UNIVERSIDADE FEDERAL DE SANTA MARIA
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PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

AVALIAÇÂO DO EUGENOL E DE ÓLEOS ESSENCIAIS NA REDUÇÃO DO ESTRESSE EM AQUICULTURA

TESE DE DOUTORADO

Thaylise Vey Parodi

Santa Maria, RS, Brasil
2014

AVALIAÇÂO DO EUGENOL E DE ÓLEOS ESSENCIAIS NA REDUÇÃO DO ESTRESSE EM AQUICULTURA

Thaylise Vey Parodi

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

Orientador: Prof. Dr. Bernardo Baldisserotto

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

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Elaborada por
Thaylise Vey Parodi

como requisito parcial para a obtenção do grau de
Doutor em Farmacologia

COMISSÃO EXAMINADORA

Bernardo Baldisserotto, Dr
(Presidente/Orientador)

Marilise Escobar Bürger, Drª. (UFSM)

Maria Amália Pavanato, Drª. (UFSM)

Levy de Carvalho Gomes, Dr. (UVV)

Luís André Nasser de Sampaio, Dr. (FURG)

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
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AVALIAÇÂO DO EUGENOL E DE ÓLEOS ESSENCIAIS NA REDUÇÃO DO ESTRESSE EM AQUICULTURA

AUTORA: THAYLISE VEY PARODI
ORIENTADOR: BERNARDO BALDISSEROTTO
Local e Data da Defesa: Santa Maria, 07 de janeiro de 2014.

O trabalho objetivou avaliar a eficácia anestésica do eugenol e óleos essenciais (OEs) de *Aloysia triphylla* e *Lippia alba* em aquicultura, bem como a eficácia dos mesmos como redutores de estresse durante o transporte de camarões e peixes e verificar efeito bloqueador de estímulos nocivos do eugenol. Camarões (sub-adultos e pós larvas), ambas variedades de jundiá e robalos-peva foram transportados em água contendo óleo essencial de *A. triphylla* em diferentes concentrações e avaliada a redução do estresse. Para eugenol foi traçado modelo experimental de avaliação da atividade analgésica em peixe-zebra (*Danio rerio*). As concentrações de eugenol e OE de *A. triphylla* e *L. alba* recomendadas para anestesia em camarões foram, respectivamente de 200, 300,750 µL L^{-1} para sub-adultos e para pós-larvas 175, 300 e 500 µL L^{-1}. Para transporte concentrações entre 20-50; 20-30 e 50 µL L^{-1} são apropriadas para sub-adultos e para pós-larvas somente eugenol e OE de *A. triphylla* 20 e 20-50 µL L^{-1}. Uma melhor capacidade antioxidante na hemolinfa de camarões foi obtida com 30 µL L^{-1} OEs de *A. triphylla* e *L. alba* e 20 µL L^{-1} para eugenol. Juvenis albinos de jundiá induziram e recuperaram da anestesia em maior tempo que os cinzas e considerou-se 200 µL L^{-1} a concentração mais adequada. No transporte, embora o OE de *A. triphylla* tenha causado aumento de cortisol corporal em jundiás, houve redução da perda de íons. Em robalos-peva o aumento da concentração do OE de *A. triphylla* proporcionalmente diminuiu e aumentou o tempo de indução e recuperação anestésica, respectivamente. A concentração de 20 µL L^{-1} reduziu os níveis de glicose sanguínea após 24h e de cortisol e lactato após 6h. O eugenol por si só não apresentou atividade analgésica, porém quando injetado ácido acético na porção anterior de zebrafish a resposta ao efeito nocivo foi bloqueada.

Palavras-chave: *Aloysia triphylla*. Aquicultura. Anestesia. Estimulo Nocivo. Parâmetros sanguíneos. Transporte, Sedação, Óleos essenciais. Estresse oxidativo.

ABSTRACT

Doctoral Thesis
Graduate Program Pharmacology
Federal University of Santa Maria

EVALUATION OF THE STRESS-REDUCING EFFECT OF EUGENOL AND ESSENTIAL OILS ON AQUACULTURE

AUTHOR: Thaylise Vey Parodi
ADVISER: Bernardo Baldisserotto
Place and date of defense: Santa Maria, January 7th, 2014.

This study evaluated the anesthetic efficacy of eugenol and essential oils (EOs) of *Aloysia triphylla* and *Lippia alba* on aquaculture, as well as their stress-reducing effect during transport of shrimp and fish. The effectiveness of eugenol on blocking noxious stimulis was also tested. Sub-adult and post-larvae of white shrimp (*Litopenaeus vannamei*), albine and grey silver catfish (*Rhamdia quelen*) and fat snook (*Centropomus paralellus*) were evaluated for anesthetic induction time and recovery during short and long exposure to the EOs and eugenol. Oxidative stress parameters were also evaluated in sub-adult shrimps. Both silver catfish strains and fat snook were transported in water containing EO of *A. triphylla* at different concentrations and the reduction of stress was evaluated. An experimental model was elaborated to test the analgesic activity of eugenol in zebrafish (*Danio rerio*). The concentrations of eugenol, EO of *A. triphylla* and *L. alba* recommended for anesthesia in shrimps were respectively 200, 300 and 750 μL L^{-1} for sub-adults and 175, 300 and 500 μL L^{-1} for post-larvae. The concentrations indicated for transport are between 20-50, 20-30 and 50 μL^{-1} for sub–adults and for post-larvae only eugenol and EO of *A. triphylla* at 20 and 20-50 μL L^{-1}. A better antioxidant capacity in the hemolymph was obtained with 30 μL L^{-1} EOs of *A. triphylla* and *L. alba* and 20 μL L^{-1} eugenol. Albine silver catfish juveniles induced and recovered form anesthesia in longer time than grey ones and 200 μL L^{-1} was considered the most suitable concentration for both strains. In the transport the OE de *A. triphylla* increased whole body cortisol but did not promote ion loss. The increase of the concentration of OE *A. triphylla* proportionately decreased and increased the time of induction and recovery from anesthesia in fat snooks. The concentration of 20 μL L^{-1} reduced blood glucose levels after 24 h and plasma cortisol and lactate levels after 6h. Eugenol itself did not presented analgesic activity, but after the injection of acetic acid in the anterior portion eugenol blocked the noxious reflex.

Keywords : *Aloysia triphylla* . Aquaculture. Anesthesia. Noxious stimulis. Blood parameters . Transportation, Sedation , Essential Oils . Oxidative stress

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SUMÁRIO

1. INTRODUÇÃO

O setor de aquicultura e comércio de organismos aquáticos é um setor rentável, tendo a pesca extrativista continental e marinha como predominante atividade. O transporte de peixes vivos é uma rotina que representa um considerável custo e risco aos piscicultores, transportadores de peixes e proprietários de pesqueiros. O uso de estratégias adequadas de transporte permitem minimizar tais riscos e custos (VIDAL, 2002).

Durante a captura e confinamento para depuração (jejum), carregamento, transporte e descarregamento os peixe sofrem pressões estressoras diversas. Tais pressões podem ser físicas ou mecânicas, fisiológicas ou químicas que resultam de injúrias físicas causadas durante a captura e confinamento. O estresse fisiológico diz respeito às alterações nos processos metabólicos, osmorregulatórios, inflamatórios e imunológicos dos peixes. O estresse fisiológico pode começar antes mesmo de completada a despesca, desencadeado por estímulos hormonais. O confinamento dos peixes na rede após a captura resulta em uma localizada depleção no oxigênio dissolvido, marcando o início do estresse fisiológico. A resposta primária é marcada pelo aumento imediato dos níveis de hormônios corticosteróides (dentre eles o cortisol) e de catecolaminas (dentre as quais a epinefrina ou adrenalina). A liberação das catecolaminas estimula a hidrólise da reservas de glicogênio no fígado, aumentando os níveis de glicose no sangue, marcando o início da resposta secundária. A taxa de batimento cardíaco e o fluxo sanguíneo nas brânquias também são acelerados nesta fase. Os corticosteróides por sua vez desencadeiam um significativo aumento da permeabilidade das membranas celulares. No caso dos peixes de água doce, ocorre um desequilíbrio osmorregulatório devido à excessiva entrada de água e perda de íons através do epitélio branquial. O desequilíbrio osmorregulatório é marcado pela alteração no balanço de eletrólitos (íons) e água no sangue e no tecido dos peixes (BONGA, 1997).

Nas últimas décadas as avaliações clássicas dos efeitos biológicos de substâncias químicas nos organismos aquáticos têm se baseado nos resultados de testes de laboratoriais para uma dada espécie. A padronização destes testes forneceu um meio eficiente e de custo moderado para o monitoramento dos efeitos adversos potenciais de algumas substâncias químicas durante o manuseio dos animais e que possam interferir na fisiologia normal (RAND, PETROCELLI,1985)

Os anestésicos reduzem a atividade e o metabolismo dos peixes durante as operações relacionadas ao transporte, reduzindo as injúrias físicas, o consumo de oxigênio e a excreção de metabólitos tóxicos. Sendo assim é possível aumentar a carga de peixes minimizando os custos do transporte. Nas operações de pesagem e carregamento, o uso de anestésicos é bastante recomendado pois ajudam a acalmar os peixes facilitando o manuseio. Nas primeiras horas de transporte o consumo de oxigênio é bastante elevado. Concentrações moderadas de anestésicos reduz este consumo resultando em grande economia de oxigênio. Ao longo do transporte os anestésicos devem ser usados em doses sedativas moderadas, ou seja, doses que não façam o peixe perder por completo o equilíbrio, mas proporcione apenas uma leve redução no batimento opercular e faça com que os peixes apresentem pouca reação ao toque ou a estímulos visuais. Peixes profundamente anestesiados podem ficar asfixiados no fundo do tanque (COOKE *et al*., 2004)

O uso de drogas terapêuticas em peixes é dificultado em função da farmacocinética em cada espécie e pela questão dos resíduos para o consumo humano, pouco estudado nas espécies aquáticas nativas, entre outros fatores. Internacionalmente os anestésicos mais utilizados na aquicultura são a tricaína metanossulfonato (MS-222, nos Estados Unidos e TMS, no Canadá), benzocaína, quinaldina, metomidato, 2-fenoxietanol, mentol, eugenol (ROSS, ROSS, 2008).

Estas questões atualmente não estão esclarecidas, porém o uso de terapêuticos nesta atividade é uma necessidade antiga e real. Baseando-se nisso seguem alguns referenciais para melhor entendimento de anestesia em animais aquáticos.

1.1 Dor: conceito e mecanismos gerais de percepção

A dor é considerada uma experiência sensorial e emocional desagradável associada a dano tissular presente ou potencial. Seu mecanismo envolve a transmissão a partir de nociceptores localizados na pele e vísceras os quais podem ser ativados por estímulos mecânicos, térmicos e químicos, e cuja resposta pode ser modulada por meio da ação de prostaglandinas, cininas, catecolaminas, íons hidrogênio (H^+), potássio (K^+) e substância P (um neurotransmissor específico das fibras condutoras do estímulo doloroso) (HUGHES *et al*., 1975). Tais estímulos são conduzidos através de dois tipos de fibras nervosas (fibras A-δ e C) até o corno dorsal da coluna espinhal, onde realizam sinapses com interneurônios medulares, podendo ser modulados por peptídeos opióides endógenos (MENDEL, WALL, 1965). Da medula espinal, os estímulos dolorosos

percorrem os tratos espinotalâmicos e espinorreticulares, alcançando estruturas nervosas centrais (formação reticular, tálamo, sistema límbico, córtex cerebral), onde são modulados novamente via receptores opióides (MELZACK, 1965; IKEDA *et al*., 2006).

A interpretação do estímulo doloroso é individual e sofre influência dos padrões culturais, do grau de medo e ansiedade e das experiências dolorosas prévias. A partir dessa percepção da dor pelo Sistema Nervoso Central (SNC), são obtidas as respostas motoras, autonômicas e comportamentais diante do estímulo doloroso (JI *et al*., 2003).

