish anesthetized with 150 and 300 mg L^{-1} MS-222 (Gressler *et al*., 2014). However, silver catfish anesthetized with 150-450 µL L^{-1} of *Hesperozygis ringens* and *Lippia alba* (EO-L) showed altered plasma Na^+ and K^+ between 30-240 min of recovery (Toni *et al*., 2014) and anesthesia of tambaqui with 20 mg L^{-1} jambu extract induced blood ionoregulatory

changes 2 h after recovery from anesthesia and Na^+ levels did not return to control values, even after 72 h (Barbas *et al.*, 2016). Apparently ionoregulatory effects of anesthesia in blood or plasma are significant only after a few hours of recovery, when they can be detected.

During fish anesthesia, opercular movement (respiration) generally decreases compared to conscious fish, explaining the lower blood pO_2 in silver catfish anesthetized with both EOs and the higher pCO_2 in those anesthetized with EO-C. Through anesthetic recovery from both EOs there was an increase in blood pO_2 levels and a reduction in pCO_2 levels in those exposed to EO-C, which is expected for the recovery period with normal return of opercular movements. These same oscillation patters in pO_2 and pCO_2 from fish anesthesia, were found for red "pacu" (*Piaractus brachypomus*) anesthetized with MS-222 (150 mg L^{-1}) (Hanley *et al.*, 2010) and "tambaqui" (*Colossoma macropomum*) anesthetized with waxy extract of "jambu" flowers (*Spilanthes acmella*) at 20 mg L^{-1} (Barbas *et al.*, 2016).

An increase in plasma levels of glucocorticoids such as cortisol is one of the main responses to stress (Barton, 2002). Plasma cortisol increases significantly in juvenile *R. quelen* 5 - 30 min after handling (Koakoski *et al.*, 2012), but surprisingly, handling was not sufficient to raise the plasma cortisol in the control group of silver catfish in our study. Toni *et al.* (2015) observed no increase in plasma cortisol levels of silver catfish exposed for 6 h to 30 and 50 µL L^{-1} EO from *Hesperozygis ringens* and they proposed that the primary stress reaction only took place in the first minutes after contact with the EO, as observed in fish exposed to EO-L in the present study.

A study by Gesto *et al.* (2014) using stressed rainbow trout (*Oncorhynchus mykiss*) showed that when catecholamines were released in the blood no changes in cortisol levels were observed as glucose levels increased. As plasma cortisol levels did not increase significantly, we suppose that the increase of blood glucose in silver catfish observed in the present study might be due to catecholamine release. According to Morgan, Iwama (1997), an increase in blood glucose occurs in response to a stressor, in order to provide most of the energy demand to cope with this stress. Our results corroborate those obtained by Inoue *et al.* (2011) and Honorato *et al.* (2014), who observed that anesthesia with eugenol increased plasma glucose compared to the sham control in "tambaqui" (20 and 60 mg L^{-1}) and Amazon catfish (10-200 µL L^{-1}). Anesthesia with 20 mg L^{-1} "jambu" extract also increased blood glucose levels in "tambaqui" (Barbas *et al.*, 2016). Several studies testing a variety of anesthetics on multiple fish species also demonstrated increased glycemia after anesthesia induction (Ortuno *et al.*, 2002; Deriggi *et al.*, 2006; Barbosa *et al.*, 2007; Park *et al.*, 2008).

An increase in plasma urea levels in silver catfish during anesthesia and recovery was observed for both EOs when compared to the control group. The same result was obtained for plasma creatinine levels, but these levels were much higher in silver catfish anesthetized with EO-C than in control fish, and these levels returned to control values after 10 min recovery in those exposed to EO-L. Anesthesia of goldfish (*Carassius auratus*) with 50 µL L^{-1} nanoencapsulated clove oil also increased serum urea levels (Gholipourkanani *et al.*, 2015). Increases in urea and creatinine levels together are probably due to lesions caused in the kidney of fish (Das, Mukherjee, 2003). Studies conducted by Borges *et al.* (2007) showed an increase in urea and creatinine levels in the serum of silver catfish exposed to cypermethrin, suggesting that these analyses can be useful for early detection of intoxication in fish. However, as nitrogen compounds are excreted as ammonia mainly through the gills (Nawata *et al.*, 2007), and time of exposure to the EOs was brief, the elevation of urea levels observed in both EO groups in our study may be due to changes in ammonia and creatinine gill excretion and not to renal lesions.

In summary, different chemotypes of *L. alba* were detected according to their place of cultivation. We suggest that the EO-L and EO-C can be safely used as anesthetics in silver catfish, because the alterations in most parameters returned to control values within 10 min. However, the EO obtained from different chemotypes of the *L. alba* presented different physiological responses in plasma creatinine and the use of EO-L is preferable because the high creatinine levels provoked by EO-C exposure. Additional studies with longer exposure and/or recovery times are necessary to improve our understanding of the effects of the EO of this chemotype on renal function.

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3.2 MANUSCRITO II

CITRAL AND LINALOOL CHEMOTYPES OF *LIPPIA ALB*A ESSENTIAL OIL AS ANESTHETICS FOR FISH: A DETAILED PHYSIOLOGICAL ANALYSIS OF SIDE EFFECTS DURING ANESTHETIC RECOVERY IN SILVER CATFISH (*RHAMDIA QUELEN*)

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Citral and linalool chemotypes of *Lippia alba* essential oil as anesthetics for fish: a detailed physiological analysis of side effects during anesthetic recovery in silver catfish (*Rhamdia quelen*)

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Abstract The viability using *Lippia alba* essential oil as an anesthetic for fish was studied, particularly with respect to physiological effects during recovery. Anesthesia of silver catfish (*Rhamdia quelen*) using 100 and 300 μL L^{-1} of two different chemotypes of *L. alba* essential oil (citral EO-C and linalool EO-L) prevented the increase of plasma cortisol levels caused by handling, but did not avoid alterations in energetic metabolism. Silver catfish did not have increased the levels of thiobarbituric acid reactive species in the kidney and liver during recovery after anesthesia with either EO, avoiding lipid damage. On the other hand, fish anesthetized with EO-C showed higher protein carbonylation levels, superoxide dismutase, catalase, and glutathione S-transferase activities and non-protein thiol group levels in both tissues compared to controls. Our results suggest that both oils show antioxidant capacity, but anesthesia with EO-L does not cause damage to lipids or proteins, only temporary changes, typical of physiological adjustments during recovery from anesthesia. Therefore, EO-L is an effective anesthetic for silver catfish with fewer side effects than EO-C.

