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ÓLEOS ESSENCIAIS COMO ANESTÉSICOS PARA PEIXES: ASPECTOS BIOQUÍMICOS E MOLECULARES

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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ÓLEOS ESSENCIAIS COMO ANESTÉSICOS PARA PEIXES: ASPECTOS BIOQUÍMICOS E MOLECULARES

Cândida Toni

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Farmacologia**

Orientador: Prof. Dr. Bernardo Baldisserotto

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A Comissão Examinadora, abaixo assinada,
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ÓLEOS ESSENCIAIS COMO ANESTÉSICOS PARA PEIXES: ASPECTOS BIOQUÍMICOS E MOLECULARES

elaborada por
Cândida Toni

como requisito parcial para obtenção do grau de
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DEDICATÓRIA

Aos meus pais, Ivo e Anita (*in memoriam*):
razões da minha vida, minha força, minha inspiração...

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
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ÓLEOS ESSENCIAIS COMO ANESTÉSICOS PARA PEIXES: ASPECTOS BIOQUÍMICOS E MOLECULARES

AUTORA: CÂNDIDA TONI
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Data e Local da Defesa: Santa Maria, 15 de janeiro de 2015.

Os óleos essenciais (OEs) extraídos das plantas *Hesperozygis ringens* e *Lippia alba* possuem propriedades anestésica e sedativa, constituindo uma alternativa aos anestésicos tradicionalmente usados em aquicultura para facilitar o manejo e/ou reduzir o estresse. Neste sentido, o estudo teve por objetivo investigar os efeitos desses OEs sobre a fisiologia de peixes, através de indicadores fisiológicos, bioquímicos e endócrinos. No artigo 1 determinou-se (a) a atividade anestésica dos OEs de *H. ringens* (OEHR) e *L. alba* (OELA) e (b) seus efeitos em jundiá (*Rhamdia quelen*) depois da indução e recuperação da anestesia. Os peixes foram submetidos a um dos seguintes tratamentos para cada OE: grupo basal, controle ou anestesiado (150, 300 ou 450 μL L^{-1} OE), avaliando-se a taxa ventilatória (TV) durante o período de indução e, posteriormente, transferidos para aquários sem anestésicos para recuperação da anestesia. Nos tempos 0, 15, 30, 60 e 240 min de recuperação foram realizadas amostragens de plasma e brânquias para medir indicadores metabólicos e enzimas ionorregulatórias, respectivamente. No artigo 2, os efeitos da exposição prolongada de jundiás a baixas concentrações do OEHR foram estudados. Após 6 h de exposição a 0 (controle), 30 ou 50 μL L^{-1} OEHR adicionado à água, analisou-se: TV, indicadores metabólicos e de estresse em plasma, atividade enzimática em fígado e expressão de hormônios hipofisários (hormônio do crescimento - GH, prolactina - PRL e somatolactina - SL). No manuscrito (a) avaliou-se a eficácia anestésica do OELA em dourada (*Sparus aurata*) e (b) investigaram-se os efeitos de 35 μL L^{-1} de OELA e 2-fenoxietanol (2-PHE) sobre a resposta ao estresse em douradas submetidos à perseguição. Após 4 h de exposição, foram amostrados plasma (para determinação dos níveis de cortisol, metabólitos e osmolalidade), cérebro e hipófise (para avaliar a expressão de indicadores endócrinos). No artigo 1, a anestesia com os OEs provocou alterações em alguns parâmetros medidos em jundiás, mas não impediu a restauração da maioria dos indicadores avaliados após 240 min de recuperação. No artigo 2, 50 μL L^{-1} do OEHR provocou a elevação dos níveis de glicose, lactato, proteína e osmolalidade, bem como aumento na atividade de enzimas metabólicas e redução na expressão do GH e SL. No manuscrito, douradas expostos ao OELA, estressados ou não, exibiram maiores níveis de cortisol, glicose, lactato e osmolalidade. A exposição ao OELA somado ao estresse reduziu os níveis de expressão de CRH-BP (hormônio liberador de corticotrofina ligado à proteína). A expressão de PRL foi reduzida no grupo controle estressado e após a exposição ao OELA e 2-PHE em peixes não estressados. Maiores expressões de pro-opiomelanocortina (POMC) “a” e “b” foram observadas em peixes estressados e expostos ao OELA e 2-PHE, respectivamente. Conclui-se que: (1) o OELA é mais eficiente para jundiás que o OEHR em concentrações para anestesia; (2) para sedar os peixes, recomenda-se 30 μL L^{-1} do OEHR (ou menos); (3) o OELA foi eficaz como anestésico para dourada entre 100-300 μL L^{-1}, mas para 4 h de exposição o 2-PHE foi mais eficiente em prevenir a resposta ao estresse.

Palavras-chave: Expressão hormonal. Dourada. Ionorregulação. Jundiá. Metabolismo energético. Resposta ao estresse. Taxa ventilatória.

ABSTRACT

Doctoral Dissertation
Post-Graduate Program in Pharmacology
Universidade Federal de Santa Maria

ESSENTIAL OILS AS ANESTHETICS FOR FISH: BIOCHEMICAL AND MOLECULAR ASPECTS

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ADVISER: BERNARDO BALDISSEROTTO
Date and Place of Defense: January 15th, 2015, Santa Maria.

The essential oil (EOs) extracted from plants *Hesperozygis ringens* and *Lippia alba* possess anesthetic and sedative properties and is an alternative to traditional anesthetics used in aquaculture for ease of handling and/or reduce stress. In this sense, the study aimed to investigate the effects of these EOs on the physiology of fish, through physiological, biochemical and endocrine indicators. In the article 1 was determined (a) the anesthetic activity of the EOs of *H. ringens* (EOHR) and *L. alba* (EOLA) and (b) its effects on silver catfish (*Rhamdia quelen*) after induction and recovery from anesthesia. Fish were subjected to one of the following treatments for each EO: basal group, control or anesthetized (150, 300 or 450 uL L^{-1} EO), evaluating the ventilatory rate (VR) during the induction period and thereafter transferred to anesthetics-free tanks for recovery from anesthesia. At 0, 15, 30, 60 and 240 min of recovery, samples of plasma and gills were collected to measure metabolic indicators and ionregulatory enzymes, respectively. In the article 2, the effects of prolonged exposure to low EOHR concentrations were studied on silver catfish. After 6 h of exposure to 0 (control), 30 or 50 uL L^{-1} EOHR added to water, it was analyzed: VR, metabolic indicators of stress in plasma, enzyme activity in liver, and expression of pituitary hormones (growth hormone - GH, prolactin - PRL and somatolactina - SL). In the manuscript, (a) evaluated the effectiveness of anesthesia EOLA on gilthead sea bream (*Sparus aurata*) and (b) we investigated the effects of 35 uL L^{-1} EOLA and 2-phenoxyethanol (2-PHE) on the stress response in gilthead sea bream undergoing persecution. After 4 h of exposure, the plasma was sampled (for the determination of cortisol, metabolites and osmolality), brain and pituitary (to evaluate the expression of endocrine indicators). In the article 1, anesthesia with EOs caused changes in some parameters measured in silver catfish, but did not prevent the restoration of most of the indicators assessed after 240 min of recovery. In the article 2, 50 uL L^{-1} EOHR led to an increase of glucose, lactate, protein and osmolality, as well as an increase in metabolic enzyme activity and reduced expression of GH and SL. In the manuscript, gilthead sea bream exposed to EOLA, stressed or not, exhibited higher levels of cortisol, glucose, lactate and osmolality. EOLA exposure added to the stress reduced the expression levels of CRH-BP (corticotropin releasing hormone bound to protein). PRL expression was reduced in the stressed control group and after exposure to EOLA and 2-PHE in fish not stressed. Higher expression of pro-opiomelanocortin (POMC) "a" and "b" were observed in fish stressed and exposed to EOLA and 2-PHE, respectively. We conclude that: (1) the EOLA is more efficient for silver catfish that EOHR in anesthesia concentrations; (2) for sedating the fish, it is recommended 30 uL EOHR L^{-1} (or less); (3) the EOLA was effective as an anesthetic for gilthead sea bream at 100-300 uL L^{-1}, but for 4 h exposure, the 2-PHE was more effective in preventing the stress response.

Keywords: Hormone expression. Gilthead sea bream. Ionorregulation. Silver catfish. Energy metabolism. Stress response. Ventilatory rate.

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INTRODUÇÃO

Aquicultura e utilização de anestésicos

A aquicultura é uma atividade crescente no cenário mundial e representa uma importante fonte de alimento para a humanidade. Segundo dados da FAO (Food and Agriculture Organization), em 2012 o consumo de pescado atingiu 136 milhões de toneladas. Desse total, 67 milhões de toneladas eram provenientes da aquicultura (FAO, 2014). Paralelamente à expansão dessa prática, a melhoria da saúde dos peixes destinados ao consumo humano tem recebido atenção. O bem-estar dos peixes é importante para a indústria não apenas pela aceitação do produto, mas também em termos de eficiência de produção, quantidade e qualidade (ASHLEY, 2007).

Ações rotineiras realizadas na aquicultura como biometria, análises patológicas, implante hormonal, manejo, captura e transporte podem resultar em prejuízos econômicos em função da interferência dessas atividades no desempenho dos peixes (BARTON, 2000). Na verdade, as práticas da aquicultura moderna frequentemente expõem os peixes a uma variedade de fatores estressantes, que podem afetar negativamente seu sistema imune e sua sobrevivência. Neste sentido, substâncias anestésicas têm sido empregadas com o intuito de minimizar os efeitos do estresse em peixes (COOKE et al., 2004; INOUE et al., 2005; BECKER et al., 2012).

O processo de anestesia envolve vários componentes, incluindo sedação, imobilização, inconsciência (narcose), amnésia e analgesia. A sedação caracteriza-se pela redução da sensibilidade, enquanto que a anestesia geral causa um estado de inconsciência, amnésia e também inclui imobilização e alívio da dor (ZAHL et al., 2012). Assim, um mesmo agente pode ser designado como sedativo (calmante) e/ou anestésico, dependendo da concentração ou dose empregada, tempo de exposição e, consequentemente, o efeito provocado no organismo vivo.

Segundo Small (2003), o processo de anestesia em peixes envolve basicamente quatro estágios, caracterizados da seguinte forma:

- estágio 1 – sedação: diminuição de reatividade a estímulo externo;

- estágio 2 – perda parcial de equilíbrio; nado errático;
- estágio 3 – perda total de equilíbrio; cessação da locomoção;
- estágio 4 – colapso medular.

Um método comum de administração de anestésicos em peixes é por imersão deste em solução anestésica. O agente anestésico disperso na água é absorvido através das brânquias, difunde-se para o sangue e alcança o sistema nervoso central (SNC) (ROSS; ROSS, 2008). O efeito geralmente é avaliado pelos tempos de indução e recuperação da anestesia, reações reflexas a estímulos externos e capacidade de resposta à manipulação (SMALL, 2003; ZAHL et al., 2012). Segundo Ross e Ross (2008), a depressão generalizada do SNC ocasionada pelos anestésicos gerais se deve por sua ação sobre o axônio do nervo, a liberação de transmissores ou a excitabilidade da membrana, ou ainda, pela combinação dessas ações. Os mecanismos de ação dos agentes anestésicos incluem (1) estabilização da propagação de impulsos nervosos em axônios aferentes e/ou eferentes, (2) bloqueio da liberação de neurotransmissores na membrana pré-sináptica, (3) bloqueio competitivo de sítios receptores na membrana pós-sináptica.

No Brasil, ainda não há legislação regulamentando o uso de anestésicos na aquicultura. As diretrizes estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) referem-se exclusivamente aos procedimentos de eutanásia (CONCEA, 2013). Assim, procura-se seguir as normas estabelecidas pela Food and Drug Administration (FDA) quando a utilização de anestésicos se faz necessária (FAÇANHA; GOMES, 2005). Em outros países, uma ampla gama de agentes anestésicos tem sido usada para peixes. Entre os mais comuns estão tricaína metano sulfonato (MS 222), benzocaína, metomidato, 2-fenoxietanol e quinaldina (NEIFFER; STAMPER, 2009). A escolha de um anestésico deve estar relacionada, entre outros fatores, com sua viabilidade econômica, praticidade no uso, eficácia para anestesiar e capacidade de reduzir o estresse (CHO; HEATH, 2000; IVERSEN et al., 2003). Além de sua disponibilidade e custo, um anestésico deve ser seguro tanto para o peixe, quanto ao seu manipulador. Neste contexto, pesquisas com anestésicos de fontes naturais vêm sendo realizadas (FAÇANHA; GOMES, 2005; GONÇALVES et al., 2008; HELDWEIN et al., 2012; BECKER et al., 2012; SILVA et al., 2012).

Caracterização das espécies estudadas

O jundiá *(Rhamdia quelen*, Heptapteridae) é uma espécie nativa da região Sul do Brasil, bem adaptada ao cultivo e comumente encontrada em cursos d'água nessa região. Caracteriza-se por ser um peixe de couro, onívoro e apresentar crescimento bastante pronunciado nos primeiros anos de vida (BALDISSEROTTO et al., 2010). Diversos trabalhos, utilizando o jundiá como modelo experimental, têm demonstrado a eficácia de produtos naturais como anestésicos (CUNHA et al., 2010a; GRESSLER et al. 2012; SILVA et al., 2013; PARODI et al., 2014). No entanto, há poucos relatos dos efeitos desses agentes sobre aspectos bioquímicos e fisiológicos do jundiá, bem como de outras espécies, o que demanda maior atenção para a utilização de tais substâncias.

O dourada (*Sparus aurata*, Sparidae) é uma espécie marinha que ocorre naturalmente na região do Mediterrâneo e do Mar Negro (raro), e no Atlântico Oriental desde as Ilhas Britânicas, Estreito de Gibraltar a Cabo Verde e ao redor das Ilhas Canárias (FROESE; PAULY, 2011). É um dos peixes marinhos mais importantes para pesca e aquicultura, especialmente na região mediterrânea, além de possuir um alto valor comercial (BASURCO; ABELLÁN, 1999; ARABACI et al., 2010). Estudos reportando as alterações fisiológicas em dorada após exposição a anestésicos são escassos (ORTUÑO et al., 2002; VERA et al., 2013), principalmente quando se trata dos efeitos de anestésicos não sintéticos (MYLONAS et al., 2005).

Resposta ao estresse

As vias neuroendócrinas do estresse em peixes são muito similares às de outros vertebrados e contam com um sistema adrenérgico e um eixo hipotálamo-hipófise-interrenal (HHI) (WENDELAAR-BONGA, 1997; WEBER, 2011). A ativação do eixo HHI, em resposta a um estímulo nocivo (estressor), leva à liberação de mediadores químicos (catecolaminas e corticosteroides) na corrente sanguínea (IWAMA et al. 1999; BARTON, 2002). As alterações fisiológicas que caracterizam a resposta ao estresse permitem que o animal responda de forma adaptativa ou compensatória desencadeando uma série de alterações fisiológicas, classificadas em respostas primárias, secundárias e terciárias (WENDELAAR-BONGA, 1997; IWAMA et al., 2004).

Entre as respostas primárias, destaca-se o aumento na secreção de catecolaminas (adrenalina e noradrenalina) e cortisol no plasma. A consequência das respostas primárias pode levar a respostas secundárias – alterações metabólicas que contribuem para a produção de energia extra, como a alteração na glicemia, no ácido lático e no glicogênio hepático e muscular. As respostas que afetam o balanço hidromineral, como alteração nas concentrações de cloreto, sódio, potássio, proteínas e na osmolaridade do plasma, também constituem respostas secundárias ao estresse. Como resultado de um estresse crônico e exaustão fisiológica, as respostas terciárias podem ter consequências em longo prazo, afetando negativamente a função imune, locomoção, reprodução, taxa de crescimento, comportamento e sobrevivência (BARTON, 2002; WEBER, 2011).

Vários autores têm investigado a resposta ao estresse em peixes e, embora difiram no agente estressor, todos avaliaram os níveis de cortisol e seu aumento como indicador da resposta ao estresse (SMALL, 2003; PIRHONEN; SCHRECK, 2003; BARCELLOS et al., 2009; MARICCHIOLO; GENOVESE, 2011; KOAKOSKI et al., 2012). Quando alguns produtos de fontes naturais foram utilizados como anestésicos e/ou sedativos para jundiá, observou-se redução nos níveis de cortisol e, possivelmente, uma atenuação da resposta ao estresse (CUNHA et al., 2010 a, b; ZEPPENFELD et al., 2014).

Somado à elevação do cortisol, geralmente o estresse provoca um aumento nos níveis de glicose e lactato no plasma (respostas secundárias). A alteração desses parâmetros metabólicos pode resultar de processos como a glicogenólise e gliconeogênese, mediada por catecolaminas e cortisol, respectivamente (PANKHURST, 2011). O incremento de corticosteroides no plasma, além de modular o metabolismo de carboidratos, interfere no metabolismo proteico e na atividade aminotransferase (MOMMSEN et al., 1999), sugerindo um papel funcional do cortisol no metabolismo intermediário. A análise de parâmetros metabólicos, não somente em plasma, mas também em tecidos, embora escassamente, tem sido empregada com o intuito de avaliar a forma como o metabolismo de peixes se comporta frente à utilização de anestésicos (CARNEIRO et al., 2002; INOUE et al., 2005; BARBOSA et al., 2007; MARICCHIOLO; GENOVESE, 2011).

As brânquias dos peixes são órgãos multifuncionais importantes para atividades vitais como as trocas gasosas, a regulação iônica e o equilíbrio ácido-base (EVANS et al., 2005). Para manutenção da homeostase interna, os peixes dispõem de mecanismos osmorregulatórios altamente eficientes, incluindo as

enzimas Na^+/K^+-ATPase e H^+-ATPase, que participam da regulação iônica (HUANG et al., 2010). O cortisol desempenha papel chave na osmorregulação por meio de seu efeito sobre a atividade da Na^+/K^+-ATPase (PANKHURST, 2011). Condições de estresse aumentam o fluxo sanguíneo e a permeabilidade paracelular nas brânquias, o que pode resultar em perda iônica (McDONALD et al., 1991).

Óleos essenciais como anestésicos para peixes

Diversos estudos relatam a utilização de produtos de fontes naturais empregados como anestésicos em peixes. Óleos essenciais obtidos do cravo (PEREIRA-DA-SILVA et al., 2009, SIMÕES et al., 2011), *Lippia alba* (CUNHA et al., 2010a; BECKER et al., 2012), *Ocimum gratissimum* (SILVA et al., 2012), *Aloysia triphylla* (GRESSLER et al., 2012), bem como compostos isolados de plantas como o mentol (FAÇANHA; GOMES, 2005; GONÇALVES et al., 2008; SIMÕES; GOMES, 2009) e o eugenol (CUNHA et al., 2010b; BECKER et al., 2012) mostraram-se eficazes para diferentes espécies de peixes.

A família Lamiaceae é composta por 220 gêneros e cerca de 3.500-4.000 espécies; constitui uma importante fonte de óleos essenciais (gêneros *Mentha*, *Rosmarinus*, *Thymusus e Lavandula*), muitos deles usados como matéria-prima em indústrias de cosméticos, perfumaria e alimentos (AGOSTINI et al., 2009). O gênero *Hesperozygis* é um membro da família Lamiaceae, com cerca de seis espécies, cinco delas restritas ao sudoeste do Brasil e uma citada para o México.

Hesperozygis ringens é uma espécie endêmica dos campos rupestres do sudoeste do Rio Grande do Sul. A planta, conhecida pelo nome vernáculo “espanta pulga”, é empregada por suas propriedades antiparasitárias e teve comprovada sua propriedade acaricida (RIBEIRO et al., 2010). Recentemente, foi relatada a propriedade anestésica do óleo essencial de *H. ringens* para jundiá (SILVA et al., 2013). Esse efeito pode estar relacionado, em parte, ao seu componente majoritário – a pulegona – que é um modulador alostérico positivo dos receptores GABA (ácido gama-aminobutírico), principal neurotransmissor inibitório no SNC de vertebrados (TONG; COATS, 2010). No entanto, os efeitos do óleo essencial de *H. ringens* sobre o organismo animal são pouco conhecidos.

A família Verbenaceae compreende cerca de 175 gêneros e 2.800 espécies distribuídas em regiões tropicais e subtropicais, em zonas temperadas do hemisfério

Sul e poucas no hemisfério Norte, cujos representantes são principalmente ervas perenes, arbustos e subarbustos (BARROSO et al., 2004). No Brasil, a família Verbenaceae é representada principalmente pelos gêneros *Aloysia*, *Clerodendrum*, *Lantana*, *Lippia* e *Verbena* (LORENZI, 1998). O gênero *Lippia* inclui aproximadamente 200 espécies de pequenos arbustos. As espécies são encontradas em todos os países da América do Sul e Central e territórios tropicais da África (SCHULTZ, 1984; TERBLANCHE; KORNELIUS, 1996).