A dor desencadeia uma série de respostas neuroendócrinas e cardiovasculares com o objetivo de preparar o organismo contra a agressão, em um tipo de resposta de "luta ou fuga". Nem sempre é fácil detectar os sinais de que a dor está pronunciada, porém muitos animais respondem à dor de forma subjetiva, alterando o comportamento e/ou com alterações fisiológicas (JI *et al*., 2003). Dependendo da intensidade da dor pronunciada existem possibilidades de amenizá-la com o uso de analgésicos, anestésicos e/ou sedativos.

1.2 **Mecanismos de controle da dor**

A intensidade com que os diferentes indivíduos sentem e reagem a situações semelhantes causadoras de dor é bastante variada. Esta variação deve-se à diferença na ativação das vias analgésicas naturais nos indivíduos. A via analgésica principal tem 4 componentes principais de modulação para a percepção da dor no ser humano:

1.2.1 As áreas cinzentas periaquedutais e periventriculares do mesencéfalo e ponte superior, em volta do aqueducto de Sylvius enviam axônios que secretam encefalinas, que são opióides naturais (atuam no receptor dos opióides) (CARVALHO, LEMÔNICA, 1998, acesso 22 outubro, 2013; GUYTON, HALL, 2008);

1.2.2 Núcleos magno da rafe e reticular gigantocelular, localizados na ponte inferior e medula superior, recebem os axônios das áreas periaquedutais, e enviam os seus para as colunas dorsolaterais da coluna espinhal, onde liberam serotonina (CARVALHO, LEMÔNICA, 1998, acesso 22 outubro, 2013; GUYTON, HALL, 2008)**;**

1.2.3 Núcleos de interneurônios na coluna espinhal dorsal, localizados na substância gelatinosa, inibem a criação de potenciais de ação ao liberar encefalinas

e endorfinas na sinapse local com os neurônios aferentes da dor (CARVALHO, LEMÔNICA, 1998, acesso 22 outubro, 2013; GUYTON, HALL, 2008).

Com a finalidade de amenizar ou mesmo abolir a dor a farmacologia apresenta diferentes classes de fármacos que utilizados individualmente ou em associação são capazes de controlar a dor por diferentes modos de ação. De modo geral os principais fármacos utilizados no controle da dor englobam atividade analgésica, sedativa e/ou anestésica.

Analgesia ocorre quando há diminuição ou interrupção das vias de transmissão nervosa, suprimindo a dor. Há dois mecanismos de ação para analgesia que dependem do grupo medicamentoso envolvido: antiinflamatórios não hormonais (AINH) produzem analgesia por bloqueio periférico da produção de prostaglandinas (quimiotáxicos do processo inflamatório) e analgésicos opióides que atuam em receptores μ (mu/mi 1 e 2), k (kappa), δ (delta) e σ (sigma) sendo os receptores μ e k responsáveis pela analgesia supra-espinal e espinal. Há alguns estudos demonstrando que os opióides também podem interferir na condução periférica da dor até a coluna espinhal e levam à depressão respiratória (CARVALHO, LEMÔNICA, 1998; FUCHS, WANNMACHER, 1998; CRAIG, STITZEL, 2006).

Anestesia é um estado farmacologicamente induzido e reversível de amnésia, analgesia, perda de responsividade, perda de reflexos musculares esqueléticos e redução na resposta ao estresse. Neste estado ocorre a inibição da transmissão sináptica (sem efeito na condução axonal) que pode ser devida à redução da liberação de neurotransmissores, inibição da ação do neurotransmissor, ou redução da excitabilidade da célula pós-sináptica. A região mais sensível ao anestésico são os núcleos talâmicos de transmissão sensoriais e pelas camadas profundas do córtex para qual se projetam estes núcleos (falta de percepção de estímulos sensoriais). A amnésia se dá por uma interferência na função hipocampal (envolvido na memória à curto prazo) enquanto a sedação se estende de mínima a profunda, variando no tempo de duração e reversão do efeito medicamentoso para tal. Há redução da ansiedade e efeito calmante, com pouco ou nenhum efeito sobre as funções motoras ou de consciência (CARVALHO, LEMÔNICA, 1998; FUCHS, WANNMACHER, 1998; CRAIG, STITZEL, 2006).

1.3 **Espécies animais utilizadas como modelos experimentais:**

1.3.1 *Rhamdia quelen*

O gênero *Rhamdia* pertence a família Heptapteridae. Popularmente este gênero é conhecido no Brasil por jundiá, jundiá-tinga, jandiá, jandiá-tinga, mandi e sapipoca (GOMES *et al*., 2000).

Morfologicamente, esta espécie caracteriza-se por possuir boca sem dentes e corpo sem escamas, possuindo barbilhões de forma cilíndrica com comprimento variando proporcionalmente ao tamanho do exemplar. Em média alcança quarenta centímetros de comprimento e dois quilogramas de peso (GUEDES, 1980). O jundiá cinza é habitante natural da maioria dos lagos, rios, lagoas e mananciais de água doce do Rio Grande do Sul. Com o avanço da reprodução artificial foi realizada uma seleção genética de indivíduos homozigotos recessivos albinos. Estes representantes, salvo o par de alelos responsáveis pela coloração, são geneticamente idênticos à espécie que pertencem, não sendo facilmente encontrados na natureza, certamente devido ao alto grau de predação que sofrem (VARGAS, MOREIRA, 1998).

1.3.2 *Centropomus parallelus*

Para o gênero *Centropomus* (robalos) foram descritas 30 espécies, sendo reconhecidas somente 12. Destas, seis ocorrem no Atlântico e seis no Pacífico. A distribuição dos centropomídeos no Atlântico vai desde Pamlico Sound na Carolina do Norte até as imediações de Porto Alegre no Rio Grande do Sul, Brasil e no Pacífico, desde a baixa Califórnia até as ilhas Galápagos (RIVAS, 1986). Pertencem à subfamília Centropominae, Família Centropomidae e Ordem Parciformes (RIVAS, 1986). São conhecidos comumente como robalo (nas regiões Sudeste e Sul) e camuri ou camurim (nas regiões Norte e Nordeste), robalo-peva, peba e camurim – corcunda e robalete (VANACOR *et al*., 1996).

Possuem corpo alongado e alto, comprido, geralmente com perfil dorsal acetuadamente convexo. A maxila inferior ultrapassa nitidamente a superior, pré-opérculo serrilhado, nadadeiras dorsais separadas e com espinhos, linha lateral bem escurecida, alcançam em média 70 cm de comprimento e 4 kg de peso (RIVAS, 1986).

A espécie *Centropomus parallelus* é marinha, eurialina, encontrada tanto no mar, habitando regiões costeiras, como nas águas salobras e interiores (CHÁVEZ, 1963;

CHAPMAN *et al*., 1982), com movimentos sazonais entre a água doce e salgada. São peixes rústicos, migratórios, diádromos e dependentes de estuários (RIVAS, 1986).

1.3.3 *Danio rerio*

A espécie *Danio rerio* pertence à família Ciprinidae e é popularmente conhecido como peixe-zebra por possuir cinco listras horizontais de coloração alternada azul e preta ao longo do comprimento do corpo (SCHILLING, 2002). Usualmente possuem menos que 5 cm de comprimento (DE TOLLA *et al*., 1995).

A maior parte da massa corpórea dos peixes é formada pela musculatura axial, que se dispõe ao longo dos flancos segmentarmente e forma o principal órgão locomotor do peixe (ROMER; PARSONS, 1985). A musculatura do tronco apresenta-se na forma de segmentos sucessivos, os miômeros, que se distribuem ao longo do flanco e correspondem ao número de vértebras (DUTTA, 1996).

A espécie exibe um padrão de ritmo circadiano com similaridade aos mamíferos (ZHDANOVA, 2005). Em laboratório estes animais são mantidos por 18 meses a dois anos, após este período são considerados de baixo valor reprodutivo enquanto na natureza há evidencias de sobrevivência maior que dois anos (GERHARD *et al*., 2002). São omnívoros, com dieta consistindo de zooplâncton, fitoplâncton, insetos, algas filamentosas, esporos, ovos de invertebrados, aracnídeos e detritos e vivem em pequenos cardumes de 2 a 30 indivíduos (SPENCE *et al*., 2008).

O uso de peixe- zebra em pesquisas aumentou no campo de biologia molecular durantes os anos sessenta. No entanto sua real expansão ocorreu com a popularidade de modelo genético (STREISINGER *et al*., 1981). O genoma da espécie está totalmente caracterizado e sua fisiologia e neuroanatomia são idênticas a de humanos. Geralmente são baratos, reproduziveis em grande escala e faceis de manipular geneticamente ou farmacologicamente. Evidenciados em experimentos, as respostas comportamentais são robustas parecendo ser evolucionariamente conservadoras e assemelham-se aqueles de espécies de mamíferos (PANULA *et al*., 2006; SPENCE *et al*., 2008; PANULA *et al*., 2010).

1.3.4 *Litopenaeus vannamei*

Litopenaeus vannamei (Boone, 1931) (classe: Crustacea, ordem: Decapoda, família: Penaeidae) tem sinonímia *Penaeus vannamei* (Boone, 1931). Conhecido popularmente como camarão-de-patas-brancas ou camarão-branco-do-pacífico, este camarão é nativo da Costa do Pacífico Oriental, encontrado ao Norte do México até o Norte do Peru, em áreas onde a temperatura é normalmente maior que 20°C durante todo o ano. De habitat marinhos tropicais, os adultos vivem e desovam no mar aberto, enquanto pós-larvas migram a região costeira durante a muda para juvenis (sub-adultos) habitando estuários, lagoas costeiras ou áreas de mangue. O rostro moderadamente longo com serrilhas dorsais (7-10) e ventrais (2-4). Espermatóforo complexo, constituído por massa de esperma encapsulado pelo invólucro. Coloração normalmente branca translúcida, mas pode mudar, dependendo do substrato, alimentação e turbidez da água. O tamanho máximo atingido é de 23 cm e da carapaça de 9 cm (HOLTHUIS, 1980, acesso 22 outubro, 2013).

O *L. vannamei* apresenta maior sobrevivência em laboratório em relação a outras espécies de camarões, além de uma excelente aceitação no mercado mundial, o que dá maior segurança aos investidores (LOTZ, 1997).

1.4 Sistema Nervoso Central e noções de bem-estar em animais aquáticos

1.4.1 Crustáceos

Nos crustáceos o sistema nervoso consta de gânglios supra-esofágicos (cérebro), gânglio subesofágico e cordão nervoso ventral duplo. O gânglio subesofágico é resultante de fusão de 5 ou 6 pares de gânglios. Os órgãos sensoriais são estruturas que colocam o animal em contato com o meio ambiente. São sensíveis ao tato, gosto, olfato e visão. A visão é dada pelos olhos compostos que são pedunculados e móveis. O tato é percebido pelos pêlos tácteis que se distribuem pelo corpo. O sentido químico, gosto mais olfato, reside em pêlos localizados nas extremidades das antenas, peças bucais e extremidade daquelas (ROSS, ROSS, 2008).

1.4.2 Peixes

Os órgãos responsáveis pelos sentidos do paladar e do olfato (quimiorreceptores) dos peixes localizam-se nas narinas, na boca e em outras partes do corpo (ROSS, ROSS, 2008).

No sistema nervoso dos peixes a função do telencéfalo ainda não está completamente entendida. Possivelmente está relacionado com a aprendizagem, memória, desempenho de tarefas sociais complexas e função olfatória (bulbo olfatório) (HOFMANN, 2001; BROGLIO, RODRIGUEZ, SALAS, 2003). Os hemisférios cerebrais são pouco desenvolvidos, formados por uma massa ganglionar basal chamada corpo estriado e por uma fina camada epitelial, dorsal (palio), o qual nos vertebrados superiores irá formar o cérebro (massa cinzenta). O diencéfalo nos peixes origina o tálamo, responsável pelos impulsos olfativos e visuais, dividido em corpo parietal (anterior) e corpo pineal (posterior). O mesencéfalo dos peixes é o centro de coordenação nervosa, enquanto no metencéfalo está o cerebelo, que coordena a atividade muscular, e o mielencéfalo (bulbo do encéfalo) está relacionado com os centros de atividades vitais, como a respiração, batimento cardíaco e metabolismo (MEEK, NIEUWENHUYS, 1998; NEW, 2001). A estrutura básica do sistema nervoso central em peixes tem todos os principais domínios encontrados no cérebro dos mamíferos e também os mesmos neurotransmissores tais como GABA, glutamato, dopamina, noradrenalina, serotonina, histamina e acetilcolina (PANULA *et al*., 2006).

