UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE ANIMAL

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ÓLEO ESSENCIAL DE *LIPPIA ALBA* COMO SEDATIVO E ANESTÉSICO EM DIVERSOS ANIMAIS AQUÁTICOS

Santa Maria, RS
2016

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

Orientador: Prof. Dr. Bernardo Baldisserotto
Co-orientador: Prof. Dr. Levy de Carvalho Gomes

Santa Maria, RS
2016

Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Biodiversidade Animal

A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

ÓLEO ESSENCIAL DE *LIPPIA ALBA* COMO SEDATIVO E ANESTÉSICO EM DIVERSOS ANIMAIS AQUÁTICOS

elaborada por
Larissa Novaes Simões

como requisito parcial para obtenção de grau de
Doutor em Biodiversidade Animal

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Santa Maria, 30 de março de 2016.

AGRADECIMENTOS

Ao meu grande Deus, por todos os momentos intensos de fé e persistência, por ser o meu melhor amigo e por me abençoar, com mais esta conquista.

Agradeço ao meu orientador o Prof. Bernardo Baldisserotto, em primeiro lugar pela honra em trabalhar ao seu lado e pelas oportunidades a mim oferecidas, pela confiança, dedicação e muita paciência. É um pesquisador e professor que todos admiram e buscam inspiração.

Também agradeço ao meu co-orientador Prof. Levy de Carvalho Gomes, com quem iniciei esta grande jornada e sempre está ao meu lado, com sua amizade, confiança, rigidez e sempre auxiliando de alguma forma aos meus trabalhos.

Agradeço por toda facilidade estrutural e apoio do Programa de Pós-Graduação em Biodiversidade Animal da Universidade Federal de Santa Maria (UFSM), ao Complexo Biopráticas da Universidade de Vila Velha (UVV) e à Universidade de Cádiz (UCA - Espanha), em especial todos colaboradores e co-autores que foram de grande importância para a realização desta tese, doando além do seu conhecimento, também muita dedicação, disponibilidade, amizade e paciência. Obrigada a bolsa de estudos disponibilizada pela CAPES (Coordenação de Aperfeiçoamento de Pessoal do Nível Superior), de extrema importância durante todo este estudo.

À minha família, pelo grande incentivo, apoio, compreensão e amor. Ao meu namorado, por estar sempre ao meu lado, pela imensa compreensão, dedicação, carinho e amor. Sem ele não sei se conseguiria finalizar esta etapa. Sua positividade muitas vezes me ergueu, obrigada por me ouvir inúmeras vezes sem ao menos entender do assunto, por tentar de todas as formas me auxiliar em todas as etapas da minha vida.

Aos amigos que caminharam comigo durante a grande e difícil jornada para realização desse trabalho e me incentivaram de alguma forma a concretizar esse sonho, o meu muito obrigada!

RESUMO

ÓLEO ESSENCIAL DE *LIPPIA ALBA* COMO SEDATIVO E ANESTÉSICO EM DIVERSOS ANIMAIS AQUÁTICOS

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A utilização de anestésicos em estações de aquicultura e pesquisa é uma ferramenta muito importante para reduzir tanto os danos e o consequente estresse causado por atividades rotineiras, como também a mortalidade dos animais. A tese está dividida em três capítulos, e tem o objetivo de avaliar o uso do óleo essencial de *Lippia alba* (EOL) como sedativo e anestésico em animais aquáticos, analisando seus possíveis efeitos estressantes. O capítulo 1 consiste na avaliação dos efeitos do EOL nos parâmetros comportamentais, metabólicos e de estresse oxidativo no mexilhão *Perna perna*. Dois experimentos foram realizados, no primeiro testou-se as concentrações de 150, 300 e 450 $\mu L\ L^{-1}$, durante uma hora no máximo. No segundo experimento, os mexilhões foram expostos primeiramente a uma concentração inicial de 100 $\mu L\ L^{-1}$ de EOL durante 30 minutos e, em seguida, foi adicionado mais anestésico para atingir as concentrações testadas no primeiro experimento, por mais 30 min no máximo. Foram observados os tempos de sedação, anestesia e recuperação, além de analisados glicogênio, lactato, proteína e glicose, e parâmetros de estresse oxidativo (CAT, GST, LPO, PC e SOD), em hemolinfa, brânquias e gônadas dos animais, com e sem recuperação, após a exposição ao anestésico. No capítulo 2, assim como no capítulo anterior, avaliou-se a eficiência do EOL em *Echinometra lucunter* (ouriço-do-mar), analisando o líquido celomático, gônadas e intestino dos animais amostrados com e sem recuperação após a exposição, para analisar os parâmetros metabólicos (proteínas e lipídios) e de estresse oxidativo (TBARS, SOD E CAT). No capítulo 3 verificou-se as respostas ao estresse em lubina (*Dicentrarchus labrax*). Primeiramente elegeu-se uma concentração ideal para sedação e em um segundo experimento utilizou-se uma situação de estresse (perseguição para captura), com diversas combinações de tratamentos e tempos de amostragem. Foram amostrados o plasma e fígado dos animais para analisar as alterações fisiológicas como cortisol, glicose, lactato, triglicerídeos, proteína e glicogênio, além das atividades enzimáticas no fígado (GPtotal, HK, FBP, G6PDH, PK, G3PDH, GDH, GPT e GOT). A exposição ao EOL não causou nenhuma mortalidade e apresentou efeito sedativo e/ou anestésico satisfatório nas espécies de animais aquáticos testados, com exceção apenas dos mexilhões, porque eles fecharam a concha e reduziram a filtração como um comportamento de defesa e bem-estar quando exposto a qualquer substância diferente detectada na água. Desta forma, o EOL apresentou um efeito favorável em todas as espécies testadas, porque ocasionou a melhora na resposta das defesas antioxidantes e reduziu o estresse oxidativo, sendo uma boa alternativa como sedativo e/ou anestésico para procedimentos de estações de aquicultura e pesquisa.

Palavras chave: Antioxidantes. Estresse. Fisiologia. Metabolismo.

ABSTRACT

ESSENTIAL OIL OF *LIPPIA ALBA* AS SEDATIVE AND ANESTHETIC IN DIVERS AQUATIC ANIMALS

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The use of anesthetics in aquaculture and research stations is a very important tool to minimize both the damage and the consequent stress caused by routine activities, as well mortality of the animals. This thesis is divided into three chapters, and aims to evaluate the use of essential oil of *Lippia alba* (EOL) as sedative and anesthetic in aquatic animals, analyzing its possible stressful effects. The chapter 1 consists in the assessment of the effects of EOL in the behavioral, metabolic and oxidative stress parameters in the mussel *Perna perna*. Two experiments were conducted, the first tested concentrations of 150, 300 and 450 μL L^{-1} for one hour at most. In the second experiment, the mussels were first exposed to an initial concentration of 100 μL L^{-1} EOL for 30 minutes and then more anesthetic was added to achieve the concentrations tested in the first experiment, for an additional 30 min. The sedation, anesthesia and recovery times were observed, as well glycogen, lactate, protein and glucose, and oxidative stress parameters (CAT, GST, LPO, PC and SOD) were analyzed in hemolymph, gills and gonads of animals, with and without recovery, following exposure to the anesthetic. The chapter 2, as in the previous chapter, evaluated the EOL efficiency in *Echinometra lucunter* (sea urchin), analyzing coelomic fluid, gonads and intestine of animals sampled with and without recovery after exposure, to analyze the metabolic (proteins and lipids) and oxidative stress parameters (TBARS, SOD and CAT). The chapter 3 verified the stress responses of sea bass (*Dicentrarchus labrax*). Firstly an ideal concentration for sedation was elected and in a second experiment fish were exposed to a stressful situation (chase to capture) with different combinations of treatments and sampling times. Plasma and liver of the animals were collected to analyze the physiological changes as cortisol, glucose, lactate, triglycerides, protein and glycogen, as well as the enzymatic activities in the liver (GPtotal, HK, BPF, G6PDH, PK, G3PDH, GDH, GPT and GOT). The exposure to EOL did not cause mortality and presented satisfactory sedative and/or anesthetic effect in the aquatic animal species tested, except mussels because they closed the shell and reduced filtration as a defensive behavior and well-being when exposed to any different substance detected in the water. Thus, the EOL has a favorable effect in all species tested, because it led to improvement in the response of antioxidant defenses and reduced oxidative stress, being a good alternative as sedative and/or anesthetic in procedures for aquaculture and research stations.

Keywords: Antioxidants. Stress. Physiology. Metabolism.

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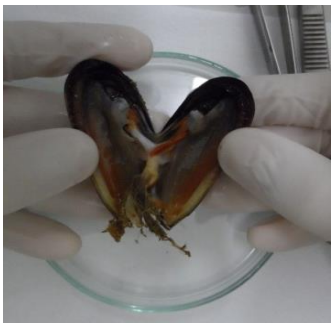
1 APRESENTAÇÃO

1.1 AQUICULTURA

É grande a procura e exploração de diversos animais aquáticos que são economicamente importantes para os fins de alimentação, pesquisa, aquariofilia e artesanato (CARNEIRO; CERQUEIRA, 2008). Diante disto, há um grande crescimento na atividade da aquicultura mundial e consequentemente as espécies mais requisitadas apresentam um forte aumento na comercialização (RORIZ et al., 2015).

Dentre estes animais está o mexilhão (*Perna perna*) (Figura 1), que entre as diferentes espécies de mexilhões, possui um crescimento relativamente rápido, tem uma alta taxa de crescimento e valor nutritivo, é facilmente coletado no ambiente (BARAJ et al., 2003) e bastante utilizado como "organismo sentinela" em diversos programas nacionais e internacionais de monitoramento ambiental do ambiente aquático (BELLOTTO et al., 2005; VIDAL-LIÑÁN; BELLAS, 2013; BESADA et al., 2014; OLIVEIRA et al., 2014; JOYCE et al., 2015), estando distribuído no Atlântico Sul, nas Américas e África. A produção mundial de moluscos em 2012, liderada pela China, foi de 15,2 milhões de toneladas (1,8 milhões de toneladas só de mexilhões), estando o Brasil em 12º lugar mundial e 2º lugar na América Latina, superado apenas pelo Chile (FAO, 2014).

Figura 1 – Mexilhão (*Perna perna*) (A) aberto para coleta de tecidos (B).

A  B 

Fonte: Larissa Novaes Simões

O ouriço do mar da espécie *Echinometra lucunter* (Figura 2) é outro organismo de grande procura e exploração, possuindo ampla distribuição ao longo das Índias Ocidentais e do Oceano Atlântico ocidental, entre a Flórida e Bermuda até a costa sul do Brasil (MCPHERSON, 1969; LEWIS; STOREY, 1984). Esta espécie de ouriço é utilizada como

alimento por populações de baixa renda, principalmente no Nordeste do Brasil (ALVES et al., 2006), mas também é comercializada inteira ou apenas as gônadas, com preços elevados, para restaurantes de origem europeia e asiática. Organismos vivos podem ser ainda mais valiosos, ao serem comercializados para aquários marinhos ou utilizados como modelos experimentais em estudos de evolução e toxicologia (MICAEL et al., 2009).

Figura 2 - Ouriço (*Echinometra lucunter*) (A) aberto para coleta dos tecidos (B).

A  B 

Fonte: Larissa Novaes Simões

Dicentrarchus labrax (lubina) (Figura 3) também é uma espécie economicamente importante e encontra-se distribuída ao longo do Atlântico da Noruega ao Marrocos, incluindo as ilhas Canárias e Senegal, também presente na região do Mediterrâneo e do Mar Negro (KOTTELAT; FREYHOF, 2007). A produção desta espécie de peixe tem crescido fortemente e é altamente valorizada no comércio europeu, com mais de 148 000 t produzidas em 2014 (FEAP, 2015).

Figura 3 - Lubina (*Dicentrarchus labrax*) (A) e coleta de sangue (B).

A  B 

Fonte: Larissa Novaes Simões

Um dos requisitos mais importantes para o bom desenvolvimento da atividade de aquicultura é o conhecimento adequado da biologia das espécies utilizadas para cultivo, onde a fisiologia das espécies, o funcionamento dos sistemas e as interações e respostas permitam melhores condições de cultivo. Os fatores estressantes têm sido a principal causa das perdas de lucros na aquicultura, pois afetam o metabolismo, prejudicam o estado de saúde e aumentam a suscetibilidade a doenças (RORIZ et al., 2015).

1.2 ESTRESSE

Cada espécie possui sua importância econômica distinta, porém todas necessitam de melhorias no bem-estar nas instalações aquícolas e de pesquisa, onde procedimentos comuns muitas vezes podem causar uma situação específica de estresse. As taxas de mortalidade destes animais estão vinculadas às práticas intensas de manejo como captura, estocagem, pesagem, mudanças na qualidade da água e transporte, as quais levam ao estresse dos animais (LEGAT; LEGAT, 2009). De certa forma, o estresse agudo pode obter uma resposta benéfica para o animal, pois é uma função fisiológica adaptativa, que responde a uma ameaça percebida à homeostase, preservando a viabilidade do indivíduo estressado permitindo a sua sobrevivência a esta situação (ASHLEY, 2007), mas o estresse crônico (intensidade ou duração) pode levar a alterações fisiológicas que resultem em consequências indesejáveis, como doenças e/ou mortalidade (IWAMA et al., 2004).

Quando, por exemplo, um peixe é exposto a um agente estressor, ocorre a ativação de dois eixos neuroendócrinos, o eixo hipotálamo-sistema nervoso simpático-células cromafins (HSC), que resulta na liberação de catecolaminas (adrenalina e noradrenalina) como produtos finais (PERRY; CAPALDO, 2011), e o eixo hipotálamo-hipófise-interrenal (HHI) que libera os corticosteroides (cortisol e cortisona) (WANDERLAAR-BONGA, 1997). Sendo assim, a ativação destes eixos é uma característica da resposta ao estresse, e estão envolvidos principalmente com a demanda energética do animal, ou seja, a mobilização de energia por meio da liberação de catecolaminas e cortisol que podem interferir de certa forma no metabolismo intermediário alterando as concentrações de parâmetros metabólicos (VIJAYAN et al., 1997; MOMMSEN et al., 1999).

No entanto, é evidente que todos os animais vertebrados reagem às mudanças anormais no seu ambiente (estressores) por ações motoras mediados pelos nervos e pela liberação de hormônios, mas em invertebrados as respostas hormonais ao estresse não são

bem compreendidas, e a maioria destes animais possui receptores especiais para tais estímulos, chamados de nociceptores (ROSS; ROSS, 2008).

Invertebrados mais avançadas são bem desenvolvidos, com sistema nervoso constituídos por conjuntos de neurônios ligados aos gânglios com diferentes níveis de complexidade, entretanto, os invertebrados mais simples não têm quaisquer estruturas semelhantes a um sistema nervoso central, mas alguns têm uma série de gânglios conectados por vias nervosas (ROSS; ROSS, 2008). Em bivalves o sistema nervoso inclui dois pares de nervo e três pares de gânglios. Não há cefalização óbvia e o sistema nervoso parece bastante simples. Neurônios mecanosensoriais são ativados durante o reflexo de retirada do pé com uma navalha, por exemplo, mas não se sabe se estes são nociceptores (OLIVO, 1970).

1.3 RESPOSTAS METABÓLICAS

As ações dos corticosteroides (cortisol) e das catecolaminas são consideradas respostas primárias em diversos órgãos-alvo, e resultam em modificações bioquímicas e fisiológicas denominadas respostas secundárias ao estresse (WENDELAAR-BONGA, 1997), que incluem o aumento da frequência cardíaca, da tomada de oxigênio e da demanda de energia e, ainda, a perturbação do balanço hidromineral. As respostas terciárias se estendem para o nível de organismo e populacional, apresentando comprometimento no desempenho, efeitos de inibição da resposta imune, aumentando a suscetibilidade a doenças, além da redução da capacidade de tolerância a agentes estressores adicionais (LIMA et al., 2006).

Desta forma, o aumento significativo destes hormônios pode resultar em uma redução no conteúdo de glicogênio hepático e aumento dos níveis de lactato e glicose no plasma, este que por sua vez, fornecem glicose para os tecidos para compensar a grande demanda de energia necessária para recuperar a homeostase (BARTON, 2002; BARCELLOS et al., 2000). A glicose é a principal fonte energética para as células dos peixes e por isso o aumento da sua produção é vital durante condições de estresse, o que torna as alterações metabólicas importantes respostas adaptativas. Diante disto, é de extrema importância ter conhecimento das variações nos parâmetros sanguíneos e hepáticos destes animais, por meio de indicadores secundários, como a glicose, lactato, glicogênio, triglicerídeos, proteína entre outros, para permitir a avaliação da resposta ao estresse, bem como a capacidade destes animais para superar a perturbação (MOMMSEN et al., 1999; ACERETE et al., 2004).

Já os mexilhões quando expostos ao ar (fechamento das valvas, resposta semelhante quando expostos ao anestésico), o fluxo de oxigênio das brânquias para os tecidos cai

bruscamente devido a um decréscimo continuo na concentração de oxigênio dissolvido na água contida no interior das valvas, o que é rapidamente consumido (LARADE; STOREY, 2002). Mas apesar de manterem suas valvas fechadas, os mexilhões realizam movimentos periódicos de abrir e fechar as valvas com objetivo de forma bolhas de ar na água que fica acumulada no seu interior. Desta forma, eles conseguem captar oxigênio do ar e manter uma baixa taxa de respiração, além de expelir metabólitos do seu metabolismo anaeróbicos (SANDEE et al., 1996).

Diferentes aspectos dos equinodermos vêm sendo avaliados, dentre os quais destacam-se os imunológicos, que envolvem o estudo de mecanismos de defesa exercidos por seus celomócitos, presentes no celoma perivisceral, e principais responsáveis pela resposta imune inata (GROSS et al., 1999).

A composição do líquido celomático assemelha-se à da água do mar, diferindo um pouco na composição de alguns compostos como potássio, lipídios, proteínas e açúcares dissolvidos (CHIA; XING, 1996). Os celomócitos, presentes no liquido celomático, são classificados basicamente em quatro tipos diferentes, sendo eles: amebócitos fagocíticos, esferulócitos vermelhos, esferulócitos incolores e células vibráteis. Estes possuem diferentes funções, e são responsáveis pela defesa do organismo (CHIA; XING, 1996; BORGES et al., 2010). A proporção dos tipos celulares pode variar de acordo com a espécie, ou até mesmo entre indivíduos da mesma espécie, levando em conta as condições fisiológicas (MATRANGA et al., 2005).

1.4 METABOLISMO ENERGÉTICO

Quando a glicose é ingerida acima do necessário, ocorre a polimerização a glicogênio, o qual é armazenado no fígado e nos músculos, sendo a mobilização da glicose controlada pela ação dos hormônios e enzimas, ou convertida à gordura. Para manter a homeostase energética, o glicogênio é mobilizado e transportado como glicose e seus valores no sangue são mantidos constantes, garantindo o suprimento de energia às células nas várias situações em que os animais estejam submetidos (SILVEIRA et al., 2009).

As catecolaminas causam aumento dos níveis de glicose no plasma pela mobilização das reservas de glicogênio hepático (glicogenólise), enquanto os corticosteroides mantêm a hiperglicemia estimulando o catabolismo proteico e a gliconeogênese, por meio da conversão de compostos aglicanos (não açucares e não carboidratos), sendo os principais precursores o lactato, a alanina (aminoácido) e o glicerol (PANKHURST, 2011). Outras vias metabólicas

também utilizadas são a glicogênese, que é a reação de síntese da glicose que ocorre no fígado e nos músculos e a glicólise, que é a sequência metabólica de várias reações enzimáticas em que a glicose é oxidada e o produto final são duas moléculas de ATP (SILVEIRA et al., 2009).

1.5 ATIVIDADES ENZIMÁTICAS

Cada via metabólica é regulada continuamente dentro das células, no entanto, situações de estresse podem alterar o metabolismo intermediário alterando as atividades das enzimas-chave no metabolismo de carboidratos (glicogênio fosforilase total - GPtotal, hexoquinase - HK, frutose-bisfosfato - FBP, glucose-6-fosfatodesidrogenase - G6PDH, piruvatocinase - PK), lipídios (glicerol-3-fosfato desidrogenase - G3PDH) e aminoácidos (glutamate desidrogenase - GDH, transaminase glutâmico-pirúvica - GPT, e transaminase glutâmico-oxalacética - GOT) (MOMMSEN et al., 1999; LAIZ-CARRÓN et al., 2003).

O metabolismo tem como uma de suas funções a de proporcionar a energia necessária para os processos vitais, para compensar as perdas de substâncias resultantes dos desgastes ou exercícios e atender ao desenvolvimento e crescimento do organismo (MOMMSEN et al., 1999). As vias metabólicas são necessárias para que o organismo mantenha a sua homeostase. Diferentes enzimas catalisam diferentes passos de vias metabólicas, agindo de forma correta de modo a não interromper o fluxo nessas vias. Dessa forma, as atividades das enzimas envolvidas no metabolismo de carboidratos, lipídios e proteínas podem dar pistas sobre a forma como o animal reorganiza seu estado energético depois de experimentar um evento estressante (TONI et al., 2015).

1.6 ESTRESSE OXIDATIVO

O estresse pode desencadear a produção de espécies reativas de oxigênio (ERO), resultante da redução de O_2, que podem ser extremamente tóxicas para a célula e, desta forma, diversos mecanismos de defesa são ativados com o objetivo de prevenir tais danos, os chamados sistemas antioxidantes. Os animais possuem um sistema de defesa bioquímica consistindo de antioxidantes enzimáticos e não enzimáticos, que combatem quaisquer espécies reativas, como por exemplo, a superóxido dismutase (SOD) que converte o ânion superóxido em peróxido de hidrogênio, a catalase (CAT) converte peróxido de hidrogênio em

água e oxigênio (VERNON; TANG, 2013) e a glutationa-*S*-transferase (GST) catalisa a conjugação da glutationa com substratos eletrófilos (MODESTO; MARTINEZ, 2010).