Lippia alba é uma espécie originária da América do Sul, sendo conhecida por diversos nomes populares, como erva-cidreira-do-campo, alecrim-selvagem, cidreira-brava, falsa-melissa, dentre outros (BIASI; COSTA, 2003). O óleo essencial de *L. alba* mostrou ser eficaz como anestésico para jundiá (CUNHA et al., 2010a). Esse efeito se deve principalmente pelo envolvimento do sistema GABAérgico, embora a existência de outros possíveis mecanismos neuroquímicos não possa ser descartada (HELDWEIN et al., 2012).

Dado o exposto, a utilização de produtos derivados de fontes naturais, que apresentam propriedade anestésica para peixes, pode constituir uma alternativa aos anestésicos tradicionais empregados em aquicultura. Neste sentido, investigar as possíveis alterações em processos fisiológicos de peixes expostos aos óleos essenciais de *H. ringens* e *L. alba,* bem como avaliar se ocorre recuperação dos parâmetros avaliados se faz necessário, a fim de determinar a viabilidade dessas substâncias como anestésicos eficazes e, sobretudo, seguros.

ARTIGO 1

Publicado no periódico Fish Physiology and Biochemistry

Fish anesthesia: effects of the essential oils of *Hesperozygis ringens* and *Lippia alba* on the biochemistry and physiology of silver catfish (*Rhamdia quelen*)

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Abstract

The anesthetic activities of the essential oils (EOs) of *Hesperozygis ringens* (EOHR) and *Lippia alba* (EOLA) and their effects in silver catfish (*Rhamdia quelen*) after anesthesia and recovery were investigated. Fish (32.19 ± 1.24 g) were submitted to one of the following treatments for each EO: basal group, control or anesthesia (150, 300 or 450 μL L^{-1} EO). After that the anesthesia was induced or simulated and the biometric measurements were completed, fish were transferred to anesthetic-free aquaria to allow for recovery. Fish were sampled at 0, 15, 30, 60 and 240 min after recovery. At time 0 of recovery, the ventilatory rate was lower in the groups anesthetized with either EO. In comparison with the basal group, control fish showed an increase in plasma glucose, aspartate aminotransferase (AST) and Na^+ levels and a reduction in Na^+/K^+-ATPase activity at 0 min of recovery. Plasma levels of ammonia and Na^+ were lower in the fish anesthetized with EOLA (450 μL L^{-1}) and EOHR (all concentrations), respectively, than in the control fish. Additionally, lactate, AST, alanine aminotransferase and K^+ plasma levels and gill Na^+/K^+-ATPase and H^+-ATPase activities were higher in the fish anesthetized with either EOHR or EOLA than in the control fish. The EOs promoted slight changes in silver catfish that enabled both an adaptive response and the recovery of most of the measured parameters after 240 min regardless of concentration or EO that was used. These findings support the use of EOHR and EOLA as anesthetics for fish.

Keywords Anesthetics Energy metabolism Ionoregulation Recovery Ventilatory rate

Introduction

Fish used in farming or research are exposed to many situations that are stressful or painful to the animals, including handling, confinement, netting, weighing, vaccination, blood sampling, transport and surgical procedures (Kiessling et al. 2009). Though anesthetics can be a valuable tool to ensure animal welfare during these events, these agents can also have unwanted side effects and should therefore be used with caution (Zahl et al. 2012).

The stress response is an adaptive physiological function that responds to a perceived threat to homeostasis, which in the short term preserves the health and viability of the stressed individual (Ashley 2007). However, when the stress is excessive in either intensity or duration, the stress response can result in undesirable consequences, such as illness or mortality (Iwama et al. 2004). The activation of the hypothalamic–pituitary–interrenal (HPI) axis is a characteristic feature of the stress response, and this activation results in the mobilization of a series of energy sources. Thus, the levels of cortisol, glucose, lactate, and other biochemical and ionoregulatory parameters can provide important information about the internal environment of the organism. This information may allow an assessment of the stress response in stressed fish as well as the capacity of the animals to overcome the disturbance (Mommsen et al. 1999; Acerete et al. 2004).

To minimize the effects of stress on fish, investigators worldwide have begun to examine the use of natural products with anesthetic properties. Examinations of Chinook salmon (*Oncorhynchus tshawytscha*), rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) anesthetized with clove oil (Cho and Heath 2000; Wagner et al. 2003; Velisek et al. 2005) and silver catfish (*Rhamdia quelen*) anesthetized with essential oil (EO) of *Lippia alba* (Cunha et al. 2010; Heldwein et al. 2012), *Ocimum gratissimum* (Silva et al. 2012), *Aloysia triphylla* (Gressler et al. 2012) and *Hesperozygis ringens* (Silva et al. 2013) have led to the development of anesthesia protocols in which products extracted from natural sources were employed with efficacy to treat fish.

The genus *Hesperozygis* belongs to the family Lamiaceae, which is an important source of EOs (Agostini et al. 2009). *Hesperozygis ringens* is a woody herb native to the rocky fields of southwest Rio Grande do Sul, Brazil. This plant is used because of its antiparasitic and acaricide properties (Ribeiro et al. 2010). *Lippia alba*

(Verbenaceae) is a species that originated from South America (Biasi and Costa 2003) with EO anesthetic activity demonstrated in silver catfish (Cunha et al. 2010) and *Hippocampus reidi* (Cunha et al. 2011).

Silver catfish is a native fish of southern Brazil that is apparently well adapted to different environments, is widely used in fish ponds and is well accepted by the consumer market (Marchioro and Baldisserotto 1999). As previously mentioned, this species has been used as an animal model in research investigating the anesthetic activity of EOs. However, the effects of these products on the physiology and biochemistry of fish remain poorly understood. Thus, the aim of this study was to assess the effects of EOs from *H. ringens* and *L. alba* on the metabolic, enzymatic and ionoregulatory parameters of silver catfish after anesthesia induction and recovery. For the first time, the interference of products used in fish anesthesia with the activity of enzymes involved in ionoregulation was investigated.

Materials and methods

Animals

Silver catfish were obtained from the Fish Culture sector at the Universidade Federal de Santa Maria (RS, Brazil) and transported to the Laboratory of Fish Physiology, where they were maintained in continuously aerated 250 L tanks with controlled water parameters. Dissolved oxygen (6.2 ± 0.2 mg L^{-1}) and temperature (22.6 ± 0.23°C) were measured with a YSI oxygen meter (Model Y5512). The pH (7.04 ± 0.21) was determined with a DMPH-2 pH meter. Total ammonia nitrogen (TAN) levels (0.64 ± 0.37 mg L^{-1}) were measured with the salicylate method (Verdouw et al. 1978). Water hardness (23.7 ± 1.4 mg L^{-1} $CaCO_3$) was analyzed using the EDTA titrimetric method (Eaton et al. 2005). The nitrite concentration (1.05 ± 0.37 mg L^{-1}) and alkalinity (31.1 ± 2.7 mg L^{-1} $CaCO_3$) were measured using the method of Boyd and Tucker (1992). A semi-static system was used, and 50% of the water volume was changed daily. Fish were fed once a day with commercial feed (28% crude protein). Fish were fasted for a period of 24 h prior to the experiments.

Experimental methodologies were approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process nº 46/2010).

Plant materials

Aerial parts of *Hesperozygis ringens* were collected in São Francisco de Assis (Rio Grande do Sul, Brazil). The species was identified by Dr. Solon Jonas Longhi, and a voucher specimen (SMDB nº 13427) was deposited in the herbarium of the Department of Biology at the Universidade Federal de Santa Maria (UFSM). *Lippia alba* was cultivated in Frederico Westphalen (RS, Brazil). The plant material was identified by botanist Dr. Gilberto Dolejal Zanetti (Department of Industrial Pharmacy, UFSM), and a voucher specimen (SMDB n° 10050) was deposited in the herbarium of the Department of Biology at the UFSM.

Essential oil extraction and analysis

The extraction of the EOs from the fresh leaves of *H. ringens* and *L. alba* was performed by hydrodistillation for 3 h and 2 h, respectively, using a Clevenger type apparatus (European Pharmacopoeia 2007). Essential oils were stored at -4ºC in amber glass bottles until the composition analysis by gas chromatography coupled to mass spectrometry (GC-MS; as described by Silva et al. 2012) and other biological tests could be performed.

Experimental design

Experiments were carried out for different time periods but with the same procedure for the EOs from *H. ringens* (EOHR) and *L. alba* (EOLA).

Silver catfish (15.55 ± 0.17 cm; 32.19 ± 1.24 g) were distributed into the following groups for each EO: basal (only sampled), control (simulated anesthesia) or anesthesia (150, 300 or 450 μL L^{-1} EO). These EO concentrations were chosen based on previous reports (Cunha et al. 2010; Silva et al. 2013) showing that these concentrations led fish to stage III of anesthesia (Small 2003). The EOs were diluted in ethanol (1:10) to enable better dissolution in water.

Fish were individually captured from the maintenance tanks and were then transferred to aquaria containing 1 L of water lacking (control) or containing the EO. The fish remained exposed to these conditions for 900 seconds (control) or until the

fish reached stage III of anesthesia (EO). After the anesthesia induction procedure was complete, fish were handled for biometric measurements and were then exposed to air for 1 min. Aerial exposure is a method that is able to induce stress in silver catfish (Barcellos et al. 2006). The fish were subsequently transferred to anesthetic-free aquaria containing 1 L of water to allow for recovery from the anesthesia. Fish were sampled after 0, 15, 30, 60 and 240 min of recovery (n = 7 fish/sampling/treatment). Each fish was sampled only once.

Ventilatory rate (VR)

The VR was quantified by visually counting 20 successive opercular or buccal movements, measuring the elapsed time with a chronometer (adapted from Alvarenga and Volpato 1995). The VR was determined at four different periods during the anesthesia procedure: immediately after transfer to anesthesia aquaria (time 0), 30 seconds after transfer, 5 min after transfer (control) or when stage I was reached (EO) and 15 min after transfer (control) or when stage III was reached (EO).

Sampling and analyses

Blood was collected from the caudal vein of each fish using heparinized 3-mL syringes. Plasma was obtained after blood centrifugation (3000 *g*, 10 min, 4°C) and was then frozen at -80°C until metabolic and ion analyses could be performed. Using commercial kits, metabolic parameters that were analyzed in plasma included glucose levels (Cat. 434, Analisa, Brazil), lactate levels (Ref. 116, Labtest, Brazil), total protein concentration (Cat. 418, Analisa, Brazil) and alanine aminotransferase (ALT) (Cat. 353, Analisa, Brazil) and aspartate aminotransferase (AST) (Cat. 352, Analisa, Brazil) activities. Plasma ammonia levels were measured according to Verdouw et al. (1978). Chloride levels were determined as described by Zall et al. (1956), and Na^+ and K^+ levels were determined with a B262 Micronal flame spectrophotometer.

Fish were killed by medullar section after blood collection. The gills were then collected and stored at -80°C until the analysis of ATPase activities could be performed. Na^+/K^+-ATPase and H^+-ATPase activities were measured simultaneously using the method described by Gibbs and Somero (1989), with some modifications

adapted for use with a microplate. The gills (100 mg) were homogenized in 1 mL of homogenization buffer (150 mM sucrose, 50 mM imidazole and 10 mM EDTA pH 7.5). Five microliters of homogenate and 200 µL of reaction solution (30 mM imidazole, 45 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.4 mM KCN 1 mM ATP, 0.2 mM NADH, 0.1 mM fructose 1,6 diphosphate and 2 mM phosphoenolpyruvate) were added to each sample. Ouabain (2 mM) and NEM (N-ethylmaleimide, 2 mM) were used as inhibitors. All assays were run in quadruplicate. Protein concentration was determined using the Coomassie blue method, according to Bradford et al. (1976).

Statistical analysis

Data are presented as means ± SEM. The homogeneity of variances between different groups was calculated with Levene's test. Because the data exhibited homogeneous variances, comparisons between different groups and times were made using two-way ANOVA and Tukey's test. Analyses were performed using the software Statistica ver. 7.0 with the minimum significance level set at $P<0.05$.

Results

The major components of EOHR were determined to be pulegone (95.18%) and limonene (1.28%), and the principal components of EOLA were linalool (54.38%) and 1,8-cineole (5.92%) (Table 1).

No mortality occurred during the experiments. Anesthesia induction times were found to be negatively correlated with the anesthesia concentration for both EOs (Fig. 1). Behavioral anesthesia recovery occurred in less than 5 min for all anesthetized fish.

Effects of the EOs

During the first 30 s of anesthesia, the effect of EOHR on the VR was concentration-dependent, with higher concentrations of EOHR associated with higher VRs. Fish anesthetized with EOLA showed a significant increase in the VR compared to control fish. The VR remained significantly higher in the fish anesthetized with EOHR than in

the control group after 5 min of exposure. During stage III of anesthesia, fish anesthetized with 300 or 450 μL L^{-1} EOHR or with any concentration of EOLA displayed a significantly lower VR than the control group. The VR of the anesthetized fish with EOHR was reduced by 41.2 – 53.9% between time 0 and stage III of anesthesia, while the VR of the control group was reduced by only 19.9%. When EOLA was used as anesthetic, reduction of 52.6 – 57.8% in the VR was verified between time 0 and stage III of anesthesia, while in the control group did not change was observed (Table 2).

Manipulation of control fish triggered an immediate (time 0) rise in plasma glucose levels compared to the basal group. EOHR was not able to prevent this increase. An increase in plasma glucose was observed after 30 and 60 min of recovery in fish anesthetized with 450 and 300 μL L^{-1} EOHR, respectively. Recovery of plasma glucose to basal levels occurred only after 240 min in fish anesthetized with 150 μL L^{-1} EOHR (Fig. 2A). When EOLA was used as the anesthetic, an increase in plasma glucose levels was prevented, regardless of the EOLA concentration (Fig. 2B).

Plasma lactate levels in control fish were not significantly different than in fish in the basal group at the beginning of the recovery period. In contrast, fish exposed to either EOHR or EOLA exhibited significantly higher lactate levels than the control fish. After 30 min of recovery, fish anesthetized with 150 or 300 μL L^{-1} EOHR or with 150 or 450 μL L^{-1} EOLA displayed plasma lactate levels near the basal values. Restoration of the basal lactate level was observed after 240 min of recovery, regardless of the concentration or EO used as the anesthetic (Fig. 2C and 2D).

Plasma protein levels were not affected by handling or by either anesthesia (Fig. 3A and 3B). However, fish anesthetized with 150 μL L^{-1} EOLA showed an increase in plasma protein levels after 60 min of recovery when compared to the 15 min recovery time point, and these levels were significantly higher than those recovering from exposure to 450 μL L^{-1} EOLA (Fig. 3B). Plasma ammonia levels in the handled, control fish and in the fish anesthetized with EOHR or EOLA did not differ from the basal group (Fig. 3C and 3D). At the start of recovery from anesthesia, lower ammonia levels were found in the plasma of the fish anesthetized with 450 μL L^{-1} EOLA compared to the other groups at the same time point. However, after complete recovery, no significant differences in ammonia levels between the groups were observed (Fig. 3D).

At the beginning of the recovery phase, the AST levels did not differ between the control and basal groups, but anesthesia with EOHR resulted in an increase in plasma AST levels at all tested EOHR concentrations. However, during recovery from anesthesia, these levels returned to values similar to the basal levels (Fig. 4A). Manipulation and anesthesia with EOLA triggered an increase in the levels of AST compared to the basal group at the start of the recovery phase (time 0). After 240 min of recovery, the AST levels in the plasma of anesthetized fish remained higher than in the control and basal groups (Fig. 4B). No differences in the ALT levels were found when comparing the control and basal groups at the beginning of the recovery phase. At this time point, the highest levels of ALT were found in the plasma of fish anesthetized with EOHR, independent of the concentration of EOHR. Although the ALT levels in these groups varied during the recovery time, after 240 min of recovery, only the fish anesthetized with 300 μL L^{-1} EOHR displayed ALT levels similar to the control group (Fig. 4C). The levels of ALT were not affected by anesthesia with EOLA (Fig. 4D).

Plasma Na^+ levels were higher in the control group in comparison with the basal group at the start of the recovery period. In fish anesthetized with EOHR, an increase in plasma Na^+ levels was prevented, except in fish treated with 300 μL L^{-1} EOHR. After 15 min of recovery, fish anesthetized with either 300 or 450 μL L^{-1} EOHR displayed plasma Na^+ levels similar to the basal group, while the control and 150 μL L^{-1} EOHR treatment groups showed elevated levels of Na^+ even after 240 min of recovery. Anesthesia with all concentrations of EOHR raised plasma K^+ levels at the beginning of the recovery period compared to the control and basal groups, and these levels remained elevated after up to 240 min following anesthesia recovery in fish anesthetized with 150 or 450 μL L^{-1} EOHR (Table 3). After 240 min of recovery, the restoration of plasma K^+ to basal levels occurred only in fish anesthetized with 300 μL L^{-1} EOHR. Chloride levels were not affected by manipulation with EOHR anesthesia. Both fish handling and anesthesia treatment with EOLA no changed the levels of Na^+, K^+ and Cl^- at the start of the recovery period. Although slight variations were observed during anesthesia recovery, after 240 min of recovery, the plasma levels of these ions were similar to the basal levels (Table 3).

Fish in the control group exhibited lower Na^+/K^+-ATPase activity at the beginning of the recovery phase in comparison with both the basal and anesthetized groups with EOHR. The enzymatic activity was not affected by any EOHR

concentration after 0 or 240 min of recovery in comparison with the basal group (Fig. 5A). Fish anesthetized with 300 or 450 μL L^{-1} EOLA exhibited higher Na^+/K^+-ATPase activity than the control group at the beginning of the recovery period. After 240 min of recovery, the enzymatic activity was similar to the basal group, but fish anesthetized with 300 μL L^{-1} EOLA showed a significantly higher activity than the control fish (Fig. 5B). Higher H^+-ATPase activity was observed in the gills of fish anesthetized with 300 or 450 μL L^{-1} EOHR in comparison with the control and basal groups after both 0 and 240 min of recovery. Fish anesthetized with 150 μL L^{-1} EOLA exhibited higher enzymatic activity than the control group at the start of the recovery period. After 240 min of recovery, the enzymatic activity was similar in all treatment groups (Fig. 5D).

Discussion

In this study, the chemical composition of EOHR was found to agree both quantitatively and qualitatively with results obtained by von Poser et al. (1996) and Ribeiro et al. (2010) in which pulegone was the predominant constituent (79.2% and 86%, respectively). In addition to the antimicrobial activity that has already been reported (Ribeiro et al. 2010), the anesthetic efficacy of EOHR in fish has been previously investigated (Silva et al. 2013). The chemical composition of the EOLA used in this study was similar to that observed in previous studies that evaluated the anesthetic effect and mechanism of action of this EO in silver catfish (Cunha et al. 2010; Heldwein et al. 2012). The concentration of linalool observed in this study was higher than that used by Cunha et al. (2010) and lower than that reported by Heldwein et al. (2012). In contrast, these studies reported the opposite relative concentrations of 1,8-cineole. Furthermore, slight variations in the time required for the fish to reach deep anesthesia were found in these three studies. These variations may be due to differences in the abundance of certain compounds or due to differences in fish size, which influence the anesthetic response (Gomes et al. 2011; Zahl et al. 2012). Nevertheless, the anesthetic efficacy of both EOLA and EOHR was as expected.

While fish anesthesia is known to help minimize the effects of handling procedures, the results of the present study indicate that the use of EOHR and EOLA

does not necessarily attenuate certain biochemical responses, particularly changes in plasma lactate and glucose concentrations. On the other hand, all of the biochemical changes provoked by the EOs returned to basal levels after 240 min of recovery, with the exception of glucose and ALT levels in fish exposed to EOHR and AST levels in fish exposed to EOLA.

Hyperglycemia observed in the plasma of manipulated control fish is a typical result of stress (Pankhurst 2011). Contrary to expectations, EOHR was unable to attenuate this effect of stress, as was observed when EOLA was used for anesthesia. Other authors have also reported that plasma glucose levels increase in Chinook salmon, rainbow trout and greater amberjack (*Seriola dumerilii*) after clove oil anesthesia (Cho and Heath 2000; Wagner et al. 2003; Maricchiolo and Genovese 2011). This increase in plasma glucose levels, possibly due to catecholamine-mediated glycogenolysis and cortisol-mediated gluconeogenesis, may represent an adaptive response during a stressful event (Pankhurst 2011).

When oxygen is not available for aerobic cell metabolism, an increase in lactate levels occurs. The results of lactate levels in the current study are in agreement with Iversen et al. (2003) and Inoue et al. (2011), who reported higher lactate levels in tambaqui (*Colossoma macropomum*) and Atlantic salmon (*Salmo salar*) anesthetized with eugenol and clove oil, respectively, when compared to non-anesthetized fish. The higher plasma lactate levels are most likely due to the lower VR observed in anesthetized silver catfish. This lower VR may have resulted in hypoxia.