Como o bem-estar animal está voltado para a qualidade de vida de um organismo, há um crescente interesse social e científico no de bem-estar dos peixes usados em escala comercial. A questão da existência ou não da capacidade de estados conscientes dos peixes devem ser abordadas a fim de avaliar o seu estado de bem-estar. Surpreendentemente, não há uma definição universal de consciência aplicado em todo o espectro do filo de vertebrados (SEARLE, 2000). No entanto, é consenso geral entre os pesquisadores que a consciência se refere a um estado mental de consciência interna e os estímulos externos (LINDAHL, 1997). A probabilidade de existir a consciência em peixes é normalmente avaliada por comparação de características neuroanatômicas, comportamentais e fisiológicas com uma matriz humana (ou outros mamíferos) associando consciência e estado emocional. Estudos comparativos tem sido propostos para peixes (ROSE 2002; SNEDDON 2003; CHANDROO, YUE, MOCCIA, 2004).

A comprovação científica sobre consciência em peixes não tem sido esclarecida devido à falta de pesquisa comprobatória sobre a etiologia da consciência humana,

limitando as comparações entre as estruturas cerebrais e cognitivas entre as espécies. Contudo pesquisas investigativas da base neurofisiológica da consciência incluem a região do telencéfalo, a qual em peixes tem sido investigada e fornece informações básicas que permitem associar a traços de consciência (BAARS, 2002).

A região do neocórtex do córtex cerebral é responsável por diferentes habilidades cognitivas na formação de consciência (BAARS, 2002). ROSE (2002) propôs que para ter consciência o animal precisa apresentar a região do neocórtex e como os peixes não o possuem não sentiriam dor. Isto porque a região do neocórtex está associada às sensações como medo e aprendizagem e isto agrega-se a consciência, a qual interfere na nocicepção, ou seja, a dor é uma experiência a estímulos prejudiciais ocasionando reações comportamentais.

A definição de dor comumente utilizada para peixes proposta por ZIMMERMAN (1986) afirma que a dor é uma experiência sensorial adversa causada por um estimulo gerando reações: a) motora - danos teciduais promovendo afastamento do estimulo agressor, vegetativa - estados inflamatórios e respostas cardiovasculares; b) comportamental – alteração do estado comportamental normal. Evidências experimentais de possível percepção da dor em peixes foram verificadas pela demonstração da presença de nociceptores utilizando técnicas de neuroanatomia e eletrofisiologia (SNEDDON, 2002; SNEDDON, BRAITHWAITE, GENTLE, 2002). Os nociceptores são receptores que preferencialmente detectam estímulos nocivos, prejudiciais e tem sido caracterizados em uma ampla variedade de animais e em seres humanos (LYNN, 1994). Em truta arco-íris (*Oncorhynchus mykiss*) os nociceptores foram encontrados na cabeça do peixe, ao redor da boca e na superfície das brânquias (SNEDDON, GENTIL, 2002) e apresentam propriedades idênticas às encontradas em mamíferos (SNEDDON, 2003). Terminações nervosas delta A e fibras C atuam como nociceptores em vertebrados superiores, as quais são responsáveis pela detecção de estímulos dolorosos em humanos (LYNN, 1994) e estas estruturas foram encontradas no nervo trigêmeo de truta arco-íris (SNEDDON, 2002).

Se um evento nocivo tem suficientemente efeitos adversos sobre o comportamento e fisiologia em um animal e esta experiência é dolorosa em seres humanos, então é susceptível de ser dolorosa para o animal. Para demonstrar que o animal é capaz de percepção da dor, o animal deve perceber o estímulo adverso sensorial e reagir para evitá-lo. O que um animal "sente" é, possivelmente, mínimo comparado à experiência de seres humanos com uma estrutura do cérebro mais complexa. No entanto, a experiência

do animal pode ser desagradável ou causar sofrimento e seu desconforto não é menos importante em termos de biologia ou ética (SNEDDON, 2003).

1.5 Óleos essenciais

Óleos essenciais, também conhecidos como óleos voláteis, são produtos naturais concentrados obtidos de plantas como metabólitos secundários, sendo constituidos por compostos aromáticos voláteis. São encontrados em pequenas bolsas (glândulas secretoras), canais ou cavidades chamados células epidérmicas ou tricomas glandulares sintetizados em todos os órgãos de plantas como brotos, galhos, sementes, frutos, na superfície de folhas, flores ou no interior de talos, madeira ou cascas, raízes. São extraídos por meio de destilação a vapor de água (hidrodestilação), microondas, extração por solventes orgânicos voláteis, por gorduras a frio ou a quente, adsorventes (sílica, carvão ativado) ou por pressão (expressão) e são populares como ingredientes de perfumes, cosméticos e produtos de limpeza doméstica, bem como usados para aromatização e conservação de comidas e bebidas (BAKKALI, 2008).

A composição dos óleos essenciais compreende uma complexa mistura de substâncias, podendo chegar até várias centenas delas. Porém, sempre há a predominância de uma até três substâncias que caracterizam a espécie vegetal em questão, lhe conferindo um aroma característico (EDRIS et al., 2007). Estas misturas de compostos orgânicos voláteis são principalmente terpenos (mono e sesquiterpenos) e compostos aromáticos (ADORJAN, BUCHBAUER, 2010).

Os terpenos formam diferentes classes de compostos estrutural e funcionalmente diferentes. São formados apartir de combinações de várias unidades de cinco carbonos (C_5) chamadas isopreno. Os principais são os monoterpenos (C_{10}) e sesquiterpenos (C_{15}), mas hemiterpenos (C_5) , diterpenos (C_{20}) , triterpenos (C_{30}) e tetraterpenes (C_{40}) também existem. Um terpeno que contém oxigênio é chamado um terpenóide. Compostos aromáticos são derivados de fenilpropano, ocorrendo com menor frequência do que os terpenos. A rota biossintética destes compostos diz respeito a via de terpenos e derivados fenilpropânicos. Em geral, são separados em plantas, mas podem coexistir em algumas plantas com uma via predominante. Adicionalmente componentes nitrogenados ou sulforados como glicosinolatos ou derivados do isotiocianato são também característicos de metabólitos secundários (BAKKALI *et al*., 2008).

Os óleos voláteis exercem diferentes ações biológicas em seres humanos, animais e outras plantas. Apresentam-se úteis para o tratamento de diferentes doenças

e sua aplicação medicinal tornou-se muito popular, sendo isso também válido para muitos dos seus constituintes, como os compostos de fragâncias únicas. Os principais focos encontrados na literatura são dirigidas às atividades antinociceptiva, antitumoral, antiinflamatória, antimicrobiana, anestésica, sedativa repelentes, inseticidas, antiviral e antioxidante. Muitos óleos essenciais têm o potencial de melhorar a absorção transdérmica da droga. Eles são conhecidos como promotores ou aceleradores da penetração por facilitarem a absorção dos constituintes de uma formulação ou por si só (BAKKALI *et al*., 2008; ADORJAN, BUCHBAUER, 2010).

1.6 Eugenol

Eugenol (4-alil-2-metoxifenol) é um fenilpropeno, membro dos fenilpropanóides de ocorrência natural no óleo essencial obtido do cravo, noz moscada, canela, manjericão e louro. É um liquido oleoso, amarelo claro, ligeiramente solúvel em água e solventes orgânicos (JIROVETS *et al*., 2006). É relatado na literatura sua atividade antioxidante (OGATA *et al*., 2007); neuroprotetora (KABUTO et al., 2007); bactericida (BURT, 2004; GILL, HOLLY, 2006), sedativo e anestésico para peixes (CUNHA *et al*., 2010).

1.7 Espécie vegetal experimental

1.7.1 *Aloysia triphylla* (L'Hér.) Britton

Pertence à família Verbenaceae, englobando cerca de quarenta espécies (OLIVEIRA, 2005), a qual no Brasil é representada principalmente pelos gêneros *Aloysia*, *Clerodendrum*, *Lantana*, *Lippia* e *Verbena*. As regiões de maior ocorrência são ao Sul, Sudeste, Bahia e Centro-Oeste (LORENZI, 1998), sendo que no Rio Grande do Sul há grande utilização dos gêneros *Aloysia* e *Lippia* na medicina popular (VENDRUSCULO *et al*., 2005; PICCININI, 2008)

Aloysia triphylla (L'Hér.) Britton recebe esta denominação por ser a mais difundida e enquadrar-se na classificação de Moldenke (PASCUAL, 2007), tendo por seu descritor (L'Hér.) Britton (FLORA DIGITAL, acesso 20 julho, 2009).

Sinonímias: *Aloysia citriodora* Ortega ex Pers.; *Aloysia citriodora* Paláu; *Aloysia sleumeri* Moldenke; *Lippia citriodora* H.B.K.; *Lippia citriodora* (Lam.) Kunth; *Lippia triphylla* (L'Her.) Kuntze; *Verbena citriodora* Cav.; *Verbena triphylla* L'Hér.; *Zapania citriodora* Lam. (CARNAT *et al*., 1999; VALENTÃO *et al*.,1999; PASCUAL *et al*., 2001; SANTOS-GOMES

et al., 2005; DUKE, GODWIN, OTTESEN, 2008). Conhecida popularmente no Brasil por cidró, cidrão, erva-luísa (MORGAN, 1997; DUARTE, 2006; JORGE, 2007)

É um arbusto de até 3 a 6 m de altura, com folhas verdes dentadas, oval-lanceoladas, estando agrupadas em número de 3 a 4 em verticilos florais em torno de um caule lenhoso muito ramificado. Suas minúsculas flores, brancas, lilases estão agrupadas formando espigas verticiladas (MORGAN, 1997). Tanto as folhas como as flores são aromáticas, com odor semelhante ao do limão. As inflorescências são solitárias e ramificadas (GATUSSO *et al*., 2008).

Não há muitos estudos sobre as propriedades farmacológicas de *A. triphylla*, exceto alguns estudos da atividade antimicrobiana (OHNO *et al*., 2003; DUSCHATZKY *et al*., 2004; SARTORATTO *et al*., 2004; OSKAY, *et al*., 2005; DUARTE *et al*., 2006; ROSSI *et al*., 2007; PARODI *et al*., 2013). Além disso, possui longo histórico no tratamento popular insônia e ansiedade (CARNAT *et al*., 1999) e como antioxidante (VALENTÃO *et al*., 2002; ONO *et al*., 2008; GRESSLER *et al*., 2012).

Devido ao intenso aroma de limão que emite de suas folhas, apresentam características sensoriais atrativas, é muito popular em termos de demanda comercial, o que a torna de interesse especial na indústria de aroma e fragrâncias (ARGYROPOULOU *et al*., 2007; BANDONI *et al*., 2008;) agitação, enxaqueca (CUNHA, SILVA, ROQUE, 2003). Também são mencionadas atividades acaricida, anticolinérgica, antiséptico, bactericida, fungicida e estimulante (DUKE, 2008). Seu uso comestível está na utilização das folhas como condimentos, aromatizantes em saladas e frutas, conservantes, bebidas ou chá e seus óleos essenciais na produção de essências para a indústria farmacêutica. As folhas ocasionalmente são cozidas, sendo comumente preparadas como uma infusão ou decocção e administradas oralmente (PASCUAL *et al*., 2001).

Quanto à composição química vale ressaltar que a mesma pode sofrer alterações tanto na composição quanto na percentagem dos constituintes dependendo da região cultivada, tipos de cultivo e de épocas do ano que coincidem com mudanças de estações (CRABAS *et al*., 2003; DUSCHATZKY et al., 2004; SARTORATTO *et al*., 2004; SANTOS-GOMES, VICENTE, 2005; ARGYROPOULOU *et al*., 2007; PEREIRA, MEIRELES, 2007; ROSSI *et al*., 2007; BANDONI *et al*., 2008).