No âmbito de um estado fisiológico normal, as ERO geradas em tecidos e compartimentos subcelulares são eficientemente eliminados pelo sistema de defesa antioxidante (LUSHCHAK; BAGNYUKOVA, 2006), mas em várias outras situações, pode ocorrer um desequilíbrio entre oxidantes e antioxidantes em favor dos oxidantes, conduzindo à ruptura do controle redox (reações de redução-oxidação) e/ou danos moleculares, situação essa chamada de estresse oxidativo (SIES; JONES, 2007), que causa danos oxidativos, tal como a peroxidação lipídica (LPO), a qual pode ser avaliada pela formação de substâncias reativas ao ácido tiobarbitúrico (TBARS) e utilizada como indicador do estresse oxidativo celular (LIMA; ABDALLA, 2001); a oxidação da proteína, onde ocorre a formação da proteína carbonilada (PC) que pode ser quantificada para medir a extensão do dano oxidativo; alterações metabólicas e enzimáticas; e danos ao DNA (BEAL, 2003; DALLE-DONNE et al., 2003).

Sugere-se que quando os mexilhões fecham as valvas, um baixo fluxo de oxigênio nos tecidos aliado a um possível decréscimo na eficiência dos sistemas de defesa e/ou reparo, podem aumentar a susceptibilidade dos tecidos à lesões oxidativas. Após abertura das valvas (re-oxigenação), a atividade de enzimas antioxidantes mantida sob níveis basais pode amenizar os efeitos causados por um possível aumento na produção de ERO (JONES, 1986).

Nos invertebrados marinhos, inclusive nos ouriços-do-mar, o desequilíbrio na produção de espécies reativas de oxigênio sob situações de estresse pode estar relacionado com alterações na capacidade fagocítica dos celomócitos, quando os animais estão submetidos ao estresse (BORGES et al., 2010).

1.7 ANESTÉSICOS

Para prevenir o estresse em decorrência das atividades rotineiras do cultivo, os anestésicos são uma ferramenta importante, pois reduzem o metabolismo dos animais e, com isto, reduzir o consumo de oxigênio (PALIC et al., 2006), além de evitar mortalidades (FAÇANHA; GOMES, 2005). De acordo com Ross e Ross (2008), é essencial o conhecimento da concentração ideal do anestésico que é necessária para a indução ao estágio desejado, assim como os tempos de exposição, a fim de garantir a eficácia do processo de anestesia, uma vez que, essas concentrações variam conforme a espécie e o tamanho do animal. Embora os anestésicos possam ser uma ferramenta valiosa para assegurar a proteção

dos animais durante um evento estressante, estes agentes também podem ter efeitos colaterais indesejáveis e, por isso devem ser utilizados com precaução (ZAHL et al., 2012).

Para determinados procedimentos em estações de aquicultura e pesquisa, a sedação (definido como um efeito calmante e estado preliminar a anestesia) é suficiente para minimizar o estresse ou danos físicos causados pela densidade, captura, pequeno manuseio e pesagem (ROSS; ROSS, 2008). Além disso, segundo Bosworth et al. (2007), o peixe sedado no processo de captura, antes do abate, evita o estresse que supostamente ocorreria durante estes processos, consequentemente melhorando a qualidade da carne.

Mas em procedimentos que demandem mais cautela ou maior manejo ou maior tempo de exposição ao ar, o mais aconselhado é utilização dos animais anestesiados (estado reversível da perda total de estímulos externos e do equilíbrio), ou seja, sem nenhum movimento, para alcançar o objetivo, sem causar danos ao animal (ROSS; ROSS, 2008). A anestesia é um procedimento muitas vezes indispensável no manejo, para assegurar que tanto o animal quanto o operador não sofra ferimentos (SMALL, 2004).

Procedimentos comuns como manuseio e transporte causam estresse nos ouriços do mar, levando a uma não planejada desova (LUIS et al., 2005; ARAFA et al., 2007), que reduz o tamanho das gônadas, diminuindo a sua qualidade e valor comercial, e às vezes leva até a morte. A anestesia, além de evitar este incidente, também facilita o desprendimento do animal a partir do substrato, que é outro problema comum encontrado por pesquisadores e produtores (HAGEN, 2003). No entanto, pouco se sabe sobre o papel dos anestésicos contra o estresse de ouriços do mar (ARAFA et al., 2007).

O uso de anestésicos permite o mínimo de danos e reduz o estresse em bivalves durante a manipulação experimental, como a coleta de amostras biológicas, avaliação do estado reprodutivo, etc. (CULLOTY; MULCAHY, 1992; LELLIS et al., 2000). No entanto, quando os bivalves detectam partículas desconhecidas ou tóxicas na água, a primeira possível defesa é fechar as conchas e reduzir a filtração para minimizar o contato com os tecidos (GAINEY; SHUMWAY, 1988). Daí a necessidade de compreender a correlação do anestésico com o processo de defesa do animal.

1.8 *LIPPIA ALBA*

Pesquisas que utilizam anestésicos naturais vêm aumentando consideravelmente nos últimos anos (MOREIRA et al., 2010). Um dos exemplos é a *Lippia alba* ((Mill.) BROWN 1925) (Verbenaceae), popularmente conhecida como erva cidreira, planta nativa amplamente

distribuída na América do Sul, América Central e África (ZOGHBI et al., 1998; BIASI; COSTA, 2003). Estudos farmacológicos têm evidenciado sua atividade sedativa e anestésica em peixes (CUNHA et al., 2010, 2011; SACCOL et al., 2013; TONI et al., 2014) e atividade antioxidante em peixes (AZAMBUJA et al., 2011; SALBEGO et al., 2014) e camarões (PARODI et al., 2012).

Estudos da composição química do óleo essencial de *L. alba* relatam que os resultados nem sempre são uniformes (CASTRO et al., 2002). Os constituintes predominantes nos óleos essenciais podem variar qualitativa e quantitativamente, em função de diversos fatores, tais como: estações do ano, época de floração, idade da planta, quantidade da água circulante, resultante da precipitação, fatores geográficos e climáticos, bem como a metodologia de extração (CÔRREA, 1992).

A composição de um óleo essencial de uma planta é determinada geneticamente, sendo geralmente específica para um determinado órgão e característica do estágio de desenvolvimento, mas as condições ambientais são capazes de causar variações significativas, dando origem aos quimiotipos ou raças químicas tão freqüentes em plantas ricas em óleos essenciais. Ou seja, as diferenças na composição dos diferentes quimiotipos da espécie *L. alba* não constituem um produto só da influência de fatores ambientais, mas refletem também a variação genotípica destas plantas (TAVARES et al., 2005).

No Brasil, diversos estudos abordam as diferenças nos componentes majoritários do óleo essencial de *L. alba*. No Paraná foi encontrado γ-terpineno; em São Paulo, o citral; no Ceará e Maranhão, o β-cariofileno (CASTRO et al., 2002) e no Rio Grande do Sul, o linalool (ATTI-SERAFINI et al., 2002).

Até a presente data, as informações sobre os efeitos da exposição a anestésicos sobre a atividade das enzimas associadas com o metabolismo dos carboidratos, lipídios e proteínas de peixe são escassas, assim como quase não existem trabalhos testando o efeito do óleo essencial de *L. alba* em invertebrados. Desta forma, é necessário melhorar o conhecimento sobre a forma como o animal reorganiza seu estado energético na busca da homeostase após um evento estressante, como também testar invertebrados aquáticos.

O sucesso produtivo de espécies aquáticas economicamente importantes é diretamente associado com a fisiologia do estresse destes animais, sendo assim o entendimento básico das alterações internas do organismo possibilita a identificação das condições adversas e o desenvolvimento de métodos que atenuem os seus efeitos na sua saúde. Diante disto, há necessidade da produção de trabalhos científicos que correlacionam o efeito sedativo e/ou anestésico com as atividades metabólicas e energéticas e parâmetros de estresse oxidativo em

animais aquáticos, principalmente em invertebrados, com o objetivo geral de avaliar o uso do óleo essencial de *L. alba* (EOL) como sedativo e anestésico em animais aquáticos, analisando seus possíveis efeitos estressantes, sendo os objetivos específicos: avaliar os efeitos do EOL nos parâmetros comportamentais, metabólicos e de estresse oxidativo no mexilhão *Perna perna*; analisar o uso do EOL como sedativo e anestésico no ouriço-do-mar *Echinometra lucunter*, verificando os parâmetros de estresse oxidativo; e determinar a eficiência do EOL como sedativo e anestésico na lubina (*Dicentrarchus labrax*), e suas respostas ao estresse.

Sedation and anesthesia with the essential oil of *Lippia alba* affects behavioral, metabolic, and oxidative stress parameters in the mussel *Perna perna*

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Running title: Anesthesia effects with essential oil of *Lippia alba* in mussel *Perna Perna*.

ms. has 27 pages, 6 figures, 5 tables

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Abstract

The objective of this study was to evaluate the effects of the essential oil of *Lippia alba* (EOL) on behavioral, metabolic, and oxidative stress parameters in the mussel *Perna perna*, an important comestible species worldwide. To determine the times required for anesthesia induction (maximum 1 hour) and recovery (maximum 30 min), in the first experiment, three different concentrations of EOL (150, 300, and 450 μL L^{-1}) were tested. An ethanol only and a negative control (no substance added to the water) group were also included. In the second experiment, the mussels were first exposed to an initial concentration of 100 μL L^{-1} EOL for 30 min and then more anesthetic was added to reach the concentrations tested in the first experiment for an additional 30 min. Sedation, anesthesia, and recovery times were observed in both behavioral experiments. Gills and gonads of mussels exposed to EOL presented increased catalase, glutathione-*S*-transferase (GST) and superoxide dismutase activities, except for GST in the gonads, which remained unchanged, while lipoperoxidation and protein carbonyl values decreased. The EOL did not produce a satisfactory anesthetic effect, taking into account the shell closing as animal behavior observed in the face the EOL. However, the addition of EOL, and consequently, the multifunctional hypoxia by closing the valves, improved the response to oxidative stress and may be useful in handling *P. perna*.

Key words: bivalve, cidreira herb, lipid peroxidation, antioxidants, carbohydrate metabolism.

1. Introduction

Due to the growth in world aquaculture, most consumed species have seen a reduction in price and a strong increase in commercialization. The world production of mollusks in 2012 was 15.2 million tons (1.8 million tons of mussels alone), with China being the largest producer and Brazil, the 12th, but second in Latin America, surpassed only by Chile (FAO, 2014). Among the different species of mussels, *Perna perna* (LINNAEUS 1758) grows relatively fast, has a high growth rate and nutritive value, and is easily collected (Baraj et al., 2003).

The use of anesthetics allows minimal damage and reduces stress and mortality in bivalves during experimental manipulation, such as the collection of biological samples, assessment of reproductive status, etc. (Culloty and Mulcahy, 1992; Lellis et al., 2000). However, when bivalves detect unknown or toxic particles in the water, the first possible defense is to close the valves and reduce filtration to minimize contact with the tissues (Gainey and Shumway, 1988).

Due to the difficulties of acquiring and high cost synthetic anesthetics, the use of plant-derived essential oils has been proven to be a viable alternative (Palic et al., 2006; Silva et al., 2012; Souza et al., 2012). A good example is the essential oil of *Lippia alba* ((Mill.) BROWN 1925) (Verbenaceae) (EOL), a plant widely distributed in Brazil and commonly known as cidreira herb, which has sedative

and anesthetic efficacy (Cunha et al., 2010, 2011; Toni et al., 2014). EOL also has antioxidant activity in fish (Azambuja et al., 2011; Salbego et al., 2014) and shrimp (Parodi et al., 2012).

Anesthesia of bivalves is not developed as fish anesthesia, but it is essential for management in aquaculture and research stations. Hence, the objective of this study was to evaluate, the effects of EOL in the sedation and anesthesia, on behavioral, metabolic and oxidative stress parameters in *P. perna*.

2. Material and methods

2.1 Acquisition of animals and acclimation

P. perna mussels were collected from Itapoã Beach, Vila Velha, Espírito Santo state, southeast Brazil, and transferred to the Laboratory of Applied Ichthyology at the University of Vila Velha (UVV). The animals were acclimated individually in continuously aerated polyethylene aquaria (without substrate) for five days. Water quality was monitored for temperature, conductivity, and salinity using the YSI conductivity (EC 300, Yellow Springs Inc., OH, USA), dissolved oxygen saturation with a YSI oximeter (OD200), pH with a pH meter YSI (pH 100), and alkalinity and total ammonia (indophenol method) following APHA, (1998). Animal collections from the environment were conducted with authorization from the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (nº 33571) (Anexo D). The methodology of this experiment was approved by the Ethical Committee on the Use of Animals of the University of Vila Velha (Process no 218/2012) (Anexo E).

2.2 Plant material

L. alba was cultivated in Frederico Westphalen, Rio Grande do Sul state, southern Brazil. The plant material was identified by botanist Dr. Gilberto Dolejal Zanetti (Department of Industrial Pharmacy, UFSM). A voucher specimen (SMDB n. 10050) was deposited in the herbarium of the Department of Biology (UFSM). The EOL was obtained from fresh plant leaves by steam distillation for 2 h using a Clevenger type apparatus (European Pharmacopeia, 2007) and stored at -20 °C until composition analysis and biological assays.

2.3 Essential oil analysis

The analysis was carried out on an Agilent 7890A GC coupled to a 5975C mass spectrometer using a non-polar HP5-MS fused silica capillary column (5 % phenyl – 95 % methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 mm film thickness) and EI-MS of 70 eV. The operating conditions were: carrier gas: He; flow rate: 1 mL min^{-1}; split inlet: 1:100; injector and detector temperature: 250 ºC; analysis program: 40 ºC for 4 min and 40 ºC - 320 ºC at 4 ºC min^{-1}. The constituents of the EO were identified by comparison of the mass spectra and Kovats retention index with the literature and a mass spectral data bank (NIST, 2008; Adams, 2009).

Quantitative analysis was performed on a Agilent 7890A gas chromatograph equipped with a flame ionization detector using a non-polar HP-5 fused silica capillary column (5 % phenyl – 95 % methylsiloxane 30 m x 0.25 mm i.d. x 0.25 µm film thickness). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1} in splitless mode. Both injector and detector temperatures were set at 300 ºC. A sample of EOL was injected in triplicate, and the analysis program corresponded to that described for CG-MS.

2.4 Experimental procedures

Before use, EOL was diluted 1:10 in 99.5 % ethanol. The anesthetic was placed in the water where the mussels were already acclimated, and diluted by smooth movements with the aid of a glass rod. The anesthetic was added to the water only if animals presented their natural behavior with semi-open valves, closed only by the edge of the mantle. Experiments were also performed using aquaria containing only ethanol at a concentration equivalent to the dilution used at the highest anesthetic concentration tested. Control groups consisted of animals submitted to the same procedure, but in anesthetic-free water. To evaluate the time required for anesthesia induction, 10 animals, each placed in individual aquaria with 1 L of water, were used for each concentration tested, and each one was used only once to observe the different anesthesia stages following criteria adapted from Lellis et al. (2000) (Table 1). The mussels were considered sedated when they showed semi-open valves (with the edge of the mantle open) and responded to touch by closing the valves and anesthetized when they had wide open valves (also with the edge of the mantle open) and did not respond to touch. Mussels were considered recovered when they had the ability to respond to touch by closing the valves. Upon detection of anesthesia or after a predetermined period of 1 h of exposure to EOL, animals were rinsed in clean water and individually transferred to continuously aerated 4 L aquaria containing 2 L of water to measure recovery time.

2.5 Experiment 1 - Single application of the anesthetic

Fifty mussels (30 males and 20 females, weight: 14.39 ± 0.47 g; length: 52.23 ± 0.58 mm; height: 24.83 ± 0.35 mm and width: 19.24 ± 0.25 mm) were used. In this study, mussels of both sexes were used, to rule out any possible effect resulting from sex-based differences. Preliminary tests were performed with 20, 30, 50, 80, 100, 120, 150, 180, 200, 250, 300, 400, 450, 500, 600, 700, 800, 900, 1000, 1200, 2000, and 2500 µL L^{-1} EOL to choose the concentrations to be used in the experiment. Three concentrations were chosen: 150, 300, and 450 µL L^{-1}, since, lower concentrations than the 150 µL L^{-1} remained normal behavior within 4 hours of exposure and concentrations above 450 µL L^{-1} no differences in behavior with respect to the chosen concentrations.

In this experiment, the water parameters were: temperature: 23.89 ± 0.09 ºC; conductivity: 49.90 ± 1.66 mS cm^{-1}; salinity: 34.70 ± 0.17 ppt; dissolved oxygen: 6.76 ± 0.09 mg L^{-1} (78.48 ± 0.99 %

saturation); pH: 7.93 ± 0.04; alkalinity: 127.35 ± 3.50 mg $CaCO_3$ L^{-1}; and total ammonia: 0.02 ± 0.01 mg L^{-1}.

2.6 Experiment 2 - Gradual increase in the concentration of anesthetic

Fifty mussels (weight: 12.00 ± 0.80 g; length: 48.76 ± 0.80 mm; height: 23.69 ± 0.52 mm and width: 17.26 ± 0.47 mm), including 30 males and 20 females, were used. Animals exposed to high concentrations of EOL (above 200 μL L^{-1}) in the previous tests closed their valves on first contact with the anesthetic. Therefore, in this experiment, the mussels were initially exposed to 100 μL L^{-1} for 30 min (during which the animals did not show this behavior). Then, more anesthetic (50, 200, or 350 μL L^{-1}) was added to achieve the same concentrations as those tested in the first experiment (150, 300, and 450 μL L^{-1}, respectively) for an additional 30 min, totaling 1 h of exposure, as in the first experiment. The ethanol group was subjected to the same procedure: the animals were initially exposed to 900 μL L^{-1} ethanol (the concentration used for dilution of 100 μL L^{-1} anesthetic) and after 30 min, more ethanol was added (3150 μL L^{-1}) to reach the concentration of ethanol (4050 μL L^{-1}) used to dilute the highest concentration of the anesthetic. In the control group, the animals remained in the aquaria for 1 h and then were transferred to the recovery aquaria where they remained for 30 min. Mussels that did not reach the stage of anesthesia were also placed in the recovery aquaria for 30 min. The water parameters were also monitored (temperature: 24.56 ± 0.10 ºC; conductivity: 54.31 ± 0.13 mS cm^{-1}; salinity: 36.30 ± 0.12 ppt; dissolved oxygen: 6.04 ± 0.07 mg L^{-1} (71.00 ± 0.84 % saturation); pH: 7.73 ± 0.06; alkalinity: 120.12 ± 4.30 mg $CaCO_3$ L^{-1}; and total ammonia: 0.11 ± 0.01 mg L^{-1}). In both experiments, the length, height, and width of the animals were measured with a digital Caliper (Maxwell) and weighed with a precision balance Adventurer Pro (Ohaus), after recovery (n = 5 in each group). Five other animals did not go through the recovery process. Subsequently, the ten animals were killed by hypothermia (20 min submerged in ice), and hemolymph was collected directly from the adductor muscle using disposable syringes. The mussels were then opened by muscle section with a scalpel inserted between the valves. The sex of each animal was defined and the gonad and gills were collected and stored at -80 ºC, as well as the hemolymph, for analysis of metabolic and oxidative stress parameters.

2.7 Analysis of metabolic parameters

Tissues (gills and hemolymph) were dissolved in an equal volume of 20 % TCA using a Potter–Elvehjem homogenizer. The acid homogenate was centrifuged for 10 min at 10,000 *g* and the supernatant was used for metabolic determinations. Glycogen was determined using the method described by Dubois et al., (1956) after addition of KOH and ethanol (1 mL and 3 mL, respectively) for precipitation of glycogen. Homogenate was used to estimate protein levels according to Lowry et al., (1951), lactate by Harrower and Brown, (1972), glucose by Dubois et al., (1956). Glycogen and

lactate values were analyzed in the gills and hemolymph, while in the hemolymph, glucose and protein levels were also evaluated.

2.8 Analysis of oxidative stress parameters

Tris-Hepes buffer (20 mM) was used for homogenizing the tissues 1:5 (v/v). Catalase (CAT) activity was measured in the gonad and gills according to Nelson and Kiesow, (1972) and was expressed in U mg protein^{-1}. The activity of glutathione-*S*-transferase (GST) was determined in the gonad and gills according to the method described by Habig et al., (1974) and was expressed in U mg protein^{-1}. The activity of superoxide dismutase (SOD) was determined in the gonad and gills according to the method of Misra and Fridovich, (1972) and the results were expressed as SOD U mg protein^{-1}. One unit of SOD is defined as the amount of enzyme that inhibits the rate of formation of adenochrome by 50 %.

The level of lipid peroxidation (LPO) was determined in the gonad and gills through the malondialdehyde (MDA) reaction with thiobarbituric acid (TBA) according to Buege and Aust, (1978). The amount of lipid peroxidation was expressed as nmol of MDA mg protein^{-1}.

The tissues (gill and gonad) were homogenized in 10 volumes (w/v) of 10 mM Tris-HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl (PC) content was assayed by the method described by Yan et al., (1995), with some modifications. Soluble protein (0.5 mL) was reacted with 10 mM DNPH in 2 N hydrochloric acid. After incubation at room temperature for 1 h in the dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate, pH 6.8, containing SDS 3.0 %), 2.0 mL of heptane (99.5 %) and 2.0 mL of ethanol (99.8 %) were added sequentially, vortexed for 40 s and centrifuged at 10,000 *g* for 15 min. The protein extracted from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1) and suspended in 1 mL of denaturing buffer. The carbonyl content was then measured spectrophotometrically at 370 nm. The total carbonylation was calculated using a molar extinction coefficient of 22,000 M cm^{-1}. The protein carbonyl content was expressed in nmol of protein carbonyl mg protein^{-1}.

2.9 Statistical analysis

All data are presented as mean ± SEM. Homogeneity of variances among treatments was tested by Levene's test. Data exhibited homogeneous variances, so comparisons between different treatments were made using two-way ANOVA and Tukey's test. Analysis was performed using GraphPad Prism 6 and the minimum significance level was set at $p = 0.05$.