Most fish anesthetics have an inhibitory effect on the respiratory system, which results in a lower VR (Keene et al. 1998). At the beginning of anesthesia, hyperventilation is common and an increase in oxygen consumption takes place, possibly in response to the presence of the anesthetic in the water (McFarland 1959; Summerfelt and Smith 1990). However, hypoventilation and a reduction in oxygen consumption occur as a result of the anesthesia induction (Houston et al. 1971; Dixon and Milton 1978). Thus, the depressor effect of both EOHR and EOLA on the respiratory system of silver catfish appears to not be extended. Although the VR was not measured throughout the anesthesia recovery period in the present study, plasma lactate levels returned to basal levels within 240 min of recovery, demonstrating that the oxygen supply was restored.

In response to stress and plasma corticosteroid levels increased, the carbohydrate and protein metabolism (increased protein turnover, production of ammonia) and aminotransferase activity can be altered (Mommsen et al. 1999). Anesthesia with EOHR and EOLA increased the ALT and AST activities, respectively, but an increase in both of the transaminases was not observed with the same anesthetic exposure. The increased activities of ALT and AST in silver catfish anesthetized with EOs are unlikely to be due to an increase in the transamination pathway as suggested by Velisek et al. (2011), who used rainbow trout anesthetized with clove oil, since the total protein and ammonia levels were not altered by the anesthesia treatment. Although 240 min of recovery in anesthetic-free water was not enough to restore the basal levels of transaminases, additional tests of liver function should be performed to confirm whether the increase in transaminase activities is due to injury of hepatic tissue or to a transient response after a stressful event.

Ionoregulatory homeostasis is essential to ensure proper cell function (Hwang et al. 2011). Additionally, stress due to routine aquaculture procedures is known to affect fish ionoregulation (Ashley 2007). Furthermore, the addition of sedative agents to water can reduce ion loss in silver catfish after transport (Becker et al. 2012). The ionoregulation in silver catfish is not strongly affected by anesthesia with the EOs used in this study because plasma Na^+ and Cl^- levels did not change.

The enzymes Na^+/K^+-ATPase and H^+-ATPase that are present in fish gills contribute to the maintenance of internal homeostasis and therefore participate in important processes, including ionic regulation and acid-base balance in fish (Huang et al. 2010). Fenwick et al. (1999) have shown that Na^+ uptake in freshwater fish is associated with a proton-motive force created by a proton pump (H^+-ATPase). We are not aware of any studies that have evaluated the effects of fish anesthesia on the activity of these enzymes.

The anesthesia with either EOHR or EOLA prevented the reduction of the Na^+-K^+-ATPase activity caused by handling of silver catfish. However, because this is a key enzyme involved in many physiological activities, the effects of prolonged exposure to sedative and anesthetic substances deserve further examination. Regarding a possible role for a H^+-ATPase in Na^+ regulation, the higher activity observed in fish anesthetized with 300 or 450 μL L^{-1} EOHR indicates that this enzyme may contribute to maintaining plasma Na^+ levels in silver catfish.

The energetic metabolism of silver catfish was slightly altered by EOHR and EOLA anesthesia, and most indices returned to basal levels within a short recovery time of up to 240 min. Moreover, these anesthetics did not impair ionoregulation. These EOs may be possible alternatives for use in aquaculture procedures that require the use of anesthetics. This potential use may be particularly true for EOLA, which presented fewer side effects after anesthesia.

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Table 1 Chemical composition of the essential oils *Hesperozygis ringens* (EOHR) and *Lippia alba* (EOLA).

RI[a] experimental				**Percent composition**	
EOHR	EOLA	**RI literature[b]**	**Compound**	EOHR	EOLA
-	924	925[1]	á-thujene	-	0.049
928	929	935[1]	á-pinene	0.287	0.144
-	943	946[1]	Camphene	-	0.109
967	969	964[1]	Sabinene	0.142	1.056
968	971	970[1]	β-pinene	0.252	0.103
977	979	975[2]	1-octen-3-ol	0.036	0.508
-	987	986[2]	6-methyl-5-hepten-2-one	-	0.089
987	990	991[2]	â-myrcene	0.24	0.449
-	996	994[1]	3-octanol	-	0.038
1023	1025	**1022[1]**	**Limonene**	**1.284**	0.205
-	**1028**	**1031[2]**	**1,8-cineole**	-	**5.925**
-	1037	1037[1,2]	*Z*-â-ocimene	-	0.087
-	1047	1050[1,2]	*E*-â-ocimene	-	0.65
1045	-	1045[1]	β-*E*-ocimene	0.537	-
-	1066	1070[2]	*Z*-sabinene hydrate	-	0.164
-	1072	1073[1,2]	*E*-linalool oxide (furanoid)	-	0.104
-	1085	1088[2]	Isoterpinolene	-	0.104
1098	**1111**	**1103[1]**	**Linalool**	0.539	**54.381**
-	1111	1104[1]	Hotrienol	-	0.201
-	1122	1110[2]	1,3,8-p-menthatriene	-	0.199
-	1130	-	NI	-	0.397
-	1142	1146[2]	Camphor	-	1.358
-	1149	1150[2]	camphene hydrate	-	0.077
-	1151	-	NI	-	0.046
-	1154	-	NI	-	0.043
-	1160	1165[2]	Pinocarvone	-	0.147
-	1163	1169[1,2]	Borneol	-	0.155
-	1165	1170[2]	p-mentha-1,5-dien-8-ol	-	0.471
-	1172	-	NI	-	0.04
-	1174	1177[1,2]	1-terpinen-4-ol	-	0.108
1173	-	1171[1]	neoisopulegol	0.31	-
-	1188	1189[1,2]	á-terpineol	-	0.259
-	1193	1196[1,2]	Myrtenol	-	0.084
-	1201	1190[3]	*Z,Z*-2,6-dimethyl-3,5,7-octatrien-2-ol	-	2.019
-	1209	1197[3]	*E,E*-2,6-dimethyl-3,5,7-octatrien-2-ol	-	3.766
1208	-	-	NI	0.307	-
-	1227	1226[1,2]	Citronellol	-	0.189
-	1239	1238[1,2]	*Z*-citral	-	0.534
1248	-	**1239**	**Pulegone**	**95.177**	-
-	1253	1253[2]	Geraniol	-	0.127
-	1268	1267[2]	*E*-citral	-	0.622
-	1340	1354[1]	exo-2-hydroxycineole acetate	-	0.212
-	1363	-	NI	-	0.101

-	1372	1377[1,2]	á-copaene	-	0.345
-	1381	1388[1,2]	â-bourbonene	-	0.275
-	1389	-	NI	-	0.948
-	1402	1400[1]	eugenol methyl ether	-	0.062
-	1406	1404[1]	á-gurjunene	-	0.06
1402	-	-	NI	0.619	-
1416	1416	1419[1,2]	â-caryophyllene	0.27	2.383
-	1426	1432[2]	â-copaene	-	0.161
-	1431	1437[2]	ã-elemene	-	0.455
-	1450	1455[1,2]	á-caryophyllene	-	0.416
-	1454	1457[2]	*E*-â-farnesene	-	0.343
-	1457	1460[2]	allo-aromadendrene	-	0.2
-	1479	1480[2]	ã-muurolene	-	4.032
-	1491	1494[2]	epi-cubebol	-	0.416
-	1497	1500[1,2]	á-muurolene	-	0.486
-	1501	1509[2]	germacrene A	-	0.519
-	1512	1515[2]	Cubebol	-	1.05
-	1520	1523[2]	ä-cadinene	-	0.533
-	1536	1533[2]	*Z*-nerolidol	-	0.346
-	1555	1561[2]	germacrene B	-	2.26
-	1561	1563[2]	*E*-nerolidol	-	0.535
-	1574	1576[1,2]	germacrene D-4-ol	-	2.323
-	1582	1583[1,2]	caryophyllene oxide	-	0.876
-	1620	-	NI	-	2.173
-	1627	1629[2]	1-epi-cubenol	-	0.226
-	1641	1642[2]	ô-muurolol	-	0.698
-	1646	1647[2]	Cubenol	-	0.254
-	1648	-	NI	-	0.261
-	1654	1654[2]	á-cadinol	-	0.704
Identified components				**99.07**	**94.65**
Unidentified components				**0.93**	**6.35**

[a] RI experimental: calculated Kovats retention index; [b] RI literature: reference Kovats retention index, NI: not identified constituent. [1] NIST (2002); [2] Adams (2001); [3] Heldwein et al. (2012).

Table 2 Ventilatory rate (opercular or buccal movements min^{-1}) measured in silver catfish submitted to anesthesia with the essential oil of *Hesperozygis ringens* (EOHR) or *Lippia alba* (EOLA). Stages of anesthesia induction are according to Small (2003).

Stage of anesthesia or time of exposure	EOHR (µL L^{-1})			
	Control	150	300	450
0	67.63 ± 2.75^{Ca}	80.29 ± 3.01^{Ba}	91.73 ± 2.06^{Aa}	93.77 ± 3.16^{Aa}
30 seconds	54.42 ± 2.26^{Cb}	78.57 ± 2.91^{Ba}	89.87 ± 1.95^{Aa}	90.89 ± 2.14^{Aa}
5 minutes or Stage I	53.20 ± 2.56^{Cb}	77.99 ± 2.84^{Aa}	83.41 ± 2.13^{Aa}	66.48 ± 2.46^{Bb}
15 minutes or Stage III	54.16 ± 2.29^{Ab}	47.17 ± 2.20^{ABb}	42.26 ± 2.50^{Bb}	46.54 ± 2.32^{Bc}
	EOLA (µL L^{-1})			
	Control	150	300	450
0	65.32 ± 3.04^{Aa}	65.83 ± 1.93^{Ab}	71.51 ± 2.20^{Ab}	72.01 ± 1.42^{Ab}
30 seconds	68.64 ± 2.52^{Ca}	84.74 ± 2.46^{Ba}	103.62 ± 2.92^{Aa}	87.21 ± 2.06^{Ba}
5 minutes or Stage I	70.20 ± 3.26^{Aa}	66.73 ± 2.15^{Ab}	66.30 ± 1.65^{Ab}	53.41 ± 1.87^{Bc}
15 minutes or Stage III	72.29 ± 2.89^{Aa}	31.22 ± 1.37^{Bc}	32.03 ± 1.36^{Bc}	30.41 ± 1.37^{Bd}

Values are represented as the mean ± SEM. Different capital letters in the rows indicate significant differences between treatments at the same time. ($P < 0.05$). Different lowercase letters in the columns indicate significant differences between times in the same treatment ($P < 0.05$).

Table 3 Plasma Na^+, K^+ and Cl^- levels (mmol L^{-1}) of silver catfish submitted to anesthesia with the essential oil of *Hesperozygis ringens* (EOHR) or *Lippia alba* (EOLA).

		EOHR			EOLA		
Recovery time (min)	Treatments	Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
	Basal	161.16 ± 3.30	3.15 ± 0.31	122.22 ± 5.78	178.37 ± 5.59	6.00 ± 0.56	103.04 ± 5.56
0	Control	183.46 ± 3.98^{*Cb}	2.67 ± 0.18^{Aa}	132.35 ± 2.52^{Ab}	176.27 ± 5.75^{Aab}	5.01 ± 0.31^{Aa}	106.81 ± 7.90^{ABa}
	150 µL L^{-1}	146.50 ± 4.51^{ABa}	8.05 ± 0.53^{*Bb}	144.79 ± 6.93^{Aa}	174.16 ± 7.17^{Aa}	4.85 ± 0.30^{Aa}	91.34 ± 4.09^{Aa}
	300 µL L^{-1}	126.00 ± 8.12^{*Aa}	5.65 ± 0.36^{*Ba}	125.11 ± 4.07^{Aab}	137.74 ± 8.26^{Aa}	4.74 ± 0.39^{Aa}	123.22 ± 6.34^{Ba}
	450 µL L^{-1}	161.14 ± 2.58^{Bab}	6.10 ± 0.51^{*Ba}	136.69 ± 5.30^{Aa}	172.06 ± 8.21^{Aa}	3.97 ± 0.35^{Aa}	78.42 ± 5.05^{Aa}
15	Control	180.91 ± 2.14^{*Bb}	2.96 ± 0.37^{Aa}	99.36 ± 5.35^{Aa}	163.66 ± 10.95^{Aa}	6.11 ± 0.29^{Ba}	104.79 ± 7.85^{Ba}
	150 µL L^{-1}	181.54 ± 3.85^{*Bb}	7.91 ± 0.61^{*Cb}	141.90 ± 3.27^{Ba}	148.95 ± 6.59^{Aa}	5.07 ± 0.44^{ABa}	60.12 ± 5.82^{*Aa}
	300 µL L^{-1}	155.14 ± 3.64^{Ab}	4.59 ± 0.16^{ABa}	103.85 ± 6.24^{ABa}	133.53 ± 6.32^{*Aa}	5.84 ± 0.41^{Aba}	110.31 ± 7.44^{Ba}
	450 µL L^{-1}	164.00 ± 0.76^{ABb}	4.76 ± 0.37^{Ba}	128.44 ± 6.76^{Ba}	154.55 ± 8.26^{Aa}	3.64 ± 0.43^{*Aa}	60.93 ± 4.89^{*Aa}
30	Control	157.96 ± 2.87^{Aa}	2.48 ± 0.18^{Aa}	124.97 ± 3.74^{Aab}	221.80 ± 10.62^{Bb}	5.73 ± 0.44^{Ba}	111.92 ± 7.38^{Ba}
	150 µL L^{-1}	192.37 ± 4.88^{*Bb}	5.67 ± 0.55^{*Ba}	151.74 ± 5.92^{*Aa}	135.64 ± 4.86^{Aa}	5.51 ± 0.52^{ABab}	69.81 ± 6.87^{*Aa}
	300 µL L^{-1}	150.57 ± 2.34^{Ab}	5.00 ± 0.49^{*ABa}	136.11 ± 4.76^{Ab}	194.48 ± 12.76^{Bb}	4.96 ± 0.35^{ABa}	99.95 ± 4.55^{ABa}
	450 µL L^{-1}	142.86 ± 4.68^{Aa}	5.54 ± 0.50^{*Ba}	134.52 ± 5.87^{Aa}	144.05 ± 8.90^{Aa}	3.32 ± 0.30^{*Aa}	78.69 ± 8.14^{Aa}
60	Control	150.96 ± 2.71^{Aa}	2.62 ± 0.25^{Aa}	130.47 ± 5.29^{Ab}	181.17 ± 9.35^{BCab}	4.90 ± 0.36^{Aa}	110.04 ± 6.41^{BCa}
	150 µL L^{-1}	196.83 ± 4.27^{*Cb}	7.05 ± 0.82^{*Bab}	157.67 ± 6.54^{*Aa}	132.13 ± 6.66^{*Aa}	7.48 ± 0.35^{Bb}	78.55 ± 5.10^{ABa}
	300 µL L^{-1}	150.28 ± 2.51^{Ab}	5.74 ± 0.68^{*Ba}	148.55 ± 4.82^{Ab}	197.59 ± 7.68^{Cb}	4.63 ± 0.33^{Aa}	115.15 ± 5.04^{Ca}
	450 µL L^{-1}	175.42 ± 7.15^{*Bb}	4.85 ± 0.50^{Ba}	134.23 ± 5.40^{Aa}	142.65 ± 10.08^{ABa}	3.70 ± 0.48^{*Aa}	74.11 ± 6.76^{Aa}
240	Control	190.89 ± 4.26^{*Bb}	2.86 ± 0.30^{Aa}	144.79 ± 5.69^{ABb}	175.57 ± 7.76^{ABab}	6.44 ± 0.42^{Ca}	104.39 ± 7.06^{ABa}
	150 µL L^{-1}	190.46 ± 3.21^{*Bb}	6.15 ± 0.69^{*Bab}	156.22 ± 5.24^{*Ba}	136.34 ± 6.81^{Aa}	6.11 ± 0.32^{BCab}	80.44 ± 4.41^{Aa}
	300 µL L^{-1}	145.14 ± 3.29^{Aab}	4.58 ± 0.44^{Aa}	123.96 ± 5.07^{Aab}	207.07 ± 7.87^{Bb}	4.08 ± 0.44^{ABa}	131.43 ± 5.38^{Ba}
	450 µL L^{-1}	142.86 ± 5.50^{Aa}	6.32 ± 0.66^{*Ba}	129.74 ± 5.66^{Aa}	163.66 ± 11.92^{ABa}	4.00 ± 0.44^{Aa}	83.26 ± 6.43^{Aa}

Values are represented as the mean ± SEM. * Indicates significant differences from the basal group ($P < 0.05$). Different capital letters indicate significant differences between treatments at the same time ($P < 0.05$). Different lowercase letters indicate significant differences between times at the same treatment ($P < 0.05$).

Figure captions

Fig. 1 Time required for the induction of anesthesia in silver catfish (*Rhamdia quelen*) using the essential oils of *Hesperozygis ringens* (EOHR) ($y = 155.1 + 1577.0e^{(-0.0099x)}$; $r^2 = 1.00$) or *Lippia alba* (EOLA) ($y = 167.6 + 945.3e^{(-0.0057x)}$; $r^2 = 1.00$, where y represents the time for anesthesia induction (s) and x indicates the EO concentration ($\mu L\ L^{-1}$))

Fig. 2 Biochemical plasma profiles of silver catfish (*Rhamdia quelen*) anesthetized with the essential oils of *Hesperozygis ringens* ((a) glucose, (c) lactate) or *Lippia alba* ((b) glucose, (d) lactate). An asterisk (*) indicates a significant difference compared to the basal group. Capital letters indicate significant differences between treatments at the same time point. Lowercase letters indicate significant differences between time points within the same treatment ($P < 0.05$)

Fig. 3 Biochemical plasma profiles of silver catfish (*Rhamdia quelen*) anesthetized with the essential oils of *Hesperozygis ringens* ((a) protein, (c) ammonia) or *Lippia alba* ((b) protein, (d) ammonia). An asterisk (*) indicates a significant difference compared to the basal group. Capital letters indicate significant differences between treatments at the same time point. Lowercase letters indicate significant differences between time points within the same treatment. ($P < 0.05$)

Fig. 4 Transaminase activities in the plasma of silver catfish (*Rhamdia quelen*) anesthetized with the essential oils of *Hesperozygis ringens* ((a) aspartate aminotransferase (AST), (c) alanine aminotransferase (ALT)) or *Lippia alba* ((b) AST (d) ALT). An asterisk (*) indicates a significant difference compared to the basal group. Capital letters indicate significant differences between treatments at the same time point. Lowercase letters indicate significant differences between time points within the same treatment ($P < 0.05$)

Fig. 5 Enzymatic activities in gills of silver catfish (*Rhamdia quelen*) after 0 and 240 min of recovery from anesthesia with the essential oils of *Hesperozygis ringens* ((a) Na^+/K^+-ATPase, (c) H^+-ATPase) or *Lippia alba* ((b) Na^+/K^+-ATPase, (d) H^+-ATPase). 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 points ($P < 0.05$)

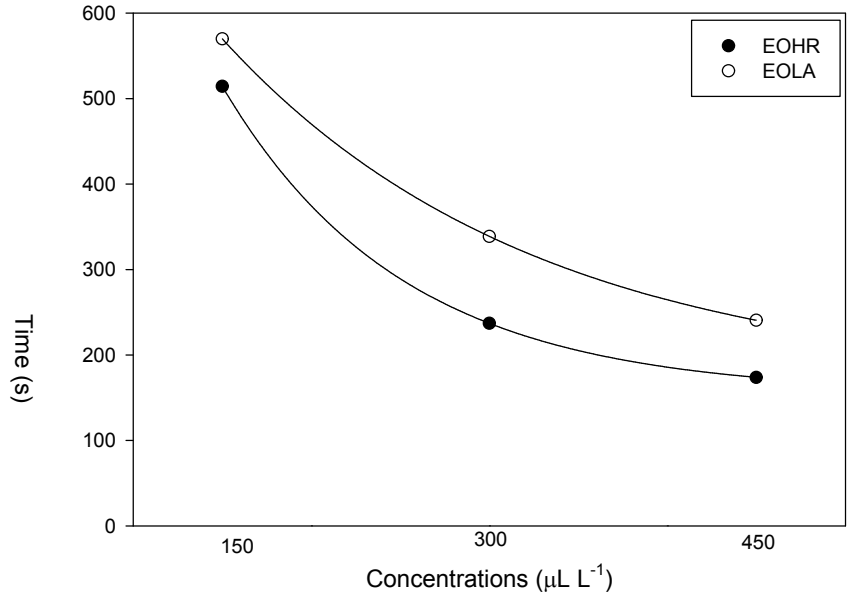
600
500
400
300
200
100
0
Time (s)
150
300
450
Concentrations (μL L-1)
EOHR
EOLA