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Introduction

Anesthetics are commonly used in aquaculture and experimental procedures to facilitate handling and transport and to reduce fish stress. In addition to preventing physical injury, some anesthetics may even prevent or reduce secondary effects caused by stress, such as increased plasma cortisol, glucose, and lactate levels, alterations that are linked to oxidative stress and, consequently, tissue damage (Rotllant et al. 2001; Skjervold et al. 1999; Neiffer and Stamper 2009; Tort 2011; Saccol et al. 2016). Moreover, at low concentrations, anesthetics may be used as sedatives in fish transport (HSA 2005; Azambuja et al. 2011; Becker et al. 2012; Salbego et al. 2014), reducing metabolism and stress (Tort et al. 2002; Becker et al. 2012; Toni et al. 2014).

In recent years, the properties of a variety of anesthetics have been investigated to provide effective fish anesthesia. However, each anesthetic presents advantages and disadvantages, such as the ratio between the therapeutic and toxic effects and causing disturbances in fish physiology (Velisek et al., 2006). Recently, studies showed that low concentrations of 2-phenoxyethanol induced a similar stress response to handling in bighead carp (*Hypophthalmichthys nobilis*) (Akbary et al. 2016) and reared meager (*Argyrosomus regius*) (Barata et al. 2016). A similar response was found for sunshine bass (*Morone chrysops* × *Morone saxatilis*) exposed to low concentrations of tricaine methanesulfonate (MS-222) and Aqui-S®, which presented an increase of important stress biomarkers, such as seric glucose and cortisol levels even 24 h after exposure (Davis and Griffin 2004). These data demonstrate the importance of continuing to develop and approve compounds for use in aquaculture.

In order to minimize the negative effects caused by synthetic anesthetics, several researchers have examined the use of natural products with anesthetic properties. Studies using rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and tambaqui (*Colossoma macropomum*) anesthetized with clove oil (Wagner et al. 2003; Velisek et al. 2005; Boijinka et al. 2016) and silver catfish (*Rhamdia quelen*) anesthetized with essential oils (EOs) of *Lippia alba* (Cunha et al. 2010), *Aloysia triphylla* (Gressler et al. 2014; Parodi et al. 2014), *Hesperozygis ringens* (Toni et al. 2014), and *Ocimum americanum* (Silva et al. 2015) have shown promising results due to their effectiveness, low incidence of side effects and ability to mitigate stress effects.

Lippia alba (Mill.) N.E. Brown (Verbenaceae) EO is a novel anesthetic and sedative whose action has been established for silver catfish *Rhamdia quelen* (Cunha et al. 2010; Becker et al. 2012; Heldwein et al. 2012; Salbego et al. 2014; Toni et al. 2014), tambacu (*Piaractus mesopotamicus* × *Colossoma macropomum*) (Sena et al. 2016), Nile tilapia (*Oreochromis niloticus*) (Hohlenwerger et al. 2017) and sea bream (*Sparus aurata*) (Toni et al. 2014). These studies used only the linalool chemotype essential oil (EO-L), but *L. alba* shows a very diverse chemical composition, which may vary according to the chemotype (Pascual et al. 2001; Hennebelle et al. 2008; Maffei et al. 2011; Teles et al. 2012).

Recently, a study conducted by Souza et al. (2017) demonstrated that EO-L and the *L. alba* citral chemotype essential oil (EO-C) are effective anesthetics for silver catfish; however, increased biomarkers of hepatic and renal damage are observed, such as plasma glucose, creatinine, and urea. Based on these facts, the aim of this study was to evaluate in detail whether anesthesia with EO-L and EO-C can cause liver and/or renal damage in silver catfish after induction and recovery from anesthesia.

Materials and methods

Animals

One hundred forty-four juvenile silver catfish (41.34 ± 8.23 g and 19.22 ± 1.41 cm) were obtained from a local fish farm and taken to the Fish Physiology Laboratory (LAFIPE) at the Universidade Federal de Santa Maria (UFSM). They were maintained for 1 week in continuously aerated 250 L tanks (50 fish/tank) for acclimation, with temperature around 21 °C, pH 6.5–7.5 and dissolved oxygen above 5.5 mg L^{-1}. The Committee on Animal Experimentation – UFSM, under the registration number 074/2014, approved the experimental protocol.

Essential oil extraction and constituents

Extraction of the EO from the fresh leaves of *L. alba* and the composition analysis was described previously

(Souza et al. 2017). These authors verified that the major component in the EO-L was β-linalool (50.56%), while the major compounds in the EO-C were *E*-citral (29.84%) and *Z*-citral (24.41%).

Anesthetic tests

Silver catfish were placed in aquaria containing 2 L of the EOs at 100 μL L^{-1} for up to 5 min or 300 μL L^{-1} for up to 2 min. These concentrations and times led to sedation and deep anesthesia, respectively (Cunha et al. 2010; Souza et al. 2017). In addition, there were ethanol (2700 μL L^{-1}, concentration used to dilute the highest EO concentration) and control (only water) groups maintained in the aquaria for up to 5 min. Fish from the basal group were immediately removed from the tank and sampled. Each animal was used only once. After anesthesia, fish were handled for biometric measurements, which provided exposure to air for 1 min, and then fish from all groups were transferred to 40 L aquaria with only water for recovery for up to 480 min.

Sample collection

Total blood was collected from the caudal vein with heparinized sterile syringes after 0, 10, 240, and 480 min of recovery (n = 6 for each EO concentration and collection time). Plasma was obtained after centrifugation (3000×g, 10 min, −4 °C), and stored at −80 °C until metabolic analysis. The fish were euthanized by sectioning of the spinal cord, and the liver and kidney tissues were collected and stored at −80 °C until analysis.

Plasma analysis

Plasma cortisol levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Diagnostics Biochem Canada Inc., Canada). This analysis was previously validated by Souza et al. (2015). Absorbance was determined in a spectrophotometer at 450 nm, and intra- and inter-assay coefficients of variation were 6.3 and 5.2%, respectively. All tests were carried out in duplicate. Plasma was used to determine glucose levels using a commercial kit (Bioclin®, MG, Brazil) according to the manufacturer's recommendation. The cortisol levels were expressed as nanograms per milliliter.

Metabolites in the liver

One portion of liver was homogenized at a proportion of 50 mg mL^{-1} of 10% TCA (trichloroacetic acid) using a Turrax homogenizer. Afterwards, the homogenates were centrifuged at 1000×g for 10 min, and the supernatants were used for the determination of glucose and glycogen levels according the method described by Dubois et al. (1956), while lactate levels were measured according Harrower and Brown (1972). All data were expressed as micromole per gram of tissue.