3. Results

3.1 Chemical composition of EOL

Of the total chemical composition of EOL, 98.68 % was identified. Among the 30 detected constituents, 18 were monoterpenoids (peaks 1-18, 86.45 %) and 12 were sesquiterpenoids (peaks 19-30, 12.23 %). The main component of EOL was determined to be linalool (48.69 %), followed by eucalyptol (10.51 %) and β-myrcene (9.74 %) (Table 2).

3.2 Unique application of EOL

Ethanol did not induce any stage of sedation or anesthesia in any of the tested animals. The mussels of this group and the control retained their normal behavior (semi-open valves, closed only by the edge of the mantle) in the recovery aquaria. Animals exposed to the lowest EOL concentrations (below 150 μL L^{-1}) in the preliminary tests remained with their valves semi-open (their natural behavior) and did not reach sedation or anesthesia. Mussels subjected to EOL at concentrations of 150 μL L^{-1} or higher closed their valves at first contact with the anesthetic and only opened them when mussels placed in clean water for recovery. Only 10% of animals exposed to 150 μL L^{-1} EOL reached sedation and anesthesia was not observed at any concentration tested. One mussel exposed to 150 μL L^{-1} EOL not even reaching sedation or anesthesia kept its valves fully closed for a long time during recovery (Table 3).

3.3 Gradual increase in the concentration of EOL

Animals in the control and ethanol groups did not show any stage of sedation or anesthesia and retained their normal behavior in the recovery aquaria, as in the first experiment. The concentration increase to 300 μL L^{-1} EOL led to sedation in 10 % of animals, but the other tested concentrations did not induce any stage of anesthesia. As in the first experiment, two animals exposed to 150 μL L^{-1} EOL not even reaching sedation or anesthesia kept their valves fully closed for a long time through recovery (Table 4). Most animals closed the shells after adding the anesthetic in the initial concentration, same before to supplement the final concentration of EOL. There was no mortality in either series of experiments.

3.4 Metabolic parameters

Exposure to all EOL concentrations (both unique application and gradual increase of concentration) increased glycogen levels in the gills and reduced them in the hemolymph compared with control and ethanol groups. The largest glycogen changes were observed in the hemolymph of mussels exposed to the higher EOL concentrations (300 and 450 μL L^{-1}). Glycogen levels in the hemolymph returned to control values in the mussels exposed to these higher concentrations, after recovery. These levels decreased significantly in the gills of recovered mussels, but were still higher than those of control and ethanol groups (Table 5).

Lactate decreased in the gills and hemolymph of mussels exposed to all EOL concentrations (both single application and gradual increase in concentration) compared with control and ethanol groups.

However, mussels exposed to a single application of ethanol presented higher lactate levels in the hemolymph when compared with controls. Lactate levels increased in the gills of recovered mussels when compared with exposed animals irrespective of treatment, but those exposed to all EOL concentrations presented higher values. Lactate levels in the hemolymph also increased in mussels that recovered from exposure to a gradual increase in concentration to 150 and 300 μL L^{-1} EOL, but these values remained lower than in mussels that recovered from control and ethanol treatments. Mussels exposed to all other EOL treatments kept lactate levels in the hemolymph lower than control and ethanol groups. Mussels exposed to a single application of ethanol returned lactate levels in the hemolymph to control values after recovery (Table 5).

Glucose values in the hemolymph of mussels exposed to all EOL concentrations (both single application and gradual increase in concentration) were lower than in control and ethanol-exposed mussels. After recovery, glucose values were reduced in all groups and remained lower in those that were exposed to all EOL concentrations (Figures 1A and 1C). The protein values in the hemolymph of animals exposed to a single application of 300 μL L^{-1} EOL were significantly higher than in those exposed to ethanol. The gradual increase in the EOL concentration did not change protein levels. Protein values were lower in control animals after recovery than in those exposed to a single concentration. Mussels recovered from exposure to single and gradual increases in concentration to 300 and 450 μL L^{-1} EOL increased protein levels, but all recovered EOL-exposed mussels showed higher protein levels than recovered control animals (and compared with ethanol-exposed mussels in those exposed to unique and gradual increase of EOL concentration) (Figure 1B and 1D).

3.5 Oxidative stress parameters

The effects of EOL on oxidative stress parameters were similar in mussels exposed to a unique application of anesthetic and to a gradual increase in concentration. The activities of CAT and SOD were higher in gills and gonads of mussels exposed to and recovered from all EOL concentrations compared with control and ethanol treatments. Recovered animals in all treatment groups presented lower activities of these enzymes than exposed ones, except in the gonads of control and ethanol groups (Figures 2 and 3). Significantly higher GST values were observed in the gills of mussels exposed to all EOL concentrations compared with control and ethanol groups, and when compared with recovered animals. Recovered mussels did not show any significant effect of treatment on GST levels, but control and ethanol groups obtained significantly higher values compared with the same treatments in exposed animals (Figures 4A and 4C). The GST values in the gonads of mussels exposed to all treatments were significantly lower relative to the recovered animals, but no significant difference was observed between the treatments at either sampling time (exposed or recovered) (Figures 4B and 4D).

Lipoperoxidation and PC were significantly lower in the gills and gonads of mussels exposed to all EOL concentrations than in the control and ethanol groups at both sampling times (exposed or recovered). Mussels exposed to EOL showed significantly lower values than did recovered animals (Figures 5 and 6). In the control and ethanol groups, significantly higher LPO and PC values were obtained for the gills of exposed animals than for the recovered ones in the same treatments, except for LPO in the gills of animals that received a single application (Figures 5A, 5C, 6A and 6C).

4. Discussion

To identify the chemotype is considered the major compound, with concentrations above 10% (Jannuzzi et al., 2011). Thus, in our studies, *Lippia alba* belongs to chemotype Linalool (48.69 %). Some monoterpene of the essential oils, as linalool, possess local anesthetic activity (Ghelardini et al., 1999; Galeotti et al., 2001), which could be responsible, at least in part, to their muscle relaxant properties (Ghelardini et al., 2001). It is believed that the linalool possesses a number of pharmacological properties, including anticonvulsant, anxiolytic, anti-inflammatory, antinociceptive (Kamatou and Viljoen, 2008).

EOL at concentrations lower than 150 µL L^{-1} did not induce any behavioral alterations in animals, but at higher EOL concentrations, *P. perna* closed their valves immediately when EOL was added to the water and opened them only after a relatively long recovery time. Aquilina and Roberts, (2000) demonstrated that the abalone *Haliotis iris* (GMELIN 1791) exposed to 2500 µL L^{-1} phenoxytol propylene presented excessive mucus secretion and considerable loss of pigment in the foot. Also in the same study, the anesthetic action of benzocaine may have been partially caused by the ethanol used to dilute this anesthetic (Aquilina and Roberts, 2000). In the present study, *P. perna* from control and ethanol groups retained their normal behavior (semi-open valves, closed only by the edge of the mantle), contrary to Noble et al., (2009), who observed that *Dicathais orbita* (GMELIN 1791) closed their valves upon exposure to ethanol (4050 µL L^{-1}). In view of this, the current result is very satisfactory, since it was observed that ethanol is very efficient as a vehicle of administration of EOL, without causing any change to the behavior of animals.

There is considerable variation in the response to anesthetics among the mollusk an species tested (Lellis et al., 2000; Acosta-Salmón et al., 2005; Noble et al., 2009; Suquet et al., 2010; Torres-Martinez et al., 2012). Thus, successful use in a species does not indicate a positive response in others and products must be tested at different concentrations (Norton et al., 1996).

Despite the positive results achieved with EOL in inducing anesthesia and sedation in fish (Cunha et al., 2010, 2011; Becker et al., 2012; Heldwein et al., 2012; Salbego et al., 2014; Toni et al., 2014) and white shrimp *Litopenaeus vannamei* (BOONE 1931) (Parodi et al., 2012), this oil showed no efficacy as an anesthetic in *P. perna* at any of the concentrations tested. Only 10 % sedation was observed in

328 mussels exposed directly to 150 μL L^{-1} and when added gradually to the water up to 300 μL L^{-1}.
329 Similar to the present study, exposure of *Elliptio complanata* (LIGHTFOOT 1786) and *Nodipecten*
330 *subnodosus* (SOWERBY 1835) to menthol (125-500 mg L^{-1} and 250 mg L^{-1}, respectively) did not
331 achieve anesthesia within 3 h of exposure (Lellis et al., 2000; Torres-Martinez et al., 2012).
332 The distribution of contaminants and/or xenobiotics in the organs of mussels varies due to differences
333 in the contact surfaces, affinities, and rates of accumulation and excretion among tissues (Yap et al.,
334 2008; Verlecar et al., 2008). In this context, the gills represent the first line of contact (Livingstone et
335 al., 1992; Almeida et al., 2005) and their high sensitivity is the result of direct exposure to oxygen
336 during breathing (Cossu et al., 1997). The gills reflect the state of the aquatic environment and
337 changes in antioxidant enzyme activities do not depend on the physiological state as they do in the
338 gonads. On the other hand, the antioxidant complex of the gonads is affected not only by the
339 environment but also by certain internal factors (e.g. spawning) (Vidal-Linan et al., 2010).
340 Physiological stress detection in invertebrates such as clams is difficult because their endocrine organs
341 are not clearly differentiated. Thus, the gills and gonads are important organs in bivalves and were
342 chosen to investigate the mechanisms of antioxidant defense relative to exposure to EOL.
343 Glycogen is an important energy reserve for most animal species (Greenberg et al., 2006) and from its
344 degradation, glucose becomes readily available to the tissues when required (Berthelin et al., 2000;
345 Zeng et al., 2013). In bivalves, glucose is essential for gamete development and tissue maintenance
346 (Mathieu and Lubet, 1993; Giacomin et al., 2014).
347 The closure of the shell and concomitant reduction of the oxygen consumption rate can suggest that
348 possibly less energy being used for breathing and feeding activities (Kim et al., 2001), thus justifying
349 why glycogen values were higher in the gills of *P. perna* exposed to EOL compared with controls and
350 reduced after recovery. The results showed that these bivalves mobilized stored energy (glycogen) and
351 may use protein breakdown to deal with the exposure to EOL. The glycogen values in the hemolymph
352 were significantly lower in animals exposed to EOL and these values increased after recovery, the
353 reverse of what happened in the gills. The results of the present study show that an energy reserve as
354 important as glycogen (Mouneyrac et al., 2008) was mobilized to maintain homeostasis (Smolders et
355 al., 2004; Voets et al., 2006) during exposure to EOL. This is in agreement with previous studies
356 showing that a reduction in glycogen in the hemolymph occurs in many aquatic organisms exposed to
357 environmental change (Hummel et al., 1996).
358 Stressed bivalves increase glucose levels in the tissues (Yusufzai et al., 2010). Apparently, exposure to
359 EOL reduced stress in *P. perna* and consequently, glucose is decreased in the hemolymph. In
360 agreement with this hypothesis, in mussels exposed to EOL, the glycogen decreased in the hemolymph
361 because it was stored in the gills (where it increased). This pattern was maintained even after recovery.
362 During air exposure (as when valves are closed) pyruvate kinase and lactate dehydrogenase activities
363 increased in *Mytilus galloprovincialis* (LAMARCK 1819), increasing lactate production (Lushchak et

al., 1997). In *P. perna* exposed to EOL, less lactate was produced in the gills and hemolymph, suggest the activation of routes alternatives in the energy production. After recovery, glucose levels decreased in all groups compared with exposed ones, but glycogen was reduced (compared with exposed ones) only in those exposed to EOL, in which there was a higher lactate increase in the gills. Apparently, after recovery, lactate was used as a source of energy for maintaining the general supply of free energy as ATP.

Protein values in tissues have been widely used as an animal health index, and are even suggested as a reliable biomarker for stress in mussels following exposure to metals or other chemicals (Pytharopoulou et al., 2006; Vijayavel et al., 2009; Almeida et al., 2014). A decrease in total protein levels can be attributed to a general decrease in metabolic activity (Baudrimont et al., 1997; Geret et al., 2003). Protein in hemolymph has an important role in hemocyte stability, oxygen transport and integrity of the cell (Vijayavel et al., 2005). There were no significant changes in the hemolymph protein levels in *P. perna* exposed to EOL compared with the control. However, animals that have undergone recovery from exposure to 300 and 450 μL L^{-1} EOL presented significantly higher values of protein in the hemolymph.

CAT is the primary defense against oxidative damage, and is widely used as a biomarker in bivalves (Romeo et al., 2003). In this study, there was an increase in CAT and SOD activity in the gills and gonads of animals exposed to EOL, demonstrating that in these tissues, an adjustment to EOL exposure occurred. Such enzymes are important components of different detoxification pathways, antioxidants and tolerance to stress (Parodi et al., 2012). Azambuja et al., (2011) also observed an increase in CAT activity in silver catfish (*Rhamdia quelen*, QUOY AND GAIMARD 1824) transported with EOL added to the water. *P. perna* exposed to air for 4 h increased SOD activity and 1 h after reoxygenation, this activity decreased to baseline values (Almeida et al., 2005). The decreased CAT and SOD activity of mussels after recovery (but not enough to reach control levels) suggest that oxidative stress likely decreased.

Comparison of the antioxidant defense system between the bivalve tissues presents some discrepancies between species (Almeida et al., 2005). In this study, the GST activity values present in the gills were higher than in the gonads of *P. perna*. This was likely related to the gills being a more active site than the gonads because of their direct contact with the environment and exposure to oxygen due to respiratory function, providing the removal of unwanted compounds from the body (Almeida et al., 2005). Increased GST in gills compared with other tissues, such as the digestive gland, was also found in *P. perna* mussels exposed to air (Almeida et al., 2005).

In the current study, the GST activity in the gills increased with exposure to EOL and in recovered animals the values decreased, returning to values significantly similar to those of control mussels. This result is in accordance with a study by Parodi et al., (2012), where the hemolymph of shrimp (*L. vannamei*) displayed increased GST values when exposed to EO of *Aloysia triphylla* ((L'Hér.) BRITTON 1925), EOL, and eugenol, which may be a response designed to mitigate the toxic effects

of these anesthetics or to neutralize the damaging effects of free radicals generated directly or indirectly by them.

The *P. perna* tested in this study decreased LPO and PC when exposed to EOL compared with the ethanol-exposed and control mussels. The levels of these parameters returned to near control values similar after recovery from almost all EOL concentrations. Apparently, EOL is effective in combating the oxidative stress induced by closure of the valves, because Almeida et al., (2005) verified that *P. perna* exposed to air (valves closed) increased TBARS (or LPO).

The EOL did not produce a satisfactory anesthetic effect, taking into account the shell closing as animal welfare behavior in the face of a xenobiotic. On the other hand, exposure to EOL had a positive effect because increased activity of the antioxidant enzymes CAT, GST, and SOD decreased LPO and PC and were not lethal. This may suggest that the EOL and consequently, the multifunctional hypoxia by closing the valves, improved the response of antioxidant defenses and reduced oxidative stress, and could be useful in handling *P. perna*.

Acknowledgments. L.N. Simões received Ph.D. fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). B. Baldisserotto, Vania Loro and L.C. Gomes received research fellowships from CNPq.

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596

597 **Legends**

598

599 **Figure 1. Glucose and protein levels in the hemolymph of *Perna perna* exposed to a single (A and**
600 **B, respectively) and gradual increase (C and D, respectively) in concentration of the essential oil**
601 **of *Lippia alba*.** Different letters indicate significant differences between treatments at the same
602 sampling times (exposed or recovered). *Indicates significant difference from exposed mussels ($p <$
603 0.05). Results are expressed as mean ± SEM.

604

605 **Figure 2. Activities of catalase (CAT) in gills and gonads of *Perna perna* in response to a single**
606 **(A and B, respectively) and gradual increase (C and D, respectively) in concentration of the**
607 **essential oil of *Lippia alba*.** Different letters indicate significant differences between treatments at the
608 same sampling times (exposed or recovered). *Indicates significant difference from exposed mussels
609 ($p < 0.05$). Results are expressed as mean ± SEM.

610

611 **Figure 3. Activities of superoxide dismutase (SOD) in gills and gonads of *Perna perna* to a single**
612 **(A and B, respectively) and gradual increase (C and D, respectively) in concentration of the**
613 **essential oil of *Lippia alba*.** Different letters indicate significant differences between treatments at the
614 same sampling times (exposed or recovered). *Indicates significant difference from exposed mussels
615 ($p < 0.05$). Results are expressed as mean ± SEM.

616

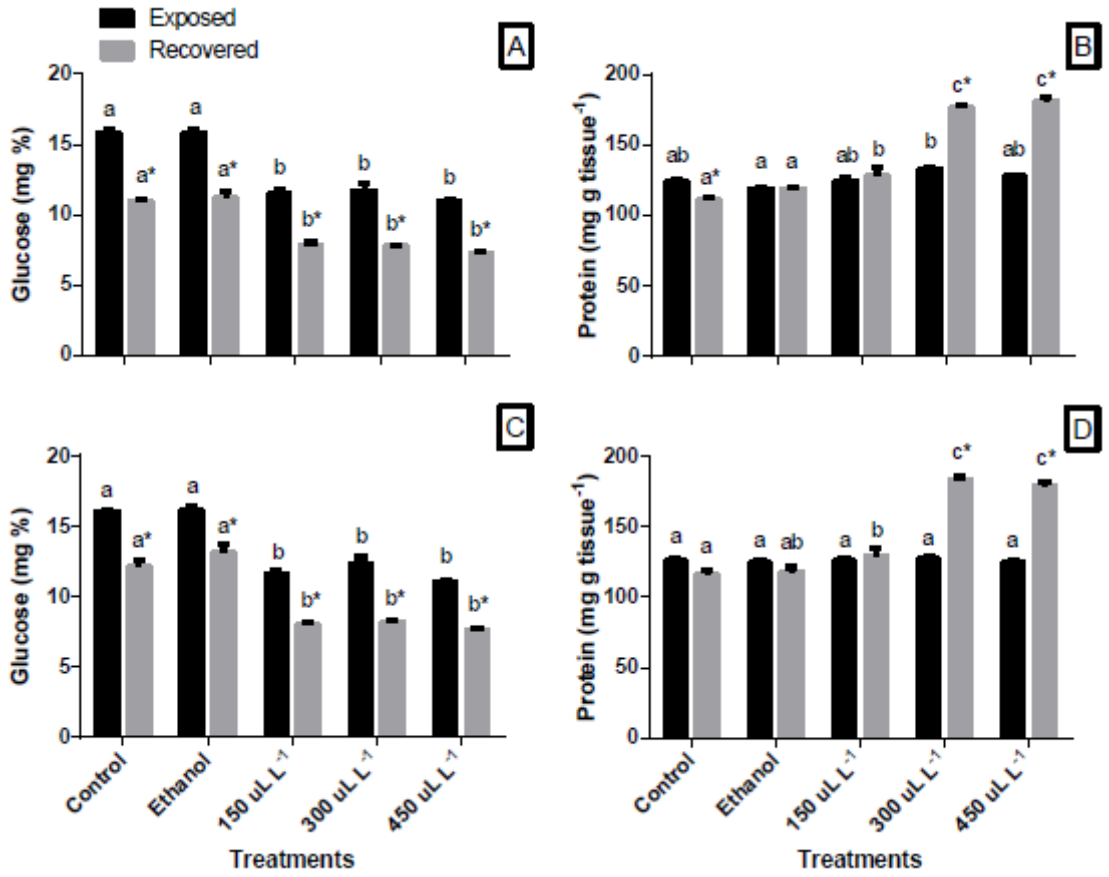
617 **Figure 4. Activities of glutathione-*S*-transferase (GST) in gills and gonads of *Perna perna* in**
618 **response to a single (A and B, respectively) and gradual increase (C and D, respectively) in**
619 **concentration of the essential oil of *Lippia alba*.** Different letters indicate significant differences
620 between treatments at the same sampling times (exposed or recovered). *Indicates significant
621 difference from exposed mussels ($p < 0.05$). Results are expressed as mean ± SEM.

622

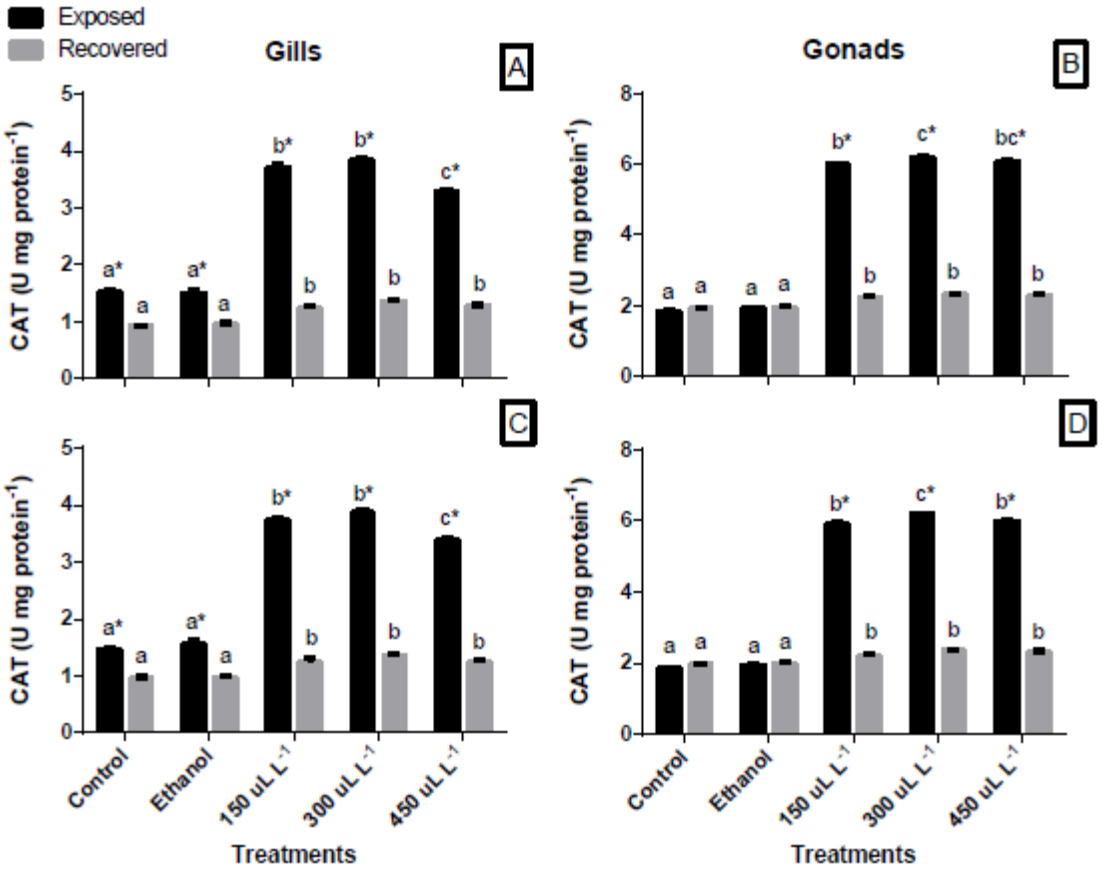
Figure 5. Lipid peroxidation (LPO) in gills and gonads of *Perna perna* in response to a single (A and B, respectively) and gradual increase (C and D, respectively) in concentration of the essential oil of *Lippia alba*. Different letters indicate significant differences between treatments at the same sampling times (exposed or recovered). *Indicates significant difference from exposed mussels ($p < 0.05$). Results are expressed as mean ± SEM.