Fig. 1

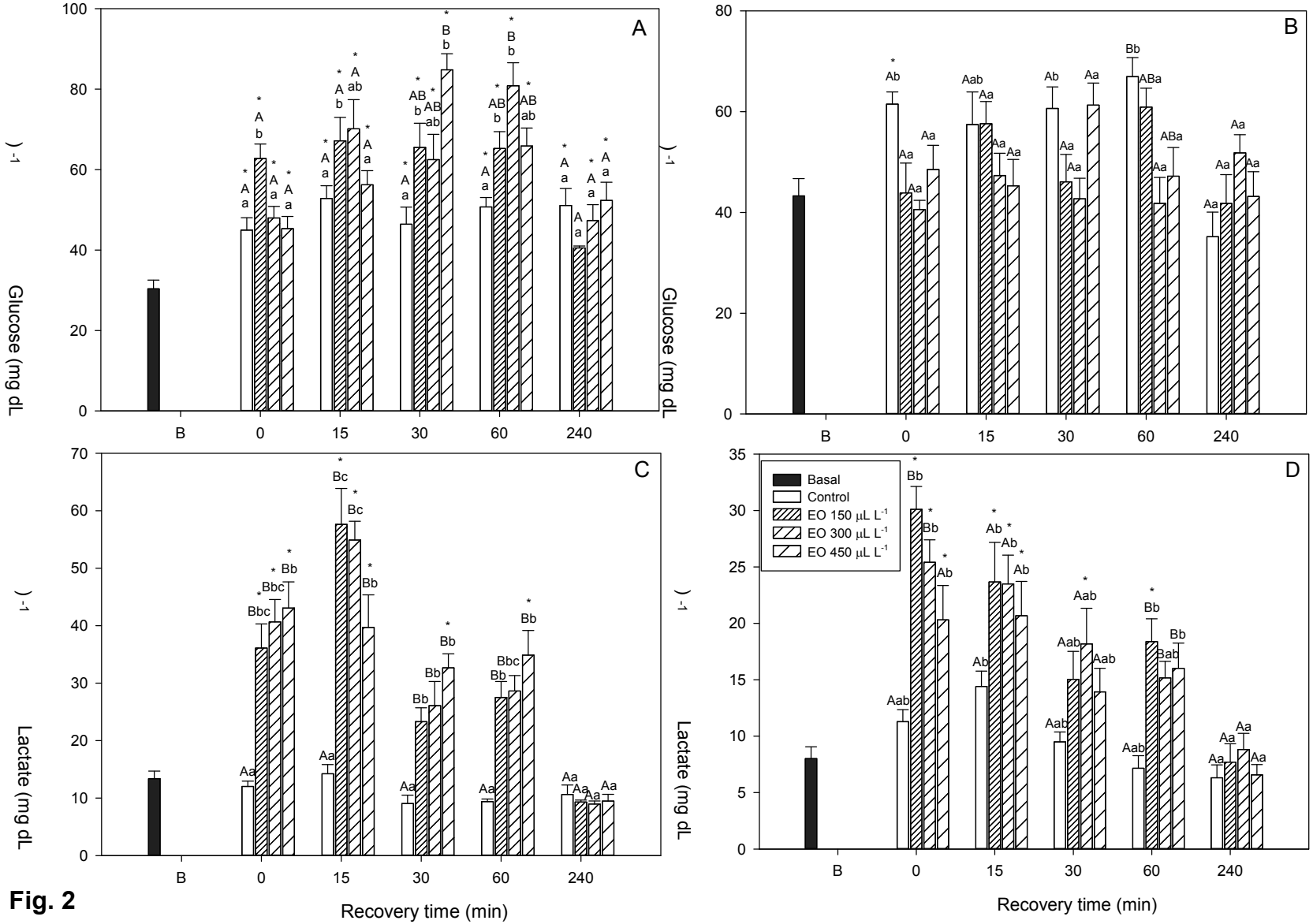
A
B
C
D
Glucose (mg dL) -1
Lactate (mg dL) -1
Recovery time (min)
B
0
15
30
60
240
Basal
Control
EO 150 µL L-1
EO 300 µL L-1
EO 450 µL L-1

Fig. 2

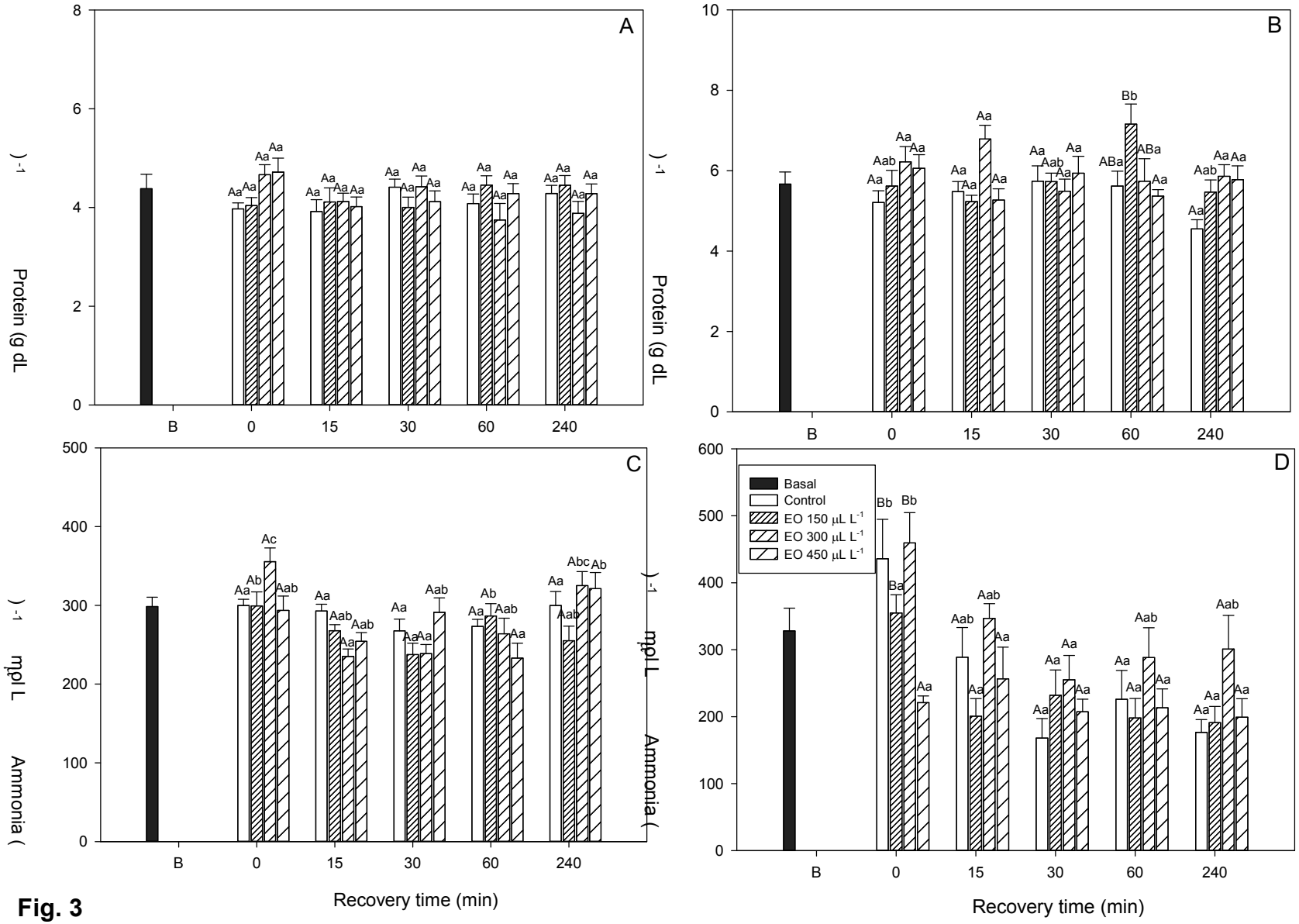
A
B
C
D
Protein (g dL-1)
Ammonia (mg dL-1)
Basal
Control
EO 150 µL L-1
EO 300 µL L-1
EO 450 µL L-1
B
0
15
30
60
240
Recovery time (min)

Fig. 3

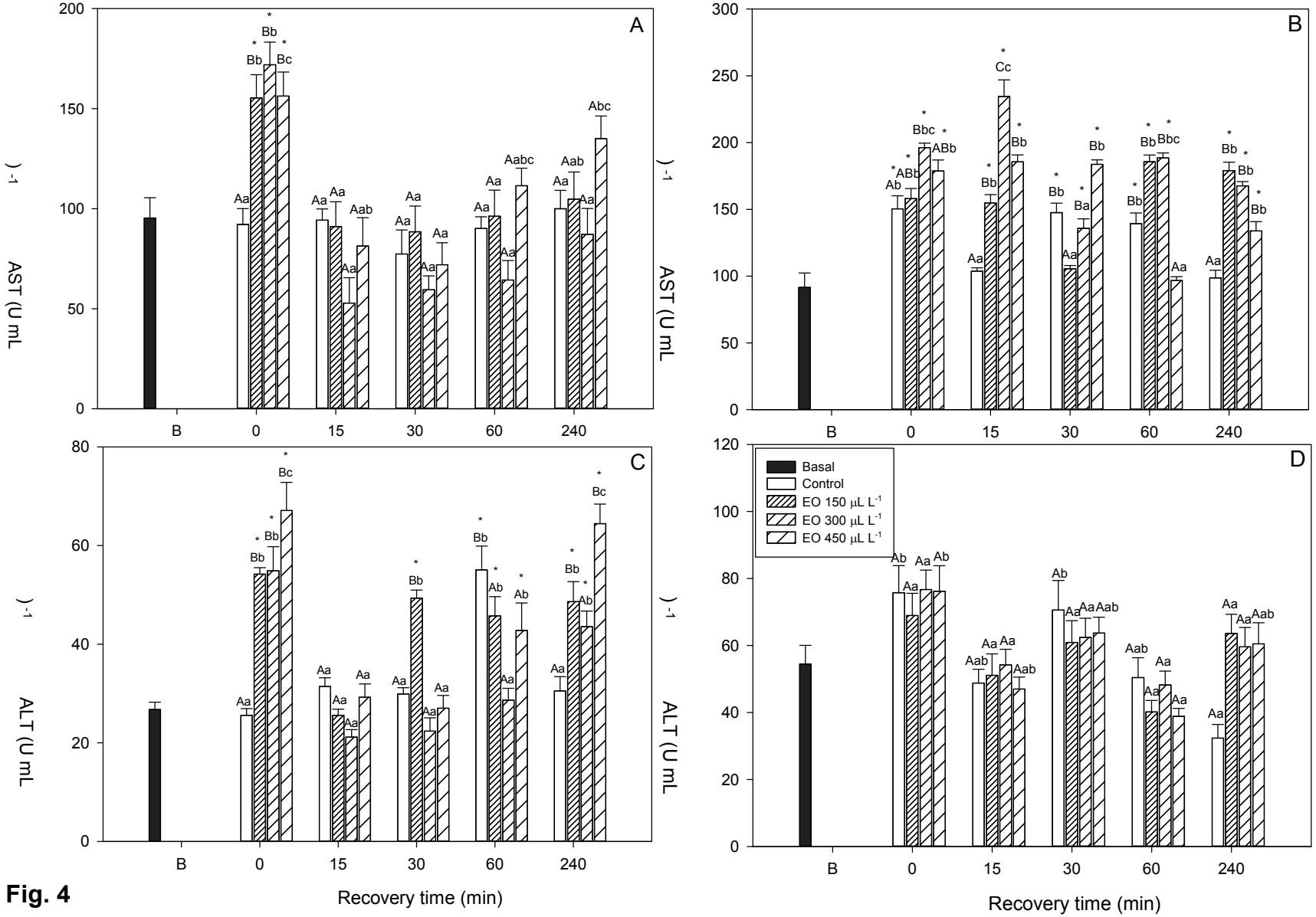
A
B
C
D
AST (U mL) -1
ALT (U mL) -1
Recovery time (min)
B
0
15
30
60
240
Basal
Control
EO 150 µL L-1
EO 300 µL L-1
EO 450 µL L-1

Fig. 4

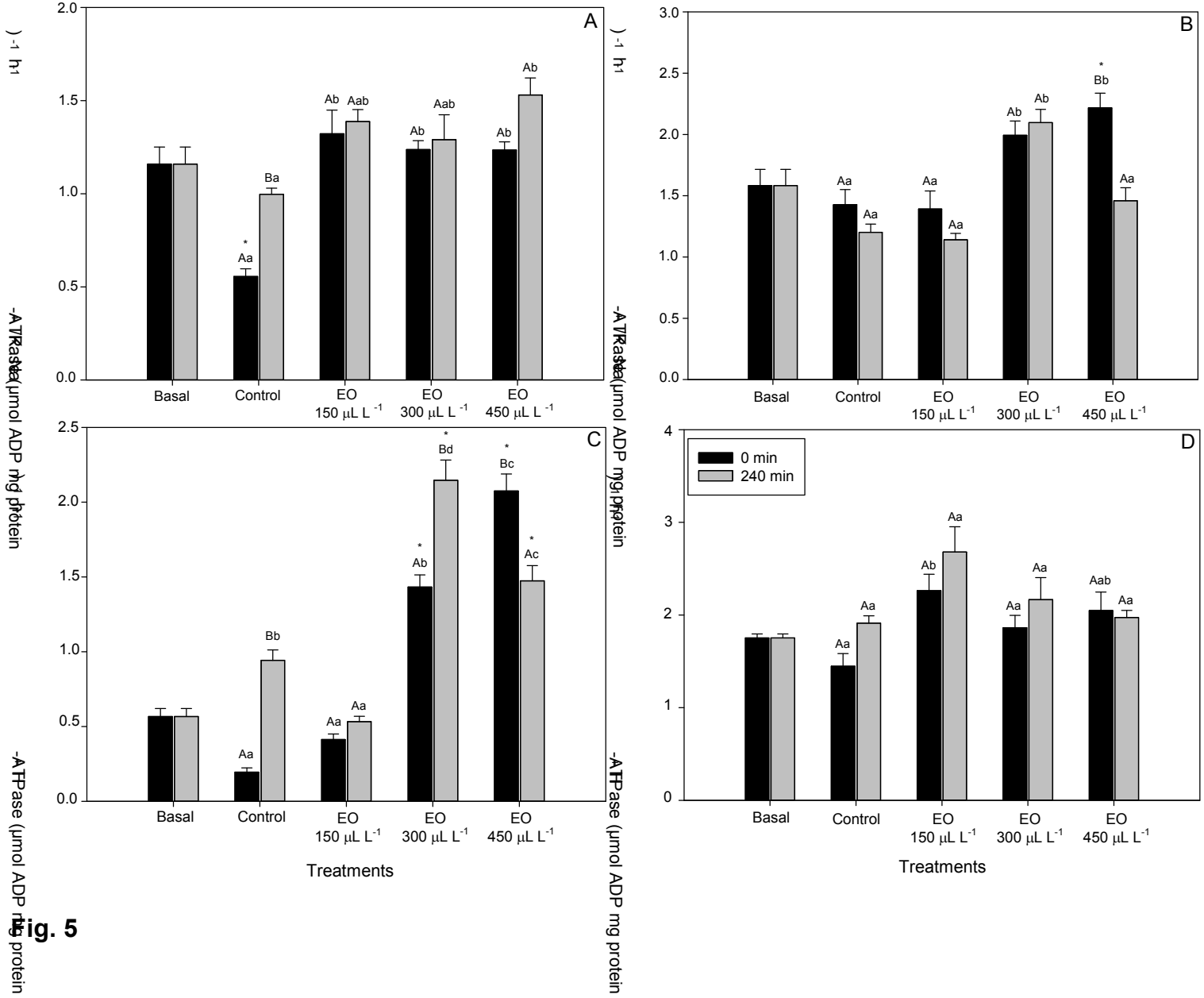
A
B
C
D
0 min
240 min
Basal
Control
EO 150 μL L-1
EO 300 μL L-1
EO 450 μL L-1
Treatments
-ATPase (μmol ADP mg protein-1 h-1)

Fig. 5

ARTIGO 2

Publicado no periódico Fish Physiology and Biochemistry

Stress response in silver catfish *(Rhamdia quelen)* exposed to the essential oil of *Hesperozygis ringens*

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Abstract

This study investigated the effects of prolonged exposure of silver catfish (*Rhamdia quelen*) to the essential oil (EO) of *Hesperozygis ringens*. Ventilatory rate (VR), stress and metabolic indicators, energy enzyme activities and mRNA expression of adenohypophyseal hormones were examined in specimens that were exposed for 6 h to 0 (control), 30 or 50 μL L^{-1} EO of *H. ringens* in water. Reduction in VR was observed in response to each treatment, but no differences were found between treatments. Plasma glucose, protein and osmolality increased in fish exposed to 50 μL L^{-1}. Moreover, lactate levels increased after exposure to both EO concentrations. Plasma cortisol levels were not changed by EO exposure. Fish exposed to 30 μL L^{-1} EO exhibited higher glycerol-3-phosphate dehydrogenase (G3PDH) activity, while exposure to 50 μL L^{-1} EO elicited an increase in glucose-6-phosphate dehydrogenase (G6PDH), fructose-biphosphatase (FBP) and 3-hydroxyacyl-CoA-dehydrogenase (HOAD) activities compared to the control group. Expression of growth hormone (GH) only decreased in fish exposed to 50 μL L^{-1} EO, while somatolactin (SL) expression decreased in fish exposed to both concentrations of EO. Exposure to EO did not change prolactin expression. The results indicate that GH and SL are associated with energy reorganization in silver catfish. Fish were only slightly affected by 30 μL L^{-1} EO of *H. ringens*, suggesting that it could be used in practices where a reduction in the movement of fish for prolonged periods is beneficial, i.e., such as during fish transportation.

Keywords: Anesthetic; fish; hormones expression; metabolism; ventilatory rate.

Introduction

The use of anesthetics for fish is widespread in field studies, laboratory research and commercial aquaculture (Cotter and Rodnick 2006). Live fish transport, as well as other practices in aquaculture, often induces a physiological stress response that may result in undesirable outcomes such as immune system depression and growth impairment (Rotllant et al. 2001). The addition of sedative agents to the water during transport has the positive effect of improving osmotic and ionic balance, metabolism and oxidative stress parameters (Inoue et al. 2005; Azambuja et al. 2011; Becker et al. 2012, 2013).

Stressors can invoke primary, secondary and tertiary responses in fish (Wendelaar-Bonga 1997). Fish neuroendocrine stress pathways rely on the adrenergic system and the hypothalamus-pituitary-interrenal (HPI) axis, whose stimulation culminates in the release of catecholamines and corticosteroids into the circulation (Pankurst 2011; Weber et al. 2011). As a result, metabolic, ionic, cardiac, and respiratory changes could occur, undertaken the fish survival if these stressors persist for a long time (Barton 2002; Schreck 2010).

The activity of enzymes involved in the metabolism of carbohydrates, lipids and proteins can give clues as to how the animal reorganizes its energy status after experiencing a stressful event. However, to date, studies addressing these indicators have been related to the energy demand required for osmoregulatory adjustments that arise due to changes in salinity (Láiz-Carrion et al. 2005; Sangiao-Alvarellos et al. 2005; Polakof et al. 2006). The relationship between exposure to anesthetic and/or sedative agents and activity of metabolic enzymes remains unknown.

The endocrine system plays a key role in the maintenance of homeostasis of a number of functional activities that are related to short or long-term changes in the environment (Laiz-Carrión et al. 2009). The pituitary is considered to be the master gland of the endocrine system and is implicated in the control of a large variety of physiological processes mediated by several hormones and factors released into the bloodstream. Pituitary hormones that are members of the GH/PRL family include prolactin (PRL), growth hormone (GH) and somatolactin (SL). These hormones have been implicated in the control of pleiotropic biological functions in teleosts (Sudo et al. 2013).

Hesperozygis ringens is a Brazilian native plant whose essential oil (EO) exhibits anesthetic and sedative properties for silver catfish (*Rhamdia quelen*) (Silva et al. 2013), which is an economically important species to southern Brazil (Baldisserotto 2009). Although the consequences of anesthetic use of EO of *H. ringens* on the physiology and biochemistry of silver catfish has recently been reported (Toni et al. 2014), very little is known about the effects of low concentrations of this EO on fish. Thus, in the present study, we investigated the consequences of long-term exposure to EO of *H. ringens* on expression of adenohypophyseal hormones, plasmatic cortisol, metabolic indicators and osmolality levels in silver catfish. The information obtained in this study could be critical to determining the feasibility and safety of using this EO in aquaculture practices that require a longer handling time of fish, such as during transport.