Oxidative stress parameters

One portion of liver and kidney were homogenized (1 : 10 w v^{-1}) in a medium containing 120 mM potassium chloride and 30 mM buffer phosphate (pH 7.4), and the supernatant fraction obtained was frozen at −80 °C.

Prooxidants

The supernatants were used for the estimation of lipid peroxidation, established by thiobarbituric acid reactive species (TBARS) production, performed by reaction of malondialdehyde (MDA) with 2-thiobarbituric acid, which was optically measured according to Buege and Aust (1978) at 532 nm. TBARS levels were expressed as nanomole MDA per milligram of protein. Protein carbonyl content was assayed by the method described by Yan et al. (1995), with some modifications. The carbonyl content was then measured spectrophotometrically at 370 nm and was expressed as nanomole carbonyl per milligram of protein.

The protein concentration was determined by the Coomassie Blue method following Bradford (1976) using bovine serum albumin as a standard.

Enzymatic antioxidant activity

Superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adrenochrome generation at 480 nm (Misra and Fridovich 1972). Catalase (CAT) activity was measured using the method described by Nelson and Kiesow (1972). The change of H_2O_2 absorbance after 60 s was measured at 240 nm by ultraviolet spectrophotometry. CAT activity was calculated and expressed in micromoles per minute per milligram of protein. Glutathione S-transferase (GST) activity was measured based on the method described by

Habig et al. (1974). The extinction coefficient used for 2,4-dinitrochlorobenzene (DNCB) was 9.6 mM^{-1} cm^{-1}, and the activity was expressed as micromole GS-DNB per minute per milligram of protein.

Nonenzymatic antioxidants

Non-protein thiol groups (NPSH) were determined by the method of Ellman (1959) with 0.05 mL of 10 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 0.7 mL of 0.5 mM phosphate buffer (pH 6.8) added to 0.25 mL of supernatant. The results were expressed as micromole non-protein thiols per gram of tissue.

Statistical analysis

Data are reported as mean ± SE. The homogeneity of variances among groups was determined with the Levene test. All treatment groups were compared by two-way analysis of variance (time × concentration) and Tukey's test, or when homogeneity of variances was not obtained, by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test and the Nemenyi test. Analyses were performed using the STATISTICA software package, version 5.1 (StatSoft, Tulsa, OK, USA), and the minimum significance level was set at $p < 0.05$.

Results

No mortality occurred during the experiments. Behavioral anesthesia recovery occurred in around 5 min for fish anesthetized with EO-L and in around 10 min for fish anesthetized with EO-C, according to what have observed by Souza et al. (2017).

Effects of the essential oils

Silver catfish from the control and ethanol groups had significantly higher plasma cortisol levels 240 min after anesthesia than the basal and control groups at time zero of anesthesia. The plasma cortisol levels of silver catfish anesthetized with EO-L and EO-C were significantly lower than those from the control group after 240 min (Fig. 1a, b). Manipulation of control fish triggered an immediate (time 0) reduction, followed by an increase after 10 min, of plasma glucose levels compared to the basal group. Fish exposed to ethanol had increased plasma glucose levels from time zero and neither EO was able to prevent this increase. Recovery of plasma glucose to basal levels occurred only after 480 min in fish from all treatments, except those anesthetized with 100 μL L^{-1} EO-C (Fig. 1c, d).

Hepatic glucose levels increased after 10 min of recovery in the control and ethanol groups compared to basal and time zero levels. Fish anesthetized with both EOs presented higher hepatic glucose levels after 0 and 10 min of recovery than the control and ethanol groups. After 240 and 480 min of recovery, all groups presented a significant decrease in hepatic glucose levels, with fish recovering from anesthesia with both EOs having lower levels than the control and ethanol groups (Fig. 2a, b). Hepatic glycogen levels decreased in fish anesthetized with both EOs at time zero of recovery (except 100 μL L^{-1} EO-L). After 10 min of recovery, hepatic glycogen levels returned to basal values in fish anesthetized with EO-C as the control group, while those anesthetized with EO-L presented a significant increase of these levels. After 240 and 480 min of recovery, all treatments (except fish exposed to 100 μL L^{-1} EO-L at 240 min and 300 μL L^{-1} EO-C at 240 and 480 min) showed lower glycogen levels compared to basal values (Fig. 2c, d). Hepatic lactate levels exhibited significantly higher values after 10 and 480 min of anesthetic recovery in all treatments compared to basal and time zero levels. No significant differences were observed between groups (Fig. 2e, f).

In control fish, TBARS levels increased progressively during recovery in the liver, while in the kidney this increase was observed only after 10 min of recovery. Overall, TBARS levels were lower throughout recovery in the liver of fish anesthetized with EO-L or EO-C compared to the control and ethanol groups. Immediately after anesthesia with both EOs, TBARS levels in the kidney increased in treated animals compared to the control and ethanol groups, but after 10 min these values reduced, with only the fish anesthetized with 100 μL L^{-1} EO-L maintaining values similar to the control group. At the end of 480 min, all fish returned to basal TBARS values in the kidney, but those exposed to both EOs kept TBARS levels in the kidney lower than the control and ethanol groups (Fig. 3).

Protein carbonyl (PC) levels in the liver of the control and ethanol groups were significantly higher than the basal group at the beginning of the recovery period (time zero) (Fig. 4a, b), while in the kidney, this increase was observed only after 10 min of recovery, as observed for TBARS (Fig. 4c, d). Fish exposed to both EO-L