Figure 6. Protein carbonyl (PC) in gills and gonads of *Perna perna* in response to a single (A and B, respectively) and gradual increase (C and D, respectively) in concentration of the essential oil of *Lippia alba*. Different letters indicate significant differences between treatments at the same sampling times (exposed or recovered). *Indicates significant difference from exposed mussels ($p < 0.05$). Results are expressed as mean ± SEM.

Figure 1


Exposed
Recovered
A
B
C
D
Glucose (mg %)
Protein (mg g tissue-1)
Control
Ethanol
150 uL L-1
300 uL L-1
450 uL L-1
Treatments

654 Figure 2



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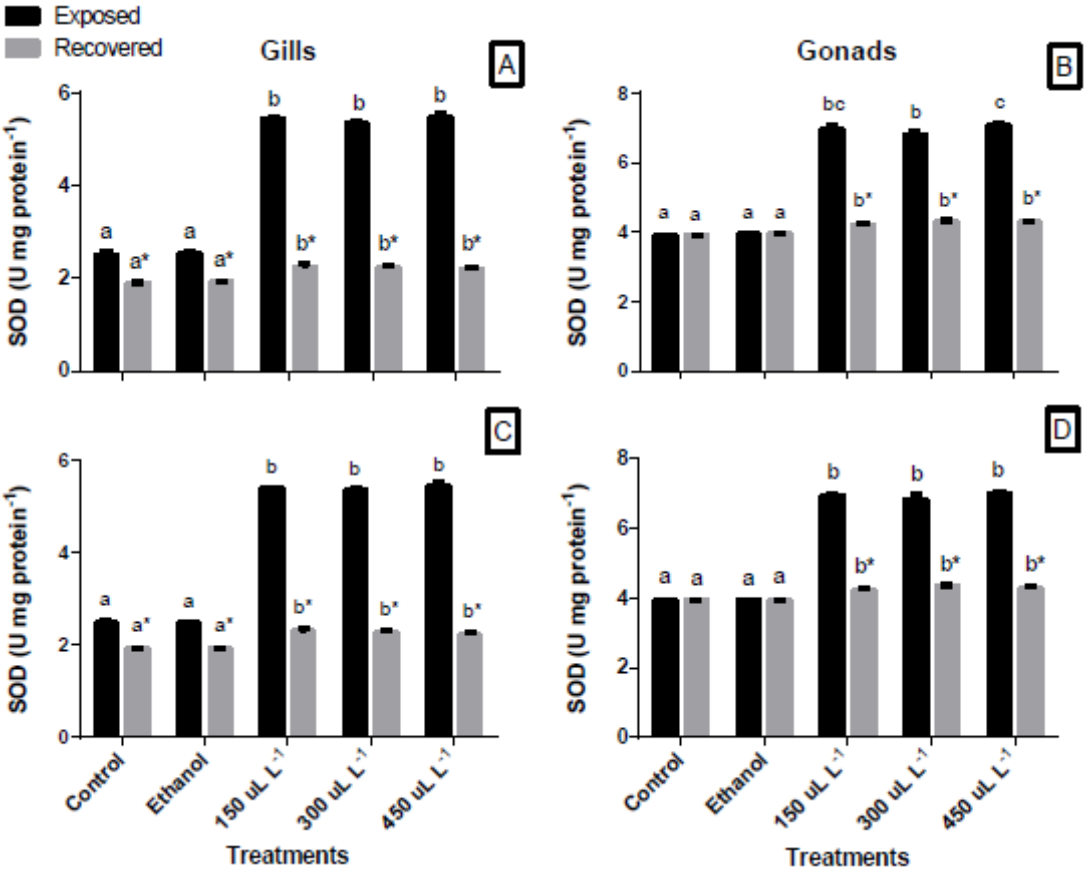
673 Figure 3



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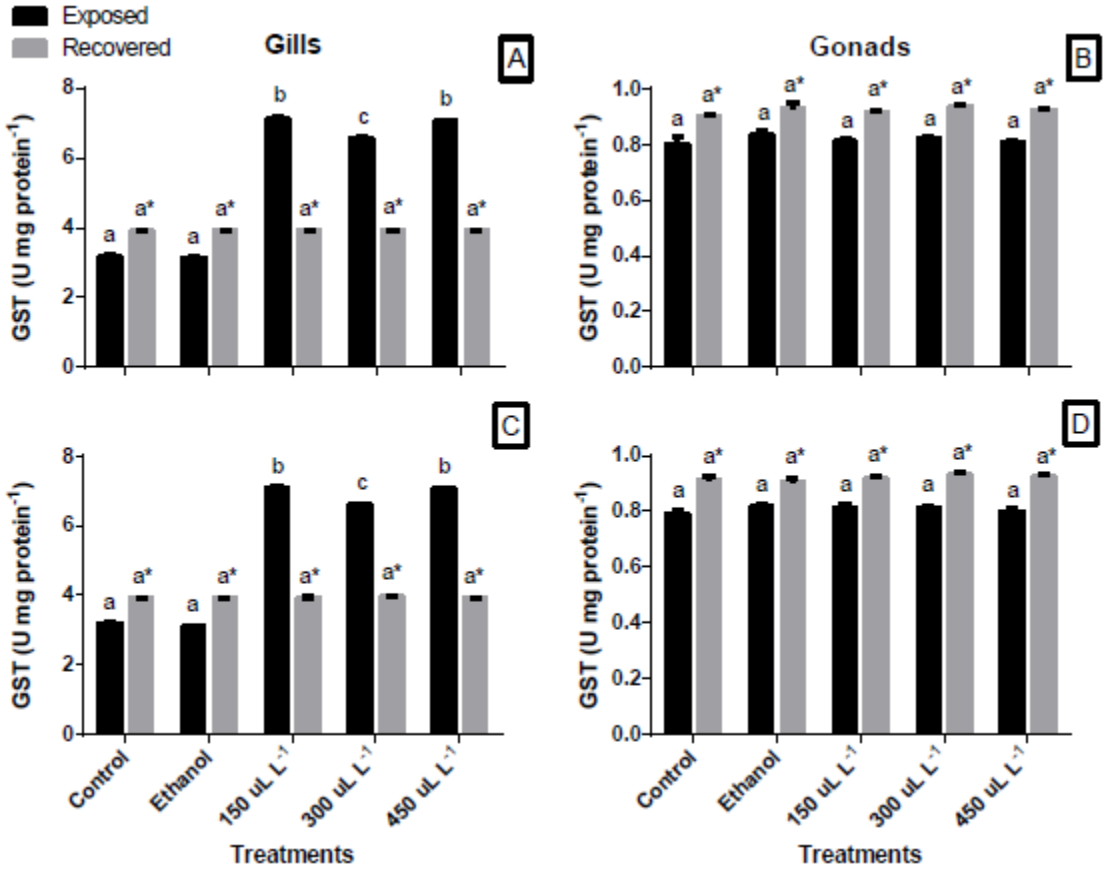
692 Figure 4



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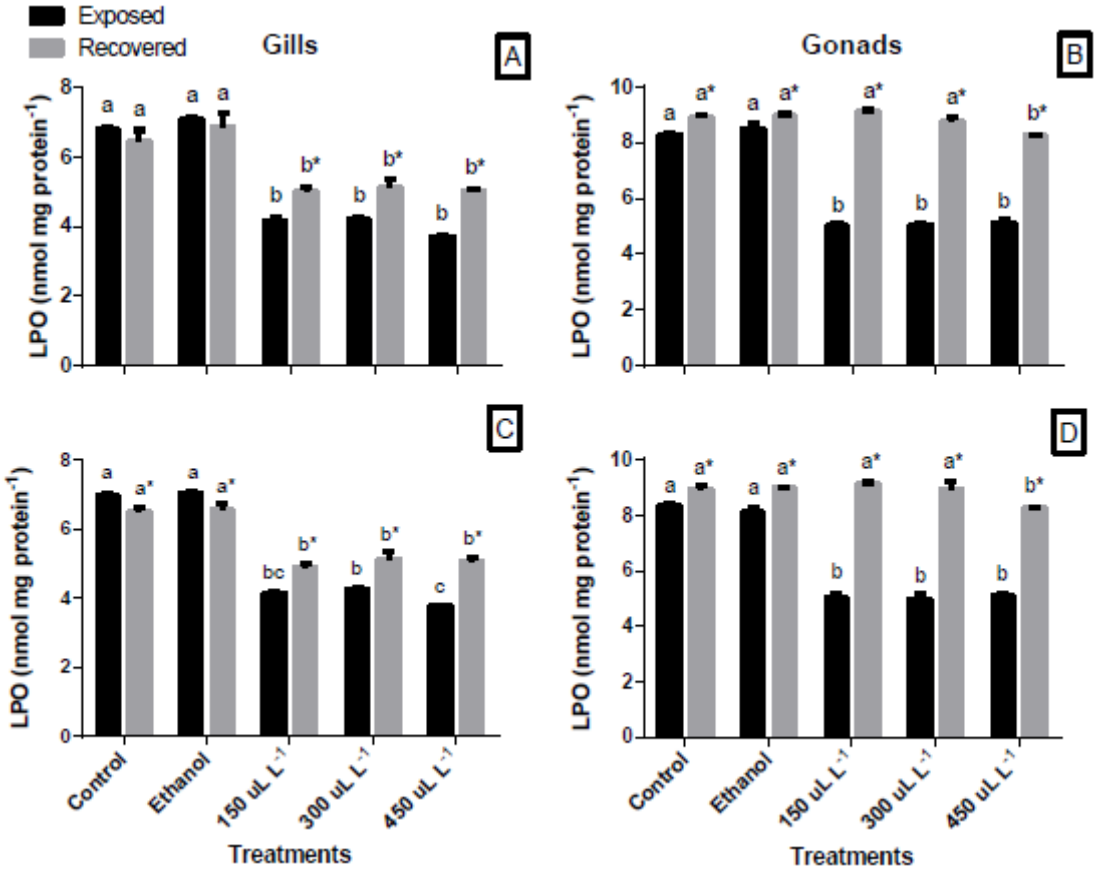
711 Figure 5



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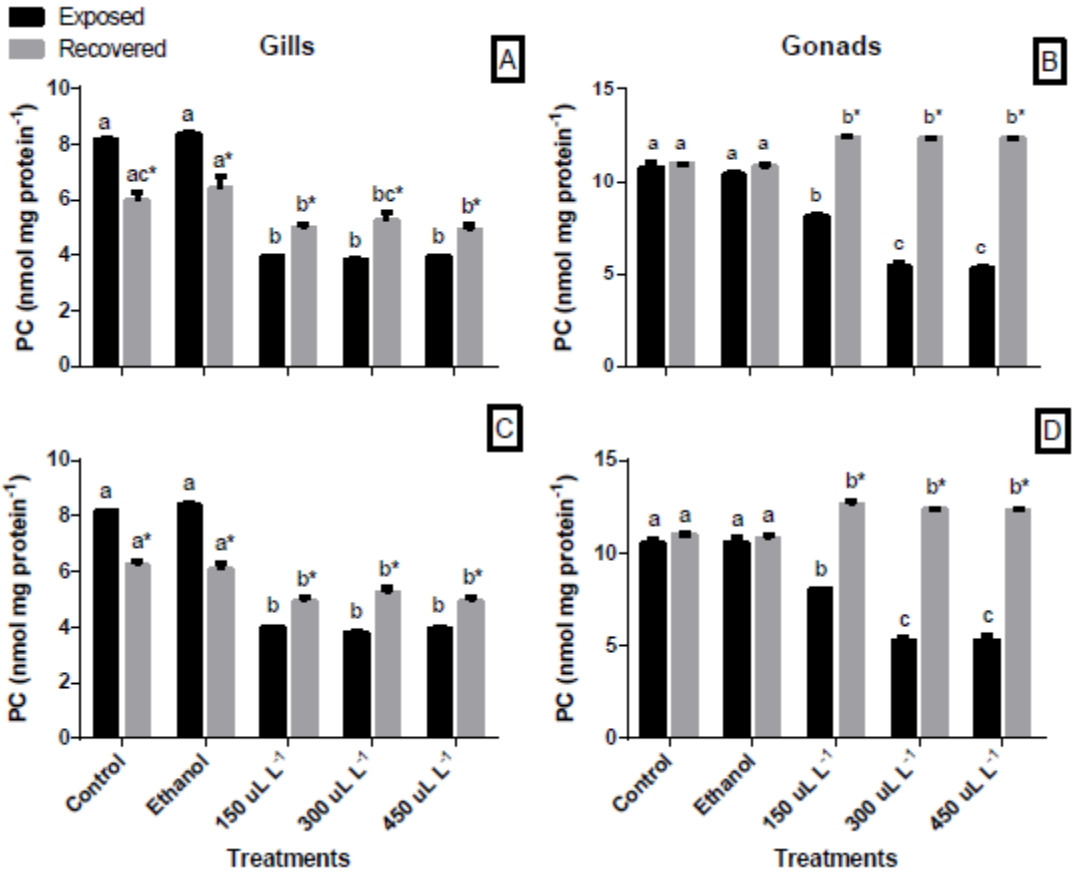
730 Figure 6



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Tables

Table 1. Stages of anesthesia in mussel *Perna perna*, adapted from Lellis et al. (2000).

Stages of anesthesia	Behavioral response
Stage I	Semi-open valves, closed only by the edge of the mantle (natural behavior)
Stage II	Semi-open valves, edge of the mantle open and responded to touch by closing the valves (sedation)
Stage III	Open valves and did not respond to touch (anesthesia)
Recovery	Ability to respond to touch by closing the valves

Table 2. Chemical components of the essential oil of *Lippia alba*. RT: Retention time; RI Calc: Calculated retention index; RI Lit: Literature retention index (NIST, 2008; Adams, 2009).

Peak	RT	Compound	RI Calc	RI Lit	%
1	10.37	α-pinene	931	932	0.52
2	11.91	5-hepten-2one-4,6-dimethyl	969	973	1.32
3	11.99	sabinene	971	973	0.30
4	12.08	β-pinene	974	975	0.61
5	12.79	β-myrcene	991	992	9.74
6	14.20	limonene	1027	1028	0.53
7	14.30	eucalyptol	1029	1030	10.51
8	15.07	β-*E*-ocimene	1049	1048	4.86
9	17.06	linalool	1100	1100	48.69
10	18.17	2.6-dimethyl-1,3,5,7 –octatretaene, *E*,*E*	1130	1134	2.81
11	18.70	camphor	1144	1143	1.86
12	20.46	α-terpineol	1191	1190	0.19
13	20.68	*E*-dihydrocarvone	1197	1195	0.47
14	20.85	γ-terpineol	1202	1199	0.48
15	20.96	*Z*-dihydrocarvone	1205	1205	0.70
16	21.11	*E*-carveol	1209	1207	0.86
17	22.28	neral	1243	1240	0.77
18	23.31	geranial	1273	1270	1.23
		Total of Monoterpenoids			**86.45**
19	26.79	copaene	1377	1377	0.33
20	27.31	β-elemene	1393	1391	2.06
21	28.18	β-caryophyllene	1421	1418	4.19
22	29.24	α-caryophyllene	1455	1454	1.30
23	29.32	β-*Z*-farnesene	1458	1457	0.22
24	30.10	γ-muurolene	1483	1480	1.00
25	30.83	germacrene A	1507	1509	0.78
26	31.13	γ-cadinene	1517	1518	0.49
27	31.38	δ-cadinene	1525	1524	0.86
28	32.37	germacrene B	1559	1561	0.22
29	32.54	*E*-nerolidol	1565	1563	0.38
30	33.15	caryophyllene oxide	1586	1583	0.40
		Total of Sesquiterpenoids			**12.23**
		Total identified			**98.68**

Table 3. Effect of essential oil of *Lippia alba* (unique concentration) on sedation, anesthesia and recovery (time in seconds) of *Perna perna*. Valves: semi-open valves, closed only by the edge of the mantle (natural behavior - NB), semi-open valves (SO) and closed valves (C). Recovery: with recovery (+) or without recovery (-). Each concentration n = 10.

Anesthetic	Concentration (µL L^{-1})	Valves	Sedation (s)	Anesthesia (s)	Recovery	Recovery time (s)
Lippia alba	150	NB	-	-	+	0
		C	-	-	+	5032
		C	-	-	+	0
		C	-	-	+	0
		NB	-	-	+	0
		SO	2155	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
	300	C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
	450	C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-

Table 4. Effect of gradual increase in essential oil of *Lippia alba* concentration on sedation, anesthesia and recovery (time in seconds) of *Perna perna*. Valves: semi-open valves, closed only by the edge of the mantle (natural behavior - NB), semi-open valves (SO), and closed valves (C). Recovery: with recovery (+) or without recovery (-). Each concentration n = 10.

Anesthetic	Final concentration ($\mu L\ L^{-1}$)	Valves	Sedation (s)	Anesthesia (s)	Recovery	Recovery time (s)
Lippia alba	150	C	-	-	+	0
		C	-	-	+	3968
		C	-	-	+	2253
		C	-	-	+	0
		C	-	-	+	0
		NB	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
	300	C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		SO	1455	-	+	3320
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
	450	C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	+	0
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-
		C	-	-	-	-

814 Table 5. Levels of glycogen and lactate in gills (µmol g tissue^{-1}) and hemolymph (µmol mL^{-1}) of
815 *Perna perna* exposed to essential oil of *Lippia alba*. Means followed by different letters in the
816 columns indicate significant differences between treatments at the same sampling times (with or
817 without recovery). *Indicates significant difference from exposed mussels ($p < 0.05$). Results are mean
818 ± SEM.

	Unique application of the anesthetic		**Gradual increase in the concentration of the anesthetic**	
	Exposed	**Recovered**	**Exposed**	**Recovered**
Glycogen				
Gills				
Control	110.72±1.63^{a}	114.42±2.54^{a}	111.20±1.28^{a}	112.14±0.86^{a}
Ethanol	112.00±1.79^{a}	114.42±2.54^{a}	110.80±2.85^{a}	112.14±0.86^{a}
150 µL L^{-1}	139.20±2.31^{b}	128.86±0.93^{b*}	138.00±2.95^{b}	130.60±1.20^{b*}
300 µL L^{-1}	146.60±3.03^{b}	133.46±0.52^{b*}	146.60±2.56^{c}	133.26±0.87^{b*}
450 µL L^{-1}	141.40±3.06^{b}	131.42±0.67^{b*}	141.00±2.14^{bc}	131.20±0.43^{b*}
Hemolymph				
Control	10.56±0.05^{a}	8.33±0.11^{a*}	11.05±0.32^{a}	8.16±0.11^{a*}
Ethanol	10.24±0.20^{a}	8.33±0.25^{a*}	10.79±0.28^{a}	8.24±0.26^{a*}
150 µL L^{-1}	7.85±0.09^{b}	9.14±0.15^{b*}	7.90±0.08^{b}	9.05±0.04^{b*}
300 µL L^{-1}	6.52±0.15^{c}	8.24±0.19^{a*}	6.60±0.07^{c}	8.37±0.16^{ab*}
450 µL L^{-1}	6.40±0.04^{c}	8.09±0.07^{a*}	6.41±0.05^{c}	8.18±0.11^{a*}
Lactate				
Gills				
Control	17.32±0.48^{a}	21.54±0.31^{a*}	17.70±0.34^{a}	21.84±0.37^{a*}
Ethanol	17.34±0.50^{a}	22.34±0.45^{a*}	17.48±0.55^{a}	22.08±0.44^{a*}
150 µL L^{-1}	13.46±0.09^{b}	28.84±0.66^{b*}	14.12±0.34^{b}	28.72±0.54^{b*}
300 µL L^{-1}	12.96±0.19^{b}	32.28±0.39^{c*}	12.88±0.33^{b}	32.08±0.47^{c*}
450 µL L^{-1}	14.04±0.25^{b}	30.52±0.36^{d*}	14.10±0.33^{b}	31.22±0.50^{c*}
Hemolymph				
Control	1.38±0.02^{a}	1.30±0.01^{a}	1.38±0.03^{a}	1.45±0.03^{a}
Ethanol	1.54±0.04^{b}	1.26±0.03^{a*}	1.48±0.04^{a}	1.37±0.06^{a}
150 µL L^{-1}	0.86±0.03^{c}	0.95±0.03^{b}	0.86±0.03^{b}	1.19±0.04^{b*}
300 µL L^{-1}	0.87±0.03^{c}	0.91±0.05^{bc}	0.88±0.01^{b}	1.07±0.07^{b*}
450 µL L^{-1}	0.86±0.01^{c}	0.82±0.02^{c}	0.86±0.02^{b}	0.90±0.03^{c}

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Essential oil of *Lippia alba* as sedative and anesthetic for sea urchin *Echinometra lucunter* (Linnaeus, 1758)

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Essential oil of *Lippia alba* as sedative and anesthetic for sea urchin *Echinometra lucunter* (Linnaeus, 1758)

This study evaluated the use of the essential oil of *Lippia alba* (EOL) as sedative and anesthetic in the sea urchin *Echinometra lucunter*. Three different concentrations were tested (50, 100, and 150 μL L^{-1}), as well as an ethanol and control groups, totaling five treatments. After anesthesia (maximum 15 min) and recovery to the different treatments, coelomic fluid, gonads and intestine were collected for analysis of oxidative stress parameters. The highest concentration tested (150 μL L^{-1}) was chosen as the optimal concentration for anesthesia and the lowest one (50 μL L^{-1}) did not induce anesthesia, being chosen the ideal for sedation. Anesthesia with the EOL decreased TBARS levels and increased significantly the activity of the antioxidant enzymes SOD and CAT in the coelomic fluid and gonads (except that only 100 μL L^{-1} EOL increased CAT in the gonads), demonstrating that EOL increases the antioxidant activity and improves the response to oxidative stress, compared to control animals.