Material and methods

Plant material and essential oil extraction

Aerial parts of *Hesperozygis ringens* were collected in São Francisco de Assis (Rio Grande do Sul, Brazil). The species was identified by Dr. Solon Jonas Longhi, and a voucher specimen (SMDB nº 13427) was deposited in the herbarium of the Department of Biology, Universidade Federal de Santa Maria (UFSM). Extraction of the EO of the dry leaves of *H. ringens* was performed by hydrodistillation for 3 h using a Clevenger type apparatus (European Pharmacopoeia 2007). Extract was stored at -4 °C in amber glass bottles. Analysis of the EO extract composition by gas chromatography coupled to mass spectrometry demonstrated that it was similar to the composition of EO used by Toni et al. (2014), with pulegone being the major constituent (81.4 %) (Table 1).

Animals and experimental protocol

Silver catfish (93.9 ± 3.9 g, 25.0 ± 0.5 cm) were obtained from Fish Culture Laboratory at the UFSM (RS, Brazil) and transported to the Laboratory of Fish Physiology. Catfish were maintained in continuously aerated 250 L tanks under

natural photoperiod and controlled temperature (21.8 ± 0.5 °C) for one week. A semi-static system was used, and 50 % of the water volume was changed daily. Fish were fed once a day with commercial feed (28 % crude protein). Fish were fasted for 24 h prior to the experiments. Experimental methodologies were approved by the Ethical and Animal Welfare Committee of the UFSM (Process nº 46/2010).

Fish (n = 30) were captured from the tanks and allocated to 40 L aquaria, with 5 fish per aquaria. Fish were treated with control (without EO), 30 or 50 μL L^{-1} EO of *H. ringens* added to water (2 aquaria per treatment). The EO was previously diluted in ethanol (1:10) for better dissolution in water. Concentrations used in this study do not cause deep anesthesia in silver catfish (Silva et al. 2013). Fish remained in stage 1 (sedation) of anesthesia, which is characterized by decreased reactivity to external stimuli according to Small (2003). The fish remained under these conditions for 6 h.

Ventilatory rate (VR)

The VR was quantified by visually counting 20 successive opercular movements and measuring the elapsed time with a chronometer (adapted from Alvarenga and Volpato 1995). VR was measured 0, 1, 2, 4 and 6 h after treatment exposure.

Sampling

After 6 h of treatment, fish were removed by dip-net and blood was collected into heparinized syringes by caudal puncture. Plasma obtained after blood centrifugation (1000 *g*, 10 min at 4 °C) was immediately stored at -80 °C until assayed. After euthanasia by spinal section, the liver and pituitary were removed quickly from each fish. Liver was stored at -80 °C until the assessment of enzyme activities, while pituitary was immersed in RNAlater (Ambion) solution and stored at -20 °C prior to RNA extraction and hormone expression measurement.

Analytical techniques

Plasma cortisol levels (expressed in ng mL^{-1}) were measured by indirect enzyme immunoassay (ELISA) adapted to microplate as described previously by Rodríguez et al. (2000) for testosterone. Steroids were extracted from 3.5 μL of plasma in 100

µL RB (10 % v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01 % w/v NaN_3, 2.34 % w/v NaCl, 0.037 % w/v EDTA, 0.1 % w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac) and evaporated for 48-72 h at 37 ºC. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. The standard curve range was 2.5 ng/mL to 39.1 pg mL^{-1} (R^2= 0.986). The lower limit of detection (90.4 % of binding, ED90.4) was 78.1 pg mL^{-1}. The percentage of recovery was 95 %. The intra-assay coefficient of variation (calculated from the sample duplicates) was 4.29 ± 0.48 %. The cross-reactivity of specific antibodies with intermediate products involved in steroids synthesis was given by the supplier cortexolone (1.6 %), 11-deoxycorticosterone (0.23 %), 17-hydroxyprogesterone (0.23 %), cortisol glucurinoide (0.15 %), corticosterone (0.14 %), cortisone (0.13 %), androstenedione (<0.01 %), 17-hydroxypregnenolone (<0.01 %), testosterone (<0.01 %).

Glucose, lactate and triglyceride concentrations were measured in plasma using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides Ref. 1001311) adapted to 96-well microplates. Plasma protein was analyzed by diluting plasma 50 times and measuring protein concentration. Protein concentration was measured using the bicinchonic acid method with a BCA protein kit (Pierce P.O., Rockford, USA), using bovine serum albumin as standard. All of these assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by KCjunior™ program. Standards and all samples were run in quadruplicate and duplicate, respectively. Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske-VT, USA) and expressed as mOsm kg^{-1}.

Frozen liver was finely minced on an ice-cold Petri dish and homogenized by ultrasonic disruption (Misonix inc., Microson Ultrasonic liquid processor XL-2000) with 10 volumes of ice-cold stopping-buffer containing 50 mM imidazole (pH 7.5), 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM PMSF, and 250 mM sucrose. The homogenate was centrifuged at 10 000 *g*, 30 min at 4 °C (Centrifuge 5810R, Eppendorf) and the supernatant was immediately frozen using liquid nitrogen and

kept at -80 ºC until enzyme assays were performed. Enzyme activities were analyzed using a Bio-Tek PowerWave 340 Microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using KCjunior Data Analysis Software for Microsoft Windows XP. Reaction rates of enzymes were calculated from the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (15 µL) in duplicate, at a pre-established protein concentration, omitting the substrate in control wells (final volume of 275-295 µL, depending on the enzyme tested), and allowing the reactions to proceed at 37 ºC. The specific conditions for the enzymes hexokinase (HK, EC 2.7.1.11), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), pyruvate kinase (PK, EC 2.7.1.40), phosphorilase (total and active GPase, EC 2.4.1.1), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), fructose-biphosphatase (FBP, EC 3.1.3.11), glutamate dehydrogenase (GDH, EC 1.4.1.2), glutamic-pyruvic transaminase (GPT, EC 2.6.1.2), 3-Hydroxyacyl-CoA-dehydrogenase (HOAD, EC. 1.1.1.35), are previously described (Sangiao-Alvarellos et al. 2005; Polakof et al. 2006). Enzymatic analyses were carried out at conditions meeting requirements for optimal velocities for *R. quelen* (data not shown). Protein levels were assayed in triplicate as performed with plasma samples.

Pituitary expression of GH, PRL and SL mRNA

Total RNA was isolated from pituitary gland using NucleoSpin®RNA XS kit (Macherey-Nagel) and the on-column RNase-free DNase digestion, according to manufacturer's protocol. The amount of RNA was spectrophotometrically measured at 260 nm with the BioPhotometer Plus (Eppendorf) and its quality was measured in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used for qPCR. Total RNA (250 ng) was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences).

Real-time PCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep *realplex* ^{2} S) as previously described by Baldisserotto et al. (2014). Each reaction mixture (10 µL) contained 0.5 µL of each specific forward and reverse primers at a final concentration of 200 nM, 5 µL of PerfeCTa SYBR® Green FastMix™ (Quanta Biosciences), and 4 µL of cDNA. The

nucleotide sequences of specific primers used for semi-quantitative PCR are in Table 2. The PCR profile was obtained as follows: (95 ºC, 10 min; [95 ºC, 30 sec; 60 ºC, 45 sec] X 40 cycles; *melting curve* [60 ºC to 95 ºC, 20 min], 95 ºC, 15 sec). β-actin was used like a housekeeping gene.

Statistical analysis

Homogeneity of variance between groups was tested with Levene's test. Data exhibited homogeneous variance, so comparisons between treatments were made using one-way ANOVA followed by the Tukey's test. Analysis was performed using Statistica v. 7.0 software. The minimum significance level was set at $P < 0.05$.

Results

No mortality was registered for the duration of the experiments as a consequence of the exposure to EO.

After 6 h, the VR was reduced in all treatments compared to time 0, but this parameter did not show difference between treatments (Fig. 1).

Glucose and protein levels showed a significant increase when EO was increased from 0 (control) to 50 μL L^{-1}, being only different between the lowest (0 μL L^{-1}) and highest (50 μL L^{-1}) concentrations (Fig. 2a and 2c, respectively). Moreover, plasma lactate and osmolality increased in the specimens exposed to 50 μL L^{-1} EO when compared to control and 30 μL L^{-1} groups (Fig. 2b and 2e, respectively). In addition, triglycerides and cortisol levels were not affected by EO exposure (Fig. 2d and 2f, respectively).

The activities of G6PDH, FBP and HOAD significantly increased in fish exposed to EO in a dose-dependent manner, with a remarkable enhancement at the highest EO concentration. In addition, EO increased the hepatic G3PDH activity in both experimental groups, although the highest values were achieved in the 30 μL L^{-1} EO group (Table 3).

Expression of GH showed a clear inverse relationship with EO concentration, with a significant decrease if this parameter in specimens exposed to 50 μL L^{-1} (Fig. 3a), whereas SL was significantly decreased in fish exposed to EO independently of

the concentration administered (30 or 50 μL L^{-1} EO, Fig. 3c). Furthermore, PRL expression was not affected by EO exposure (Fig. 3b).

Discussion

As expected, the chemical composition of the EO agree with the description provided by Toni et al. (2014), being the pulegone the major component (81.4 %). The sedative effect of EO observed in the present study, as well as anesthetic activity observed in previous studies may be due, in part, to pulegone, that is a positive allosteric modulator of GABA (gamma-aminobutyric acid) receptors, major inhibitory neurotransmitter in the Central Nervous System of vertebrates (Tong and Coats 2010). Low concentrations of anesthetics in aquaculture practices provide a useful means of reducing physical damage preventing the exacerbation of handling stress (Khalil et al. 2012). In this sense, is important to assess the extent of physiological changes related to stress processes in fish exposed to an anesthetic agent.

Given the importance of the cortisol as a modulator of various physiological processes (Mommsen et al. 1999), changes in plasma cortisol are considered to be a primary response to a potential stressor. In the present study, levels of this hormone did not change after 6 h of exposure to EO of *H. ringens*. As plasma cortisol concentrations reflect the effect of net production and plasma clearance of the hormone (Mommsen et al. 1999), we propose that the primary reaction of stress processes started just in the first minutes of contact with EO. Thus, our results are in agreement with Barcellos et al. (2012), who found that specimens of *R. quelen* of similar size to those used in the present study experienced elevated cortisol levels from 5 to 30 min after stressor exposure, returning to baseline concentrations within 60 min. In the present study, even though the fish may still be responding to the stressor after 6 h of exposure to EO, plasma cortisol levels were at baseline, suggesting that other parameters could be playing an important role to cope the putative stress situation.

Although most studies attribute the physiological changes associated with stress based primarily on the elevated circulating level of the cortisol, some studies have demonstrated that plasmatic levels of metabolites might change without changes in that hormone (Svobodova et al. 1999; Thomas et al. 1999; Park et al.

2008). That would explain the elevated plasma levels of glucose, lactate, protein and osmolality observed in fish exposed to higher EO concentration.

In addition, the increase observed in plasma lactate levels verified that fish exposed to both EO concentrations are submitted to tissue hypoxia. This phenomenon occurs when cells receive too little oxygen to function normally. In the absence of oxygen, or even when the aerobic pathways are insufficient to produce the required energy supply, anaerobic glycolysis is the principal energy-generating pathway that results in lactate accumulation (Bickler and Buck 2007). The highest plasma lactate levels observed in fish exposed to EO would result of the hypoventilation caused by decreased breathing amplitude and not by reduced ventilation rate.

To our knowledge, this is the first report about the effects of exposure to anesthetics agents on the activity of enzymes associated with the metabolism of carbohydrates, lipids and protein in fish. The increase in the activity of FBP, G6PDH and HOAD observed in silver catfish demonstrate that intermediary metabolism was affected after 6 h of exposure to 50 μL L^{-1} EO of *H. ringens*. In addition, lipids and plasma lactate appeared to be used as energy sources because the activities of enzymes involved in protein metabolism were not altered. These results are indicative of gluconeogenesis. Other authors have reported similar gluconeogenic potential in the liver of brook charr *(Salvelinus fontinalis)* and gilthead sea bream *(Sparus aurata)* after the experience of stressful conditions (Vijayan et al. 1991; Polakof et al. 2006). In the present study, the gluconeogenic pathway could be the energetic mechanism that the animal was using in an attempt to restore its homeostasis.

Adenohypophyseal hormone expression related to growth, metabolism, and osmoregulation were also investigated in this study. Growth hormone (GH) is an important pituitary hormone known to regulate body growth and metabolism and its release in teleost fish is regulated directly at the pituitary level by a multitude of neuroendocrine factors (Wong et al. 2006). In this study, the lowest levels of GH were found in the pituitary of silver catfish exposed to 50 μL L^{-1} EO of *H. ringens.* Although the consequences of stress responses are dependent on a variety of factors, it is known that tertiary responses to stress (especially chronic stress) include effects on fish growth (Barton 2002). Reduction in GH expression, as observed in this

study, indicates that fish growth may be compromised to face an adverse situation, once energy is required to restore homeostasis.

Since its discovery, the effects of SL on energy metabolism have been widely investigated. These studies demonstrate an active role for this hormone as a marker of energy surplus (availability). Uchida et al. (2009) observed that fasting for 4 weeks produced significant reductions in pituitary SL expression of Mozambique tilapia *(Oreochromis mossambicus)* both in freshwater and in seawater. Another study observed that plasma SL levels decreased in response to reduced nutritive value of diet in gilthead sea bream (Vega-Rubín de Celis et al. 2004). The reduction in SL expression in silver catfish exposed to EO of *H. ringens* is consistent with the increased metabolic activity of enzymes that oxidize organic molecules to the detriment of energy reserves, especially lipids.

In summary, these results confirm that GH and SL are associated with the energy reorganization required for homeostatic balance in silver catfish after sedation with EO of *H. ringens.* Furthermore, this study shows greater alterations in metabolic parameters after exposure to 50 μL L^{-1} EO, suggesting that a 30 μL L^{-1} concentration of EO is better and may be advisable for situations that require a long-term exposure, i.e., such as during fish transportation.

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Table 1. Chemical composition of *Hesperozygis ringens* essential oil.

Peak	Rt (min)	Compounds	%	RI calc	RI ref
1	9.718	α-Pinene	0.61	931	930[2]
2	11.355	Sabinene	0.37	970	971[2]
3	11.419	β-Pinene	0.51	972	971[2]
4	12.176	β-Myrcene	0.36	990	991[2]
5	13.613	Limonene	1.92	1025	1026[2]
6	13.705	Eucalyptol	0.18	1027	1027[2]
7	14.454	NI	0.19	1046	
8	16.621	Linalool	1.33	1099	1099[2]
9	17.177	Octen-3-yl acetate <1->	0.09	1113	1113[1]
10	18.690	Menthone	0.04	1152	1154[2]
11	18.749	(-)- Isopulegol	0.06	1153	1156[2]
12	19.061	Menthofurane	0.16	1161	1163[2]
13	19.547	Isopulegone	0.93	1174	1177[2]
14	20.154	α-Terpineol	0.39	1189	1189[2]
15	20.889	Verbenone	0.51	1209	1207[2]
16	21.140	NI*	0.29	1215	
17	21.666	NI	0.17	1230	
18	22.188	Pulegone	81.37	1244	1244[2]
19	22.383	NI	0.39	1250	
20	22.876	NI*	0.13	1263	
21	23.471	NI	0.28	1279	
22	23.613	NI	0.39	1283	
23	23.817	NI	3.32	1288	
24	25.620	Eucarvone	0.19	1340	1343[2]
25	25.913	NI	0.24	1348	
26	27.197	NI	0.27	1385	
27	27.519	NI	0.18	1394	
28	27.825	NI	2.32	1403	
29	28.291	Caryophyllene	0.39	1418	1418[2]
30	28.633	NI	0.14	1428	
31	28.806	NI	0.31	1433	
32	29.136	NI	0.23	1443	
33	32.678	NI	0.11	1555	
34	33.370	(-)-Spathulenol	0.65	1577	1578[2]
35	33.538	Caryophyllene oxide	0.98	1583	1581[2]
		Total	99.99		
		Identified	91.03		

Rt: retention time; %: percentage; RI calc.: calculated Kovats retention index; RI ref.: reference Kovats retention index; NI: not identified. [1]Adams (2001), [2]NIST (2002).
*Compounds with fragmentation model indicative of structure derived from oxidation of pulegone.

Table 2. Sequences for the primers of *Rhamdia quelen* used in real-time PCR.

Gene	Primer	Nucleotide sequence
GH	Fw	5'-GGACAAACCACCCTAGACGAG-3'
	Rv	5'-TTCTTGAAGCAGGACAGCAGA-3'
PRL	Fw	5'-CCTGTCTCTGGTTCGCTCTCT-3'
	Rv	5'-GTCCTGCAGCTCTCTGGTCTT-3'
SL	Fw	5'-TCCAGCACGCTGAGCTGATCT-3'
	Rv	5'-AAGAGTTTCCCCCATGACCTT-3'
β-ACTIN	Fw	5'-GAAGTGTGACGTCGATATCCG-3'
	Rv	5'-CCTGAACCTCTCATTGCCAAT-3'

Table 3. Enzymatic activity in the liver of *Rhamdia quelen* after 6 h of exposure to essential oil of *Hesperozygis ringens*.

Enzyme	Metabolism involved	Treatments		
		Control	30 µL L^{-1}	50 µL L^{-1}
HK	Carbohydrate	0.13 ± 0.02	0.18 ± 0.03	0.14 ± 0.02
G3PDH	Carbohydrate/Lipid	8.00 ± 0.49^{a}	11.23 ± 0.43^{b}	10.65 ± 1.11^{ab}
PK	Carbohydrate	9.90 ± 0.86	10.59 ± 0.80	10.90 ± 0.77
GPtotal	Carbohydrate	3.60 ± 0.39	3.32 ± 0.31	3.28 ± 0.31
GPactive	Carbohydrate	2.61 ± 0.24	1.98 ± 0.38	3.01 ± 0.26
G6PDH	Carbohydrate	5.66 ± 0.58^{a}	6.59 ± 0.78^{ab}	8.18 ± 0.83^{b}
FBP	Carbohydrate	1.56 ± 0.16^{a}	2.01 ± 0.21^{ab}	2.26 ± 0.29^{b}
GDH	Amino acid	7.25 ± 1.07	9.43 ± 1.80	10.51 ± 2.37
GPT	Amino acid	19.50 ± 1.72	17.71 ± 2.12	17.32 ± 2.53
HOAD	Lipid	0.31 ± 0.07^{a}	0.68 ± 0.12^{ab}	0.89 ± 0.22^{b}

Data represent the mean ± SEM (n = 10).
Different letters indicate significant differences ($P < 0.05$) between treatments.
Enzymatic activity expressed by U mg^{-1} protein.

Figure captions

Figure 1 Effects of the essential oil of *Hesperozygis ringens* on ventilatory rate of *Rhamdia quelen* through 6 h of exposure. Data represent the mean ± SEM. An asterisk (*) indicates a significant difference ($P < 0.05$) from 0 h in the same treatment.

Figure 2 Effects of the essential oil of *Hesperozygis ringens* on plasma levels of glucose (a), lactate (b), protein (c), triglycerides (d), osmolality (e) and cortisol (f) of *Rhamdia quelen* after six hours of exposure. Data represent the mean ± SEM (n = 10). Different letters indicate significant differences (one way-ANOVA, $P < 0.05$) between treatments.

Figure 3 Effects of the essential oil of *Hesperozygis ringens* on expression of growth hormone (GH) (a), prolactin (PRL) (b) and somatolactin (SL) (c) in the pituitary of *Rhamdia quelen* after six hours of exposure. Data represent the mean ± SEM (n = 10). Different letters indicate significant differences (one way-ANOVA, $P < 0.05$) between treatments.