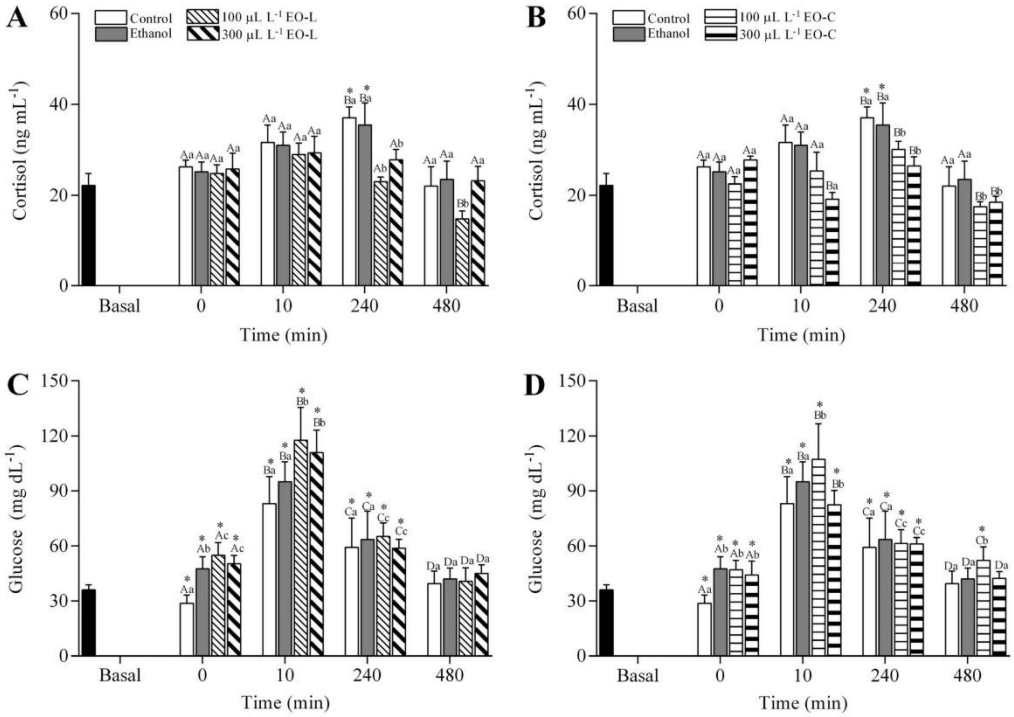

Fig. 1 Plasma cortisol (**a**, **b**) and glucose (**c**, **d**) levels in silver catfish (*Rhamdia quelen*) ($n = 6$) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$)

concentrations exhibited significantly lower PC levels in the liver than control fish up to 10 min of recovery, and these reduced PC levels were still lower than controls up to 480 min. In contrast, fish anesthetized with EO-C presented lower PC values in the liver at time zero of recovery but after that, in general, PC values increased up to 480 min of recovery. In the kidney, anesthesia with EO-L reduced PC levels after 10 min of recovery (and 480 min at the highest concentration), but fish anesthetized with EO-C exhibited an increase in PC levels throughout the whole recovery period (Fig. 4c, d).

SOD and CAT activities in the liver were not altered significantly by handling of the control group compared to the basal group. Anesthesia with EO-L increased SOD activity from 10 to 480 min of recovery compared to the control and ethanol groups. Fish exposed to both EO-C concentrations had increased hepatic SOD after 10 min of recovery, while those anesthetized with the highest concentration (300 µL L^{-1}) kept SOD activity higher than the control and ethanol groups throughout the whole recovery period (Fig. 5a, b). Overall, CAT activity was higher in the liver of silver catfish anesthetized with both EOs than in the control group (Fig. 5c, d) from 10 min up to 480 min of recovery. In the kidney, the CAT activity of control fish was higher than the basal group only after 240 min of recovery. This parameter was higher in the kidney of fish anesthetized with both concentrations of EO-L after 10 and 480 min of recovery in relation to control and basal groups (Fig. 5e, f).

GST activity was higher in the liver after 10 and 480 min of anesthetic recovery from both EOs compared to the control and ethanol groups (Fig. 6a, b). In the kidney, GST activity was higher in all treatments

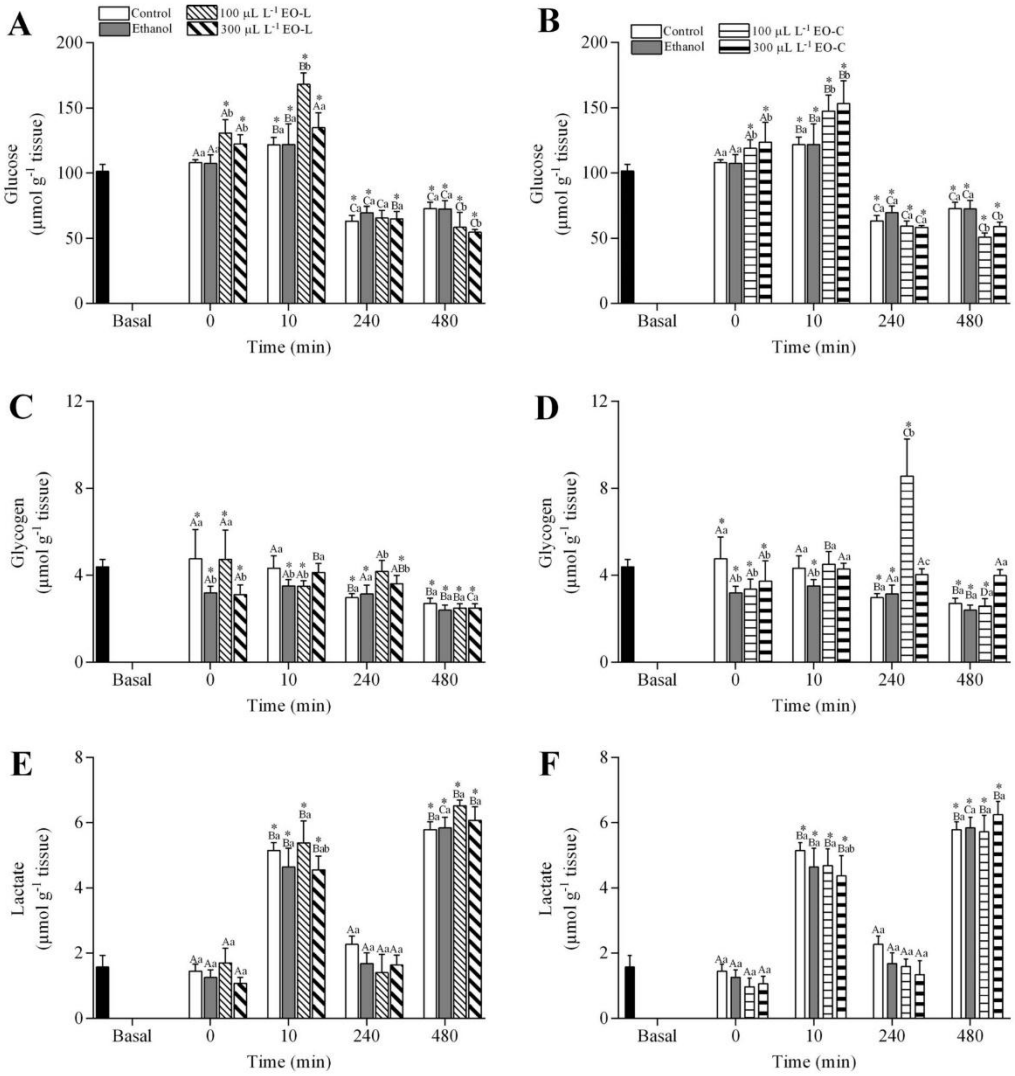

Fig. 2 Glucose (**a**, **b**), glycogen (**c**, **d**), and lactate (**e**, **f**) levels in the liver of silver catfish (*Rhamdia quelen*) ($n = 6$) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$)