Keywords: cidreira herb, echinoderms, handling, oxidative stress, recovery.

1 Introduction

The commercialization and exportation of echinoderms has been increasing in several countries (Micael et al. 2009). The sea urchin *Echinometra lucunter* has wide distribution throughout the West Indies and the western Atlantic Ocean between Florida and Bermuda to southern coast of Brazil (McPherson 1969; Lewis and Storey 1984). In the Brazilian coast this species is found on rocks, occupying dens or crevices, is exposed to the air during low tide and also habits constantly submerged areas with depths up to 45 m (Grünbaum et al. 1978).

This species is consumed as food by fishermen, the low-income population (such as in the Northeast of Brazil) (Alves et al. 2006) and sold whole or only the gonads by high prices to restaurants of European and Asian origin. They are also sold to marine aquariums (where live animals may be even more expensive), or used as experimental models in studies (Micael et al. 2009) and until the carapaces can be used as decorative artifacts (Alves et al. 2006).

During echinoculture the animals undergo common procedures as handling and transportation which often cause stress, leading to unplanned (spontaneous) spawning and mortality (Luis et al. 2005; Arafa et al. 2007). Spontaneous spawning of sea urchin reduces the size of the gonads, decreasing their quality and commercial value. Stress can trigger production of reactive oxygen species and to occurs an imbalance between oxidants and

antioxidants in favor of oxidants, leading the break of the redox control (lowering-oxidation reactions) and/or damages molecular, situation called of oxidative stress (Sies and Jones 2007). The study of different systems related to oxidative stress, in these organisms, can give important information about their physiological status.

Anesthesia facilitates the detachment of the animal from the substrate, which is another common problem encountered by researchers and producers (Hagen 2003). Thus, anesthesia can be a valuable technique to handle sea urchins and avoid such drastic consequences. However, little is known about the role of anesthetics against stress of sea urchins (Arafa et al. 2007). Studies with natural anesthetics have increased considerably in recent years (Moreira et al. 2010). *Lippia alba* (Verbenaceae), popularly known as cidreira herb, is a native plant widely distributed in Brazil (Terblanche and Kornelius 1996), whose essential oil has sedative and anesthetic activity (Cunha et al. 2010, 2011; Parodi et al. 2012; Toni et al. 2014) and antioxidant activity in fish (Azambuja et al. 2011; Salbego et al. 2014) and shrimps (Parodi et al. 2012). Hence, the objective of this study was to evaluate the use of essential oil of *L. alba* (EOL) as sedative and anesthetic in *E. lucunter*.

2 Materials and methods

2.1 Acquisition of animals and acclimation

Eighty animals (56.07 ±1.12 mm in diameter; 128.48 ± 3.51 g; and 39.41 ± 0.94 mm in height) were collected close to the shore of the beach at Ilha do Boi, Vitória, Espírito Santo (ES) state, southeast Brazil, and transferred to the Laboratory of Applied Ichthyology at the University Vila Velha (UVV). The animals were acclimated in continuously aerated 310 L tanks (without substrate) for five days.

Water quality was monitored: temperature, conductivity and salinity by YSI conductivity (EC 300, Yellow Springs Inc. Ohio, EUA), and dissolved oxygen saturation were monitored using a YSI oximeter (OD 200), pH with a YSI pH meter (pH 100), alkalinity by titulation and total ammonia (indophenols method) according to APHA (1998). The collections of the animals from the environment were conducted with due authorization from Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (nº 33571) (Anexo D).The methodology of this experiment was approved by the Ethical Committee on Use of Animals of the University Vila Velha (Process no 218/2012) (Anexo E).

2.2 Plant material

Lippia alba was cultivated in Frederico Westphalen, Rio Grande do Sul state, southern Brazil. A voucher specimen (SMDB n. 10050) was deposited in the herbarium of the Department of Biology (UFSM). The EOL was obtained from fresh plant leaves by hydrodistillation for 2 h using a Clevenger type apparatus (European Pharmacopeia 2007) and was stored at -20 °C until composition analysis and biological assays.

2.3 Essential oil analysis

The analysis was carried out on an Agilent 7890A GC coupled to a 5975C mass spectrometer using a non-polar HP5-MS fused silica capillary column (5 % phenyl – 95 % methylsiloxane, 30 m x 0.25 mm i.d. x 0.25 mm film thickness) and EI-MS of 70 eV. The operating conditions were: carrier gas: He, flow rate: 1 mL min^{-1}; split inlet: 1:100; injector and detector temperature: 250 ºC; analysis program: 40 ºC for 4 min and 40 ºC - 320 ºC at 4 ºC min^{-1}. The constituents of the EO were identified by comparison of the mass spectra and Kovats retention index with the literature and a mass spectral data bank (NIST 2008; Adams 2009).

Quantitative analysis was performed on a Agilent 7890A gas chromatograph equipped with a flame ionization detector using a non-polar HP-5 fused silica capillary column (5 % phenyl – 95 % methylsiloxane 30 m x 0.25 mm i.d. x 0.25 µm film thickness). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}; splitless mode; both injector temperature and detector temperature were set at 300 ºC. Sample of EOL was injected in triplicate, and the analysis program corresponded to that described for CG-MS.

2.4 Experimental procedures

The EOL was previously diluted 1:10 in 99.5 % ethanol and then added to 2 L aquaria containing 1 L of water. Subsequently, the animal was transferred from the acclimation tank to these individual aquaria to assess the behavioral stages. The water parameters were: temperature: 27.08 ± 0.14 ºC; conductivity: 57.44 ± 0.20 mS cm^{-1}; salinity: 36.63 ± 0.19 ppt; dissolved oxygen: 6.18 ± 0.13 mg L^{-1} (73.58 ± 2.09 % saturation); pH: 8.06 ± 0.05; alkalinity: 132.57 ± 3.97 mg $CaCO_3$ L^{-1}; and total ammonia: 0.05 ± 0.02 mg L^{-1}.

Previous tests were performed with 25, 50, 80, 100, 150, 200, 250, 300 and 400 µL L^{-1} EOL to choose the concentrations to be used in the experiment. Three concentrations were chosen (50, 100 and 150 µL L^{-1}), since, lower concentrations than the 50 µL L^{-1} remained normal behavior within 1 hour of exposure and concentrations above 150 µL L^{-1} no

differences in behavior with respect to the chosen concentrations. Experiments were also performed using aquaria containing only ethanol at a concentration (1350 μL L^{-1}) equivalent to the dilution used at the highest anesthetic concentration tested. The control group consisted of animals submitted to the same procedure, but in anesthetic-free water. To evaluate the time required for sedation and anesthesia induction, sixteen animals, each being placed in individual aquaria, were used for each concentration tested, and each one was used only once to observe the different behavioral stages following criteria adapted from Santos (2010) (Table 1). Upon detection of anesthesia or after the predetermined period of anesthetic exposure (average time of anesthesia - 15 min), the animals were rinsed in clean water and transferred to continuously aerated 6 L aquaria containing 3 L of water to assess recovery time.

Animals that did not anesthetize and control and ethanol groups remained in the recovery aquaria for the mean time of recovery of anesthetized animals (10 min). The diameter and height of the carapace were measured with a digital caliper Caliper (Maxwell) and they were weighed with a precision balance Adventurer Pro (Ohaus), after recovery (n=8) (sampling time recovered). Eight other animals have not gone through the recovery process (sampling time exposed). Subsequently, all animals were killed by hypothermia (20 minutes submerged in ice) and the coelomic fluid that surrounds the lantern of Aristotle was collected with disposable syringes. Sea urchin were then opened by sawing the shell, with the help of a saw Dremel, when gonad and intestine were collected and stored at -80 ºC for analysis of oxidative stress parameters.

2.5 Analysis of metabolic parameters

Tissues (gonads and intestine) were dissolved in an equal volume of 20 % TCA using a Potter–Elvehjem homogenizer. The acid homogenate was centrifuged for 10 min at 10,000 × g and the supernatant was used for the metabolic determinations. Homogenate was used to estimate the protein level according to Lowry et al. (1951) and total lipid was analyzed by commercial kit (Labtest).

2.6 Analysis of oxidative stress parameters

2.6.1 Lipid peroxidation (TBARS)

Lipid peroxidation was estimated in the coelomic fluid, gonad and intestine by TBARS (thiobarbituric acid-reactive substances) assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured according to Buege

and Aust (1978). Aliquots of supernatants (0.25 mL) were mixed with 10 % trichloroacetic acid (TCA) (0.25 mL) and 0.67 % thiobarbituric acid (0.5 mL) to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 15 min at 95 °C. After cooling, it was centrifuged at 5000 × g for 15 min, and optical density was measured by a spectrophotometer at 532 nm. TBARS levels were expressed as nmol mg protein^{-1}.

2.6.2 Superoxide dismutase (SOD)

The activity of superoxide dismutase was determined in the coelomic fluid, gonad, intestine and ambulacral feet according to the method of Misra and Fridovich (1972), which is based on inhibition of the reaction of radical superoxide with epinephrine (1 mM). The oxidation of adrenalin leads to the formation of a colored product, the adenochrome, detected spectrophotometrically at 480 nm. The results are expressed as SOD units per milligram of protein. One unit of SOD is defined as the amount of enzyme that inhibits by 50 % the speed of formation of adenochrome. Superoxide dismutase activity is expressed in U mg protein^{-1}.

2.6.3 Catalase (CAT)

Catalase activity was measured in the coelomic fluid, gonad, intestine and ambulacral feet according as method of Nelson and Kiesow (1972) by the monitoring the consumption of H_2O_2 by measuring the decrease in absorbance at 240 nm, in a reaction mixture containing H_2O_2 (15 mM) in Tris-Hepes buffer (20 mM) and 10 µL of enzyme material. Catalase activity is expressed in U mg protein^{-1}. All assays was used Tris-Hepes buffer (20 mM) and for homogenizing the tissue 1:5 (v/v).

2.7 Statistical Analyses

All data are expressed as mean ± SEM. Homogeneity of variances among treatments was tested by Levene's test. Data exhibited homogeneous variances, so the time to reach the stage of sedation, anesthesia and recovery and metabolic and oxidative stress parameters at the different anesthetic concentrations were analyzed by one-way ANOVA and Tukey's test. Analysis was performed using the software GraphPad Prism 6, and the minimum significance level was set at $p < 0.05$.

3 Results

3.1 Chemical composition of EOL

From EOL total chemical composition, 98.68 % were identified, and among the 30 detected constituents, 18 were monoterpenoids (peaks 1-18, 86.45 %) and 12 were sesquiterpenoids (peaks 19-30, 12.23 %). The main component of the essential oil of *Lippia alba* was determined as linalool (48.69 %), followed by eucalyptol (10.51 %) and β-myrcene (9.74 %) (Table 2).

3.2 Induction to sedation and anesthesia

Animals exposed to the lowest EOL concentration (25 μL L^{-1}) in the previous tests did not reach sedation. The concentration of 50 μL L^{-1} EOL led to sedation but was the only tested that did not induce anesthesia. The concentration of 150 μL L^{-1} led to anesthesia significantly faster than 100 μL L^{-1}, but there was no significant difference in recovery time between both concentrations (Table 3). There was no mortality through the experiments.

3.4 Metabolic parameters

Sea urchins anesthetized with 100 and 150 μL L^{-1} EOL had lower lipid values in gonads at both sampling times compared to other treatments (Figure 1A). The lipid values in the intestine of sea urchins anesthetized with 100 μL L^{-1} EOL were significantly lower in the recovered animals than the control ones (Figure 1B).

The protein values in the gonads were higher in recovered urchins than in those exposed to50 and 100 μl L^{-1} EOL. Treatments with EOL resulted in the increase of protein values in the gonads compared to control and ethanol groups at both sampling times (exposed or recovered), except animals exposed to 100 μL L^{-1} EOL (Figure 1C). Urchins recovered from ethanol and all EOL concentrations exposure had higher amounts of protein in the intestine compared to controls and those exposed. In addition, animals exposed to 100 and 150 μL L^{-1} EOL showed lower protein values in the intestine when compared to the controls (Figure 1D).

3.5 Oxidative stress parameters

Urchins exposed to all EOL concentrations had lower TBARS values in coelomic fluid and gonads at both sampling times compared to control and ethanol groups (Figure 2A and B). The gonads of urchins exposed to ethanol had significantly higher TBARS values than those from the recovered (Figure 2B). The intestine of urchins exposed to all EOL

concentrations presented lower TBARS values compared to the control and ethanol groups and then the recovered animals (Figure 2C).

The exposure to all EOL concentrations at both sampling times led to significantly higher SOD activity in the coelomic fluid, gonads and intestine compared with the control and ethanol groups, except in the intestine of those exposed to 100 μL L^{-1} EOL (Figure 2D, E and F). The gonads of urchins exposed to 100 and 150 μL L^{-1} EOL presented significantly lower SOD activity than the recovered ones (Figure 2E). The intestine of animals exposed to all tested EOL concentrations also had significantly lower SOD activity than in the recovered ones (Figure 2F).

The coelomic fluid of urchins exposed to all EOL concentrations showed significantly higher CAT activity at both sampling times than the control and ethanol treatments (Figure 2G). The gonads of animals exposed and recovered from100 μL L^{-1} EOL exposure presented significantly higher CAT activity than in those exposed and recovered from ethanol exposure, respectively (Figure 2H). The intestine of urchins recovered from150 μL L^{-1} EOL exposure had significantly higher CAT activity than the control group. The CAT activity in the intestine of animals exposed to all EOL concentrations was significantly lower than in recovered ones (Figure 2I).

4 Discussion

To identify the chemotype is considered the major compound, with concentrations above 10% (Jannuzzi et al. 2011). Thus, in our studies, *Lippia alba* belongs to chemotype Linalool (48.69 %). Some monoterpene of the essential oils, as linalool, possess local anesthetic activity (Ghelardini et al. 1999; Galeotti et al. 2001), which could be responsible, at least in part, to their muscle relaxant properties (Ghelardini et al. 2001). It is believed that the linalool possesses a number of pharmacological properties, including anticonvulsant, anxiolytic, anti-inflammatory, antinociceptive (Kamatou and Viljoen 2008).

The usual anesthetic techniques have limited viability in sea urchins, making it uncertain to determine sedation and anesthesia because unlike fish and shellfish, it is difficult to determine their average anesthesia and recovery time. They are always connected to the substrate and can also remain stationary for a long time. When disturbed, the animal shows no response, only adheres more strongly to the surface (Hagen 2003; Arafa et al. 2007). Such

difficulties were not found in the present study: the stages of anesthesia achieved by sea urchins were observed with clarity and precision.

Concentrations of 100 and 150 μL L^{-1} of the EOL achieved 100 % of anesthesia in the animals tested, similar to the study by Hagen (2003), in which 25-100 μL L^{-1} potassium chloride (KCl) induced 100 % of anesthesia in green juvenile sea urchins (*Strongylocentrotus droebachiensis*). The same authors verified that $MgCl_2$, benzocaine and 2-phenoxyethanol were less effective. The same authors stated that the cost of $MgCl_2$ exceeds that of KCl and has an effective concentration at least 10 times higher. Benzocaine was ineffective at very high concentrations (at least 100 times stronger than the recommended) and 2-phenoxyethanol has no safety margin because the effective and lethal concentrations are the same.

A good anesthetic must be easy to administer and have high-efficiency relaxing activity, easily accessible and cheap to use in large-scale commercial applications, nontoxic to sea urchins either the operator (Arafa et al. 2010). The EOL has these advantages and it is a natural product that can be easily applied in the commercial culture of sea urchins, including organic cultures.

Glycogen and lipids, important energy reserves (Mouneyrac et al. 2008), are usually mobilized to maintain homeostasis during exposure to a xenobiotic. This in turn can affect the energy balance of animals because of the rising cost of maintenance, such as the activation of defense and repair mechanisms (Smolders et al. 2004; Voets et al. 2006). The lipid values found in the gonads of *E. lucunter* were significantly lower at the highest EOL concentrations (100 and 150 μL L^{-1}) compared to the control even after recovery. The same was observed in the intestine of those exposed to 100 μL L^{-1} EOL compared to control ones.

When the glycogen reserves and lipids are exhausted an alternative is the use of proteins as an energy source (Mouneyrac et al. 2008), which was confirmed in this study because in the intestine of *E. lucunter* exposed to 100 and 150 μL L^{-1} EOL lower protein values were observed compared to control animals. In addition, urchins recovered from all exposures (EOL and ethanol) increased protein values. However, studies by Arafa et al. (2010) reported that carbohydrates and proteins are used as the initial energy sources in sea urchins (*P. lividus*) instead of lipids. Protein catabolism plays an important role in the production of metabolic energy (David et al. 2004), therefore the decrease of protein levels in urchins exposed to the EOL may be due to increased protein catabolism. In view of this, energy reserves apparently are affected by exposure to EOL.

Is not very clear the difference in response between the analyzed tissues, but according to Almeida et al. (2005), it may be due to structural differences, possibly in relation to the

lipid content and antioxidants, as well as the mechanism of action of the evaluated substance. As the cells of the immune system of sea urchins, responsible for the degradation of phagocytic particles through the production of reactive oxygen species (ROS), are located in coelomic fluid (Gross et al. 2000), the significant increase in SOD activity and CAT in this tissue may indicate detection of the presence of the EOL. The enzyme SOD can convert the superoxide anion into hydrogen peroxide, which subsequently undergoes action of the enzyme catalase (CAT) which, in turn, converts water and oxygen (Vernon and Tang 2013). Such enzymes are important components of different detoxification pathways, antioxidants and tolerance to stress (Parodi et al. 2012). Wang et al. (2008) found changes in SOD and CAT activities in the coelomic fluid, of *Apostichopus japonicas* under heat stress and these changes were associated with the death of the organisms tested. The lower TBARS levels and higher SOD and CAT activities in the coelomic fluid and gonads of *E. lucunter* exposed to EOL and that in general were maintained even after recovery, demonstrating that EOL improved the response to oxidative stress and, maybe, may be useful in the treatment of sea urchin. This is in agreement with the improvement of the antioxidant system of silver catfish *Rhamdia quelen* (Azambuja et al. 2011) and white shrimp (*Litopenaeus vannamei*) Parodi et al. (2012) exposed to EOL.

In conclusion, EOL is an efficient anesthetic to sea urchin *E. lucunter*. The best concentration for anesthesia of this species is 150 μL L^{-1} EOL and not caused mortality. Despite the effects on energy reserves, it is believed that the time was too small to cause such effects therefore, suggest that the EOL has improved the response to oxidative stress and is a very promising anesthetic for sea urchins.

Acknowledgement

L.N. Simões received Ph.D. fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). B. Baldisserotto, V.L. Loro and L.C. Gomes received a research fellowship from CNPq.

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Tables

Table 1. Stages of anesthesia used to determine the effectiveness of anesthetics in sea urchin *Echinometra lucunter* (adapted from Santos 2010).

Stages of anesthesia	Behavioral response
Stage I	Ambulacral feet with slow movements and/or some retracted
Stage II	Ambulacral feet with little or no prehensile ability (sedation)
Stage III	Retracts all ambulacral feet, losing prehensile full capacity, but the thorns respond to touch
Stage IV	Stops moving and the thorns stop responding to touch (anesthesia)
Recovery	The ambulacral feet retake the prehensile ability and the sea urchin starts moving

Table 2. Chemical components of the essential oil of *Lippia alba*. RT: Retention time; RI Calc: Calculated retention index; RI Lit: Literature retention index (NIST 2008; Adams 2009).

Peak	RT	Compound	RI Calc	RI Lit	%
1	10.37	α-pinene	931	932	0.52
2	11.91	5-hepten-2one-4,6-dimethyl	969	973	1.32
3	11.99	sabinene	971	973	0.30
4	12.08	β-pinene	974	975	0.61
5	12.79	β-myrcene	991	992	9.74
6	14.20	limonene	1027	1028	0.53
7	14.30	eucalyptol	1029	1030	10.51
8	15.07	β-*E*-ocimene	1049	1048	4.86
9	17.06	linalool	1100	1100	48.69
10	18.17	2.6-dimethyl-1,3,5,7 –octatretaene, *E, E*	1130	1134	2.81
11	18.70	camphor	1144	1143	1.86
12	20.46	α-terpineol	1191	1190	0.19
13	20.68	*E*-dihydrocarvone	1197	1195	0.47
14	20.85	γ-terpineol	1202	1199	0.48
15	20.96	*Z*-dihydrocarvone	1205	1205	0.70
16	21.11	*E*-carveol	1209	1207	0.86
17	22.28	neral	1243	1240	0.77
18	23.31	geranial	1273	1270	1.23
		Total of Monoterpenoids			**86.45**
19	26.79	copaene	1377	1377	0.33
20	27.31	β-elemene	1393	1391	2.06
21	28.18	β-caryophyllene	1421	1418	4.19
22	29.24	α-caryophyllene	1455	1454	1.30
23	29.32	β-*Z*-farnesene	1458	1457	0.22
24	30.10	γ-muurolene	1483	1480	1.00
25	30.83	germacrene A	1507	1509	0.78
26	31.13	γ-cadinene	1517	1518	0.49
27	31.38	δ-cadinene	1525	1524	0.86
28	32.37	germacrene B	1559	1561	0.22
29	32.54	*E*-nerolidol	1565	1563	0.38
30	33.15	caryophyllene oxide	1586	1583	0.40
		Total of Sesquiterpenoids			**12.23**
		Total identified			**98.68**

Table 3. Time, in seconds, to induce the main behavioral stages of sea urchin (*Echinometra lucunter*) (n = 16) exposed to different concentrations of essential oil of *Lippia alba* (μL L^{-1}). Means followed by different letters in the columns indicate significant differences between concentrations at the same stage and same anesthetic by ANOVA and Tukey test ($p < 0.05$). There were no significant differences between sampling times in the same concentration and stage by two-way ANOVA ($p < 0.05$). Results are mean ± standard error. – This concentration did not induce the behavioral stage.