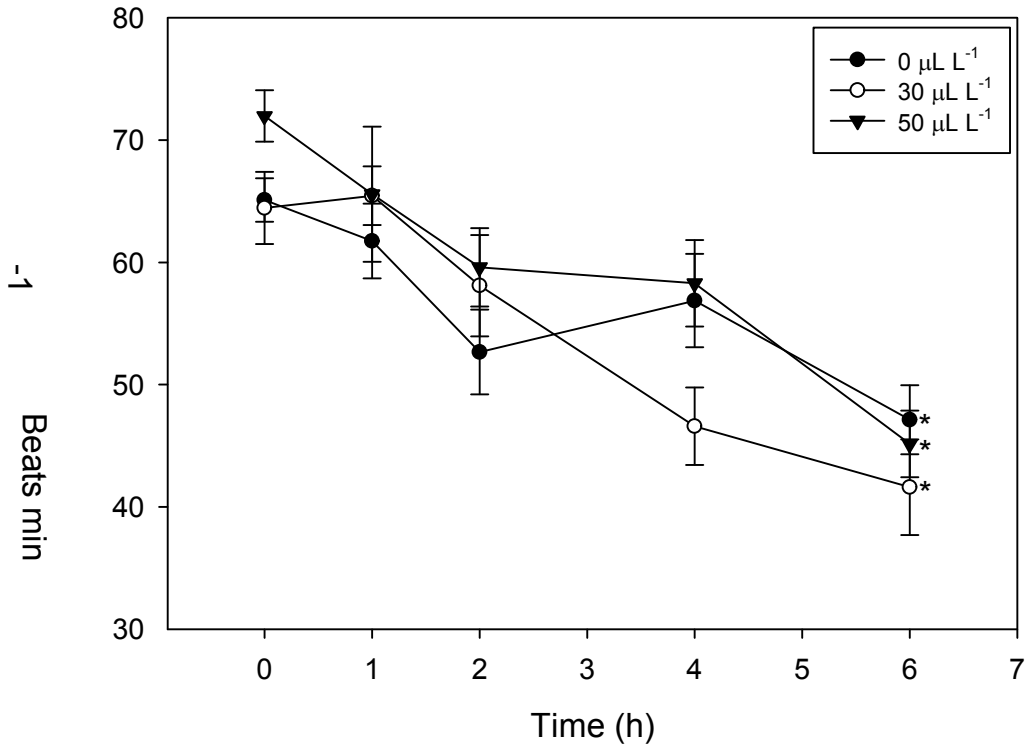
80
70
60
50
40
30
Beats min -1
0
1
2
3
4
5
6
7
Time (h)
0 μL L-1
30 μL L-1
50 μL L-1
*
*
*

Fig. 1

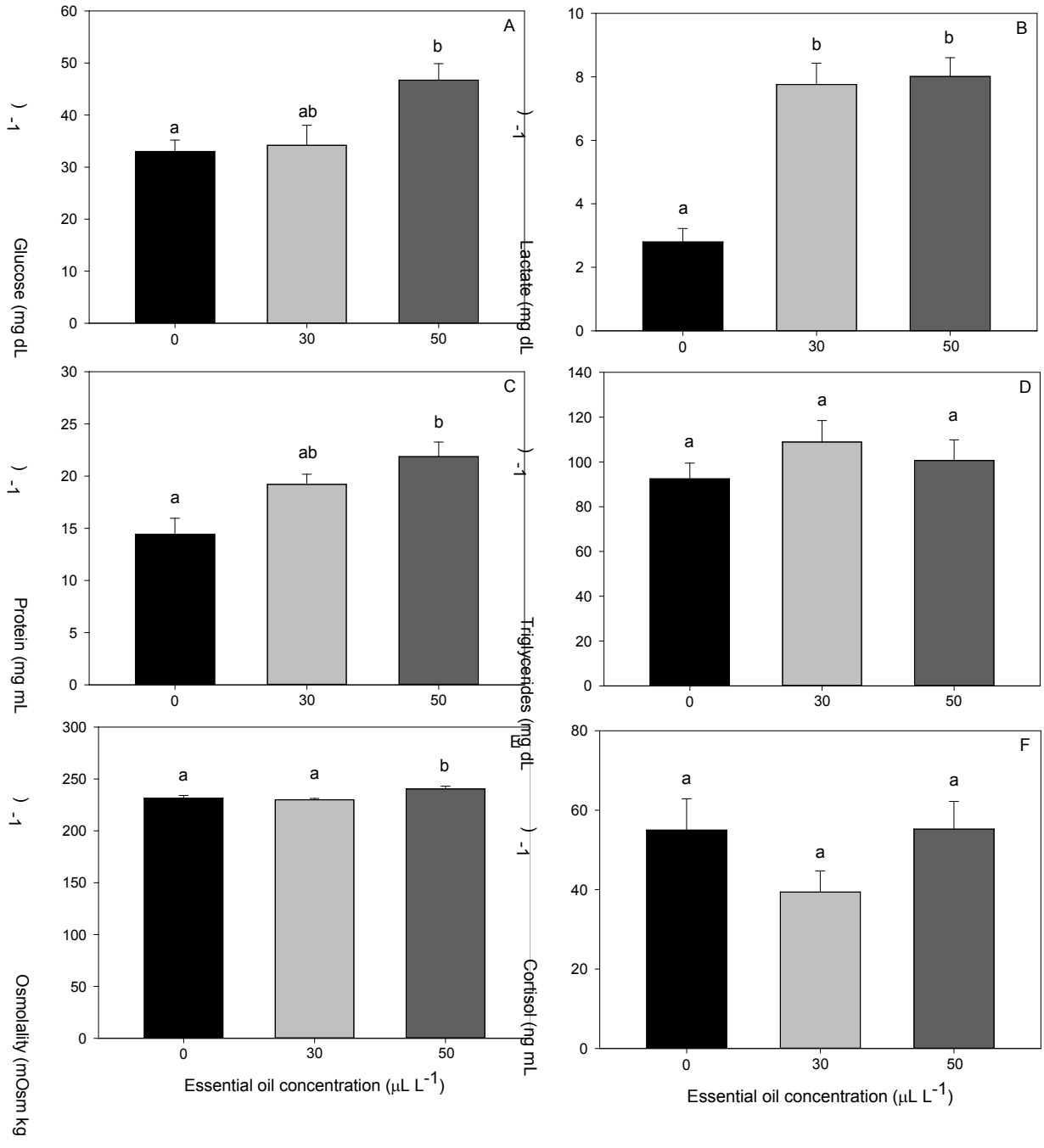
A
B
C
D
F
Glucose (mg dL) -1
Lactate (mg dL) -1
Protein (mg mL) -1
Triglycerides (mg dL) -1
Osmolality (mOsm kg) -1
Cortisol (ng mL) -1
Essential oil concentration (μL L-1)
Essential oil concentration (μL L-1)
0
30
50
a
ab
b

Fig. 2

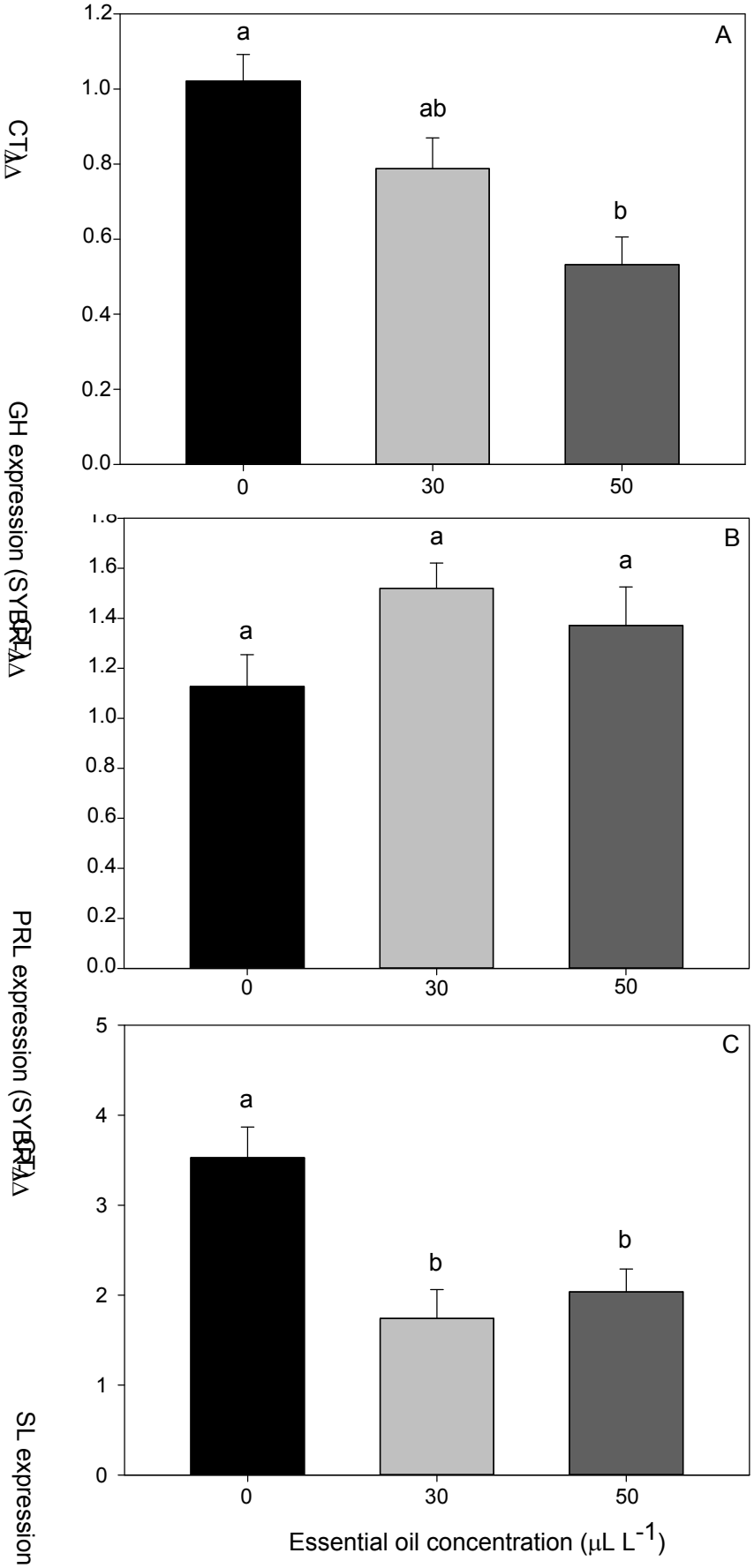
A
B
C
a
ab
b
a
a
a
a
b
b
0
30
50
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5
4
3
2
1
0
Essential oil concentration (μL L-1)
GH expression (SYBR-ΔΔCT)
PRL expression (SYBR-ΔΔCT)
SL expression (SYBR-ΔΔCT)

Fig. 3

MANUSCRITO

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Sedative effect of 2-phenoxyethanol and essential oil of *Lippia alba* on stress response in gilthead sea bream (*Sparus aurata*)

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Abstract

The use of anesthetics agents is a useful alternative to reduce stress during routine practices in aquaculture, since a safe and effective concentration is used. We investigated the effects of low concentrations of two anesthetics on stress response in gilthead sea bream (*Sparus aurata*). Previously, we evaluated the anesthetic efficacy of essential oil of *Lippia alba* (EOLA), induction and recovery times of anesthesia. Secondly, fish were exposed to i) water (control), ii) 35 μL L^{-1} of EOLA or iii) 35 μL L^{-1} of 2-phenoxyethanol (2-PHE) during 4 h, subjected to i) unstressed or ii) stress conditions. Plasma was used to cortisol, glucose, lactate, triglycerides, proteins and osmolality determinations. Corticotropin-releasing hormone (CRH) and CRH-binding protein (CRH-BP) expression were analyzed in brain, as well as proopiomelanocortin (POMC), growth hormone (GH) and prolactin (PRL) in pituitary. Cortisol, glucose, lactate and osmolality levels were higher in plasma of fish exposed to EOLA, both undisturbed and stressed. Protein and triglycerides levels were not affected significantly by the treatments. Similarly, expressions of CRH and GH did not change after anesthetics and stress exposition. Lower CRH-BP expression levels were verified in stressed fish exposed to EOLA compared with 2-PHE/stress combination or respect to EOLA undisturbed. Stress caused the reduction of the PRL expression in control fish, without differences between treatments. In addition, undisturbed fish exhibited reduced PRL expression after exposure to both anesthetics. POMCa expression was higher in stressed fish exposed to EOLA in compare to control and undisturbed EOLA, while the POMCb expression was higher after 2-PHE exposure in stressed fish compared to control. Thus, the EOLA was effective as anesthetic to gilthead sea bream at 100-300 μL L^{-1}, but for 4 h of exposition, the 2-PHE was more effective than EOLA to prevent the stress response in gilthead sea bream at the concentrations used in the present investigation.

Keywords: anesthetics, animal welfare, fish, hormones, metabolism.

1. Introduction

In aquaculture, fish are subjected to routine practices (i.e. handling, capture, biometrics, blood collection or transportation) that activated the stress system and may affect adversely their health and welfare (Kiessling et al., 2009). The loss of sensation is important during these procedures, and in an attempt to reduce the stress, the use of anesthetic substances is advisable provided that adequate concentrations are established for a specific purpose, i.e. sedation or deep anesthesia (Becker et al., 2012; Toni et al., 2014). The 2-phenoxyethanol is one of the most commonly used anesthetics in aquaculture and has been evaluated experimentally, but its use is restricted to non-food fish and to research (Coyle et al., 2004). Essential oil (EO) of *Lippia alba* is obtained from the Brazilian “false-melissa” plant and its efficacy as anesthetic for fish has been confirmed (Cunha et al., 2010, 2011; Toni et al., 2014).

Fish neuroendocrine stress pathways rely on the adrenergic system and the hypothalamus-pituitary-interrenal (HPI) axis, whose stimulation culminates in the release of catecholamines and corticosteroids into the circulation (Pankurst, 2011; Weber et al., 2011). In the HPI cascade, corticotropin-releasing hormone (CRH) synthesized in the hypothalamus acts on pituitary corticotropic cells to stimulate the synthesis of proopiomelanocortin (POMC), resulting in the control of adrenocorticotrophic hormone (ACTH) release, which in turn will stimulate production and release of cortisol in the interrenal cells (Mommsen et al., 1999). At the hypothalamic level, CRH-binding protein (CRH-BP) is considered as other important player in the HPI axis with antagonistic roles to CRH in the control of stress pathways (Huising et al., 2004). Even so, the CRH/CRH-BP system has been recently suggested to induce differential responses depending on the stressor applied (Martos-Sitcha et al., 2014).

Besides the stress response, cortisol is involved in several processes in fish physiology, such as regulation of metabolic pathways, behavior, growth, reproduction and osmoregulation (Mommsen et al., 1999). Furthermore, the expression of pituitary hormones as growth hormone (GH) and prolactin (PRL), involved on growth and osmoregulation control, respectively, was also modified in response to different stressors (Laiz-Carrión et al., 2009).

Gilthead sea bream (*Sparus aurata* L., Sparidae) is a marine teleost of high commercial value (Basurco and Abellán, 1999). This species inhabits the Atlantic coasts of Europe, Mediterranean Sea and Black Sea (rare) and is one of the most important marine fish in fishery and aquaculture, especially in the Mediterranean area (Arabaci et al., 2010).

The aim of this study was to test the ability of 2-phenoxyethanol and EO of *L. alba* to suppress normal stress response in gilthead sea bream. Effects of low concentrations were evaluated on hormonal expression, plasma cortisol, metabolites and osmolality levels. Comparisons of these parameters between the two anesthetics will provide information about which one produces fewer adverse effects to fish.

2. Material and methods

2.1 Anesthetics

The 2-phenoxyethanol was purchased from Sigma (St. Louis, MO, USA). The EO of *L. alba* (EOLA) was extracted from the fresh leaves of the plant by hydrodistillation for 2 h, using a Clevenger type apparatus (European Pharmacopoeia, 2007). The EO was stored at –4 ºC in amber glass bottles until the composition analysis by gas chromatography coupled to mass spectrometry (GC-MS; as described by Silva et al., 2012) and other biological tests could be performed. The EOLA composition is shown in Table 1.

2.2 Fish

Juvenile specimens of gilthead sea bream, *S. aurata* (48.5 ± 3.9·g body weight, 14.1 ± 0.4 cm), were provided by Servicios Centrales de Investigación de Cultivos Marinos (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Operational Code REGA ES11028000312) and transferred to the laboratory at Faculty of Marine Science (Puerto Real, Cádiz). They were kept in 400 L tanks in a re-circulating system containing seawater (34 ppt salinity) and constant aeration for a 2-week acclimation period. During this period, fish were maintained under natural photoperiod (Feb/Mar, 2013) for our latitude (36º 31’ 44’’ N) and constant temperature (18 °C). Fish were fed daily with commercial dry pellets at 1% body mass.

2.3 Experiment I – Anesthetic efficacy, induction and recovery times

The anesthetic efficacy of different concentrations (25, 35, 50, 100, 200 and 300 μL L^{-1}) of the EOLA were tested on gilthead sea bream. Each fish (n = 9 per concentration) was transferred to continuously aerated aquaria containing the test concentration (previously diluted in ethanol (1:10)) added to water. Control experiments were performed using aquaria containing ethanol alone at the same concentration used for dilution of the highest EOLA concentration. The anesthesia stages observed in the present study are according to Small (2003). The induction times were recorded in the stage I (sedation), which is characterized by decreased reactivity to external stimuli, and in the stage III, when occurs total loss of equilibrium and cessation of locomotion. The maximum observation time was 30 min. Then, fish were transferred to anesthetic-free aquaria to evaluate the recovery time of the anesthesia. Fish were considered recovered when they returned to normal swimming and react to external stimuli. Each fish was used only once.

2.4 Experiment II – Anesthetics exposure with additional stress

Fish were placed in one of the following treatments (n = 8 each): control (water), 35 μL L^{-1} EOLA or 35 μL L^{-1} 2-phenoxyethanol (2-PHE), in undisturbed or stressed groups. These concentrations were chosen based on results of the experiment I (for EOLA) and Ghanawi et al. (2013) (for 2-PHE), after previous tests on gilthead sea bream (data not shown). Throughout the experimental period of 4 h, the stressed groups were chased out every 50 min: persecution for 1 min; then broke off the persecution for 30 s to reset it again for 1 min. After 4 h, all fish were sampled. Blood was obtained by caudal puncture, centrifuged at 3 000 *g* for 3 min to separate the plasma, and then stored at –20 ºC for subsequent analysis. Pituitaries were collected in an appropriate volume of RNA*later*® (Life Technologies, USA), maintained 24 h at 4 ºC and stored at –20 ºC, while brain was sampled and immediately frozen in liquid nitrogen prior RNA total extraction. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of laboratory animals.

2.5 Analytical techniques

Plasma cortisol levels (expressed in ng mL^{-1}) were measured by indirect enzyme immunoassay (EIA) adapted to microplate as described previously by Martos-Sitcha et al. (2014) for this species. Steroids were extracted from 5 µL of plasma in 100 µL RB (10% v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01% w/v NaN_3, 2.34% w/v NaCl, 0.037% w/v EDTA, 0.1% w/v BSA (Bovine Serum Albumin)) and 1.2 mL methanol (Panreac) and evaporated for 48-72 h at 37 ºC. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. The standard curve range was 2.5 ng mL^{-1} to 19.5 pg mL^{-1} (R^2= 0.998). The lower limit of detection (92% of binding, ED92) was 39.1 pg mL^{-1}. The percentage of recovery was 95%. The inter- and intra-assay coefficients of variation (calculated from the duplicate samples) were 0.64 ± 0.56% and 4.26 ± 0.84%, respectively.

Glucose, lactate and triglyceride concentrations were measured in plasma using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides Ref. 1001311) adapted to 96-well microplates. Plasma protein was analyzed by diluting plasma 50 times and measuring protein concentration using the bicinchonic acid method with a BCA protein kit (Pierce P.O., Rockford, USA), using bovine serum albumin as standard. All of these assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by KCjunior™ program. Standards and all samples were run in quadruplicate and duplicate, respectively. Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske-VT, USA) and expressed as mOsm kg^{-1}.

2.6 Total RNA extraction and qPCR primers optimization

Total RNA was isolated from pituitary gland and brain using NucleoSpin®RNA XS and RNA II kits (Macherey-Nagel), respectively, and the on-column RNase-free DNase digestion, according to manufacturer's protocol. The amount of RNA was spectrophotometrically measured at 260 nm with the BioPhotometer Plus (Eppendorf) and its quality was measured in a 2100 Bioanalyzer using the RNA 6000

Nano Kit (Agilent Technologies). Total RNA (500 ng) was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences).

Then, total RNA (500 ng) was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences). Briefly, the reaction was performed using qScript Reaction Mix (1 x final concentration) and qScript Reverse Transcriptase (2.5 x final concentration). The reverse transcription program consisted of 5 min at 22 ºC, 30 min at 42 ºC, and 5 min at 85 ºC. cDNA was diluted 10-fold in sterile Milli-Q water to a final estimated concentration of 2.5 ng μL^{-1}. Second, to optimize the qPCR conditions of several primers (POMCa and POMCb) not used previously by our Research Group, primer concentrations (100, 200, 400, and 600 nM) and a temperature gradient (from 54 to 64 ºC) were used. Different cDNA template concentrations estimated from total input RNA were applied in triplicate (1 ng μL^{-1} [10 ng/reaction], 250 pg μL^{-1}, 62.5 pg μL^{-1}, 15.62 pg μL^{-1}, 3.90 pg μL^{-1}, and 976 fg μL^{-1}) to assess the assay linearity and amplification efficiency (POMCa (400 nM): r^2 = 0.997, efficiency (E) = 1.02; POMCb (600 nM): r^2 = 0.994, E = 0.96).

2.7 Quantification of gene expression levels (qPCR)

Total RNA was isolated and analyzed as described above, and 250 ng of each sample was reverse-transcribed in a 20 µL reaction using the qScript™ cDNA synthesis kit (Quanta BioSciences). Only samples with a RNA Integrity Number (RIN) higher than 8.5 were used for qPCR.