with EO-C than the basal group and only the control and ethanol groups returned to baseline values at the end of 480 min of recovery. Fish anesthesia with EO-L and EO-C showed higher GST activity in the kidney than the control and ethanol groups after 10 and 240 min. After 480 min of recovery, fish anesthetized with EO-C and 100 μL L^{-1} EO-L maintained an increase in GST activity (Fig. 6c, d).

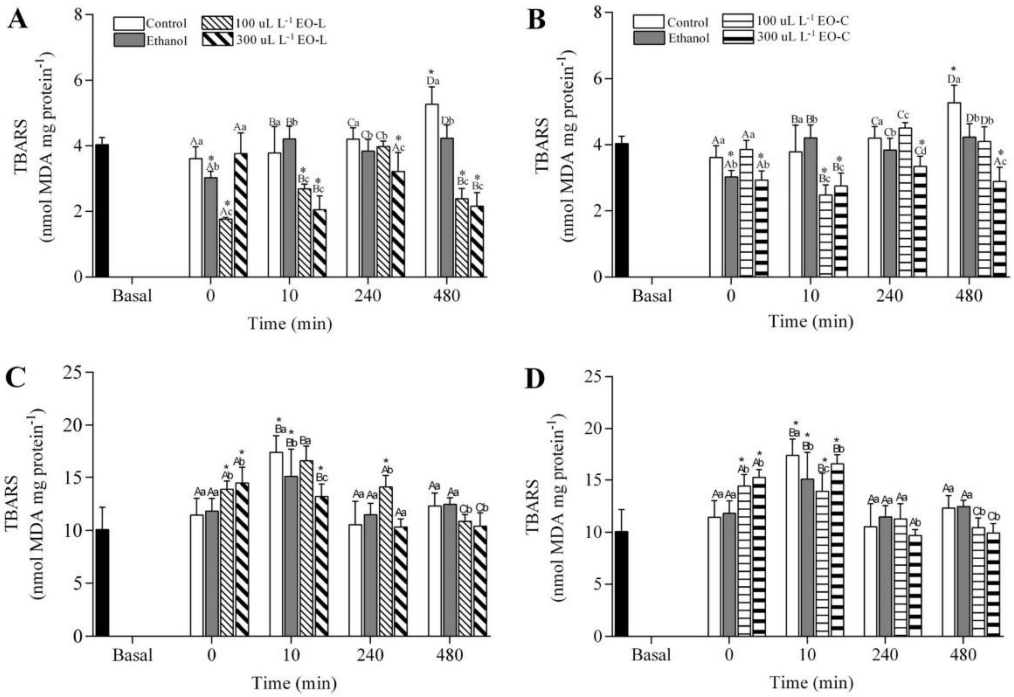

Fig. 3 TBARS in the liver (**a**, **b**) and kidney (**c**, **d**) of silver catfish (*Rhamdia quelen*) ($n = 6$) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point ($p < 0.05$). Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance

NPSH content was higher in the kidney and liver of fish anesthetized with EO-C than the control and ethanol groups after 480 min of recovery. Fish anesthetized with both EO-C concentrations presented higher NPSH content in both organs at time zero compared to basal group, and after 240 and 480 min of recovery in those anesthetized with 300 μL L^{-1} (Fig. 7).

Discussion

In the present study, the results confirmed that fish handling causes stress, in accordance with Saccol et al. (2016). Cortisol and glucose are known as primary and secondary stress responses, respectively, and their release occurs in adverse situations (Barton 2002). A significant increase in plasma cortisol levels was detected after 240 min of recovery for control silver catfish, which corresponds to a pattern previously described for this species after 1 min of aerial exposure (Cunha et al. 2010). Anesthesia with both EOs prevented or reduced the increase of plasma cortisol levels in silver catfish, reducing the stress caused by manipulation. Plasma glucose increases in response to a stressor to meet the higher energy demand (Morgan and Iwama 1997; Pankhurst 2011). Anesthesia with EO-L and EO-C was not able to prevent the increase of plasma glucose levels, and values returned to baseline levels only 480 min after exposure to air, with the exception of fish anesthetized with 100 μL L^{-1} EO-C, whose values remained higher than the basal group at all times. An increase in plasma glucose after anesthesia was also observed in tambaqui (*Colossoma macropomum*) anesthetized with clove oil (Inoue et al. 2011; Honorato et al.