1.7.2 *Lippia alba*

A espécie *Lippia alba* é um arbusto pertencente a família Verbenaceae, cultivada nas regiões quentes da America Central e do Sul, existente praticamente todas as regiões

do Brasil, onde é muito empregada com finalidade medicinal, pelas suas propriedades anestésica, sedativa (CUNHA *et al*., 2010), carminativa, espasmolítica e emenagoga (MATOS *et al*., 1996; ZÉTOLA *et al*., 2002). A composição de seu óleo essencial apresenta variação quantitativa e qualitativa, levando a separação em quimiotipos (MATOS *et al*.,1996), os quais poderiam apresentar atividades farmacológicas distintas, bem como diferenças morfológicas (CORRÊA, 1992).

1.8 Estresse oxidativo

O hipotálamo é aquela região do cérebro que produz o fator ou hormônio liberador das corticotrofinas que estimula a hipófise a liberar o hormônio adrenocorticotrófico que estimula a córtex da glândula adrenal estimulando a córtex adrenal tem a liberação do cortisol (hormônio do estresse).

O conceito de estresse baseia-se na observação de que diferentes tipos de condições físicas ou psicológicas que ameaçam a homeostase do organismo eliciam o mesmo conjunto de alterações corporais. A resposta mais característica ao estresse é a liberação do hormônio adrenocorticotrópico (ACTH) e corticóides (cortisol em humanos e cortisona em ratos) na corrente sangüínea como resultado da ativação do eixo HPA. Os estímulos ou situações que eliciam a síndrome de adaptação geral são denominados estressores e a resposta do organismo é a reação ao estresse. Além do eixo HPA, o estresse agudo também ativa a divisão simpática do sistema nervoso neurovegetativo como parte da reação de luta ou fuga. Como resultado disso, a noradrenalina das fibras nervosas simpáticas periféricas é liberada em diferentes tecidos, bem como a adrenalina da medula adrenal na corrente sanguínea.

Com relação aos últimos, os resultados relatados demonstraram consistentemente que o eixo HPA e o sistema nervoso simpático são ativados pela novidade ou indícios que sinalizem que ocorrerá punição ou ausência de uma recompensa esperada (frustração), gerando, dessa forma, ansiedade antecipatória.

Os circuitos neurais que estão envolvidos nas respostas neuroendócrinas aos estressores psicológicos incluem a ativação cortical do núcleo basolateral da amídala que, por sua vez, ativa o seu núcleo central. A mensagem é então transmitida aos neurônios hipotalâmicos por meio de diferentes caminhos: um direto; um indireto, por meio do núcleo da estria terminal; e outro ainda, por meio da serotonina do tronco cerebral (5-HT) e dos neurônios que contêm catecolamina. Os neurônios do núcleo hipotalâmico paraventricular secretam o hormônio liberador de corticotropina (CRH) na circulação

portal da glândula pituitária. Na pituitária anterior, o CRH estimula as células secretoras de ACTH a liberarem o ACTH na corrente sanguínea. O ACTH atua no córtex adrenal promovendo a liberação de cortisol na corrente sanguínea. Além do ACTH, a prolactina é liberada consistentemente pela pituitária anterior em condições de estresse.

As espécies reativas de oxigênio são átomos, íons ou moléculas que contêm oxigênio com um elétron não pareado em sua órbita externa. São caracterizadas por grande instabilidade e por isso elevada reatividade, e tendem a ligar o elétron não pareado com outros presentes em estruturas próximas de sua formação, comportando-se como receptores (oxidantes) ou como doadores (redutores) de elétrons. As espécies reativas de oxigênio são constantemente formadas no organismo, tal como ocorre, por exemplo, durante a fagocitose realizada pelos neutrófilos, monócitos e macrófagos, no combate a microorganismos invasores. Formados continuamente durante os processos metabólicos – normais ou patogênicos – ou são provenientes de fontes exógenas físicas e químicas (LEHNINGER, NELSON, COX, 1998).

Existem espécies reativas de oxigênio e nitrogênio muito prejudiciais, tais como o radical hidroxila (OH), oxigênio singlete ($^1O_2$), radical superóxido (O_2^-), óxido nítrico (NO^-), peroxinitrito ($ONOO^-$), radical semiquinona (Q^-) (POMPELLA, 1997) que reagem indiscriminadamente com as biomoléculas, exercendo efeitos biológicos prejudiciais. Os radicais livres são moléculas liberadas pelo metabolismo do corpo com elétrons altamente instáveis e reativos, que podem causar doenças degenerativas de envelhecimento e morte celular. Os radicais livres podem combinar com outras moléculas do corpo e, com isso, serem aniquilados rapidamente, caso a produção deles seja pequena. No caso de uma grande quantidade liberada pelo organismo em diferentes situações, como por exemplo - excesso de exercícios físicos de grande intensidade e duração, exposição ao sol em demasiado, fumar ou ingerir alimentos com muita fritura e refinados, - podem ocorrer danos, como o envelhecimento precoce e doenças como Parkinson, Alzheimer, entre outras e podem ser gerados no citoplasma, nas mitocôndrias ou na membrana (YU, ANDERSON, 1997) e atacar todas as principais classes de biomoléculas, sendo os lipídeos os mais suscetíveis. Os ácidos graxos poliinsaturados (PUFA) das membranas celulares são rapidamente atacados por radicais oxidantes. A destruição oxidativa dos PUFA, conhecida como peroxidação lipídica, é bastante lesiva por ser uma reação de auto-propagação na membrana (COMPORTI *et al*., 2002).

O sistema de defesa antioxidante do organismo tem como principal função inibir ou reduzir os danos causados às células pelas espécies reativas de oxigênio. O desequilíbrio entre a liberação de espécies reativas de oxigênio e a capacidade de ação dos sistemas

de defesa antioxidante promove o estresse oxidativo (STAHL, SIES, 1997). Os antioxidantes são um conjunto heterogêneo de substâncias formadas por vitaminas, minerais, pigmentos naturais e outros compostos vegetais e enzimas. O mecanismo de ação antioxidante pode dar-se por remoção ou inativação dos radicais livres através da doação de átomos de hidrogênio a estas moléculas, interrompendo a reação em cadeia; captura de oxigênio presente no meio, através de reações químicas estáveis, tornando-os indisponíveis para atuarem como propagadores da auto-oxidação e complexação de íons metálicos (ferro e cobre) que catalisam a oxidação lipídica (SOUSA *et al*., 2007; GUERRA, 2001).

OBJETIVO

- Determinar as concentrações de eugenol e óleos essenciais de *Aloysia tirphylla* e *Lippia alba* para indução anestésica em camarões sub-adultos e pós-larvas da espécie *Litopenaeus vannamei* e avaliar o tempo necessário para indução e recuperação anestésica, bem como estabelecer a concentração ideal para transporte e eficácia antioxidante;

- Avaliar a eficácia anestésica e tempo de indução e recuperação anestésica do óleo essencial de *Aloysia triphylla* em jundiás (*Rhamdia quelen*) cinzas e albinos e em robalos-peva (*Centropomus parallelus*) sua capacidade em amenizar e/ou reduzir o estresse durante transporte através da avaliação de parâmetros sanguíneos;

- Investigar a eficácia do eugenol como analgésico em peixe-zebra (*Danio rerio*). A partir desta investigação, propor um novo modelo de investigação da analgesia em animais aquáticos.

DESENVOLVIMENTO

Artigo 1

THE ANESTHETIC EFFICACY OF EUGENOL AND THE ESSENTIAL OILS OF *Lippia alba* AND *Aloysia triphylla* IN POST-LARVAE AND SUB-ADULTS OF *Litopenaeus vannamei* (CRUSTACEA, PENAEIDAE)

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The anesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae)

Thaylise V. Parodi [a], Mauro A. Cunha [a], Clarissa G. Heldwein [b], Diego M. de Souza [c], Átila Clivea Martins [c], Luciano de O. Garcia [c], Wilson Wasielesky Junior [c], José María Monserrat [d], Denise Schmidt [e], Braulio O. Caron [e], Berta Heinzmann [b], Bernardo Baldisserotto [a,*]

[a] Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, RS, Brazil
[b] Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, RS, Brazil
[c] Instituto de Oceanografia, Estação Marinha de Aquacultura, Universidade Federal do Rio Grande (FURG), RS, Brazil
[d] Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande (FURG), RS, Brazil
[e] Departamento de Agronomia, Centro de Educação Superior Norte do Rio Grande do Sul, Universidade Federal de Santa Maria, RS, Brazil

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ABSTRACT

The aim of this study was to evaluate the anesthesia induction and recovery times of sub-adult and post-larvae white shrimp (*Litopenaeus vannamei*) that were treated with eugenol and the essential oils (EOs) from *Lippia alba* and *Aloysia triphylla*. Oxidative stress parameters in the hemolymph of this species were also analyzed. The concentrations of eugenol, *A. triphylla* EO and *L. alba* EO recommended for anesthesia were 200, 300 and 750 μL L^{-1} for sub-adults and 175, 300 and 500 μL L^{-1} for post-larvae, respectively. The concentrations studied during the transport of sub-adults were between 20 and 50 μL L^{-1} eugenol, 20–30 μL L^{-1} *A. triphylla* EO and 50 μL L^{-1} *L. alba* EO. For post-larvae, the optimal concentrations for transport were 20 μL L^{-1} eugenol and between 20 and 50 μL L^{-1} *A. triphylla* EO. The white shrimp sub-adults that were exposed to *A. triphylla* EO (20 μL L^{-1}) showed increases in their total antioxidant capacities (150%), catalase (70%) and glutathione-S-transferase (615%) activity after 6 h. *L. alba* EO (50 μL L^{-1}) and eugenol (20 μL L^{-1}) also increased GST activity (1292 and 1315%) after 6 h, and eugenol (20 μL L^{-1}) decreased the total antioxidant capacity (100%). Moreover, concentrations above 30 μL L^{-1} for the EOs of *A. triphylla* and *L. alba* and 20 μL L^{-1} eugenol were effective at inducing anesthesia and improving the antioxidant system against reactive oxygen species (ROS) after 6 h.

1. Introduction

Aquatic animals in culture systems are susceptible to stress caused by capture, handling and transportation, among other stressors. Stress can induce behavioral and physiological changes as well as physical damage and may compromise fish production (Barton, 2002). Anesthetics may be used to reduce the effects of physiological stress in aquatic animals during handling and transport (Di Marco et al., 2011; Pawar et al., 2011).

The signals and responses to pain in shrimp and most crustaceans are not as clearly defined as in vertebrates, but evidence shows that these organisms can experience pain and stress in a manner similar to vertebrates (Elwood et al., 2009). Commonly used drugs do not affect their post-synaptic receptor sites, and they may respond differently to some anesthetics (Ross and Ross, 2008). The topical anesthetic Xylocaine™ reduces the signs of stress in the white shrimp *Litopenaeus vannamei* (Taylor et al., 2004). A few studies have addressed the anesthetic activity of substances of plant origin, such as eugenol (Coyle et al., 2005; Venarsky and Wilhelm, 2006; Akbari et al., 2010) and menthol (Saydmohammed and Pal, 2009), in shrimp. The Pacific white shrimp *L. vannamei* is a tropical species that is naturally distributed along the Pacific coast of Central and South America and is one of the most economically important species cultured worldwide (Zhou et al., 2009).

Like all organisms, crustaceans have an array of defense systems that enable them to meet diverse environmental challenges (Zhou et al., 2009), including constant attack from exogenous and endogenous free radicals, which can lead to serious cellular damage. The antioxidant defense system of this organism includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), which are important components of various detoxification, antioxidant and stress-tolerance pathways. Moreover, the integrated antioxidant

* Corresponding author at: Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil. Tel.: +55 3220 9382; fax: +55 3220 8241.
E-mail address: bbaldisserotto@hotmail.com (B. Baldisserotto).

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system is recognized as an essential component of an organism's self-maintenance ability. The equilibrium between GST and CAT, along with non-enzymatic antioxidants, is important for the effective removal of reactive oxygen species (ROS) (Andrew and Mathew, 1989; Guemouri et al., 1991). Some antioxidant enzymes such as CAT and GST are used as biomarkers of general health status, and the levels of these enzymes can discriminate between shrimp cultivated in intensive and extensive systems (Tu et al., 2008).