Sampling times	**Concentration (μL L^{-1})**	**Behavioral stages (s)**		
		Sedation	**Anesthesia**	
Exposed	50	707.86 ± 30.33[a]	-	
	100	360.86 ± 33.53[b]	1551.20 ± 60.84[a]	
	150	216.43 ± 6.46[b]	895.00 ± 78.25[b]	**Recovery**
Recovered	50	585.00 ± 108.26[a]	-	344.29 ± 29.37[a]
	100	249.00 ± 37.33[b]	1561.75 ± 57.67[a]	739.86 ± 62.87[b]
	150	257.71 ± 35.27[b]	740.00 ± 86.59[b]	534.50 ± 65.67[ab]

Figures

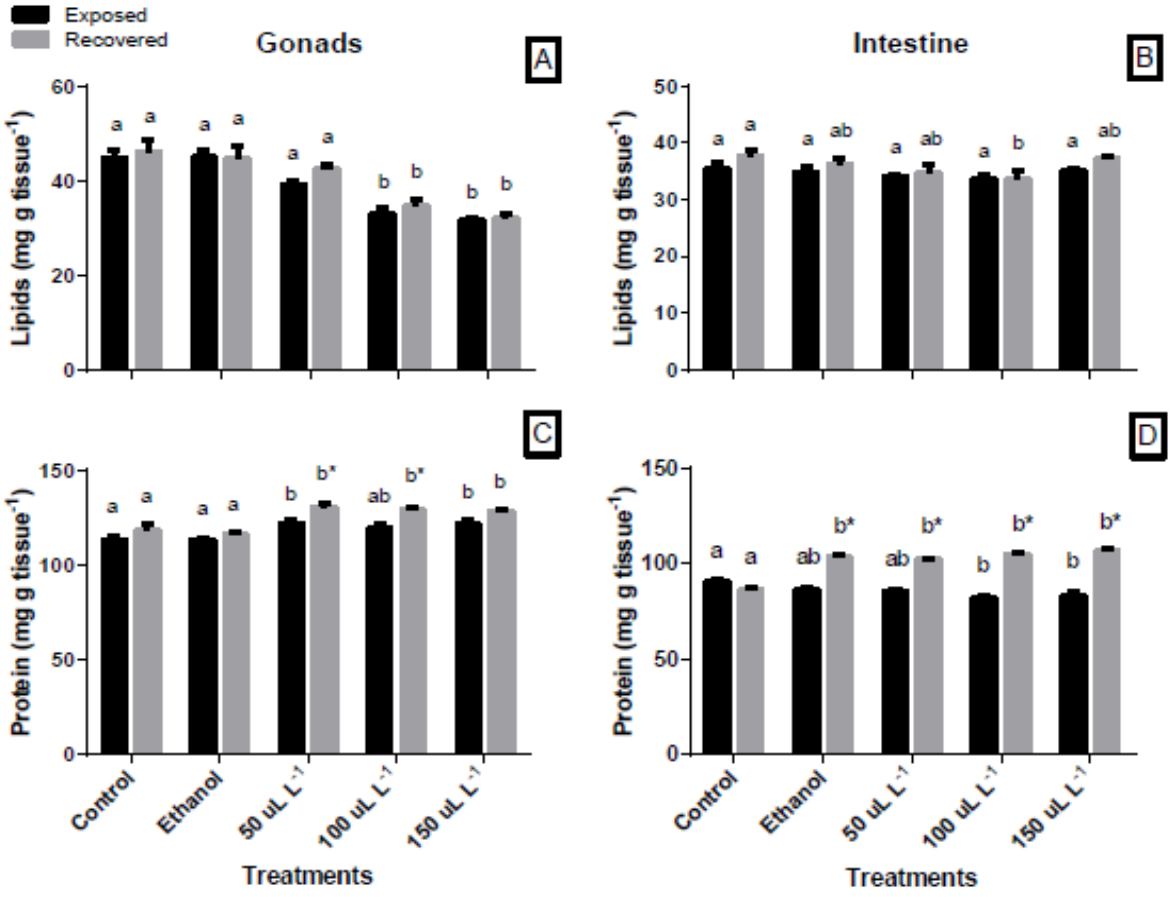

Figure 1. Levels of lipids in gonads (A) and intestine (B) and protein in gonads (C) and intestine (D) of sea urchin *Echinometra lucunter* exposed to different concentrations of the essential oil of *Lippia alba*. Different letters indicate significant differences between treatments at the same sampling times (exposed or recovered). *Indicates significant difference from exposed sea urchin ($p < 0.05$). Results are expressed as mean ± SEM.

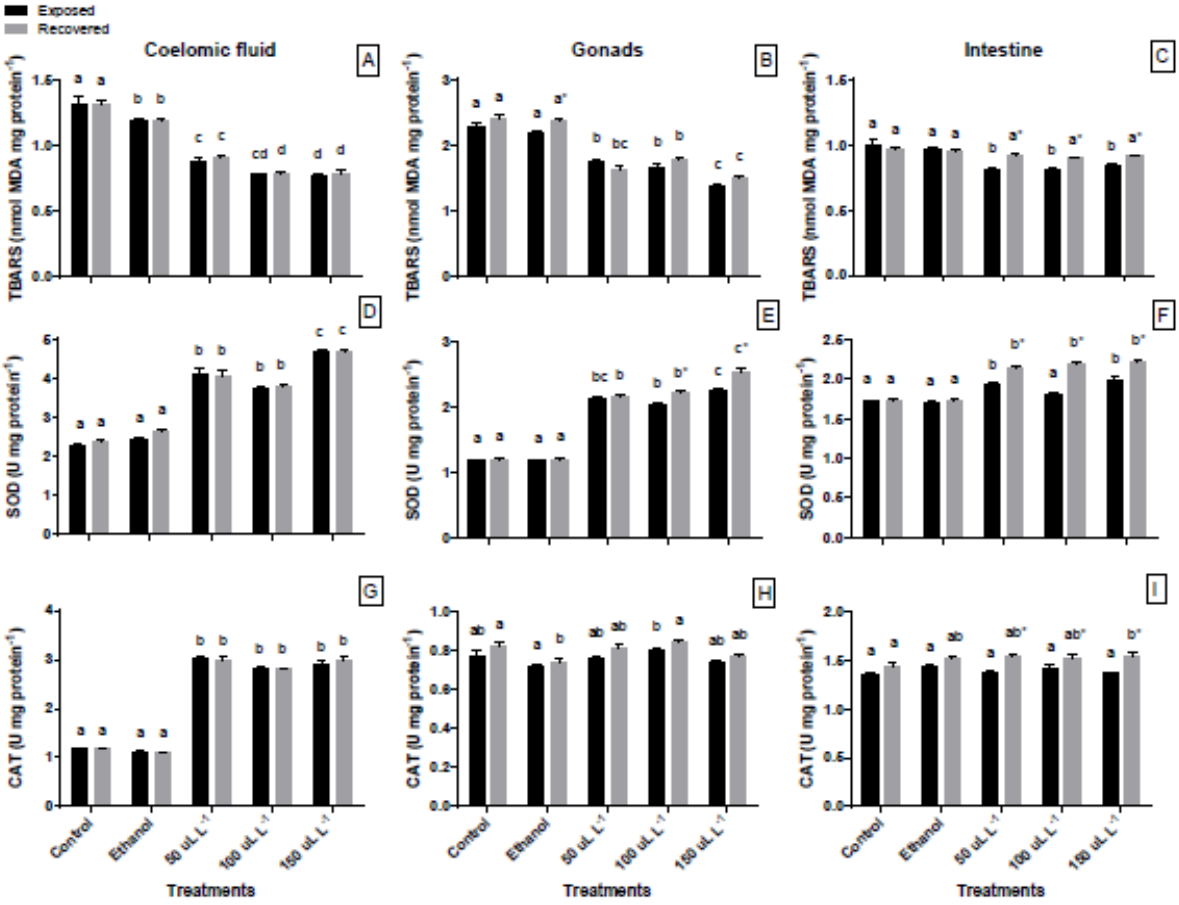

Figure 2. Levels of thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and catalase (CAT) in coelomic fluid (A, D and G, respectively), gonads (B, E and H, respectively) and intestine (C, F and I, respectively) of sea urchin *Echinometra lucunter* exposed to different concentrations of the essential oil of *Lippia alba*. Different letters indicate significant differences between treatments at the same sampling times (exposed or recovered). *Indicates significant difference from exposed sea urchin ($p < 0.05$). Results are expressed as mean ± SEM.

Effects of the essential oil of *Lippia alba* on stress response in sea bass (*Dicentrarchus labrax*)

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ABSTRACT

The aim of this study was to determine the essential oil of *Lippia alba* (EOL) efficiency as sedative and anesthetic in sea bass (*Dicentrarchus labrax*), and its response to stress. In the first experiment, different concentrations of EOL were tested and time to reach the state of sedation, anesthesia and recovery were recorded. In the second experiment, specimens were submitted to stress for 3 and 6 hours under different compounds in the water (control, 450 μL L^{-1} of ethanol, 25 and 50 μL L^{-1} of EOL). Cortisol, glucose, lactate, protein and triglycerides in plasma and glycogen, lactate, triglycerides and enzymes (glycogen phosphorilase total - GPtotal, hexokinase - HK, fructose-biphosphatase - FBP, glucose-6-phosphate dehydrogenase - G6PDH, pyruvate kinase - PK, glycerol-3-phosphate dehydrogenase - G3PDH, glutamate dehydrogenase - GDH, glutamic-pyruvic transaminase - GPT and glutamic oxaloacetic transaminase - GOT) in liver were assessed. The levels of plasma glucose, plasma lactate and liver increased during stress at the highest EOL concentration (50 μL L^{-1}), however plasma cortisol in animals exposed to EOL increased only after 6 h of stress. The ideal concentration for sedation of *D. labrax* is 25 μL L^{-1} and anesthesia is 600 μL L^{-1} EOL. Results demonstrate that EOL at 25 μL L^{-1} was effective as sedative in *D. labrax* during stress procedures. Due to observed changes in hepatic metabolism of the fish exposed to EOL, the gluconeogenic way is suggested as an energy mechanism that the animal uses to restore its homeostasis.

Key words: cidreira herb, enzymes, sedation, stress.

INTRODUTION

The fish used in aquaculture or research are subjected to numerous stressful processes, among which are handling, containment, vaccination, transport, marking, blood sampling or surgical procedures, which often can cause injury or induce physiological stress (Ross and Ross 2008; Gholipour et al. 2011). A specific situation of stress (acute stress) may obtain a beneficial response to the animal, because the physiological reactions caused by the activation of the stress system would allow it to survive this situation, but chronic stress may lead to physiological alterations that can become lethal (Iwama et al. 2004).

A feature of the stress response is the activation of the hypothalamus-pituitary-interrenal (HPI) axis, resulting in the mobilization of a series of energy sources. Thus, the metabolites can provide important information on the internal environment of the organism, allowing an evaluation of the stress response, as well as the animals' ability to overcome the disturbance (Mommsen et al. 1999; Acerete et al. 2004). The liver is the central organ of the metabolic process which is responsible for various functions associated with the metabolism of the stressors agents (Salbego et al. 2010) and plays an important role in the metabolism of carbohydrate, protein and lipid. The activities of metabolic enzymes can indicate the metabolic state and its ability to be modified against a stress event (Menezes et al. 2014).

The use of anesthetics arises as a method to reduce the stress associated with aquaculture practices, as they help reducing damage to the fish and physiological alterations (Weber et al. 2009). A variety of anesthetics have been used in aquaculture and the use of natural essential oils is becoming increasingly common and it is a viable alternative, due to the difficulties of acquiring synthetic anesthetics in some countries (Palic et al. 2006; Souza et al. 2012; Silva et al. 2012).

Lippia alba (Verbenaceae) is popularly known as cidreira herb and widely distributed in South America, Central America and Africa (Zoghbi et al. 1998; Biasi and Costa 2003). Several studies show that the essential oil of *L. alba* (EOL) is effective in inducing fish sedation and anesthesia (Cunha et al. 2010, 2011; Saccol et al. 2013; Toni et al. 2014) and has antioxidant activity (Azambuja et al. 2011; Salbego et al. 2014).

Sea bass (*Dicentrarchus labrax*) is distributed along the eastern Atlantic from Norway to Morocco, including the Canary Islands and Senegal, also present in the Mediterranean and Black seas (Kottelat and Freyhof 2007). The culture of this species has grown strongly and it is an species highly valued in trade in Europe, with more than 148 000 t produced in 2014 (FEAP 2015), hence the need to search anesthetics that can be used to aid this species in cultivation, improving its welfare in aquaculture facilities. Up to the date, information regarding the effects of exposure to anesthetics on

the activity of enzymes associated with the metabolism of carbohydrates, lipids and proteins in fish are scarce. Therefore, it is necessary improve knowledge on how the animal rearranges its energy status, in search of homeostasis after a stressful event. Thus, the objective of this study was to determine the EOL efficiency as sedative and anesthetic in sea bass (*Dicentrarchus labrax*) and analyze its effect in the metabolic and enzymatic responses under stress conditions.

MATERIALS AND METHODS

Fish

Sea bass (*Dicentrarchus labrax*) (51.4 ± 1.2 g and 16.4 ± 0.1 cm) were provided by Servicios Centrales de Investigación de Cultivos Marinos (SCI-CM) (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain) and transferred to the wet laboratory at the Faculty of Marine and Environmental Sciences (Puerto Real, Cádiz, Spain). They were kept in 400 L tanks in an open system circuit containing seawater (38 ppt salinity), under natural photoperiod, with constant temperature 19 ℃, for an acclimation period of fifteen days. Fish were fed daily with commercial dry pellets at 1 % body mass. Before each experiment fish fasted for 24 hours.

This work possesses Certificate of competency to work with experimental animals in the European Union (Anexo F).

Plant material

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). A voucher specimen (SMDB n. 10050) was deposited in the herbarium of the Department of Biology (UFSM). The EOL was obtained from fresh plant leaves by steam distillation for 2 h using a Clevenger type apparatus (European Pharmacopeia 2007) and was stored at -20 ℃ until composition analysis and biological assays.

EOL analysis

Qualitative and quantitative characterization of the EO chemical compounds occurred by chromatographic analysis using and Agilent 7890A gas chromatograph coupled to Agilent 5975C mass selective detector (GC-MS). Capillary column used was HP5-MS (Hewlett Packard, 5 % fenilmetilsiloxane, 30 m x 0,25 mm, film thickness: 0,25 μm), and energy of ionization was 70 eV. The parameters utilized for the analysis were: He as gas carrier; split inlet 1:100; temperature program: 40 ℃ for a period of 4 minutes; 40 to 320 ℃ at 4 ℃ min^{-1}; 1 mL min^{-1} of flow rate; temperatures of injection and detection of 250 ℃. The compounds identification occurred by comparison of retention indices, obtained by the utilization of a calibration curve of n-alkanes injected at the conditions mentioned for the samples, and the mass fragmentation patterns with the data of NIST (2010) and Toni et al. (2015).

Experimental procedures

Before use, EOL was diluted 1:10 in 96 % ethanol. The tests were divided into two experiments: the first rated the best EOL concentration to sedate and anesthetize sea bass and the second experiment tested the effectiveness of the optimal concentration for sedation in reducing the stress response.

Experiment 1 - Optimal concentration

Eighty-one animals were used to test the nine different concentrations chosen in the previous test (25, 35, 50, 100, 200, 300, 400, 600 and 800 μL L^{-1}). Fish were individually exposed in 3 L aquaria (n = 9 each concentration) to observe the induction time to sedation and anesthesia. The behavioral stages of anesthesia evaluated followed the criteria proposed by Small (2003), in which the sedated fish decreases the reaction to external stimuli and presents a small loss of balance (stage I), and it is anesthetized when total loss of balance and locomotion occurs (stage III). Upon detection of anesthesia or after a pre-determined period of 30 min exposure, animals were rinsed in clean water and

transferred to continuously aerated 20 L recovery tanks to estimate the recovery time. Fish was considered recovered when actively swam without imbalance.

Experiment 2 - Stress responses

Seventy-two sea bass were subjected to stressful situation (persecution for capture) for 3 and 6 h. The stressed groups were chased every 45 min, persecuted for 1 min, and then the persecution was stopped for 30 s before it was reset for a further 1 min, as previously described by Weber et al. (2009). Based on results from experiment 1, sea bass were submitted to the following treatments: exposure to 25µL L^{-1} (optimal concentration for sedation), 50 µL L^{-1} (twice the optimal concentration for sedation), ethanol - equivalent to the dilution used at the highest concentration of EOL (450 µL L^{-1}) and a control group (without exposure to any substance).

Fish were sampled at 0 (without stress exposure), 3 and 6 h in the stress (n = 8 for each combination of treatment and time). Blood was collected by caudal puncture and centrifuged (ALC mod. 42049) at 13030 g for 3 min to separate the plasma, and then stored at –80 ºC for subsequent analysis of cortisol, glucose, lactate, triglycerides and proteins. After blood collection, animals were euthanized by spinal cord section and liver was removed, immediately frozen in liquid nitrogenand after stored at –80 °C for later analysis of glycogen, lactate, triglycerides and metabolic enzymes.

Plasma physiological and biochemical assays

Plasma cortisol levels (expressed in ng mL^{-1}) were measured by indirect enzyme immunoassay adapted to microplate as described previously by Martos-Sitcha et al. (2014). The steroids were extracted as described by Baldisserotto et al. (2014). The standards and extracted plasma samples were run in duplicate. The standard curve range was 2.5 ng mL^{-1} – 9.77 pg mL^{-1} (R^2 = 0.984). The lower limit of detection (92.60 % of binding, ED 92.60) was 14.66 pg mL^{-1}. The percentage of recovery was 95 %. The inter- and intra-assay coefficients of variation (calculated from the duplicate samples) were 2.93 ± 1.02 % and 0.17 ± 0.01 %, respectively. The cross-reactivity of any specific antibodies with intermediate products involved in steroid syntheses was given by the supplier [cortexolone (1.6 %), 11-deoxycorticosterone (0.23 %), 17-hydroxyprogesterone (0.23 %), cortisol glucuronide (0.15 %), corticosterone (0.14 %), cortisone (0.13 %), androstenedione (<0.01 %), 17-hydroxypregnenolone (<0.01 %) and testosterone (<0.01 %)] (Cayman Chemical Company, Michigan, USA).

Glucose, lactate, and triglyceride concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides Ref. 1001311) adapted to microplate. 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 (ref. 23225, 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 KCjuniorTM program.

Liver metabolite levels

Frozen liver was homogenized using an Ultra-Turrax T25 basic (IKA-Werke) with a 7.5 volume of ice-cooled 0.6 N perchloric acid, neutralized (using 1 M potassium bicarbonate) and centrifuged (30 min at 3220 g, 4 ºC in an Eppendorf Centrifuge 5810R). The supernatants were stored in different aliquots at −80 °C until use in the metabolitesanalyzes. Tissue glycogen levels were assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined enzymatically using a commercial kit (Biomérieux, Spain). Lactate and triglyceride concentrations were measured using commercial kits (as previously described for the plasma samples).

Liver enzyme activities

Frozen liver was homogenized by ultrasonic disruption (Misonix Inc., Microson Ultrasonic liquid processor XL-2000) with 10 volume of ice-cold stopping-buffer containing 50 mM imidazole (Sigma I- 0125) (pH 7.5), 1 mM mercaptoethanol (Sigma M-3148), 50 mM NaF (Merck ref. 1.06449), 4 mM EDTA (Sigma ED2SS), 0.5 mM PMSF (Sigma P-7626) and 250 mM sucrose (Sigma S-9378).

The homogenate was centrifuged at 10000 g for 30 min at 4 ºC (Centrifuge 5810R, Eppendorf), and the supernatant was immediately frozen using dry ice and maintained at -80 ºC until use in the enzymes analyzes.

Enzyme activities were analyzed using a spectrophotometer (as mentioned above). 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 phosphorilase (total and active GPase, EC 2.4.1.1), hexokinase (HK, EC 2.7.1.11), fructose-biphosphatase (FBP, EC 3.1.3.11), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), pyruvate kinase (PK, EC 2.7.1.40), glycerol-3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8), glutamate dehydrogenase (GDH, EC 1.4.1.2), glutamic-pyruvic transaminase (GPT, EC 2.6.1.2), and glutamic oxaloacetic transaminase (GOT, EC. 1.1.1.35) were previously described (Sangiao-Alvarellos et al. 2005; Polakof et al. 2006). Enzymatic activities were expressed as U mg protein^{-1} and protein levels were assayed in triplicate as performed with plasma samples.

Statistical Analyses

All data are presented as mean ± SEM. Homogeneity of variances between treatments was tested by Levene's test. Data exhibited homogeneous variances, so comparisons were made by one-way ANOVA and Tukey's test. Analysis was performed using the GraphPad Prism 6 software and the minimum significance level was set at $P < 0.05$.