Real-time PCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep *realplex* ^{2} S). Each reaction mixture (10 µL) contained 0.5 µL of each specific forward and reverse primers at the best final concentration obtained above, 5 µL of PerfeCTa SYBR® Green FastMix™ (Quanta Biosciences) and 4 µL of cDNA. The PRL and GH primers were used as previously described by Vargas-Chacoff et al. (2009), whereas primer conditions of CRH and CRH-BP were retrieved from Martos-Sitcha et al. (2014). The PCR profile was obtained as follows: (95 ºC, 10 min; [95 ºC, 30 s; 60 ºC, 45 s] X 40 cycles; *melting curve* [60 ºC to 95 ºC, 20 min], 95 ºC, 15 s). β-actin was used like a housekeeping gene.

2.8 Statistical analysis

Evaluation of the anesthetic activity was performed by regression analysis (concentration x time of sedation or anesthesia induction) using the Sigma Plot 12.

Data are presented as means ± SEM. The homogeneity of variances between treatments was tested with a Levene test. Due to the presence of homogeneous variances, comparisons between treatments were performed using a two-way ANOVA followed by a Tukey test, except the data of the anesthesia recovery time, which were analyzed by one-way ANOVA. Statistica software (version 7) was used for these analyses. The minimum level of significance was 95% ($p < 0.05$).

3. Results

The major components of EOLA found were β-linalool (87.6%) and 1.8 cineol (3.6%).

Fish exposed to 25 and 35 μL L^{-1} of EOLA did not reached stage III of anesthesia through the 30 min evaluation period. Furthermore, 67 % of the fish reached stage III when exposed to 50 μL L^{-1} of EOLA. Specimens exposed to 100 to 300 μL L^{-1} of EOLA concentration range was effective in causing the stage III on all fish exposed. Ethanol alone did not produce an anesthetic effect. The anesthesia recovery time was significantly shorter following exposure to 100 and 200 μL L^{-1} of EOLA, while the lowest concentration (25 μL L^{-1}) presented longer time of recovery. There was a negative relationship between the EOLA concentration and the time required for anesthesia induction. No significant relationship was found between the EOLA concentration and the recovery time from anesthesia (Table 2). In addition, no mortality occurred during all experiments performed.

Cortisol levels did not change between undisturbed and stressed groups at each treatment. Even so, cortisol levels significantly increased its values around 12-110-fold in plasma of fish exposed to EOLA, both undisturbed and stressed, when compared with other treatments (Fig. 1).

Plasma metabolites presented a similar response pattern to cortisol values. Levels of glucose, lactate and osmolality were higher after exposure to EOLA than control and 2-PHE groups, both in undisturbed and stressed conditions (Fig. 2A, 2B

and 2E). Protein and triglycerides levels were not affected significantly by the treatments (Fig. 2C and 2D).

No significant changes were found in the CRH expression of fish exposed to different treatments (Fig. 3A). On the other hand, the CRH-BP expression was significantly reduced in stressed fish exposed to EOLA when compared to undisturbed fish at the same treatment and stressed fish exposed to 2-PHE (Fig. 3B).

mRNA expression of POMCa was higher in stressed fish exposed to EOLA than control and undisturbed fish at the same treatment (Fig. 4A). Moreover, POMCb expression did not change between groups in each treatment, but was increased in stressed fish exposed to 2-PHE when compared with stressed control fish (Fig. 4B). The exposure to both anesthetics, as well as stress processes, caused reduction in PRL expression when compared to undisturbed control fish (Fig. 4C). No significant changes in GH expression between groups or treatments were found (Fig. 4D).

4. Discussion

4.1 Anesthetic efficacy, induction and recovery times

The EOLA composition found in the present study is according to previous reports that investigated the anesthetic efficacy of this EO in silver catfish (*Rhamdia quelen*) (Cunha et al., 2010; Heldwein et al., 2012; Toni et al., 2014). Concentrations below 50 μL L^{-1} only induced sedation (stage I), and 25 μL L^{-1} presented the longest recovery time (around 6 min). Stage III (anesthesia) was reached in fish exposed to the 50-300 μL L^{-1} concentration range, with recovery time faster at 100 μL L^{-1} (about 3 min). Gilthead sea bream proved to be more sensitive to EOLA, because the anesthesia induction time was faster than silver catfish and seahorse (*Hippocampus reidi*) (Cunha et al., 2010, 2011; Toni et al., 2014). Anesthesia or sedation should be induced rapidly, preferably in less than 3 min, and with minimum hyperactivity or other stress. Recovery should be rapid, substantially complete after about 5 min in clean water (Ross and Ross, 2008). In this sense, 100 μL L^{-1} was the lowest concentration that promoted induction and recovery times of anesthesia according to the literature.

Based in the fact that in gilthead sea bream the anesthesia was caused at 100 μL L^{-1} or above, for the following experiments, the concentration 35 μL L^{-1} was

chosen because it is within the concentration range that only caused fish sedation (stage I). This stage of anesthesia is useful to for situations requiring quietness of the animal, for example, during the transport of live fish, where the reduced agitation can prevent injuries and animal death.

4.2 Anesthetics exposure with additional stress

Sedation is useful to enable a variety of procedures. This condition is characterized by a reduction in sensitivity, which results in tranquility and calmness (Zahl et al., 2012). Then, the sedation will decrease the stress and the risk of injury endured by fish during procedures such as diagnostic sampling, transport or handling. In the present work, a lack on induction of stress pathways was verified in stressed gilthead sea bream exposed to 2-PHE. However, the same was not observed after EOLA exposure. The EOLA *per se* triggered the rise of cortisol levels in gilthead sea bream, and the stress induction produced by persecution did not alter this pattern. These results were also found for glucose, lactate and osmolality levels in plasma. In addition to increases of plasma cortisol, stress typically causes increases in plasma glucose and lactate levels, as well as affect osmoregulation (Pankurst, 2011). In contrast, exposure to 2-PHE for 4 h did not change plasmatic metabolites or osmolality in gilthead sea bream.

There are few studies reporting the effects of anesthetics at low concentrations for fish handling, particularly in relation to 2-PHE (Ghanawi et al., 2013; Ortuño et al., 2002). Most research is restricted to review the times of induction and recovery from anesthesia or the effects of high concentrations (Mylonas et al., 2005; Pawar et al., 2011; Tort et al., 2002), and for our knowledge partial information regarding to the combination between anesthetics and stress induction has been reported. The EOLA (10 μL L^{-1}) improved oxidative status and prevented the ionic loss in silver catfish transported in plastic bags (Azambuja et al., 2011; Becker et al., 2012). On the other hand, oxidative stress has been reported in liver of fish transported with 30 and 40 μL L^{-1} of EOLA (Salbego et al., 2014), showing that this EO may have some undesirable effects in fish exposed. Even so, stress response verified in gilthead sea bream after 4 h of exposure to EOLA (35 μL L^{-1}), together with previous reports in other fish species, indicate that these concentrations are not recommended for prolonged exposure to this anesthetic agent.

Generally, the stress response is beneficial to an organism in a short term and also in a slight way, because energy stores are mobilized and redistributed to cope with the stressor. This fact has been previously demonstrated in larvae of carp (*Cyprinus carpio*) after a stress response to Cu, where 2-fold increase in whole-body cortisol respect to the control group promoted growth rate, but higher enhancement of this hormone produced a clear reduction in the latter (Stouthart, 1998). Thus, longer periods of stress response compromise vital body functions, as energy is allocated away from growth, reproduction and immune functions. Stress stimulates the expression and synthesis of CRH. In the present study no change on CRH expression was verified after 4 h of exposure to anesthetics. Seasholtz et al. (2002) claim that glucocorticoids act as a negative feedback regulator on the hypothalamus and the pituitary to decrease synthesis and release of CRH and adrenocorticotropin hormone (ACTH), helping to return the stress system to homeostasis. This would be a possible explanation for the observed in undisturbed fish exposed to EOLA that experienced rise in the cortisol levels but lack of response in the expression of CRH, CRH-BP and POMC. Moreover, the absence of changes in CRH mRNA levels suggests that endocrine control related to cortisol production could be coped throughout other different pathways, as has been suggested by Martos-Sitcha et al. (2014) after different stressors in the same species. In fact, several candidates, as thyrotropin releasing hormone (TRH), alpha-Melanocyte-stimulating hormone (α-MSH) or vasotocin (AVT) have been postulated as putative candidates to stimulate the stress-axis in fish (Baker et al., 1996; Moons et al., 1989; Rotlland et al., 2000; Wendelaar-Bonga et al., 1995; among others).

CRH-BP may bind CRH with high affinity and sequester ligand away from the receptor, thereby decreasing the actions of CRH at both the hormonal and synaptic levels (inhibitory activity) (Seasholtz et al., 2002). The reduced CRH-BP expression in the stressed fish and exposed to EOLA is possibly related to the kidnapping of CRH free to guarantee homeostasis. CRH-BP is a potent antagonist of CRH, being involved in negative-feedback processes triggered by high cortisol levels in order to avoid an overstimulation of HPI axis (Huising et al., 2004). Thus, our results suggest that cortisol levels can be regulated by others hypothalamic factors, as observed previously in *S. aurata* (Marthos-Sitcha et al., 2014), or even, depending on the type of applied stress (acute or chronic).

Proopiomelanocortin (POMC) is a prohormone that gives rise to several different bioactive peptides through cell-type-specific posttranslational processing. In the pituitary gland, the POMC gene is expressed in two types of cells: i) corticotrophs in the pars distalis, where POMC is processed into ACTH and β-lipotropin, and ii) melanotrophs in the pars intermedia, where it gives rise to α- MSH and β-endorphin (Souza et al., 2005). In fish, POMC-derived peptides exert a variety of functions. The regulation of skin pigmentation is mostly mediated by MSH, and ACTH plays an important role in the neuroendocrine stress response (Wunderink et al., 2012). Moreover, it had been found that fishes possess two POMC genes (POMCa and POMCb) (Cardoso et al., 2011; Souza et al., 2005; Wunderink et al., 2012). In *S. aurata*, two POMC precursors have been identified (Cardoso et al., 2011), where POMC-a is mainly expressed in melanotrophs and POMC-b is expressed in the corticotropic cells yielding, among others, ACTH. Considering the existence of these POMC paralogue genes, we could say that changes observed in POMCa expression of stressed fish exposed to EOLA are related to raised cortisol levels, while the higher expression of POMC-b in stressed fish exposed to 2-PHE was not a stress-related response. This is consistent with Wunderink et al. (2012), who predicted that POMCa (giving rise to ACTH but not to β-MSH) is mainly involved in the endocrine stress response, whereas POMCb (giving rise to α/β-MSH but no ACTH) is primarily involved in the regulation of pigmentation in Senegalese sole (*Solea senegalensis*).

In addition to the POMC family, the adenohypophyseal hormones can be classified, on the basis of structural and functional similarity, into two other groups, the glycoprotein hormone family and the GH family (Kawauchi and Sower, 2006). The latter is constituted by GH, PRL and somatolactin (SL). One of the earliest known functions of PRL in teleost fish was its role in ion uptake. More recently, GH has also been shown to have a role in teleost osmoregulation, in addition to its growth-promoting role (Sakamoto and McCormick, 2006).

Decreased PRL expression in control fish submitted to stress by persecution may indicate an inability to regulate ion absorption. Moreover, it was observed that the anesthetics per se also induced lower hormonal expression. In this case, the result may be due to the depressor effect of these agents on nervous system, once the stress did not promote additional reduction on PRL expression. However, more studies should be conducted to reveal the effects of anesthetic substances on hormone expression in fish.

Conclusion

In conclusion, anesthetic agents should be used with caution, even at low concentrations, for extended periods. In the present study, the EOLA was unable to prevent the stress response, as expected, since its sedative potential was previously proven. Thus, concentrations less than 35 μL L^{-1} are recommended for situations where a longer exposure time is required, for example, during the transport of live fish. Finally, the 2-PHE at 35 μL L^{-1} is advisable to sedate gilthead sea bream without stress produce.

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Figure captions

Fig. 1 Plasma cortisol levels of gilthead sea bream (*Sparus aurata*) after 4 h of exposure to anesthetics. Capital letters indicate significant differences between groups (undisturbed or stressed) at the same treatment (control, EOLA, 2-PHE). Lowercase letters indicate significant differences between treatments within the same group ($p < 0.05$, two-way ANOVA, Tukey test).

Fig. 2 Changes in plasma metabolites ((A) glucose, (B) lactate, (C) protein, (D) triglycerides and (E) osmolality) of gilthead sea bream (*Sparus aurata*) maintained during 4 h of exposure to anesthetics. Further details as in legend of Fig. 1.

Fig. 3 Expression levels of CRH (A) and CRH-BP (B) in brain of gilthead sea bream (*Sparus aurata*) after 4 h of exposure to anesthetics. Further details as in legend of Fig. 1.

Fig. 4 Expression levels of POMCa (A), POMCb (B), PRL (C) and GH (D) in pituitary of gilthead sea bream (*Sparus aurata*) after 4 h of exposure to anesthetics. Further details as in legend of Fig. 1.

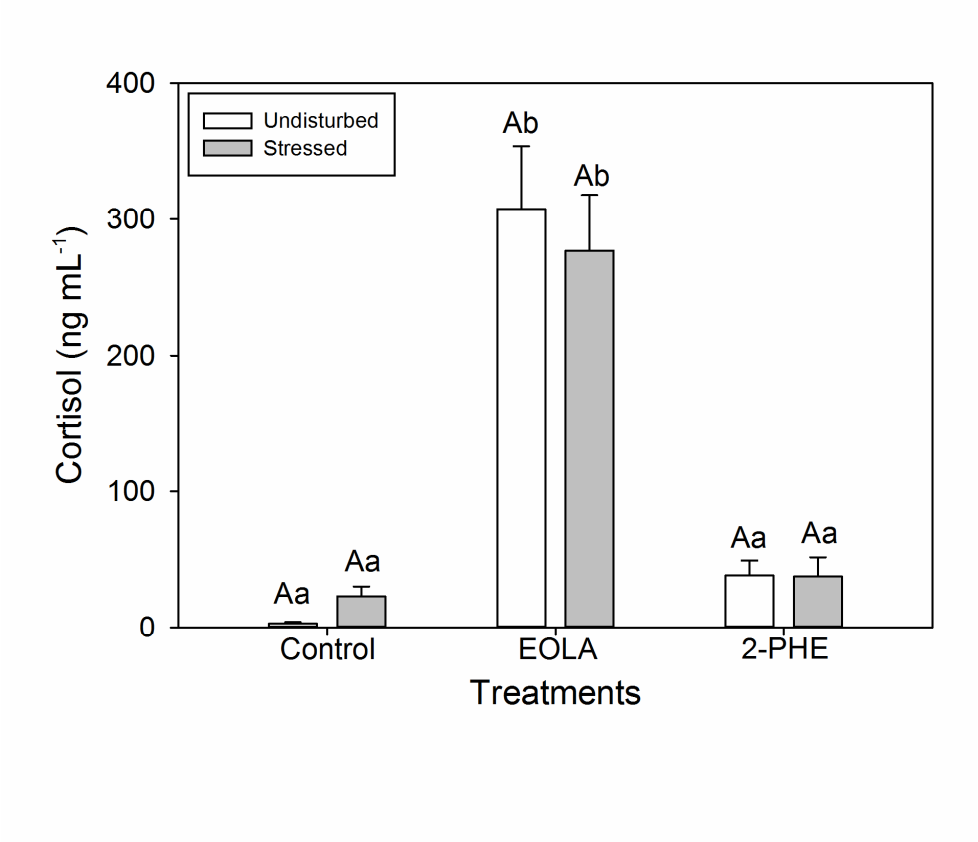
400
300
200
100
0
Cortisol (ng mL-1)
Undisturbed
Stressed
Ab
Ab
Aa
Aa
Aa
Aa
Control
EOLA
2-PHE
Treatments

Fig. 1

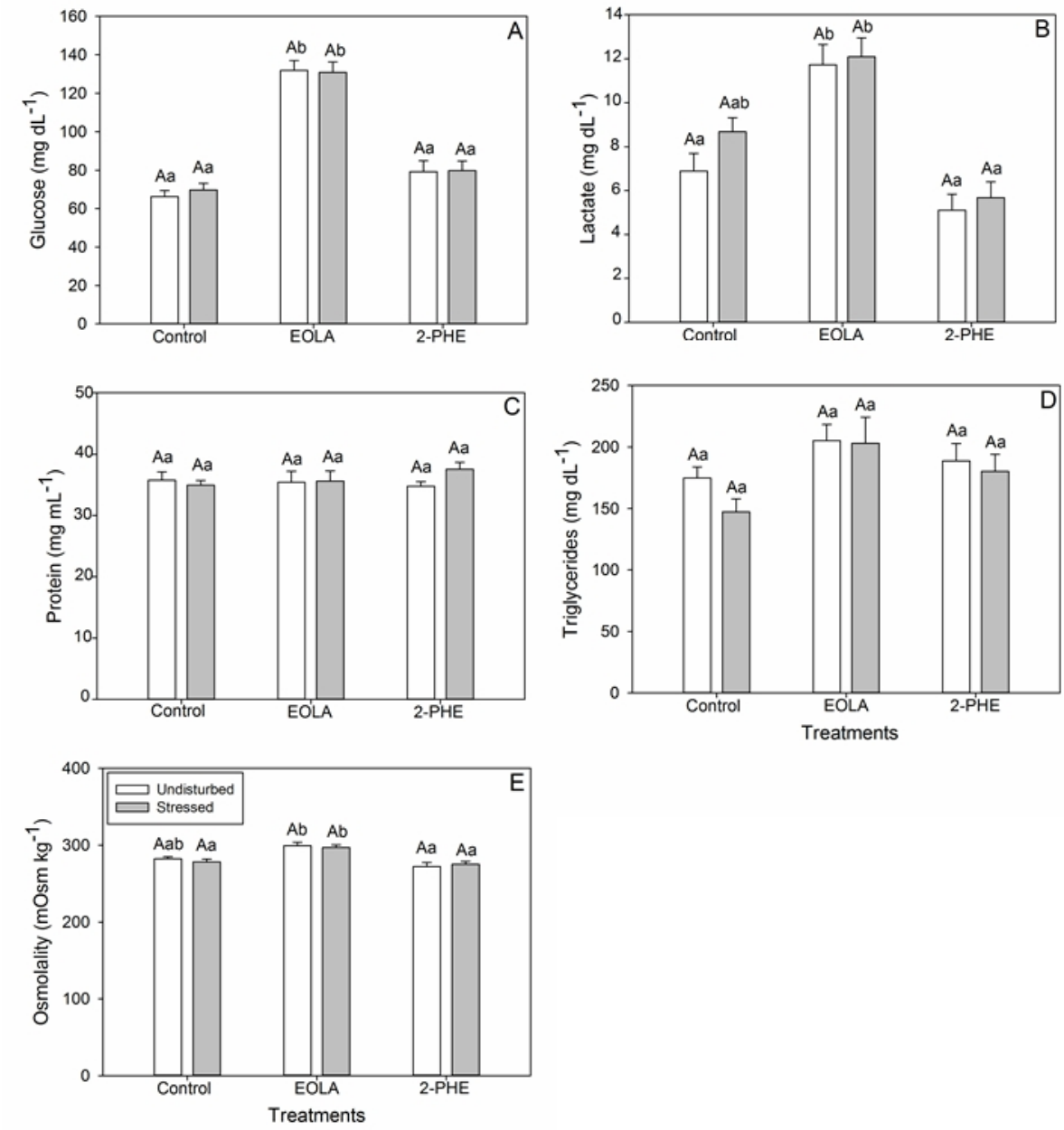
A
Glucose (mg dL-1)
Aa Aa
Ab Ab
Aa Aa
Control
EOLA
2-PHE
B
Lactate (mg dL-1)
Aa Aab
Ab Ab
Aa Aa
C
Protein (mg mL-1)
Aa Aa
Aa Aa
Aa Aa
D
Triglycerides (mg dL-1)
Aa Aa
Aa Aa
Aa Aa
Treatments
E
Undisturbed
Stressed
Osmolality (mOsm kg-1)
Aab Aa
Ab Ab
Aa Aa
Treatments