Previous studies have reported that eugenol, which is the major constituent of *Eugenia caryophyllata* (Myrtaceae) essential oil (clove oil), has anesthetic properties in some shrimp species, including *Macrobrachium rosenbergii* (Saydmohammed and Pal, 2009) and *Penaeus semisulcatus* (Soltani et al., 2004). The plant *Aloysia triphylla* (L'Herit) Britton (Verbenaceae) grows naturally in South America and was introduced in Europe in the late seventeenth century (Carnat et al., 1999). Traditional ethnobotanical applications of this species include its use in folk medicine as a treatment for insomnia and anxiety and as an analgesic and sedative (Valentão et al., 2002). Recently, the essential oil (EO) of this plant has been patented as an anesthetic for aquatic animals (Patent No PI 016090005905). The EO of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) has been indicated as an anesthetic for two fish species: the silver catfish *Rhamdia quelen* (Cunha et al., 2010) and the seahorse *Hippocampus reidi* (Cunha et al., 2011). Preparations of *A. triphylla* exhibit antioxidant properties, a powerful superoxide radical scavenging activity and a moderate hydroxyl radical scavenging activity (Valentão et al., 2002). In addition, *L. alba* EO improves the redox state of some silver catfish tissues under both hyperoxia and hypoxia (Azambuja et al., 2011).

Therefore, the aims of this study were to determine the optimal concentrations of eugenol and *A. triphylla* and *L. alba* EOs for the induction of anesthesia in *L. vannamei* sub-adults and post-larvae and to evaluate the time required for both anesthesia induction and recovery. In addition, we established the concentrations of these natural products that are suitable for the transport of this species and the effects of the isolated compound and the EOs on oxidative stress parameters in the hemolymph of *L. vannamei* sub-adults.

2. Material and methods

2.1. Animals

L. vannamei sub-adults (15 ± 0.1 g and 10 ± 1 cm) and post-larvae (0.1 ± 0.7 g and 0.5 ± 0.8 cm) were raised at the Marine Station of Aquaculture, Universidade Federal de Rio Grande, Rio Grande do Sul State, Southern Brazil. The animals were collected from rearing ponds that were equipped with biofloc technology and placed in 500 L indoor tanks with continuously aerated clean water at a salinity of 32 ppt, a pH of 8.2 and t 22 °C. The study was conducted in accordance with the ethical committee of animal welfare at the Universidade Federal de Santa Maria, RS, under protocol no. 027176.

2.2. Plant material and essential oil extraction

The plant species *L. alba* and *A. triphylla* were cultivated at São Luiz Gonzaga and Frederico Westphalen, respectively, which are cities in Rio Grande do Sul State, Brazil. The plant materials were identified by the botanist Dr. Gilberto Dolejal Zanetti of the Department of Industrial Pharmacy, UFSM, and voucher specimens (*L. alba* – SMDB no. 10050; *A. triphylla* – SMDB no. 11169) were deposited in the herbarium of the UFSM Department of Biology. The major components of *A. triphylla* EO are Z-citral (29.92%) and E-citral (42.30%), while the predominant compounds in *L. alba* EO are linalool (59.66%) and 1,8-cineole (9.11%). All other constituents occur at concentrations below 5%. Eugenol (99% purity, Odontofarma™, Porto Alegre, Brazil) was purchased commercially.

The *A. triphylla* and *L. alba* EOs were obtained from fresh plants using hydrodistillation, which was performed with a Clevenger apparatus (2 h for *L. alba* and 3 h for *A. triphylla*) according to guidelines from the European Pharmacopoeia (2007). The essential oils were stored at −20 °C in amber glass bottles. The densities were approximately 0.8 for the *L. alba* EO, 0.9 for the *A. triphylla* EO and 0.98 for eugenol.

2.3. Analysis of essential oils

GC–MS TIC analysis was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector under the following conditions: HP-5MS column (5%-phenyl–95%-methylsiloxane, 30 m × 0.25 mm × 0.25 μm); EI–MS: 70 eV; operating conditions: split inlet 1:100; temperature program, 40–260 °C; 40 °C for 4 min; ramp rate, 4 °C/min; carrier gas, He; flow rate, 1 mL min^{-1}; injector and detector temperature, 220 °C; interface temperature 250 °C; Databank NIST 2002.

The constituents of the EOs were identified by comparing their mass spectra with a mass spectral library (NIST, 2002) and by comparison of the Kovats retention index with literature data (Adams, 2001).

2.4. Anesthesia induction and recovery

The experiments involving anesthesia induction and recovery were conducted according to the procedure described by Coyle et al. (2005). Shrimp were classified as stage 1 if they demonstrated a partial loss of equilibrium but were still reactive to touch stimuli and as stage 2 if they demonstrated a complete loss of equilibrium and were not reactive to stimuli. Shrimp were considered recovered from anesthesia when they regained control of their equilibrium and attained an upright position on the bottoms of the aquaria.

The essential oils and eugenol were dissolved in ethanol at a ratio of 1:10 before being added to aquaria containing seawater. To evaluate the time required for anesthesia induction, 16 sub-adults and 30 post-larvae were used for each concentration tested. Each animal was used only once. After induction, the sub-adults and post-larvae were transferred to anesthetic-free aquaria to measure the anesthesia recovery time. The controls were added to aquaria that contained only ethanol at a concentration that was equivalent to the highest concentration used in the experimental conditions (9 mL L^{-1}). The concentrations studied were chosen based on preliminary tests.

2.4.1. Concentrations for short-term anesthesia

Sub-adult animals were transferred to 1 L aquaria (2 animals per aquarium), and the post-larvae were evaluated in 250 mL beakers (10 post-larvae per beaker). The sub-adults were exposed to the following concentrations: 250, 500, 750 or 1000 μL L^{-1} *L. alba* EO; 50, 100, 300 or 500 μL L^{-1} *A. triphylla* EO and 50, 100, 200 or 400 μL L^{-1} eugenol. The post-larvae were exposed to the following concentrations: 400, 500 or 600 μL L^{-1} *L. alba* EO; 100, 300, 400 or 500 μL L^{-1} *A. triphylla* EO and 100, 150 or 175 μL L^{-1} eugenol. The maximum observation time in this experiment was 30 min.

2.4.2. Concentrations for transport

To evaluate the anesthetic concentration range suitable for shrimp transport, the anesthetic exposure time was fixed at 6 h. Sub-adult animals and post-larvae were maintained in continuously aerated 1 L aquaria (2 animals per aquarium) or 250 mL beakers (5 post-larvae per beaker). Both the sub-adults and post-larvae had five replicates each. The sub-adults were exposed to eugenol (5, 10 or 20 μL L^{-1}), *L. alba* EO (50, 100, 200 or 250 μL L^{-1}) or *A. triphylla* EO (20, 30 or 40 μL L^{-1}). The post-larvae were also exposed to eugenol (10, 20 or 50 μL L^{-1}), *L. alba* EO (100, 200 or 250 μL L^{-1}) or *A. triphylla* EO (10, 20 or 50 μL L^{-1}). The concentrations chosen were below those that

induced deep anesthesia within 30 min in the short-term anesthesia experiment.

2.5. Oxidative stress measurements

The hemolymph of sub-adult white shrimp was collected (n = 6) 6 h after exposure to *A. triphylla* EO (control, control + ethanol or 20, 30 or 40 μL L^{-1} EO), eugenol (control, control + ethanol or 5, 10 or 20 μL L^{-1} eugenol) or *L. alba* EO (control, control + ethanol or 50, 100, 200 or 250 μL L^{-1} EO). The hemolymph was collected directly from the heart of the shrimp using sterile syringes containing an anticoagulant solution (Sotherhall and Smith, 1983), transferred to 1.5 mL polyethylene tubes and stored at −80 °C in an ultra-freezer. For protein quantification and antioxidant enzyme analysis, the hemolymph was centrifuged twice at 3000 and 9000 *g* at 4 °C for 35 and 15 min, respectively, to obtain a pellet. After the centrifugation process, the pellet was resuspended in a 4 °C buffer solution containing Tris base (20×10^{-3} M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with the pH adjusted to 7.6. All enzymatic measurements were performed at least in triplicate.

The total antioxidant competence against peroxyl radicals was analyzed by quantifying the ROS in the hemolymph as described by Amado et al. (2009). The total fluorescence production was calculated by integrating the fluorescence units (FU) along the time of the measurement after adjusting the FU data to a second order polynomial function. The results were expressed as the area difference in FU × min in the same sample with and without the addition of 2,2-azobis-2-methylpropionamidine dihydrochloride (ABAP) and were standardized to the ROS area without ABAP (background area). The relative difference between the ROS area with and without ABAP was considered to be a measurement of the antioxidant capacity. A large area of difference corresponded to a low antioxidant capacity because high fluorescence levels were obtained after the addition of ABAP, indicating a low competency for neutralizing peroxyl radicals. Catalase (CAT; EC 1.11.1.6), activity was analyzed as described by Beutler (1975) by determining the initial decomposition rate (1 min) of H_2O_2 (10 mM) at 240 nm. The results were expressed as CAT units mg $protein^{-1}$, where 1 unit (U) is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2 per min and per mg of protein at 30 °C and a pH of 8.0. Glutathione-S-transferase (GST; EC 2.5.1.18), activity was determined by monitoring (3 min) the formation of a conjugate between 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974; Habig and Jakoby, 1981). The results were expressed as GST units mg $protein^{-1}$, where 1 unit (U) is defined as the amount of enzyme that conjugates to 1 μmol of CDNB per min and per mg protein at 25 °C and a pH of 7.0. In both cases, the measurement conditions and substrate concentration were selected to guarantee maximum enzymatic activity.

2.6. Statistical analyses

The data were expressed as the mean ± standard error. The relationship between the time needed for anesthesia induction and the concentration of the anesthetic used was determined using Slide Write Plus version 4.0 software (Advanced Graphics Software, Inc., Rancho Santa Fe, CA, USA). To verify the homogeneity of variances, all data were submitted to Levene's test. The data for the anesthesia recovery times (n = 16 sub-adults and 30 post-larvae) and enzymatic activities (n = 6) were analyzed using one-way ANOVA followed by Newman–Keuls post-hoc comparisons. This analysis was performed using Statistica version 7 software (StatSoft, Tulsa, OK, USA), and the significance level was set at $P<0.05$.

3. Results

3.1. Short-term anesthesia induction and recovery

Mortality was not observed in either the sub-adults or post-larvae for any anesthetic at any concentration tested in this experiment. Ethanol alone failed to induce anesthesia.

Increasing concentrations of eugenol and the *L. alba* and *A. triphylla* EOs proportionally decreased the time required for the induction of anesthesia stages 1 and 2 in the sub-adult shrimp. Increasing concentrations of *L. alba* EO proportionally decreased the time that was required for anesthesia recovery, but this relationship was not observed with *A. triphylla* EO or eugenol (Table 1).

Eugenol induced deep anesthesia in sub-adult shrimp at 50 μL L^{-1} (23 min), and rapid (less than 4 min) anesthesia induction and recovery were observed at 400 μL L^{-1} (Fig. 1A). The lowest concentration that was needed to induce stage 2 anesthesia in post-larvae was 150 μL L^{-1}, and rapid (less than 4 min) induction and recovery from deep anesthesia were observed at 175 μL L^{-1} (Fig. 2A).

L. alba EO at a concentration of 500 μL L^{-1} promoted deep anesthesia in sub-adult shrimp after approximately 30 min, and the shortest times for the induction of stage 2 anesthesia (10 min) and recovery (7 min) were observed at 1000 μL L^{-1} *L. alba* EO (Fig. 1B). The post-larvae that were exposed to 500 μL L^{-1} of the same EO exhibited the fastest induction into deep anesthesia (9.5 min); however, the shortest recovery time (6.5 min) was observed at 600 μL L^{-1} EO. A concentration of 250 μL L^{-1} *L. alba* EO was unable to induce any stage of anesthesia in post-larvae (Fig. 2B).