RESULTS

Chemical composition of EOL

From EOL total chemical composition, 95.69 % were identified, and among the 7 detected constituents, 4 were monoterpenoids (86.21 %) and 3 were sesquiterpenoids (9.48 %). The main component of the EOL was determined as linalool (75.49 %), followed by eucalyptol (8.28 %) and germacrene D (5.10 %) (Table 1).

Experiment 1 - Optimal concentration

The lowest concentrations tested (25, 35 and 50 µL L^{-1} EOL) induced sedation but not anesthesia, and there was no significant difference in the time of sedation and recovery. The ideal concentration for sedation was considered 25 µL L^{-1} because it was the lowest concentration tested and had the same result than the higher ones (Table 2).

The highest concentrations tested (100, 200, 300, 400, 600 and 800 µL L^{-1} EOL) induced all stages of anesthesia, with 600 and 800 µL L^{-1} obtaining the lowest induction times (296.0 and 257.0 s, respectively). These same concentrations showed no significant difference in sedation (15.1 and 13.8 s, respectively) and recovery (292.7 and 304.8 s, respectively) times, so the concentration of 600 µL L^{-1} was considered ideal for anesthesia (Table 2). There was no mortality through the experiment.

Experiment 2 - Stress responses

Plasma physiological and biochemical assays

After 6 h of stress, fish exposed to 25 and 50 µL L^{-1} EOL significantly increased plasma cortisol values compared to before stress, and those exposed to 50 µL L^{-1} had the highest cortisol values (Fig. 1A). There was a continuous increase of plasma glucose values as time of stress went by in the ethanol and control groups. Plasma glucose of fish exposed to 25 µL L^{-1} EOL remained unchanged through all sampling times, but those kept at 50 µL L^{-1} EOL increased significantly these values after 3 and 6 h compared to prior stress. Fish exposed to ethanol also showed higher plasma glucose values after 6 h of stress compared to the control group (Fig. 1B). Plasma lactate in fish kept at 50 µL L^{-1} EOL were significantly highest after 3 h and decreased after 6 h of stress, returning to before stress values (but still higher than control fish), whereas those exposed to 25 µL L^{-1} EOL presented significantly lower values than control fish only after 6 h of stress (Fig. 1C). Fish exposed to ethanol and 25 µL L^{-1} EOL

presented significantly higher triglyceride levels after 6 h of stress (Fig. 1D). Plasma protein values decreased significantly after 6 h of stress (those exposed to 50 μL L^{-1} EOL also after 3 h of stress), but no significant difference between treatments was observed (Fig. 1E). Even after 3 or 6 hour of exposure, the animals did not reach the stage of anesthesia, remained only sedated.

Liver metabolite levels

Glycogen levels of control fish decreased significantly after 6 h of stress. Those exposed to 25 and 50 μL L^{-1} EOL showed significantly lower glycogen levels as time of stress went by, being significantly lower than control fish after 3 and 6 h of stress. Fish from the ethanol treatments showed lower values only after 6 h of stress (Fig. 2A). Fish exposed to 50 μL L^{-1} EOL showed the highest lactate levels 3 and 6 h after stress (Fig. 2B). Triglyceride levels significantly decreased in control, 25 and 50 μL L^{-1} EOL groups compared to before stress after 3 and 6 h, but no significant difference between groups was observed (Fig. 2C).

Liver enzyme activities

The GPtotal activity significantly increased in the control treatment after 6 h of stress compared to before stress (Fig. 3A). The HK activity showed no significant difference in the tested treatments and times (Fig. 3B). After 6 h of stress, FBP activity increased significantly in fish exposed to 25 and 50 μL L^{-1} EOL compared to the control (Fig. 3C). After 6 h of stress all treatments showed higher G6PDH activity than control group (Fig. 3D). Ethanol exposure decreased significantly PK activity after 6 h of stress compared to control and those kept at 50 μL L^{-1} EOL increased PK activity compared to before stress (Fig. 3E).

After 3 h of stress, the highest G3PDH activity was detected in the ethanol group, whereas after 6 h of stress control fish showed increased activity compared with the other sampling times and also higher than ethanol and 25 μL L^{-1} EOL groups (Fig. 4A). The ethanol treatment presented higher GDH activity than the control group after 3 h of stress, while after 6 h of stress, those exposed to 50 μL L^{-1} EOL significantly increased GDH activity compared to other sampling times and was also higher than control and ethanol groups (Fig. 4B). The highest GPT activity was obtained in fish exposed to 50 μL L^{-1} EOL after 3 h of stress (Fig. 4C). Fish exposed to 25 μL L^{-1} EOL decreased significantly GOT activity after 3 h of stress compared to before stress and was also lower than in control and ethanol groups. The ethanol group decreased GOT activity after 6 h of stress compared to 3 h, but no significant difference between treatments was observed at this time (Fig. 4D).

DISCUSSION

To identify the chemotype is considered the major compound, with concentrations above 10% (Jannuzzi et al., 2011). Thus, in our studies, Lippia alba belongs to chemotype Linalool (75.49 %). Some monoterpene of the essential oils, as linalool, possess local anesthetic activity (Ghelardini et al., 1999; Galeotti et al., 2001), which could be responsible, at least in part, to their muscle relaxant properties (Ghelardini et al., 2001). It is believed that the linalool possesses a number of pharmacological properties, including anticonvulsant, anxiolytic, anti-inflammatory, antinociceptive (Kamatou and Viljoen, 2008).

Experiment 1 - Optimal concentration

The concentration of 600 μL L^{-1} EOL was elected ideal for anesthesia in sea bass of approximately 50 g, while for *Rhamdia quelen* (6.6 g) is in the 300-500 μL L^{-1} concentration range (Cunha et al. 2010) and for *Hippocampus reidi* (2.5 g) between 150-300 μL L^{-1} (Cunha et al. 2011). The existence of factors (age, weight, sex, water temperature, nutrition, etc.) affects the anesthetic effectiveness. Despite the afore mentioned studied demonstrated a relationship between the lower animal's weight, the lower the optimum EOL concentration, these results may also indicate the existence of inter-specific variations and the need to conduct tests with each anesthetic and species studied (Mylonas et al. 2005; Ross and Ross 2008).

In the present study, the EOL caused no mortality of sea bass in any of the tested concentrations. The same was observed in previous studies with EOL (Cunha et al. 2010, 2011; Toni et al. 2014). Mylonas et al. (2005) observed no deaths in sea bass anesthetized with clove oil, but anesthesia with 50 or 60 mg L^{-1} eugenol (the main compound of clove oil) induced mortality (Filiciotto et al. 2012), demonstrated that EOL is safer for this species.

Experiment 2 - Stress responses

The use of anesthetics can reduce the damage induced by stress in fish, but the substance itself can also stand as a stressor, thus activating the response mechanism to stress (Bolasina 2006; Weber et al. 2009). This response is a physiological adjustment related to a perceived threat to homeostasis, which in the short term preserves the health and viability of stressed individual (Ashley 2007). However, when the tension is excessive in the intensity or duration, the stress response can lose its value and yield undesirable consequences, such as illness and mortality (Iwama et al. 2004).

The primary stress response is characterized by a significant increase in corticosteroid hormones (cortisol) and catecholamines (noradrenaline and adrenaline) released in the bloodstream (Barton 2002), starting metabolic processes for the production of extra energy for animal escape or adjustment to new conditions (Iwama et al. 2004). In the present study, although plasma cortisol levels at 3 h remained low and no significant difference was observed, it was possible to observe that within 6 h of stress cortisol increased in sea bass exposed to EOL compared to the time before the stress. Sedation with 35 μL L^{-1} EOL for 4 h also increased plasma cortisol levels in stressed and non-stressed gilthead sea bream, *Sparus aurata* (Toni et al. 2015).

Increases in plasma glucose levels are secondary responses to stress, but not necessarily there is a direct relationship between the increase of cortisol and glucose (Pankhurst 2011), as observed in the current study, that at 3 h glucose and lactate increased, but not cortisol, in sea bass submitted to 50 μL L^{-1} EOL. Similar results were found by Iversen et al. (2003) and Weber et al. (2009). These results suggest that other hormones related to the stress response, as catecholamines, which are released during the first phase of stress, could be responsible for such high levels of glucose and lactate (Wendelaar-Bonga 1997). The increase of glucose levels in sea bream exposed to 50 μL L^{-1} EOL after 6 h of stress, however, may be due to elevated cortisol values presented in this group, as observed by Toni et al. (2015) in sea bream exposed to EOL for 4 h.

The high levels of plasma and liver lactate in sea bass exposed to 50 μL L^{-1} EOL can result from poor availability of oxygen for aerobic cellular metabolism (Iversen et al. 2003), since most anesthetics have an inhibiting effect on fish respiratory system (Keene et al. 1998), causing hypoventilation and reducing oxygen consumption (Dixon and Milton 1978). Thus, one of the most common causes for the increase in plasma lactate levels is by activation of glycolysis in hypoxic muscle after a strong swimming stress (Bickler and Buck 2007), in the case of this study, caused by the persecution to capture. Moreover, in the present study plasma and hepatic lactate levels of sea bass submitted to stress and 50 μL L^{-1} decreased after 6 h compared to 3 h, but not reaching the baseline values. These results suggest that after 6 h the catecholamines have been consumed and therefore did not stimulate lactate production, which shows that the oxygen supply was restored, as in studies conducted by Inoue et al. (2011) and Toni et al. (2014), that observed an increase in lactate levels in Atlantic salmon (*Salmo salar*) and silver catfish (*R. quelen*) anesthetized with clove oil and EOL, respectively, and after these values returned to baseline.

Protein catabolism plays an important role in the total energy production in fish (David et al. 2004). The variation in plasma protein concentrations due to a stressful situation is also considered a secondary response (Wendelaar-Bonga 1997; Barton 2002). The mobilization of protein as an energy source is dependent on the intensity of the stress that the animal is submitted (Merighe et al. 2004). In fish exposed to the highest concentration of EOL (50 μL L^{-1}), decrease of protein levels occurred since 3 h of stress, but after 6 h of stress plasma protein levels decreased in sea bass of all treatments, indicating that EOL exposure was not the only responsible for this change in plasma protein. Silver catfish exposed to 50 μL L^{-1} of the essential oil of *H. ringens* also increased protein levels after 6 h (Toni et al. 2015).

Triglycerides are the energy source to other metabolic pathway (Merighe et al. 2004). The decrease in triglyceride levels during a stress suggests a degradation of lipids and mobilization of triglycerides to cope with the increased energy demand by stress (Menezes et al. 2015). The persecution to capture is a strong stressor that reduces energy sources, such as liver lipid reserves, which were consumed by the sea bass liver in the control and 25 μL L^{-1} EOL groups after 3 and 6 h of stress, and at 50 μL L^{-1} EOL after only 3 h of stress. However, there was an increase in plasma triglyceride level in fish from the ethanol and 25 μL L^{-1} EOL groups after 6 h of stress, indicating an oxidation of the lipid energy stores in these animals.

Secondary stress responses are characterized by reduction of hepatic glycogen content, suggesting the compensation of the great demand for energy through of the increasing glycogenolysis during stress, increasing plasma glucose levels, which provide glucose to tissues to restore homeostasis (Filiciotto et al. 2012; Dhanasiri et al. 2013; Menezes et al. 2015). The results of the present study corroborate this theory, because all treatments showed reductions in liver glycogen and glucose increased after subjecting sea bass to stress, except those exposed to 25 μL L^{-1} EOL, which did not change glucose levels. After 6 hours of stress liver glycogen levels were significantly lower in sea bass exposed to both EOL concentrations and ethanol. The use of the essential oil of *Aloysia triphylla* in the water of transport (6 h) of silver catfish also resulted in significantly lower hepatic glycogen levels compared to the control (Zeppenfeld et al. 2014).

The stress by persecution to capture (a very common procedure) and / or exposure to anesthetic (indispensable procedure) can affect many metabolic parameters and induce a significant energy cost to reorganize the whole animal metabolism in order to adapt to the stressful event. The activities of enzymes involved in hepatic metabolism can aid understanding how the fish metabolism rearranges (Toni et al. 2015).

Sea bass exposed to EOL had higher activity of FBP and G6PDH (pentose phosphate pathway) (and also PK activity in those exposed to the highest EOL concentration) after 6 h of stress. These results suggest an increase of the gluconeogenesis potential, which is a substitute pathway to glycogenolysis when glycogen levels are very low and reach their maximum consumption limits, which is consistent with glycogen results of the present study. Gluconeogenesis is a defensive or adaptive response to the decrease of glucose levels (Menezes et al. 2014). Strengthening this idea, sea bass exposed to 50 μL L^{-1} EOL after 6 h of stress also presented lower lactate levels, which possibly are due to lactate conversion to glucose (gluconeogenesis).

The use of amino acids as an energy source can help to cover the energy needs of the animal (Vargas-Chacoff et al. 2009). Regarding amino acids metabolism, there was an increase in the GDH activity after 6 h of stress in sea bass exposed to EOL, while GPT activity increased after 3 h of stress only at the highest EOL concentration (50 μL L^{-1}). These results suggest an increase in the degradation of amino acids and the GPT activity returned to baseline within 6 h of stress, a further evidence of the use of gluconeogenesis, causing the conversion of amino acids to glucose.

The ideal concentration for sedation of *D. labrax* is 25 μL L^{-1} and anesthesia is 600 μL L^{-1} EOL. Results demonstrate that EOL at 25 μL L^{-1}, even with subtle alterations it is worth using, because was effective as sedative during stress procedures, whereas 50 μL L^{-1} after 6 h of stress increases sea bass stress. Due to observed changes in hepatic metabolism of the fish exposed to EOL, the gluconeogenic pathway is suggested as an energy mechanism that the animal uses to prepare (stress response). The EOL is a good alternative as sedative and/or anesthetic to sea bass in aquaculture procedures and research stations.

ACKNOWLEDGMENTS

The authors thank Servicios Centrales de Investigación de Cultivos Marinos (SCI-CM) (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish. Experiment has been carried out at the University of Cádiz. L.N. Simões and B. Baldisserotto received Ph.D. fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), respectively.

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Table 1 Chemical composition of *Lippia alba* essential oil. * RI = Retention Index; [a]NIST 2010; [b]Toni et al. 2015

RI* Experimental	RI Literature	Chemical Compound	Composition (%)
1028	1028[a]	Eucalyptol	8.28
1099	1099[a]	Linalool	75.49
1141	1146[a]	Camphore	1.26
1209	1207[b]	*E*,*E*-2,6-Dimethyl-3,5,7-octatrien-2-ol	1.18
		Total of monoterpenoids	**86.21**
1417	1417[b]	β-Caryophyllene	3.97
1479	1480[a]	Germacrene D	5.10
1480	1480[b]	γ-Muurolene	0.41
		Total of sesquiterpenoids	**9.48**
		Total of identified compounds	**95.69**

Table 2 Induction time (seconds) to sedation, anesthesia and recovery of *D. labrax* exposed to the essential oil of *L. alba* (mean ± SEM). Different superscript letters represent significant differences between the concentrations ($P < 0.05$)

Concentration (μL L^{-1})	Sedation	Anesthesia	Recovery
25	153.3 ± 17.5^{a}	-	198.3 ± 32.9^{a}
35	153.3 ± 6.8^{a}	-	210.3 ± 26.0^{a}
50	100.9 ± 9.6^{ab}	-	224.8 ± 17.8^{ab}
100	37.2 ± 3.9^{abc}	1673.8 ± 59.48^{a}	314.6 ± 31.6^{abcd}
200	17.1 ± 0.9^{be}	902.0 ± 114.3^{b}	336.4 ± 20.3^{bcd}
300	21.1 ± 1.1^{ae}	636.6 ± 23.9^{c}	385.9 ± 18.8^{cd}
400	17.8 ± 1.0^{be}	666.8 ± 52.9^{bc}	415.1 ± 40.7^{d}
600	15.1 ± 2.7^{ce}	296.0 ± 14.6^{d}	292.7 ± 55.4^{ac}
800	13.8 ± 1.7^{e}	257.0 ± 10.3^{d}	304.8 ± 62.8^{acd}

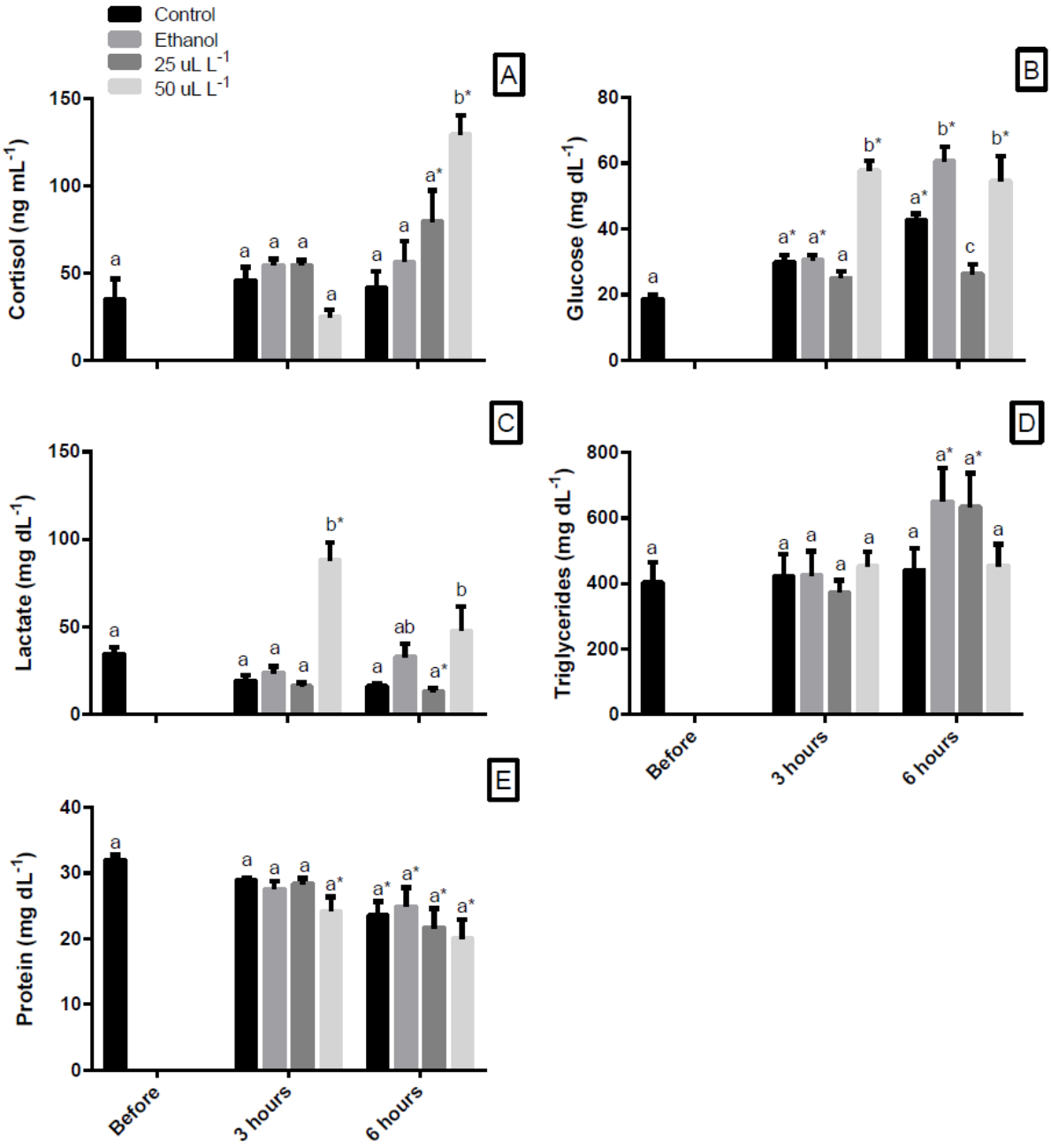


Fig. 1 Effects of the essential oil of *L. alba* on plasma levels of cortisol (a), glucose (b), lactate (c), triglycerides (d), and protein (e) of *D. labrax* subjected to stress. Lowercase letters indicate significant difference between treatments at the same sampling time ($p < 0.05$). * Indicate significant difference compared to the control group before the stress. Results presented as mean ± SEM