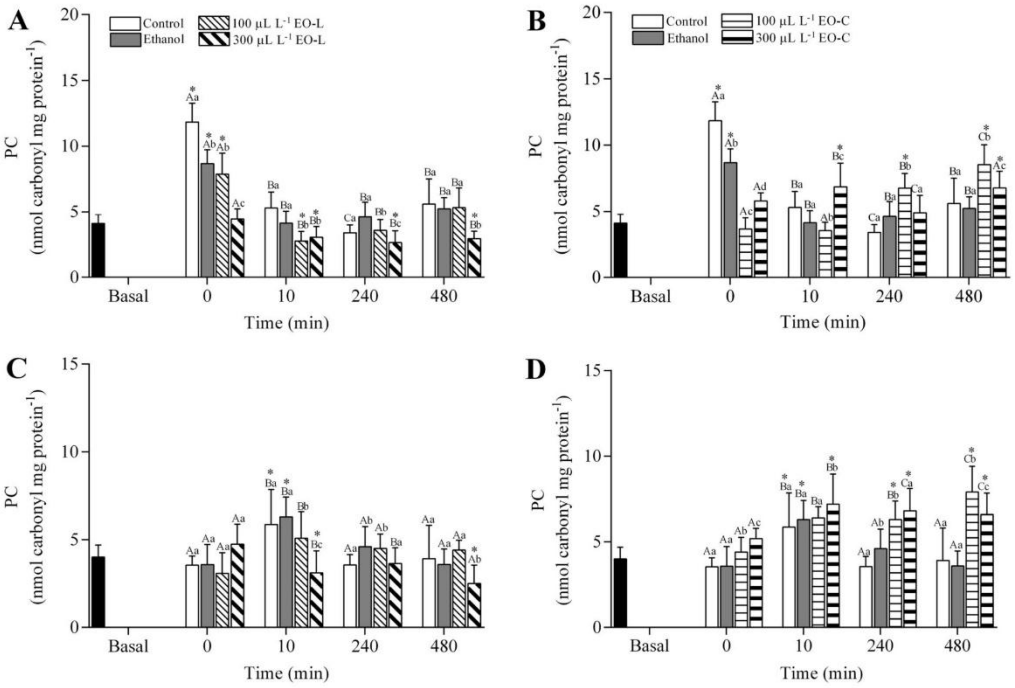

Fig. 4 Protein carbonylation levels (PC) in the liver (**a**, **b**) and kidney (**c**, **d**) of silver catfish (*Rhamdia quelen*) ($n = 6$) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point ($p < 0.05$). Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance

2014). Effects on plasma glucose, but not cortisol levels, were also detected in rainbow trout (*Oncorhynchus mykiss*) anesthetized with clove oil (Wagner et al. 2003) and silver catfish anesthetized with *O. americanum* (Silva et al. 2015). According to these authors, besides handling, this phenomenon may occur also due to fish perception of anesthetic presence. It promotes catecholamine release, which induces liver glycogenolysis, leading to higher glucose production and plasma glucose levels, providing energy to face the stressful event (Wendeelar Bonga 1997; Reid et al. 1998; Sladky et al. 2001; Lopez-Patiño et al. 2014). In accordance with this statement, hepatic glycogen levels decreased at almost all recovery times and hepatic glucose increased after 10 min of recovery for controls and anesthesia groups compared to basal values. Hepatic glucose also increased in rainbow trout 15 min after handling (Lopez-Patiño et al. 2014).

Plasma lactate increased in Atlantic salmon anesthetized with clove oil (Iversen et al. 2003), rainbow trout using clove oil (Wagner et al. 2003) and silver catfish anesthetized with EO-L (Toni et al. 2014), suggesting increased anaerobic metabolism. Deep anesthesia with both EO-L and EO-C reduced blood partial oxygen pressure up to 5 min (but not 10 min) of recovery in silver catfish, demonstrating that anesthesia with these EOs leads to transient hypoxia (Souza et al. 2017). However, in the present study, hepatic lactate levels increased in fish from all treatments after 10 and 480 min of recovery. Fish subjected to stressful situations, such as handling and air exposure, exhibit, among other responses, an increase in muscle (Tahmasebi-Kohyani et al. 2012) and hepatic lactate (Lopez-Patiño et al. 2014). Therefore, the increase of hepatic lactate observed in silver catfish in the present study was due to handling and not to anesthesia. Hepatic lactate levels

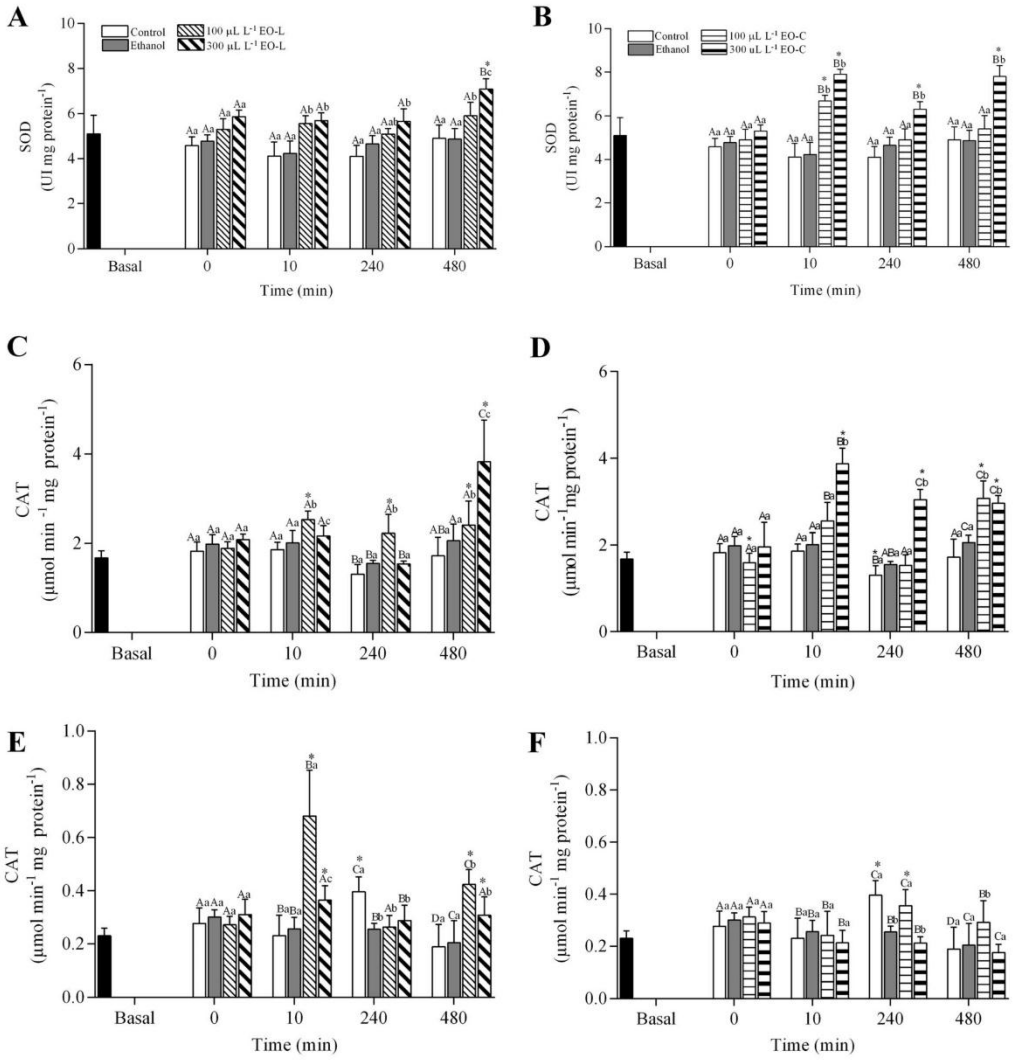

Fig. 5 Superoxide dismutase (SOD) activity in the liver (**a**, **b**) and catalase (CAT) activity in the liver (**c**, **d**), and kidney (**e**, **f**) of silver catfish (*Rhamdia quelen*) ($n = 6$) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$)

returned to basal values between 10 and 240 min after handling in silver catfish, within the same time range observed for rainbow trout (Lopez-Patiño et al. 2014). However, in silver catfish hepatic lactate increased again after 480 min. Similar tendencies, but without significant changes, were observed in the liver of rainbow trout (Lopez-Patiño et al. 2014) and the plasma of common carp (*Cyprinus carpio*) (Nematollahi et al. 2009).