Fig. 2

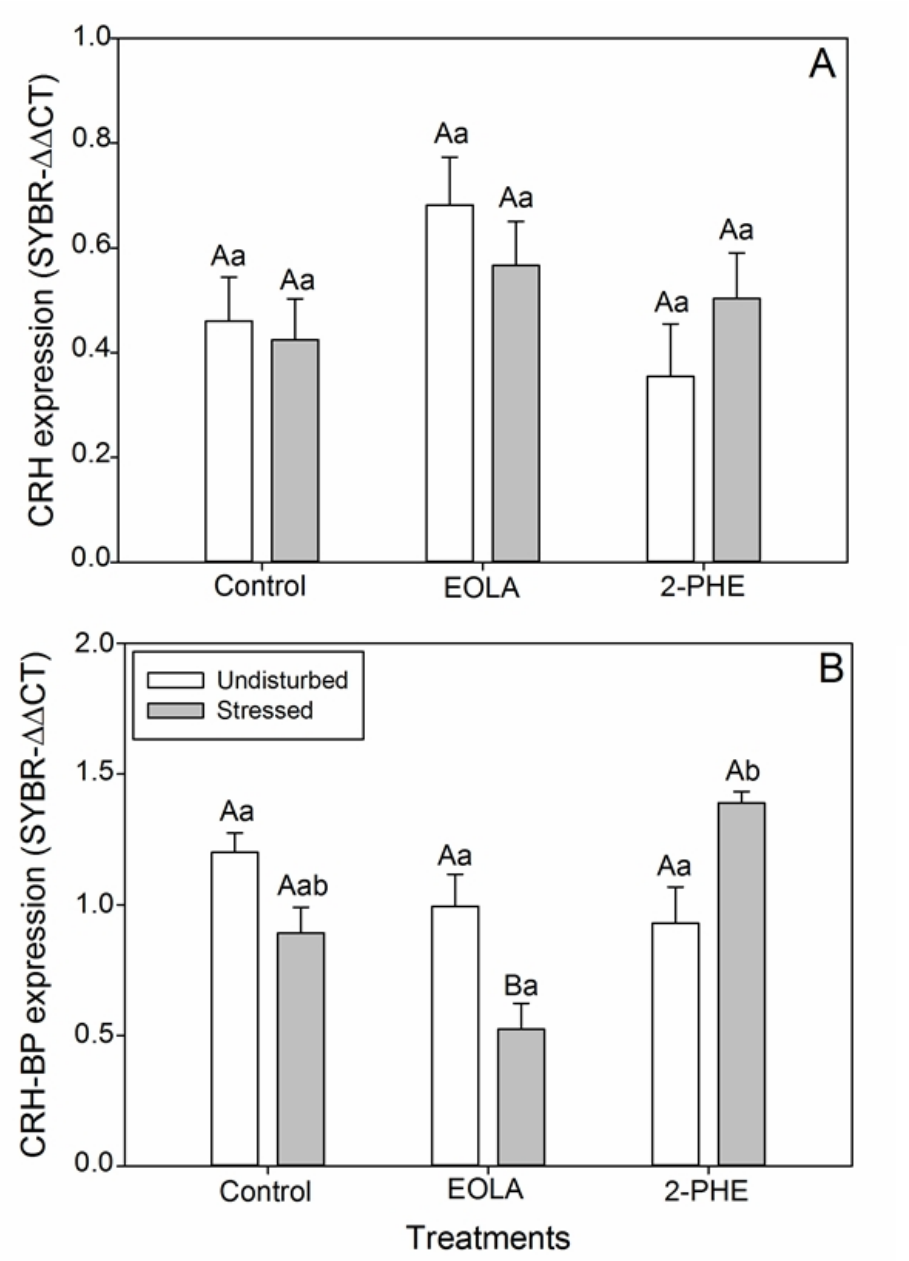
A
CRH expression (SYBR-ΔΔCT)
1.0
0.8
0.6
0.4
0.2
0.0
Aa
Aa
Aa
Aa
Aa
Aa
Control
EOLA
2-PHE
B
Undisturbed
Stressed
CRH-BP expression (SYBR-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
Aa
Aab
Aa
Ba
Aa
Ab
Control
EOLA
2-PHE
Treatments

Fig. 3

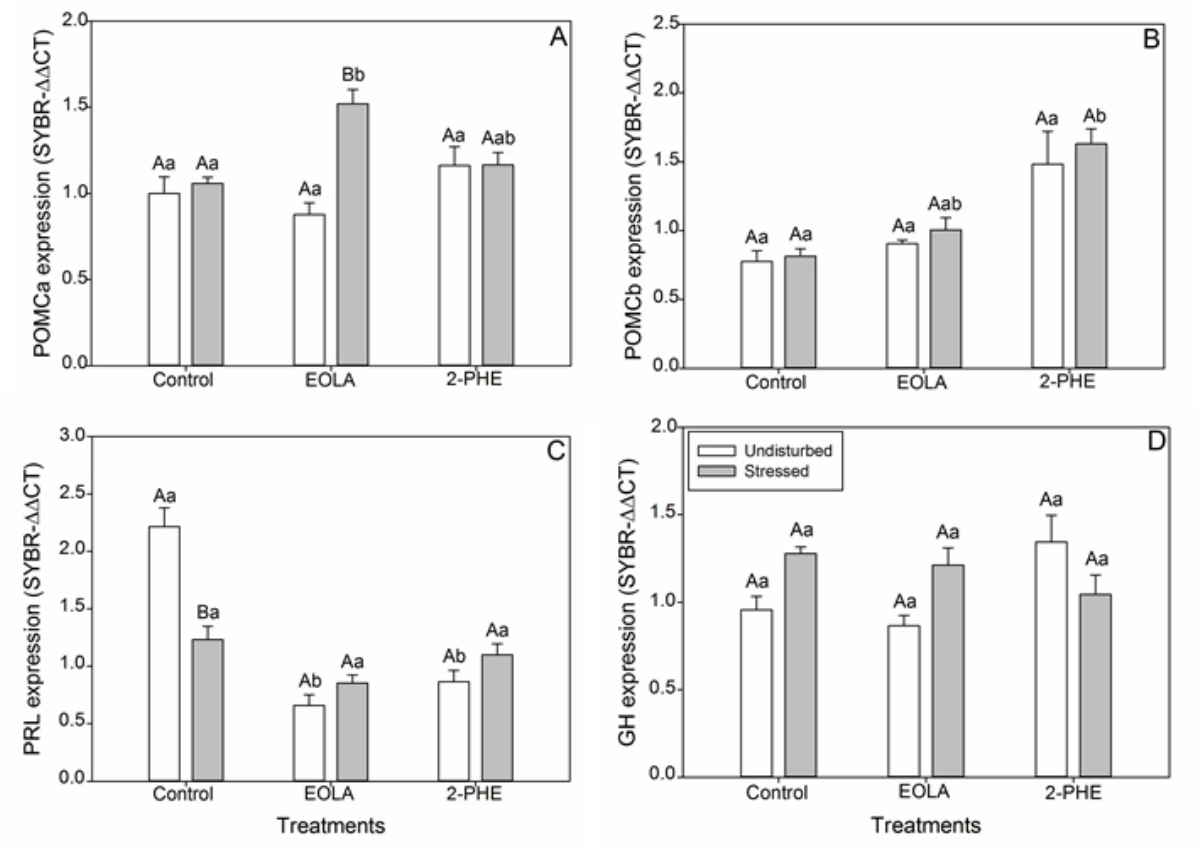
A
POMCa expression (SYBR-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
Aa
Aa
Aa
Bb
Aa
Aab
Control
EOLA
2-PHE
B
POMCb expression (SYBR-ΔΔCT)
2.5
2.0
1.5
1.0
0.5
0.0
Aa
Aa
Aa
Aab
Aa
Ab
Control
EOLA
2-PHE
C
PRL expression (SYBR-ΔΔCT)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Aa
Ba
Ab
Aa
Ab
Aa
Control
EOLA
2-PHE
Treatments
D
Undisturbed
Stressed
GH expression (SYBR-ΔΔCT)
2.0
1.5
1.0
0.5
0.0
Aa
Aa
Aa
Aa
Aa
Aa
Control
EOLA
2-PHE
Treatments

Fig. 4

Table 1 Chemical composition of the essential oil *Lippia alba* (EOLA).

Rt (min)	Constituents	(%)	RI calc	RI ref
11.329	sabinense	0.534	969	969[1]
11.72	1-octen-3-ol	0.094	978	979[1]
12.152	β-myrcene	0.152	989	991[1,2]
13.602	Limonene	0.08	1025	1029[1]
13.7	**1-8-cineol**	**3.576**	1027	1031[1,2]
14.496	E-β-ocimene	0.214	1047	1050[1,2]
15.458	E-linalool oxide (furanoid)	0.073	1070	1073[2]
16.943	**β-linalool**	**87.605**	1107	1103[1]
16.981	hotrienol	0.097	1108	1104[1]
17.47	1,3,8-p-menthatriene	0.062	1120	1110[2]
17.777	NI	0.116	1128	
18.251	camphor	0.303	1140	1146[1,2]
18.317	NI	0.067	1142	
18.977	pinocarvone	0.063	1159	1165[1,2]
19.178	p-mentha-1,5-dien-8-ol	0.148	1164	1170[2]
20.089	α-terpineol	0.219	1188	1189[1,2]
20.551	Z,Z-2,6-dimethyl-3,5,7-octatrien-2-ol	0.385	1199	1190[1,3]
20.841	E,E-2,6-dimethyl-3,5,7-octatrien-2-ol	0.779	1207	1197[1,3]
22.008	Z-citral	0.096	1239	1238[2]
23.103	E-citral	0.102	1269	1267[1,2]
25.642	exo-2-hydroxycineole acetate	0.078	1340	1354[1]
26.78	isoledene	0.063	1373	1376[2]
27.098	β-bourbonene	0.078	1382	1388[1,2]
27.351	NI	0.4	1390	
28.258	β-caryophyllene	1.019	1417	1419[1,2]
28.735	γ-elemene	0.167	1432	1437[2]
29.376	α-caryophyllene	0.143	1451	1455[1,2]
29.492	E-β-farnesene	0.097	1455	1457[2]
30.3	γ-muurolene	1.226	1480	1480[2]
31.378	NI	0.184	1513	
32.081	NI	0.106	1536	
32.68	NI	0.584	1556	
32.871	E-nerolidol	0.146	1562	1563[2]
33.497	caryophyllene oxid	0.619	1582	1583[1,2]
35.856	NI	0.203	1662	
38.594	NI	0.12	1759	
Total identified		98.218		

Rt: retention time; %: relative percentage; RI calc: calculated Kovats retention index; RI ref: reference Kovats retention index; NI: not identified. [1]NIST (2002); [2]Adams (2001); [3]Heldwein et al. (2012).

Table 2 Time required for induction and recovery of the anesthesia using the essential oil of *Lippia alba* in gilthead sea bream (n = 9 for each concentration tested). Stages are defined according to Small (2003).

Concentration	**Induction (s)**		**Recovery (s)**
(μL L^{-1})	**Stage I**	**Stage III**	
25	915 ± 11.9	-	360 ± 13.9^{a}
35	900 ± 13.1	-	277.5 ± 11^{b}
50	195.8 ± 15.8	1210.2 ± 157.5	307 ± 14.6^{ab}
100	60.3 ± 4.6	190.9 ± 9.0	212.5 ± 10.7^{c}
200	31.9 ± 2.2	96 ± 5.0	217 ± 12.6^{c}
300	-	75.7 ± 5.7	264.1 ± 14.3^{bc}
Equations	$y=5.1913+2762.7244^{(-0.0406x)}$	$y=85.2562+11949.9369^{(-0.0473x)}$	
	$r^2=0.888$	$r^2=0.999$	

Data are expressed as means ± SEM. Different letters in the rows indicate significant difference between concentrations based on one-way ANOVA and Tukey test ($p < 0.05$).

DISCUSSÃO GERAL

O conhecimento sobre a composição química de um produto natural – neste caso, óleos essenciais (OEs) utilizados como anestésicos para peixes – pode auxiliar na determinação de suas propriedades e do seu mecanismo de ação. As variações na composição química de OEs podem ocorrer em virtude de fatores genéticos, do período vegetativo das plantas ou, ainda, de fatores abióticos como temperatura, intensidade de luz, disponibilidade de água, entre outros (LIMA et al., 2003; CHALCHAT; ÖZCAN, 2008). De fato, no presente estudo, foram verificadas algumas diferenças entre os OEs utilizados como anestésicos em cada experimento, no que diz respeito à proporção dos componentes químicos majoritários presentes. Em relação ao OE de *H. ringens* (OEHR), a análise da composição química revelou 95,2% de pulegona e 1,3% de limoneno no artigo 1, ao passo que, no artigo 2, foram encontrados 81,4% e 1,9% de pulegona e limoneno, respectivamente. Essas divergências não são totalmente inesperadas, se considerarmos que o OEHR utilizado no artigo 1 foi extraído das folhas frescas e, no artigo 2, das folhas secas, resultando assim, em uma concentração menor do seu principal componente. O mesmo foi observado para o OE de *L. alba* (OELA). No artigo 1, este óleo apresentou 54,4% de linalol e 5,9% de 1,8-cineol em sua composição, enquanto que, no manuscrito, os percentuais foram de 87,6% e 3,6% de linalol e 1,8-cineol, respectivamente. Independente das variações nas quantidades de seus componentes, OEHR e OELA foram eficazes como anestésicos para peixes.

Em sistemas de cultivo intensivo, os peixes são expostos a um nível baixo, porém crônico de estresse decorrente dos efeitos combinados de confinamento, alta densidade de cultivo, bem como fatores ambientais (ambiente pobre ou parcialmente degradado, qualidade da água). Em condições laboratoriais o movimento de pessoas e ruídos também são fontes de estresse aos peixes. Além disso, tanto em aquicultura quanto em pesquisa, os peixes estão sujeitos a um estresse extra (agudo) durante práticas rotineiras como biometria, captura, marcação ou coleta de material biológico. Assim, qualquer manejo adicional pode levar ao comprometimento da saúde do animal, talvez mal alimentado por algum tempo, tornando-o menos eficiente em se adaptar às alterações ambientais (ROSS; ROSS, 2008).

A utilização de anestésicos, não apenas durante o ciclo produtivo, mas também em pesquisa, constitui uma ferramenta útil em situações que requerem um estado de maior tranquilidade do animal e/ou quando a cessação do movimento é necessária. Embora, em geral, as vantagens superem as desvantagens, anestésicos devem ser empregados com cautela, atentando para as concentrações, tempos de indução e procedimentos de manutenção adequados para o fim desejado.

As concentrações 150, 300 e 450 μL L^{-1} de OEHR e OELA foram eficazes para induzir o estágio 3 da anestesia (cessação do movimento) em jundiás (*Rhamdia quelen*). No artigo 1, embora alguns efeitos adversos (aumento dos níveis de glicose, lactato, atividade transaminase) tenham sido observados imediatamente após a anestesia (tempo 0) com OEHR, a maioria dos parâmetros analisados retornaram aos valores basais até 240 min de recuperação. A administração do OELA, além de produzir menos efeitos adversos, não impediu a restauração aos valores basais. Desse modo, comparando os óleos entre si, o OELA foi mais eficiente que o OEHR.

Quando empregado em baixas concentrações (30 ou 50 μL L^{-1}), o OEHR foi eficaz para produzir a sedação (estágio 2 da anestesia) em jundiás. Entretanto, após 6 horas de exposição à 50 μL L^{-1}, os peixes apresentaram alterações metabólicas e endócrinas indesejáveis. Sendo assim, em procedimentos de longa duração, como por exemplo, para o transporte de peixes vivos, recomenda-se a utilização de concentrações menores (30 μL L^{-1}). As concentrações de OEs reportadas nos artigos 1 e 2 estão de acordo com estudos prévios que, pela primeira vez, testaram a atividade anestésica de ambos os OEs em jundiás, avaliando, para isso, os tempos de indução a cada estágio e recuperação da anestesia (CUNHA et al., 2010; SILVA et al., 2013).

A resposta a um anestésico varia tanto entre espécies de peixes, quanto entre indivíduos da mesma espécie. Essas variações de resposta podem resultar de diferenças farmacocinéticas e/ou farmacodinâmicas individuais. Esses parâmetros, por sua vez, podem ser influenciados por fatores biológicos – peso e composição corporal, estágio de vida, taxa de crescimento, condição fisiológica, estado de saúde – e fatores ambientais como temperatura, salinidade, pH e nível de oxigênio dissolvido na água (ZAHL et al., 2012).

No manuscrito, constatou-se que o tempo para indução anestésica com OELA em dourada foi menor que o requerido para outras espécies como cavalo-marinho

(*Hippocampus reidi*) e jundiá, em concentrações similares do anestésico (CUNHA et al., 2011; TONI et al., 2014). Portanto, dourada mostrou ser mais suscetível à anestesia com OELA que outras espécies investigadas até o momento, uma vez que os componentes responsáveis pela anestesia não diferiram entre os trabalhos. Isso se deve a fatores espécie-específicos, já que os peixes anestesiados com OELA (artigo 1 e manuscrito): apresentam peso corporal e estágio de vida similares; os mecanismos fisiológicos diferem entre si, uma vez que se trata de uma espécie dulcícola e outra marinha; estudos farmacocinéticos e farmacodinâmicos não foram realizados.

Em geral, a anestesia em peixes é empregada com o intuito de facilitar algum tipo de manuseio. Além disso, aspectos como o bem-estar animal e a possível prevenção ou redução do estresse também encorajam o uso desses agentes. Todavia, a concentração 35 μL L^{-1} do OELA desencadeou o aumento dos níveis de cortisol em dorada após 4 h de exposição (manuscrito). Na verdade, o OE *per se* provocou a elevação desse conhecido indicador da resposta ao estresse.

A literatura apresenta dados controversos em relação à capacidade de um anestésico em produzir estresse. Assim como mencionado no manuscrito, Zahl et al. (2010) reportaram a elevação dos níveis de cortisol em salmão, bacalhau e alabote do Atlântico (*Salmo salar, Gadus morhua* e *Hippoglossus hippoglossus*, respectivamente) expostos a quatro anestésicos, entre eles e isoeugenol (AQUI-S®). Entretanto, quando foi administrado em adição a situações de manejo, o isoeugenol reduziu os níveis de cortisol em bagre do canal (*Ictalurus punctatus*) (SMALL; CHATAKONDI, 2005), assim como o OELA em jundiá (CUNHA et al., 2010). O contrário foi observado em doradas expostos ao MS-222 e 2-fenoxietanol ou OELA, durante o estresse de confinamento ou perseguição, respectivamente (MOLINERO; GONZALEZ, 1995; manuscrito).

Anestésicos podem ser estressantes aos peixes, pois (1) podem atuar como irritantes para a pele, causando danos na camada de muco; (2) quando o anestésico começa a exercer o seu efeito, a dificuldade em manter o equilíbrio pode também induzir estresse; (3) o modo de ação dos diferentes anestésicos pode afetar a resposta ao estresse (ZAHL et al., 2010). A segunda hipótese seria a explicação mais plausível para a resposta ao estresse desencadeada pela exposição ao OELA. Isto porque, no experimento II (manuscrito), durante o período de exposição ao anestésico (4 h), os peixes alternavam estados de perda e recuperação do equilíbrio

o que, realmente, não configura uma situação “confortável” em termos de bem-estar e ajustes fisiológicos. Além disso, estudos anteriores que utilizaram o OELA, tanto em concentrações para anestesia quanto para sedação dos peixes, apresentaram melhoria de parâmetros da resposta ao estresse (CUNHA et al., 2010; BECKER et al., 2012). Portanto, o OELA poderia ser empregado na tentativa de minimizar o estresse em peixes, dependendo da espécie-alvo e concentração utilizada. Para dorada, quando se tratar de exposição prolongada ao anestésico, recomenda-se o uso de concentrações abaixo de 35 μL L^{-1} do OELA.

CONCLUSÕES GERAIS

- Os OEs de *H. ringens* e *L. alba* foram eficazes como anestésicos para jundiás nas concentrações de 150, 300 ou 450 μL L^{-1}.

- Dourada foi mais sensível à anestesia com OELA que jundiá, uma vez que o OELA apresentou eficácia anestésica entre 100 e 300 μL L^{-1}.

- A resposta ao anestésico mostrou ser espécie-específica.

- O tempo decorrido para ambos os OEs produzirem o efeito anestésico em jundiá mostrou uma correlação negativa com a concentração utilizada, no entanto, isso não foi observado na recuperação da anestesia. O mesmo foi observado para dourada anestesiado com OELA.

- A anestesia com OEs alterou parâmetros metabólicos e ionorregulatórios em jundiás, mas a maioria dos efeitos não persistiu por mais de 4 h.

- O OELA foi mais efetivo que o OEHR para anestesia em jundiá, pois produziu menos efeitos adversos.

- Quando empregado em baixas concentrações, OEHR foi melhor na concentração de 30 μL L^{-1} que 50 μL L^{-1}.

- 35 μL L^{-1} do OELA induziu resposta ao estresse em dourada, sugerindo que menores concentrações devem ser utilizadas durante a exposição prolongada (4 h ou mais).

- 35 μL L^{-1} de 2-fenoxietanol é aconselhável para sedar dourada sem desencadear uma resposta ao estresse.

- Os OEs de *H. ringens* e *L. alba* podem ser utilizados para facilitar o manejo em práticas de aquicultura, desde que respeitadas as concentrações recomendadas e o tempo da exposição.

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