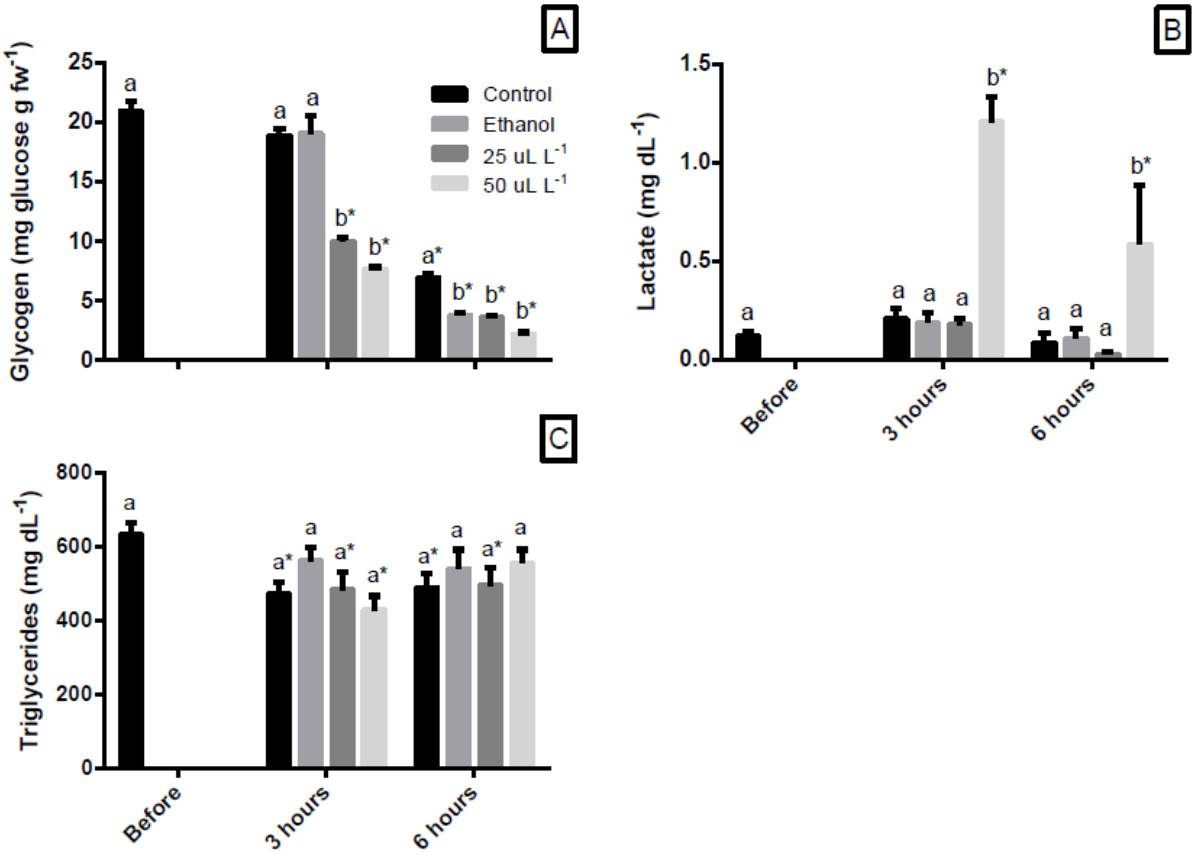


Fig. 2 Effects of the essential oil of *L. alba* on hepatic levels of glycogen (a), lactate (b), and triglycerides (c) of *D. labrax* subjected to stress. Lowercase letters indicate significant difference between treatments at the same sampling time ($p < 0.05$). * Indicate significant difference compared to the control group before the stress. Results presented as mean ± SEM

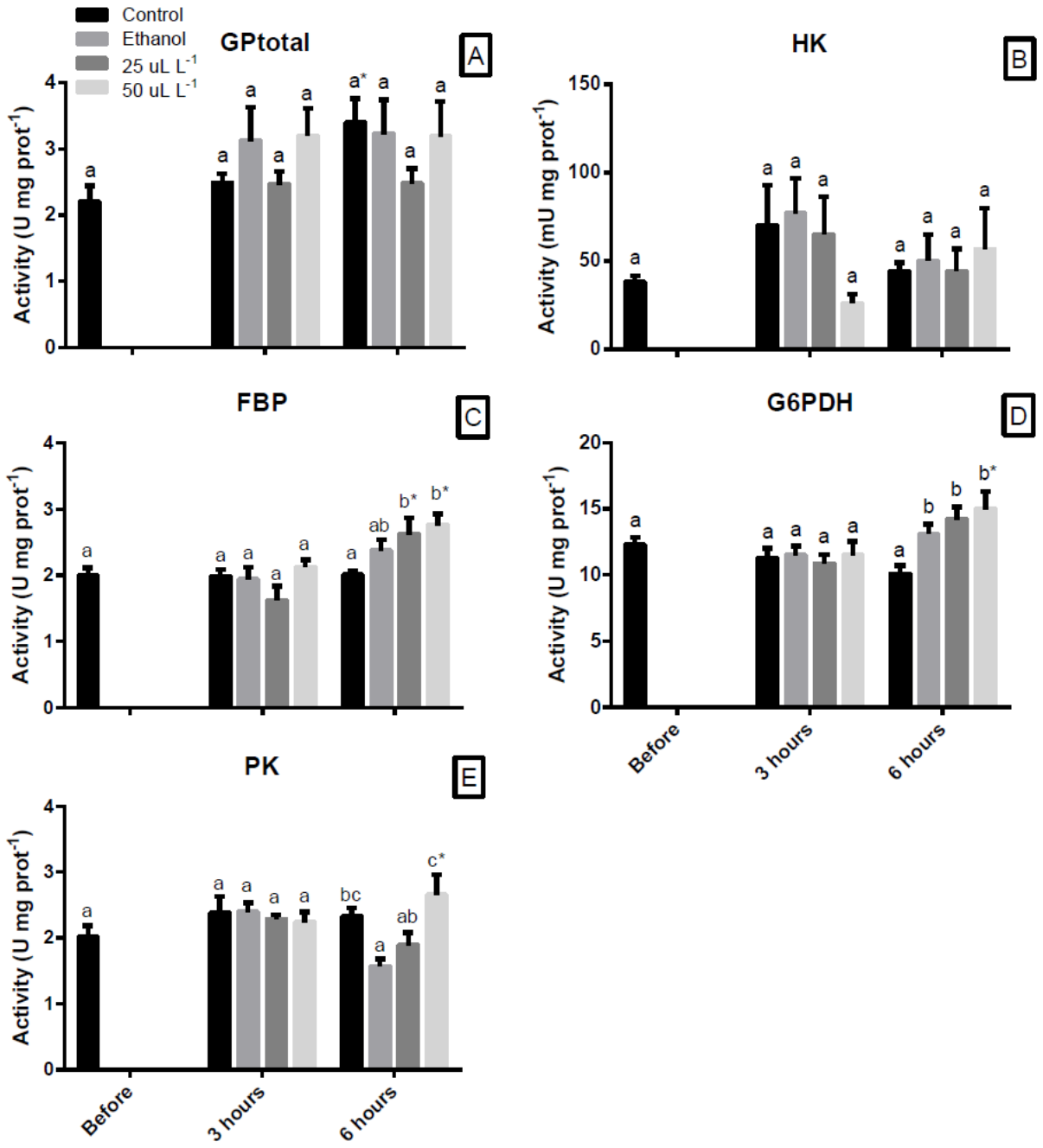
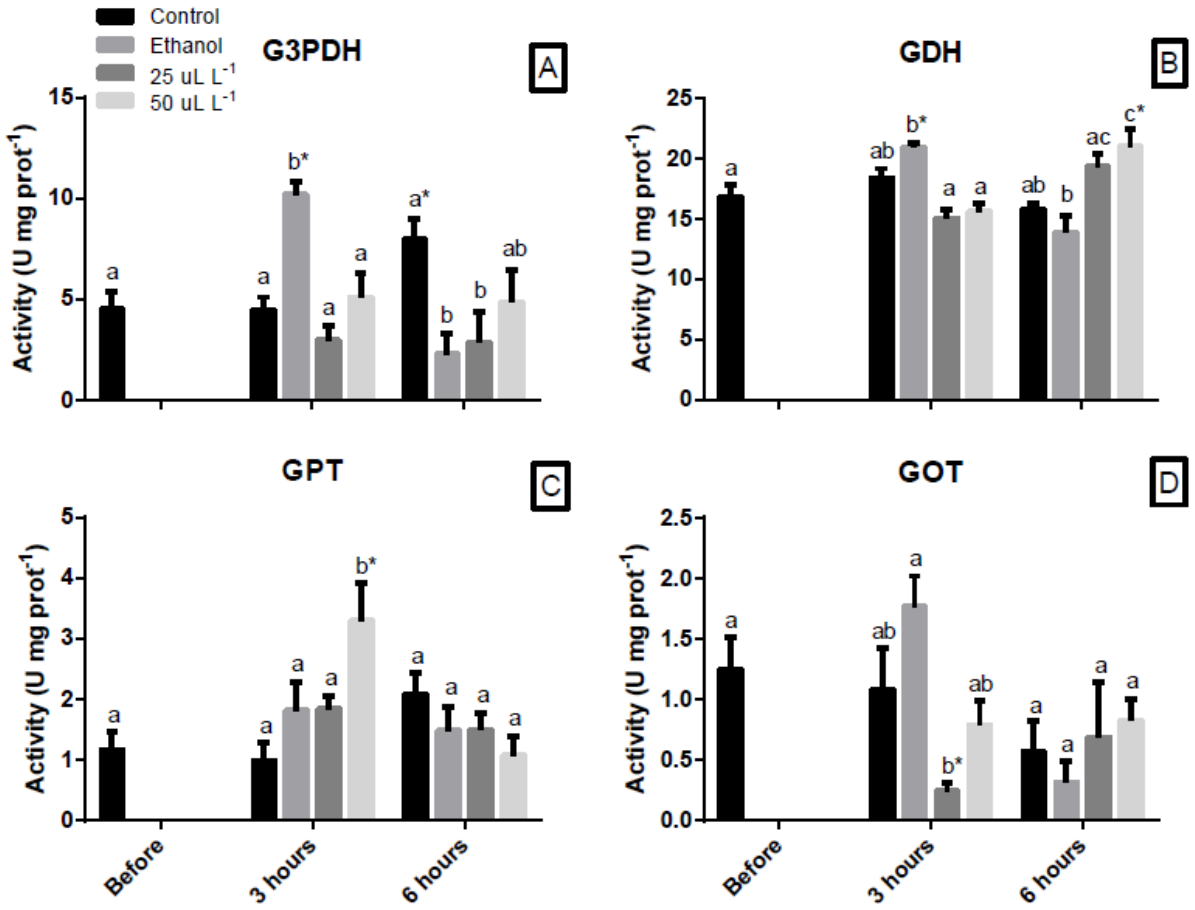


Fig. 3 Effects of the essential oil of *L. alba* in the enzymatic activity (glycogen phosphorilase total - GPtotal (A), hexokinase - HK (B), fructose-biphosphatase - FBP (C), glucose-6-phosphate dehydrogenase - G6PDH (D), and pyruvate kinase - PK (E)) in the liver of *D. labrax* subjected to stress. Lowercase letters indicate significant difference between treatments at the same sampling time ($p < 0.05$). * Indicate significant difference compared to the control group before the stress. Results presented as mean ± SEM



773
774 **Fig. 4** Effects of the essential oil of *L. alba* in the enzymatic activity (glycerol-3-phosphate
775 dehydrogenase - G3PDH (A), glutamate dehydrogenase GDH (B), glutamic-pyruvic transaminase -
776 GPT (C), and glutamic oxaloacetic transaminase - GOT (D)) in the liver of *D. labrax* subjected to
777 stress. Lowercase letters indicate significant difference between treatments at the same sampling time
778 ($p < 0.05$). * Indicate significant difference compared to the control group before the stress. Results
779 presented as mean ± SEM
780

5 CONCLUSÃO

A sedação dos animais aquáticos além de minimizar o tempo de manipulação, lesão física direta e efeitos de estresse durante procedimentos simples, é útil na redução da taxa metabólica e, consequentemente, o consumo de oxigênio, e na redução da excreção de produtos metabólicos na água. Em termos gerais, sedação (definido como um efeito calmante) é um estado preliminar de anestesia, mas no qual não há perda total de percepção sensorial ou de equilíbrio, como ocorre na anestesia. Anestesia geral pode ser definida como uma perda reversível e generalizada da percepção sensorial acompanhada de um estado ao sono induzido (Heavner, 1981)

O uso de produtos naturais, como óleos essenciais, derivados de plantas, é uma alternativa viável para procedimentos que necessitem de sedação ou anestesia de animais aquáticos, frente às dificuldades de obtenção dos químicos (Façanha e Gomes, 2005).

O atual trabalho analisando o óleo essencial de *Lippia alba* (EOL) obteve um resultado bastante positivo, além de não causar nenhuma mortalidade, apresentou o efeito sedativo e/ou anestésico satisfatório nas espécies de animais aquáticos testados, com exceção apenas do artigo 1 dos mexilhões (*Perna perna*), tendo em conta o fechamento das valvas como um comportamento do animal frente a um xenobiótico. Entretanto, ocorreu o aumento da atividade de enzimas antioxidantes CAT, GST, e SOD, e diminuição do LPO e PC, sugerindo que o EOL e consequentemente, a hipóxia multifuncional, por meio do fechamento das valvas, pode melhorar a resposta das defesas antioxidantes e estresse oxidativo em mexilhões.

Para ouriço do mar (*Echinometra lucunter*), no artigo 2, a melhor concentração eleita para a anestesia é 150 mL/L de EOL. Apesar dos efeitos sobre as reservas de energia, acredita-se que o tempo era muito curto para provocar tais efeitos, e diante do baixo nível de TBARS e alta atividade de SOD e CAT no fluido celomático e gônadas de ouriços expostos ao EOL e que em geral foram mantidos depois de recuperados, sugere-se que o EOL melhora a resposta ao estresse oxidativo e é um anestésico muito promissor para ouriços do mar.

Assim como, a exposição ao EOL demonstrou um efeito favorável em lubinas, *Dicentrarchus labrax* (artigo 3), sendo 25 mL/L eleita a concentração ideal para sedação e 600 mL/L de EOL para anestesia. Os resultados demonstram que EOL a 25 mL/L, mesmo com alterações sutis acredita-se valer a pena o seu uso, porque foi eficaz como sedativo durante os procedimentos de estresse, enquanto que 50 mL/L após 6 h de estresse aumenta o estresse da lubina. Devido às alterações observadas no metabolismo hepático do peixe exposta

a EOL, a via gliconeogênica é sugerido como um mecanismo de energia que o animal utiliza para se preparar (resposta ao estresse).

Sendo assim, o EOL demonstra ser uma boa alternativa para estas espécies de animais aquáticos em procedimentos de estações de aquicultura e pesquisa.

Além dos 3 artigos descritos nesta tese, foram gerados outros trabalhos, que se tornarão em breve artigos científicos, que seguem a mesma linha de pesquisa e os objetos dos estudos foram:

- Mexilhão (*Perna perna*) com eugenol (constituinte majoritário do óleo de cravo);
- Mexilhão (*Perna perna*) com mentol;
- Ouriço do mar (*Echinometra lucunter*) com mentol;
- Jundiá (*Rhamdia quelen*) durante o transporte (estresse) com *Lippia alba*; e
- Robalo (*Centropomus parallelus*) em diferentes salinidades (estresse) com *Lippia alba*.

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Marine and Freshwater Behaviour and Physiology

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  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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Autorização para atividades com finalidade científica

Número: 33571-1	Data da Emissão: 26/04/2012 08:38

Dados do titular

Nome: Bernardo Baldisserotto	CPF: 405.443.620-04
Título do Projeto: O USO DO ÓLEO ESSENCIAL DE Lippia alba E EUGENOL COMO ANESTÉSICOS EM ALGUNS ANIMAIS AQUÁTICOS MARINHOS	
Nome da Instituição : Prefeitura da Cidade Universitária - UFSM	CNPJ: 95.591.764/0001-05

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#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
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2	Revisão Bibliográfica	06/2012	01/2015
3	Experimentos do capítulo 1	07/2012	12/2012
4	Experimentos do capítulo 2	01/2013	12/2013
5	Experimentos do capítulo 3	01/2014	12/2014
6	Redação da Tese	01/2014	01/2015
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8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Larissa Novaes Simões	Executora	108.722.577-97	1957273 SSP-ES	Brasileira
2	Levy de Carvalho Gomes	Colaborador	024.833.107-88	10357610-4 IFP-RJ	Brasileira
3	LUCIANO DE OLIVEIRA GARCIA	Colaborador	919.132.760-15	8047330819 SSP-RS	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1		RS	Praia do Cassino	Fora de UC Federal

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº154/2007. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 33571-1	Data da Emissão: 26/04/2012 08:38

Dados do titular

Nome: Bernardo Baldisserotto	CPF: 405.443.620-04
Título do Projeto: O USO DO ÓLEO ESSENCIAL DE Lippia alba E EUGENOL COMO ANESTÉSICOS EM ALGUNS ANIMAIS AQUÁTICOS MARINHOS	
Nome da Instituição : Prefeitura da Cidade Universitária - UFSM	CNPJ: 95.591.764/0001-05

2		ES	Praia da Costa	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Captura de animais silvestres in situ	Actinopterygii, Bivalvia, Elasmobranchii, Polychaeta, Echinoidea, Malacostraca, Asteroidea
2	Coleta/transporte de espécimes da fauna silvestre in situ	Echinoidea (*Qtde: 20), Elasmobranchii (*Qtde: 35), Actinopterygii (*Qtde: 800), Asteroidea (*Qtde: 20), Malacostraca (*Qtde: 20), Polychaeta (*Qtde: 20), Bivalvia (*Qtde: 20)
3	Manutenção temporária (até 24 meses) de invertebrados silvestres em cativeiro	Echinoidea, Polychaeta, Asteroidea, Malacostraca, Bivalvia
4	Manutenção temporária (até 24 meses) de vertebrados silvestres em cativeiro	Actinopterygii

* Qtde. de indivíduos por espécie/localidade/unidade de conservação, a serem coletados durante um ano.

Material e métodos

1	Método de captura/coleta (Invertebrados Aquáticos)	Coleta manual, Draga, pegador (Van veen, Box corer, Holme, Petersen, etc.)
2	Método de captura/coleta (Peixes)	Puçá, Anzol e linha (op.manual):linha de mão,de corso,carretilha,molinete,corrico,vara e isca viva, Rede de arrasto de praia: cerco de praia (tração manual)

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	SOCIEDADE EDUCACIONAL DO ESPIRITO SANTO - UVV	
2	UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG	

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Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

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Nome da Instituição : Prefeitura da Cidade Universitária - UFSM	CNPJ: 95.591.764/0001-05

Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº154/2007, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

* Identificar o espécime no nível taxonômico possível.

Código de autenticação: 24965644

ANEXO E - APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS NA UNIVERSIDADE DE VILA VELHA (CEUA/UVV) (Nº 218/2012)

UNIVERSIDADE VILA VELHA
ESPÍRITO SANTO

Comissão de Ética no Uso de Animais (CEUA- UVV)

PARECER CONSUBSTANCIADO

Parecer Nº. 218/2012- Protocolo de Pesquisa

Pesquisador (a) Responsável: Prof. Dr. Levy de Carvalho Gomes

O uso do óleo essencial de *Lippia alba* e eugenol como anestésicos em alguns animais aquáticos.

Situação: **APROVADO**

A Comissão de Ética no Uso de Animais da Universidade Vila Velha (CEUA- UVV) analisou na sessão do dia 23 de outubro de 2013 o processo nº 292- 2013, referente o projeto: **"O uso do óleo essencial de *Lippia alba* e eugenol como anestésicos em alguns animais aquáticos"**, tendo como responsável Prof. Dr. Levy de Carvalho Gomes, sendo considerado adequado e satisfatoriamente de acordo com as exigências das Resoluções que regem esta comissão, aparado pela Portaria CFBio 148/2012, enquanto a mesma tiver validade.

Assim, mediante a importância social e científica que o projeto apresenta a sua aplicabilidade e conformidade com os requisitos éticos, somos de parecer favorável à realização do projeto classificando-o como **APROVADO**, pois o mesmo atende aos Requisitos Fundamentais das Normas de Conduta para a Utilização de Animais no Ensino, Pesquisa e Extensão da Universidade Vila Velha.

Solicita-se ao (à) pesquisador (a) o envio a esta CEUA, de relatórios parciais sempre quando houver alguma alteração no projeto, bem como o relatório final enviado em PDF.

Vila Velha, 23 de outubro de 2013.

Prof. Dr. João Luiz Rossi Junior
Coordenador da CEUA-UVV.

Universidade Vila Velha
Rua Comissário José Dantas de Melo, 21, Boa Vista, Vila Velha-ES, CEP.: 29102-770
joao.rossi@uvv.br

ANEXO F – CERTIFICADO DE COMPETÊNCIA PARA TRABALHAR COM ANIMAIS DE EXPERIMENTAÇÃO - ESPANHA

Instituto de Investigación y Formación Agraria y Pesquera
CONSEJERÍA DE AGRICULTURA , PESCA Y MEDIO AMBIENTE

Fecha: 25 de febrero de 2013
Ref. MILI/alvn
Asunto: Certificado de competencia para trabajar con animales de experimentación.

Juan Antonio Martos Sitcha
Instituto de Ciencias Marinas de Andalucía
Avda. República Árabe Saharawi, N°2
11510 Campus Universitario
Puerto Real (Cádiz).

Vista su solicitud de expedición de solicitud de certificación de competencia para trabajar con animales de experimentación (Decreto 80/2011, por el que se regula la formación en bienestar animal), se remite el correspondiente certificado de competencia para trabajar con animales de experimentación en su categoría C.

LA JEFA DE SERVICIO DE FORMACIÓN

Fdo.: María Isabel López Infante

Edificio BLUENET. Avda. Isaac Newton nº 3, Planta 2ª
Parque Tecnológico Cartuja '93. 41092 Sevilla
Tel. 954 994 600. Fax 954 994 664

Certificado de competencia de bienestar animal en animales utilizados para experimentación y otros fines científicos (categoría C).

1. IDENTIFICACIÓN

1.1 Apellidos

MARTOS SITCHA

1.2 Nombre

JUAN ANTONIO

1.3 Fecha de nacimiento	1.4 Lugar y país de nacimiento	1.5 Nacionalidad
18/03/1985	CARTAGENA, MURCIA (ESPAÑA)	ESPAÑOLA (23048928F)

2. Nº DEL CERTIFICADO: **CONV-1300100**

3. ORGANISMO QUE EXPIDE EL CERTIFICADO

3.1 Nombre y dirección del organismo que expide el certificado

CONSEJERÍA DE AGRICULTURA, PESCA Y MEDIO AMBIENTE
INSTITUTO DE INVESTIGACIÓN Y FORMACIÓN AGRARIA Y PESQUERA
Edificio BLUENET. Avda. Isaac Newton 3, 2ª
Parque C. y T. Cartuja' 93 41092 Sevilla(España)

3.2 Teléfono	3.3 Fax	3.4 Correo electrónico/Email
954 994 594	954 994 622	bienestar.ifapa@juntadeandalucia.es

3.5 Fecha	3.6 Lugar	3.7 Sello
25/02/2013	SEVILLA	

3.8 Nombre y firma

El Presidente del Instituto de Investigación y Formación Agraria y Pesquera

Fdo. Víctor Ortiz Somovilla

DECRETO 80/2011, DE 12 DE ABRIL, POR EL QUE SE REGULA LA FORMACIÓN EN BIENESTAR ANIMAL.