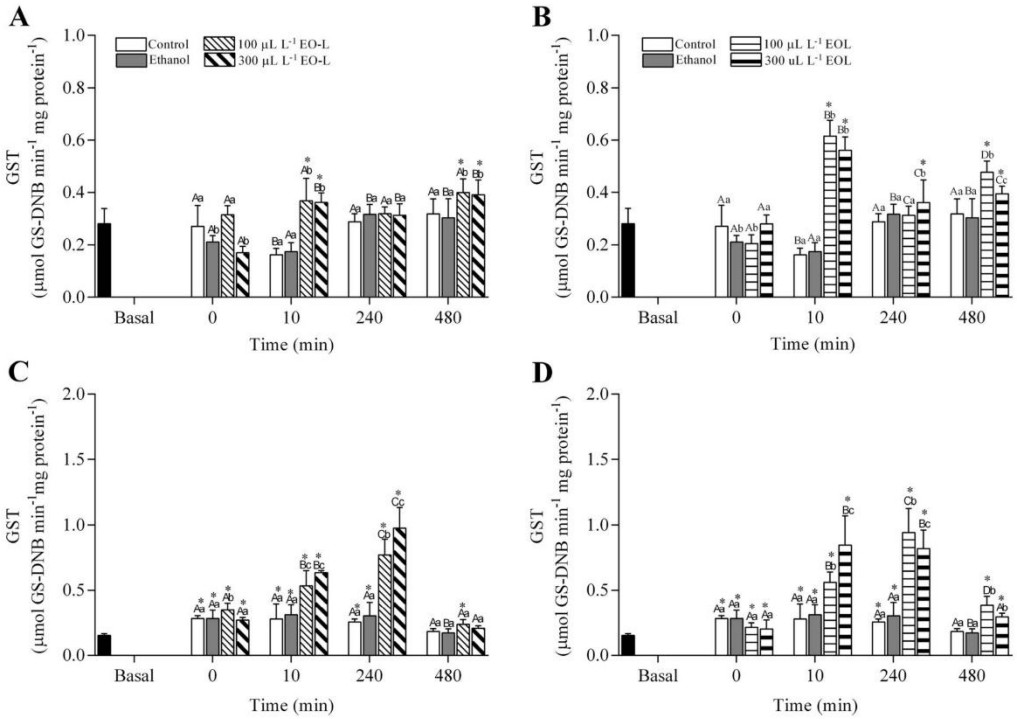

Fig. 6 Glutathione S-transferase (GST) activity in the liver (**a**, **b**) and kidney (**c**, **d**) of silver catfish (*Rhamdia quelen*) (n = 6) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$)

Analysis of TBARS and PC are popular and commonly used methods to assess oxidative damage. In order to assess possible tissue damage, lipid peroxidation has been studied as a cell lesion mechanism provoked by free oxygen radicals. Huang et al. (2003) reported that lipid peroxidation is a major contributor to the loss of cell function under oxidative stress. Anesthesia with EO-L and EO-C was able to prevent lipid damage, because after 480 min of recovery the control group showed increased TBARS, which did not occur with silver catfish anesthetized with both EOs. This result was expected because silver catfish transported with 30 or 40 µL L^{-1} EO-L avoided the formation of peroxides and TBARS in frozen fillets (Veeck et al. 2013) and those transported with 30 or 40 µL L^{-1} of *A. triphylla* EO (citral-rich EO) presented lower hepatic and muscle TBARS levels at the end of transportation (Zeppenfeld et al. 2014).

Nonetheless, in the kidney, anesthesia of silver catfish with EO-L and EO-C increased TBARS levels at the start of recovery, up to 10 min, and fish anesthetized with EO-L (100 µL L^{-1}) returned to basal values only 480 min after exposure. A similar result was found for muscle and brain of rainbow trout 24 h after anesthesia with MS 222 (100 mg L^{-1}), clove oil (30 mg L^{-1}), and 2-phenoxyethanol (0.40 mL L^{-1}) (Velisek et al. 2011) and for the liver of silver catfish after anesthesia with 300 mg L^{-1} of MS 222 (Gressler et al. 2014). EO-L and EO-C apparently cause a transient increase in TBARS levels and may be considered less harmful to lipids than clove oil, MS 222, and 2-phenoxyethanol.