The minimum *A. triphylla* EO concentration capable of inducing stage 2 anesthesia in sub-adult shrimp was 100 μL L^{-1} (approximately 16 min were required for anesthesia induction). *A. triphylla* EO at a concentration of 500 μL L^{-1} produced the shortest stage 2 anesthesia induction time (5 min), but the shortest recovery time was observed at 300 μL L^{-1} EO (approximately 10 min) (Fig. 1C). A concentration of 300 μL L^{-1} *A. triphylla* EO resulted in shorter induction and recovery times (less than 10 min) in post-larvae. The anesthesia recovery time in the shrimp that were exposed to 400 μL L^{-1} EO exceeded 30 min (Fig. 2C).

3.2. Concentrations for transport

None of the eugenol concentrations tested (5, 10 or 20 μL L^{-1}) were able to induce any stage of anesthesia within 6 h in sub-adult shrimp. Exposure to 20 μL L^{-1} *A. triphylla* EO promoted stage 2 of anesthesia in *L. vannamei* sub-adults within 96 min, but 30 and 40 μL L^{-1} of this EO only induced stage 1 of anesthesia, without a significant difference in anesthesia induction time. The time required for recovery was approximately 30 min in shrimp that were exposed to

Table 1
The relationship between the time (min) (y) required for *Litopenaeus vannamei* sub-adults to reach each stage of anesthesia and the concentration (μL L^{-1}) of each anesthetic used (x). The stages were identified following the recommendations in Coyle et al. (2005).

Substance	Stage 1	Stage 2	Recovery
A. triphylla	Ln y = 4.7 + 114/x r^2 = 0.99	1/y = (0 + 6.7) x r^2 = 0.99	–
L. alba	1/y = 0.01 − 1489/x^2 r^2 = 0.99	1/y = 0.02 − 380/x^2 r^2 = 0.99	1/y = 0 − 1.9210/x r^2 = 0.99
Eugenol	Ln y = (11.1 + 1.1) Ln (x) r^2 = 0.98	Ln y = (11 − 0.98) Ln (x) r^2 = 0.96	–

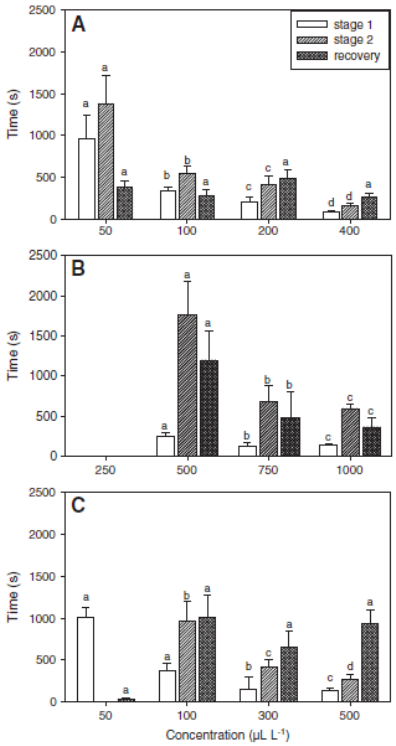

Fig. 1. The time required for induction and recovery from anesthesia in eugenol — (A), *L. alba* EO — (B) and *A. triphylla* EO — (C) treated *Litopenaeus vannamei* sub-adults. The different letters over the bars indicate significant differences in the time required for anesthesia induction and recovery between concentrations.

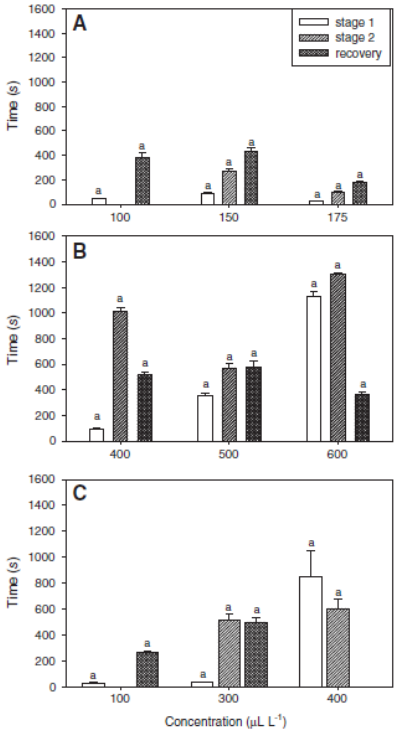

Fig. 2. The time required for induction and recovery from anesthesia in eugenol — (A), *L. alba* EO — (B) and *A. triphylla* EO — (C) treated *Litopenaeus vannamei* post-larvae. The recovery bar for 400 µL L^{-1} *A. triphylla* EO is not shown because the time exceeded 30 min. The different letters over the bars indicate significant differences in the time required for induction and recovery from anesthesia between concentrations.

all three concentrations. *L. alba* EO caused stage 2 of anesthesia at the highest concentrations studied (100, 200 and 250 µL L^{-1}), and the time required for anesthesia induction increased with increasing concentrations. At these concentrations, the recovery time was longer than 70 min. After 6 h, 37 and 25% mortality were observed in the sub-adults treated with 200 and 250 µL L^{-1} *L. alba* EO, respectively (Table 2).

In the post-larvae, both eugenol and *A. triphylla* EO (10 µL L^{-1} each) were unable to induce any stage of anesthesia. The exposure of post-larvae to 20 µL L^{-1} *A. triphylla* EO did not induce any stage of anesthesia, but eugenol induced anesthesia stage 1 in 170 min. A concentration of 50 µL L^{-1} eugenol or *A. triphylla* EO induced anesthesia up to stage 2, and recovery was faster in those anesthetized with eugenol (20 min) than with *A. triphylla* EO (over 120 min). All of the *L. alba* EO concentrations tested led to stage 2 of anesthesia (Table 2), but the post-larvae that were exposed to 250 µL L^{-1} progressed rapidly to death. The mortality at this concentration was 100%.

3.3. Oxidative stress

The white shrimp exposed to 20 µL L^{-1} *A. triphylla* EO exhibited a significantly higher total antioxidant capacity (lower relative area) than the shrimp receiving other treatments ($P<0.05$). The white shrimp exposed to 20 µL L^{-1} eugenol exhibited a significantly lower total antioxidant capacity (higher relative area) compared with the other treatments ($P<0.05$), but those shrimp exposed to *L. alba* EO did not display any significant difference from the controls ($P<0.05$) (Fig. 3A).

Exposing white shrimp to 20 µL L^{-1} *A. triphylla* EO significantly increased CAT activity compared to other treatments. The CAT activity in the white shrimp that were treated with eugenol did not differ from that resulting from the other treatments ($P>0.05$). The white shrimp exposed to 50 µL L^{-1} *L. alba* EO exhibited the lowest CAT activity, followed by those treated with 100 µL L^{-1} *L. alba* EO (Fig. 3B).

The GST activity in the white shrimp treated with 20 and 30 µL L^{-1} *A. triphylla* EO was increased compared to the GST activity in the controls ($P<0.05$), and GST activity reached a maximum at

Table 2
The time for anesthesia induction and recovery in *L. vannamei* sub-adults and post-larvae following a total exposure time of 6 h. N = 16 for sub-adults and 30 for post-larvae.

	Substance	Concentration ($\mu L\,L^{-1}$)	Time (min)		
			Stage 1	Stage 2	Recovery
Sub-adults	*A. triphylla*	20	93 ± 18	96 ± 13	30 ± 18
		30	117 ± 12	Not achieved	30 ± 9
		40	146 ± 47	Not achieved	30 ± 12
	L. alba	50	45 ± 0.1	Not achieved	45 ± 1
		100	40 ± 4	131 ± 22	70 ± 0.5
		200	103 ± 18	153 ± 7[a]	120 ± 1
		250	95 ± 16	172 ± 3[b]	120 ± 11
Post-larvae	*A. triphylla*	10	Not achieved	Not achieved	0
		20	Not achieved	Not achieved	0
		50	75 ± 5	165 ± 3	Above 120
	L. alba	100	100 ± 2	240 ± 1	70 ± 2
		200	65 ± 18	40 ± 15	65 ± 17
		250	65 ± 3	46 ± 6	No recovery[c]
	Eugenol	10	Not achieved	Not achieved	0
		20	170 ± 6	Not achieved	5 ± 10
		50	95 ± 5	175 ± 13	20 ± 2

The values represent the means ± SEM.
[a] 37% mortality.
[b] 25% mortality.
[c] When post-larvae reached stage 2, they progressed to death.

40 $\mu L\,L^{-1}$ EO. The white shrimp exposed to all of the eugenol concentrations exhibited significantly higher GST levels than the control group, and those exposed to 5 $\mu L\,L^{-1}$ eugenol displayed the highest GST activity ($P<0.05$). The GST activity in the shrimp exposed to all tested concentrations of *L. alba* EO was significantly higher than that in the controls ($P<0.05$) (Fig. 3C).

The addition of alcohol to the water did not significantly alter the TOSC, CAT and GST activities compared to the control group.

4. Discussion

4.1. Short-term anesthesia induction and recovery

An effective anesthetic must provide rapid sedation, anesthesia and recovery times (Soltani et al., 2004). In the current study, eugenol induced rapid and deep anesthesia in *L. vannamei*. At 175 $\mu L\,L^{-1}$ eugenol, post-larvae were anesthetized in 4.1 min, and sub-adults were anesthetized in 2.6 min at 400 $\mu L\,L^{-1}$. The shortest deep anesthesia induction time (approximately 21 min) that was elicited by eugenol in the shrimp *M. rosenbergii* was obtained at 800 $\mu L\,L^{-1}$ (Saydmohammed and Pal, 2009), while anesthesia stage 2 was reached by all of the animals only after 60 min of treatment with 300 $\mu L\,L^{-1}$ (Coyle et al., 2005). The same authors verified that treating this species with 25–100 $\mu L\,L^{-1}$ eugenol provoked only light sedation (stage 1) within 60 min. In juvenile (1.8–2.1 g) *P. semisulcatus*, 100, 150 and 200 $\mu L\,L^{-1}$ clove oil (80% eugenol) induced deep anesthesia in 5, 3 and 2.2 min, respectively (Soltani et al., 2004). Therefore, eugenol is more effective at anesthetizing *L. vannamei* than *M. rosenbergii*, and lower concentrations are also effective at anesthetizing *P. semisulcatus*.

There was no relationship between eugenol concentration and recovery time, but both *L. vannamei* sub-adults and post-larvae exhibited the shortest recovery times (4.3 and 3.36 min) at the highest eugenol concentrations tested (400 and 175 $\mu L\,L^{-1}$, respectively). In *M. rosenbergii* (Coyle et al., 2005; Saydmohammed and Pal, 2009) and *P. semisulcatus* (Soltani et al., 2004), increasing clove oil or eugenol concentrations correlated with longer anesthesia recovery times. The recovery of *M. rosenbergii* that were exposed to 800 $\mu L\,L^{-1}$ eugenol was longer than 55 min (Coyle et al., 2005; Saydmohammed and Pal, 2009), and the fastest recovery time in *P. semisulcatus* was observed at 50 $\mu L\,L^{-1}$ (Soltani et al., 2004).

The EOs of *L. alba* and *A. triphylla* showed concentration-dependent anesthetic activity in silver catfish (Cunha et al., 2010; Patent No PI0160900059O5), and this pattern was also be detected in sub-adult *L. vannamei*. The concentration range of *A. triphylla* EO that produced the shortest deep anesthesia induction time (3.5–1.2 min) in silver catfish was 300–800 $\mu L\,L^{-1}$, with recovery times between 9.4 and 18.3 min (Patent No PI0160900059O5). The *L. vannamei* sub-adults that were exposed to 500 $\mu L\,L^{-1}$ *A. triphylla* EO were anesthetized in 4.48 min, with recovery within 15 min. Therefore, this EO seems to have effects on *L. vannamei* at a concentration that is equivalent to the concentration that induced anesthesia in the silver catfish. However, this EO is less effective in post-larvae because it takes approximately 10 min to induce deep anesthesia. Sub-adult shrimp quickly reach stage 2 of anesthesia when exposed to 750 to 1000 $\mu L\,L^{-1}$ *L. alba* EO, recovering after 6.6 to 8.3 min. In post-larvae, the shortest stage 2 anesthesia induction time was obtained using 500 $\mu L\,L^{-1}$ *L. alba* EO. The concentrations for sub-adults (but not for post-larvae) are above the range recommended for silver catfish (300–500 $\mu L\,L^{-1}$) (Cunha et al., 2010).