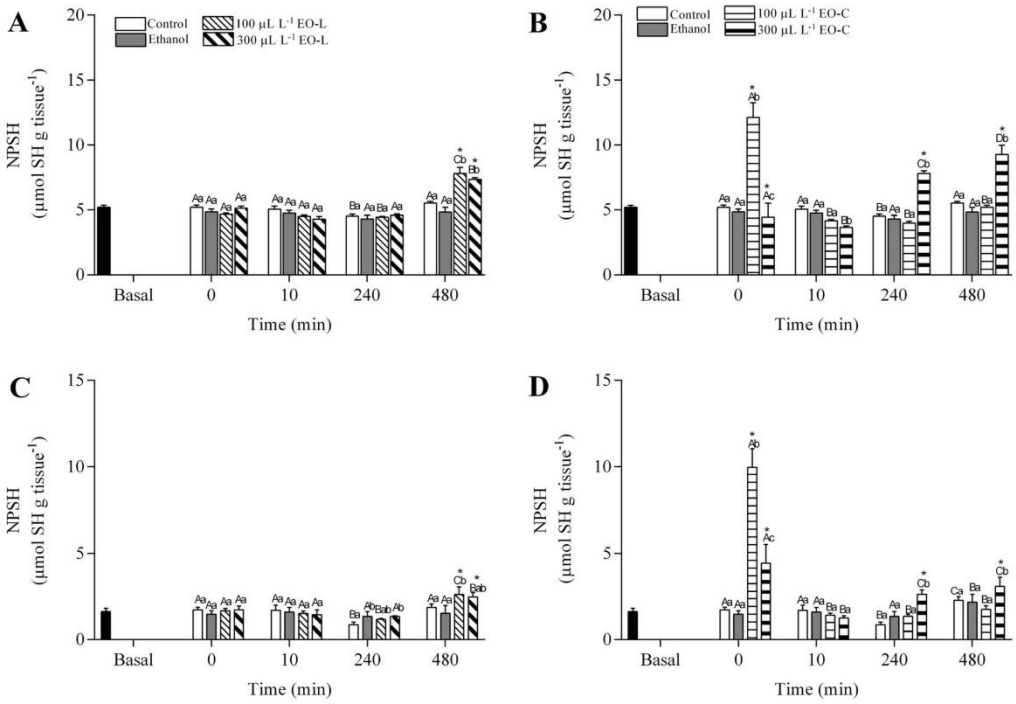

Fig. 7 Non-protein thiol group (NPSH) content in the liver (**a**, **b**) and kidney (**c**, **d**) of silver catfish (*Rhamdia quelen*) (n = 6) subjected to different concentrations of essential oils from the linalool (EO-L) and citral (EO-C) chemotypes of *Lippia alba*. Values are means ± SE. An *asterisk* indicates a significant difference compared to the basal group. *Capital letters* indicate significant differences between time points within the same treatment. *Lowercase letters* indicate significant differences between treatments at the same time point. Two-way ANOVA and Tukey's test or Scheirer–Ray–Hare extension of the Kruskal–Wallis and Nemenyi tests were used to determine statistical significance ($p < 0.05$)

Protein carbonylation is a result of protein oxidation which decreases the catalytic activity of enzymes and results in breakdown of proteins by proteases (Almroth et al. 2008). Immediately after exposure, silver catfish anesthetized with EO-L (100 μL L^{-1}) and controls had increased PC levels in the liver, but after 10 min of exposure these levels returned to baseline values. On the other hand, the liver and kidney of silver catfish anesthetized with both EO-C concentrations had increased PC levels. Higher hepatic PC levels were observed in silver catfish transported with 30 μL L^{-1} EO-L (Salbego et al. 2014). These results suggest that the antioxidant defenses promoted by both EOs tested may not be completely able to effectively eliminate the ROS produced. It is important to emphasize that these results confirm the hypothesis of renal and hepatic damage in fish anesthetized with EO-C (Souza et al. 2017).

SOD and CAT are the main enzymes in eliminating reactive oxygen species, formed during bioactivation of xenobiotics in tissues (Sk and Bhattacharya 2006); they provide protection against oxidative damage (Pandey et al. 2003). In the present study, SOD and CAT activities in the liver increased significantly in silver catfish anesthetized with 300 μL L^{-1} EO-L after 480 min of recovery. The activity of both enzymes was higher and more lasting for fish anesthetized with EO-C, keeping their levels higher than the control group for up to 480 min after exposure, probably in an attempt to minimize oxidative damage. Overall, the results suggest that the primary antioxidant defense system represented by SOD and CAT is capable of reducing oxidative stress, as at the end of 480 min of recovery to EO-L exposure, PC and TBARS values returned to basal values, suggesting an antioxidant action of this EO. Recent studies

conducted by Saccol et al. (2016) showed similar results for tambaqui anesthetized with essential oils of *Myrcia sylvatica* and *Curcuma longa*, in which the increase in SOD and CAT activity prevented lipid peroxidation. Nonetheless, the same was not observed for fish anesthetized with EO-C, which still showed damage to proteins (higher PC levels) after 480 min of recovery. Thus, these results suggest that EO-C has a lower antioxidant potential compared to EO-L.

GST is an important enzyme involved in catalyzing the conjugation of a wide variety of electrophilic substrates to reduced glutathione (van der Oost et al. 2003; Langiano and Martinez 2008). It acts in the process of biotransformation and catalyzes the conjugation of a variety of metabolites (including xenobiotic and LPO products), transforming the toxic compound into another more easily excreted (Lushchak et al. 2009). In turn, high NPSH levels may protect cellular proteins against oxidation either via the NPSH redox cycle or by directly detoxifying the ROS generated by exposure to stressor agents (Ruas et al. 2008). SH (sulfhydryl) groups also have the capacity to protect the catalytic site of some antioxidant enzymes, such as CAT, preventing its impairment. Thus, the no depletion of NPSH levels 480 min after exposure to EO-L may be an indication of beneficial effects linked to CAT activity, such as observed in this study. Silver catfish anesthetized with EO-L and EO-C showed an increase in GST activity in the liver and kidney through practically all times of anesthetic recovery. Again, the increased GST activities in the liver and kidney of fish anesthetized with EO-L contributed to the compensatory mechanisms of the antioxidant defense system, since EO-L was able to avoid oxidative stress, which did not occur for fish anesthetized with EO-C.

Conclusion

Silver catfish anesthetized with EO-L had reduced hepatic and renal damage to lipids and proteins, but the same did not occur in fish anesthetized with EO-C, demonstrating that the antioxidant defenses were not completely able to effectively scavenge the excessive ROS production or prevent tissue damage. Both EOs were efficient in avoiding an increase in plasma cortisol levels, a positive characteristic for an anesthetic. In conclusion, the use of EO-L as an anesthetic is recommended because it avoids hepatic and renal damage in silver catfish.

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4 CONCLUSÕES

O OE de *Lippia alba* apresenta distintas composições químicas que variam de acordo com o local de coleta. O OE obtido no norte do Brasil apresentou o citral como composto majoritário, enquanto o OE obtido no sul do Brasil apresentou o linalol como composto majoritário.

Embora o tempo de indução à anestesia seja semelhante para os dois OE, o processo de recuperação demonstrou-se mais lento em peixes anestesiados com o OE-C.

A anestesia com OE-C e OE-L 100 e 300 µL L^{-1} previnem o aumento nos níveis de cortisol plasmáticos causados pela manipulação.

O OE-C causa aumento nos níveis de creatina e ureia em jundiás anestesiados com 100 e 300 µL L^{-1}.

O uso do OE-C e OE-L aumentou a atividade da superóxido dismutase, catalase e glutationa-S-transferase e os níveis de tióis não proteicos, mas este aumento na capacidade antioxidante não foi capaz de evitar a carbonilação proteica em fígado e rim de jundiás anestesiados com OE-C.

Os resultados sugerem que ambos os óleos possuem capacidade antioxidante, mas anestesia com OE-L não causa danos aos lipídios ou proteínas, apenas mudanças temporárias, típicas de ajustes fisiológicos durante a recuperação da anestesia.

Portanto, OE-L é um anestésico eficaz para *R. quelen*, não apresentando efeitos indesejáveis na anestesia de peixes.