Menthol, which is the main component of the EOs of plants from the genus *Mentha*, was able to induce stage 2 of anesthesia in *M. rosenbergii* at 800 $\mu L\,L^{-1}$, but only after 207 min (Saydmohammed and Pal, 2009). This finding suggests that the EOs from *A. triphylla* and *L. alba* have superior efficacy for inducing deep anesthesia in shrimp. Anesthesia stage 2 was reached in *L. vannamei* with 300 $\mu L\,L^{-1}$ Aqui-S™, which is an isoeugenol-based anesthetic, and recovery took over 30 min. After 30 min, 100 $\mu L\,L^{-1}$ Aqui-S™ induced deep anesthesia in only 13% of the shrimp, and the percentage of anesthetized animals remained the same even after 60 min. The commercial anesthetic quinaldine (300 $mg\,L^{-1}$) was effective at inducing stage 2 of anesthesia in only 20% of the shrimp by 45 min, and recovery took approximately 18 min. Both 100 $mg\,L^{-1}$ quinaldine and 100 $mg\,L^{-1}$ MS-222 were unable to induce any stage of anesthesia in *M. rosenbergii* (Coyle et al., 2005). In *L. vannamei*, 800 $\mu L\,L^{-1}$ lidocaine induced deep anesthesia in 2.5 min, with recovery in 24 min. Halothane also promoted rapid anesthesia (2.5 min) at 2.5 $mL\,L^{-1}$, but recovery took 17 min (Guzmán-Sáenz et al., 2010).

4.2. Long-term anesthesia induction and recovery

Anesthetics have been used for fish transportation because they reduce the stress and agitation of the fish (Singh et al., 2004; Park et al., 2009). However, if the fish are too heavily sedated, they lose equilibrium, cease swimming and may experience mechanical injury from hitting the tank walls; thus, anesthetic concentrations that only

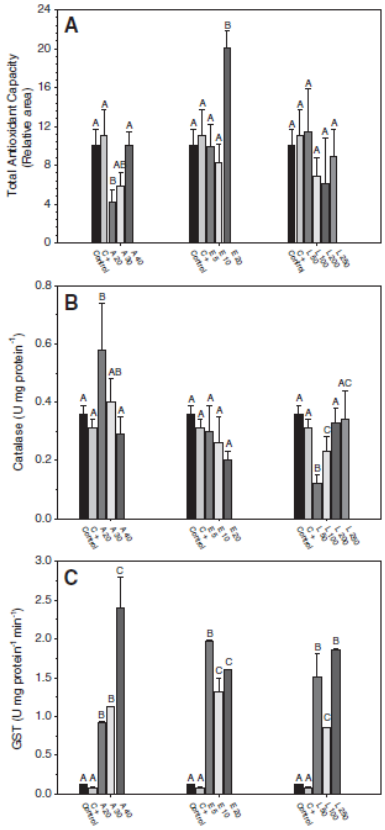

Fig. 3. The total oxyradical scavenging capacity against peroxyl radicals (A), catalase activity (B) and glutathione-S-transferase activity (C) in the hemolymph of *L. vannamei* sub-adults after 6 h of exposure to *A. triphylla* EO (A), eugenol (E) and *L. alba* EO (L). The data are expressed as the means ± SEM (n = 6). The different letters over the bars indicate significant differences between the treatments that used the same anesthetic ($P<0.05$). C+: Control plus ethanol.

induce sedation are recommended (Wagner et al., 2003). Although most operations in crustacean culture can be conducted without anesthesia, the rapid movement of shrimp may cause handling problems. In addition, their cannibalistic nature and sharp rostrum can present problems during transportation. Consequently, there has been some interest in investigating shrimp anesthetics, particularly for use in transport (Akbari et al., 2010).

Low concentrations of eugenol (5, 10 or 20 μL L^{-1}) were not sufficient to induce sedation (stage 1) in sub-adult *L. vannamei*. Because sub-adults went into deep anesthesia when exposed to 50 μL L^{-1} eugenol, the best range of eugenol for transport seems to be 20–50 μL L^{-1}. However, a concentration of 20 μL L^{-1} eugenol can be considered adequate for the post-larvae of this species because it only induced sedation over 6 h, with a 5–10 min recovery after this extended exposure time. The post-larvae of *Fenneropenaeus indicus* are more sensitive to eugenol, as the recommended concentration range for 12 h of transport is 1.3–3.7 μL L^{-1}, and 4 μL L^{-1} eugenol provoked mortality after 4 h (Akbari et al., 2010).

Concentrations of up to 5 μL L^{-1} eugenol were sufficient for 30 min of *Micropterus salmoides* transport (Cooke et al., 2004), but the recommended concentrations of eugenol for the transport (12–24 h) of the fish *Haplochromis obliquidens* are 18–20 μL L^{-1} (Kaiser et al., 2006). Therefore, the range of eugenol that is optimal for the transport of *L. vannamei* and *F. indicus* is similar to the range that is observed in fish.

A. triphylla EO at a concentration of 30–40 μL L^{-1} was sufficient to sedate *L. vannamei* sub-adults for 6 h, with complete recovery of the animals observed in 30 min. The *A. triphylla* EO concentration range that is recommended for the transport of *L. vannamei* post-larvae is 20–50 μL L^{-1}. The 10 and 20 μL L^{-1} concentrations of *A. triphylla* EO were unable to promote sedation. At 50 μL L^{-1} EO, the post-larvae progressed to stage 2 of anesthesia and exhibited long recovery times (greater than 120 min). *L. alba* EO can be used at a concentration of 50 μL L^{-1} for the transport of *L. vannamei* sub-adults, but higher concentrations cause death and therefore cannot be used. For post-larvae, the apparent concentration of this EO that is suitable for transport is below 100 μL L^{-1}.

4.3. Oxidative stress

In our study, white shrimp exhibited a rapid modulation of the antioxidant defense system after 6 h of exposure to several anesthetics, and *A. triphylla* EO (20 μL L^{-1}) augmented the total antioxidant capacity (150%) against peroxyl radicals. This natural product exhibits similar antioxidant characteristics to compounds that increase the total antioxidant capacity of human plasma by 49.4% by inhibiting lipid peroxidation during the oxidation process (Dadé et al., 2009).

In the present study, *L. vannamei* that were exposed to 20 μL L^{-1} eugenol decreased their total antioxidant capacity (100%), suggesting that this anesthetic exhibits pro-oxidant behavior. Thus, concentrations of eugenol that are lower than 20 μL L^{-1} are indicated for application in this species.

Exposure to *A. triphylla* EO (20 μL L^{-1}) increased CAT (70%) activity in white shrimp after 6 h of exposure. The increase in the activity of this antioxidant enzyme could be at least partly responsible for the higher total antioxidant capacity observed under this experimental condition. The same result occurs in *Farfantepenaeus paulensis* and *Corbicula fluminea* that are submitted to eyestalk ablation and hypoxia, respectively. The increased CAT activity observed after these treatments indicates enhanced antioxidant potential (Almeida et al., 2004; Lushchak, 2011). The catalase activity in the hemolymph of white shrimp exposed to 50 and 100 μL L^{-1} *L. alba* EO for 6 h decreased by 165 and 55%, respectively, compared to the control group. A similar effect was observed when *Litopenaeus stylirostris* was fed probiotics (*Pediococcus acidilactici*) because of the low stimulation of GST and CAT (Castex et al., 2009). The rapid modulation (i.e., reduced activity) of one of the enzymes involved in H_2O_2 processing should affect oxidative damage because this ROS is the precursor of the hydroxyl radical, which is a chemical species that triggers lipid peroxidation (Hermes-Lima, 2004). After exposure to *A. triphylla* or *L. alba* EOs or eugenol for 6 h, GST activity increased (615; 1292, and 1315%, respectively), which may be a response intended to mitigate the toxic effects of these substances or neutralize the harmful free radicals generated directly or indirectly by these anesthetics. A similar response was observed in *L. vannamei* exposed to pH stress, during which the toxic effects of acidosis increased the GST levels (Zhou et al., 2009). In crabs, exposure to the cyanotoxin microcystin for seven days also increases GST activity, indicating that the detoxification system is activated (Pinho et al., 2003).

A. triphylla EO has antioxidant properties, a powerful superoxide radical scavenging activity and moderate hydroxyl radical scavenging activity (Valentão et al., 2002). These properties can increase GST activity, which is extremely important for the detoxification of peroxyl radicals. Therefore, the EO of this plant not only produced an anesthetic effect but also increased GST activity, thus demonstrating the beneficial effects of this natural product on the transport of *L. vannamei*.

5. Conclusions

These results have demonstrated the effectiveness of eugenol and the *L. alba* and *A. triphylla* EOs in inducing anesthesia in *L. vannamei* at both the sub-adult and post-larvae stages. Compound efficacy and animal survival are dependent on the concentration, exposure time and developmental cycle of this species. These anesthetics could also be utilized in the transport of *L. vannamei* sub-adults and post-larvae. *A. triphylla* EO increased the total antioxidant capacity and CAT and GST activities in response to antioxidants. In addition, GST activity was enhanced upon treatment with all of the tested anesthetics.

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Artigo 2

ANESTHETIC ACTIVITY OF THE ESSENTIAL OIL OF *Aloysia triphylla* AND EFFECTIVENESS IN REDUCING STRESS DURING TRANSPORT OF ALBINO AND GRAY STRAINS OF SILVER CATFISH, *Rhamdia quelen*

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Anesthetic activity of the essential oil of *Aloysia triphylla* and effectiveness in reducing stress during transport of albino and gray strains of silver catfish, *Rhamdia quelen*

Thaylise V. Parodi · Mauro A. Cunha · Alexssandro G. Becker · Carla C. Zeppenfeld · Dirlaine I. Martins · Gessi Koakoski · Leonardo Gil Barcellos · Berta M. Heinzmann · Bernardo Baldisserotto

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Abstract This study investigated the efficacy of the essential oil (EO) of *Aloysia triphylla* as an anesthetic for albino and gray strains of silver catfish, *Rhamdia quelen*. Juveniles were exposed to concentrations between 20 and 800 μL L^{-1} EO of *A. triphylla* to evaluate time of induction and recovery from anesthesia. In another experiment, both strains were divided into four groups such as 0 (control), 30, 40, or 50 μL L^{-1} EO and transported for 5 h. The longest time for anesthetic induction and recovery was observed in the albinos. Both strains reached anesthesia in the 100–800 μL L^{-1} (11.1–1.24 min) range, without mortality, being 200 μL L^{-1} the best response considering time to anesthesia (5.35 min). Albinos transported with all EO concentrations showed higher values of carbon dioxide in the water of transport, but lower levels were observed in grays transported with 40 and 50 μL L^{-1} EO when compared to control fish. The same concentrations did not prevent significant whole-body cortisol rise at the end of transport in the albino strain. Juveniles of both strains transported with EO presented lower ion loss to the water compared to control fish. The EO of *A. triphylla* is an effective anesthetic for albino and gray silver catfish. This EO increases whole-body cortisol levels in the albino strain, but as it reduces net ion loss as in the gray strain, it can be also recommended for transport.

Keywords Ion fluxes · Sedation · Water parameters · Fish

T. V. Parodi · G. Koakoski
Programa de Pós Graduação em Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

M. A. Cunha · A. G. Becker · C. C. Zeppenfeld · D. I. Martins · B. Baldisserotto (✉)
Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
e-mail: bbaldisserotto@hotmail.com

L. G. Barcellos
Curso de Medicina Veterinária, Universidade de Passo Fundo, Campus Universitário do Bairro São José, Passo Fundo, RS 99001-970, Brazil

B. M. Heinzmann
Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

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Introduction

In fish culture, different operations, such as biometry and transport, are stressful and may provoke several injuries that result in loss of scales and mucus, damaging the gill epithelium, leading to bacterial and fungal infections (Moyle and Cech 1998). Handling is an inevitable precursor to any transportation and may cause mechanical abrasion and induce some

degree of stress suffered by the physical processes involved in transporting fish (Iversen et al. 1998; Carneiro and Urbinati 2002). During transportation, the mechanical stress directly causes a reaction in most animals and is known to induce a cortisol rise in fish (Barton 2002). This stress may cause not only deleterious effects to fish health, but also death (Barcellos et al. 2006). Anesthetics and sedatives may be beneficial in fish transportation by reducing physical activity and fish stress during handling (Rotllant et al. 2001; Skjervold et al. 2001).

The use of anesthetics to reduce stress and mortality is common in fish farming (Barton 2002). To choose an anesthetic, it is necessary to take into account its economic viability, practicality, and effectiveness in use (Cho and Heath 2000). Traditional anesthetics such as tricaine methanesulfonate (MS 222) (Gomes et al. 2001; Kiessling et al. 2009) and benzocaine (Gomes et al. 2001; Kiessling et al. 2009; Heo and Shin 2010), among others, are still widely used, but have many disadvantages such as gill irritation, corneal damage besides causing some inconveniences to workers due to the need of wearing gloves. More recently, less toxic anesthetics such as clove oil or its active compound, eugenol (Soto and Burhanuddin 1995; Inoue et al. 2003; Gonçalves et al. 2008; Cunha et al. 2010a), Aqui-S (isoeugenol as active compound) (Meinertz et al. 2006; Kiessling et al. 2009), menthol (Façanha and Gomes 2005; Gonçalves et al. 2008; Simões and Gomes 2009), and the essential oil (EO) of *Lippia alba* (Cunha et al. 2010b) have been suggested for use in fish.

The plant *Aloysia triphylla* (L'Herit) Britton grows naturally as a shrub in South America and was introduced in the late seventeenth century in Europe (Carnat et al. 1999). The EO of this plant is effective as anesthetic, promotes oxidative protection, and mitigate stress in the silver catfish, *Rhamdia quelen* (Gressler et al. in press). In addition, the EO of *A. triphylla* can also be used as anesthetic and improves the antioxidant system against reactive oxygen species in post-larvae and subadults of the shrimp *Litopenaeus vannamei* exposed for 6 h (Parodi et al. 2012).

The silver catfish is a native species suitable for fish culture in southern Brazil. The gray strain is the most used strain in fish culture, but several fish culturist also raise the albino strain (Baldisserotto 2003). The effect of salt (Gomes et al. 1999), temperature, load density (Golombieski et al. 2003; Carneiro et al. 2009), and anesthetics added to the water of transport (Azambuja et al. 2011; Becker et al. 2012) were studied in the gray strain. Moreover, the study of Gressler et al. (in press) verified the anesthetic effect of only two concentrations of the EO of *A. triphylla* (no concentration–response determined) in the gray strain. However, no similar studies have been conducted on the albino strain. Therefore, this study aimed at evaluating the efficacy of the EO of *A. triphylla* for induction anesthesia in both strains (gray and albino) and its effect in the transport of juveniles, considering water parameters, survival, and ionoregulatory balance.

Materials and methods

Plant material and essential oil extraction

The plant *A. triphylla* was cultivated at the Universidade Federal de Santa Maria (UFSM), southern Brazil. A voucher specimen (SMDB No. 11169) was identified by botanist Gilberto Dolejal Zanetti, from the Department of Industrial Pharmacy/UFSM, and was deposited in the herbarium of the Biology Department. The oil extraction from aerial parts of the plant was performed in August 2009, by hydrodistillation using Clevenger apparatus according to the European Pharmacopoeia (2007).

Animals

Albino (2.6 ± 1.0 g; 9.6 ± 0.7 cm) and gray (3.0 ± 0.6 g; 7.2 ± 0.8 cm) juveniles were obtained from a fish culture in São João do Polêsine, southern Brazil. The experiments were conducted in accordance with the Ethical Committee and the Animal Welfare Committee of the UFSM, under the registration no. 027176.

Anesthesia induction and recovery

Fish were maintained for 1 week in 250-L continuously aerated tanks (temperature 24 ± 1 °C, pH 7.0 ± 0.5 and dissolved oxygen 6.45 ± 1.0 mg L^{-1}), and the same water parameters were maintain during the experiment from induction and recovery anesthesia. Juveniles were then transferred with net to aquaria containing 1 L of water and the EO of *A. triphylla* at concentrations of 20, 30, 40, 50, 100, 200, 300, 400, 600, or 800 μL L^{-1} (equivalent to 18, 27, 36, 45, 90,

180, 270, 360, 540, and 720 mg L^{-1}, respectively, because the density of this EO is of approximately 0.90), first diluted on ethanol (1:10). To evaluate the time required for anesthesia induction, 10 juveniles of each strain, each being placed in individual aquaria, were used for each concentration tested, and each juvenile was used only once, based on the procedure reported by Schoettger and Julin (1967). This method involves stages, in which the following parameters were observed: deep sedation (stage 2), partial and total loss of equilibrium (stages 3a and b, respectively), and deep anesthesia (stage 4). The maximum observation time was 30 min. Control experiments were performed using aquaria containing only ethanol at a concentration equivalent to the dilution used for 800 μL L^{-1} + EO-free water. After the induction of anesthesia, juveniles were transferred to anesthetic-free aquaria to measure the recovery time. Animals were considered to have recovered when they demonstrated normal swimming and reaction to external stimuli.

Transport

Experiment 1

The albino and gray juveniles were captured from a 1,000-L tank with running water and did not undergo to a depuration period because this procedure, although recommended (Amend et al. 1982), is not followed by most fish producers in southern Brazil (Golombieski et al. 2003). Fish were transported at a load density of 162.5 g L^{-1} for 5 h in pavement roads using plastic bags with 2 L of water and 2 L of pure oxygen. All plastic bags were covered with a black plastic to reduce luminosity through transport. Fish of each strain were divided into four treatments (three replicates each): 0 (control), 30, 40, or 50 μL L^{-1} EO of *A. triphylla*. The transport time and load density were chosen to reduce mortality (Golombieski et al. 2003), and the concentrations of EO of *A. triphylla* were within the range suggested for the transport of *L. vannamei* subadults (Parodi et al. 2012).

Experiment 2

A second transport experiment with the same methodology of Experiment 1, but with a load density of 157 g L^{-1}, was performed with albino and gray juveniles to measure the whole-body cortisol levels. The whole-body cortisol was extracted using the method described by Sink et al. (2007). Each fish was weighted, minced, and placed in a disposable stomacher bag with 2 mL phosphate-buffered saline (PBS, pH 7.4) for 6 min. The contents were then transferred to a 10-mL screw top disposable test tube to which 5 mL laboratory grade ethyl ether was added. The tube was vortexed for 1 min and centrifuged for 10 min at 3,000 rpm and then immediately frozen in liquid nitrogen. The unfrozen portion (ethyl ether containing cortisol) was decanted and transferred to a new tube and completely evaporated under a gentle stream of nitrogen for 2 h, yielding a lipid extract containing the cortisol, which was stored at −20 °C. The accuracy of cortisol detection was tested by calculating the recoveries from samples spiked with known amounts of cortisol (50, 25, and 12.5 ng mL^{-1}). The mean detection accuracy of spiked samples was 94.3 %. All cortisol values were adjusted for recovery following the equation "Cortisol value = measured value × 1.0604". Tissue extracts were re-suspended in 1 mL PBS, and whole-body cortisol levels were measured in duplicate samples of each extract using a commercially available enzyme-linked immunosorbent assay kit (EIAgen^{TM}CORTISOL test, BioChem ImmunoSystems). This kit was fully validated for silver catfish tissue extracts using methodology proposed by Sink et al. (2007). Precision was tested by carrying out 12 repeated assays on 7 randomly chosen samples on the same plate and calculating the intra-assay coefficient of variation (CV), and reproducibility was tested by assaying the same samples on different plates and calculating the inter-assay CV. To test for linearity and parallelism, serial dilutions of tissue extracts were performed in the buffer provided with the kit. It was found a strong positive correlation between the curves ($R^2 = 0.8918$) and determined that the samples had low inter- and intra-assay CVs (7–10 % and 5–9 %, respectively).

Water sampling and analyses

Water samples were collected before and after transportation (within the plastic bags) and on the water from anesthesia and recovery (data showed Sect. 2.3). Dissolved oxygen and water temperature were measured using an YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA), and the pH was checked with pH meter DMPH-2 (Digimed, São Paulo, Brazil). The total ammonia nitrogen (TAN) levels were checked according to Eaton et al. (2005), and levels of unionized

ammonia were calculated according to the method of Emerson et al. (1975) based on pH and temperature. The water hardness was analyzed by the EDTA titrimetric method. Alkalinity and nitrite were measured according to Boyd and Tucker (1992), and the levels of carbon dioxide (CO_2) were calculated according to the method defined by Wurts and Durborow (1992).

Chloride levels were determined using the colorimetric method described by Zall et al. (1956). The levels of Na^+, K^+, and Ca^{2+} were determined with a flame photometer (B262 Micronal, São Paulo, Brazil). The standard solutions of all ions were made with analytical reagents (Vetec or Merck) dissolved in deionized water. Five different concentrations were set for the standard curve of each ion. The flow of ions was calculated according to Gonzalez et al. (1998):

$$\text{Jnet} = V\left([\text{ion}]_1 - [\text{ion}]_2\right) \cdot (M.t)^{-1},$$

where $[\text{ion}]_1$ and $[\text{ion}]_2$ are the ion concentrations in the water of transport at the beginning and at the end of the transport period, respectively; V is the volume of water (in L); M is fish mass (in kg); and t the duration of the transport (in h).

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 comparisons between different treatments were made using one-way ANOVA and Tukey's test. Evaluation of anesthetic activity was performed by regression analysis (concentration x time of anesthesia induction; concentration x time of recovery from anesthesia) using the Slide Write Plus software (Advanced Graphics Software, 1996). Anesthesia comparisons between albino and gray strains were made by Student's t test. Analysis was performed using the Statistical version 7.0 (StatSoft, Tulsa, OK) software, and the minimum significance level was set at $P < 0.05$.

Results

Induction and recovery times from anesthesia

The increasing concentration of EO proportionally decreased the time required for sedation and anesthesia induction but proportionally increased recovery time in both strains of silver catfish. In addition, when exposed to concentrations among 20–50 μL L^{-1}, EO of *A. triphylla* did not report any sedative or anesthetic effect during the 30-min evaluation period. The stage 4 of anesthesia only showed significant difference at 200 μL L^{-1} EO when the albino strain showed lower induction of anesthesia and longer recovery times compared to gray strain (Table 1). The ethanol *per se* did not produce anesthetic effect even at a concentration equivalent to the dilution of the highest EO concentration tested. No mortality resulted from anesthesia induction within the range tested.

Transport

The concentrations of EO of *A. triphylla* used did not induce deep anesthesia throughout the 5 h of transportation to Experiments 1 and 2. At the end of both strains transport in the Experiment 1, the dissolved oxygen, TAN, unionized ammonia levels, and pH in the water were not significantly different between treatments but these levels were significantly lower (except TAN, which was significantly higher) than at the beginning of transport. Carbon dioxide and alkalinity levels increased significantly when compared to the beginning of transport. Nitrite levels in the water of transport did not differ between treatments at the end of both strains transport, but increased significantly at the end of albino fish transport when compared to the beginning of transport (Table 2).

Albino silver catfish transported with EO in all concentrations, and gray strain with 40 and 50 μL L^{-1} EO of *A. triphylla* presented significantly higher carbon dioxide and lower alkalinity levels in the water compared to the control fish. The highest water hardness after transport was observed in albino and gray transported with 30 and 50 μL L^{-1} EO of *A. triphylla*, respectively (Table 2).

At the end of the transport in the Experiment 2, the pH, dissolved oxygen, and non-ionized ammonia decreased but there was no change between treatments for all strains. Carbon dioxide, alkalinity, water hardness, and TAN values were higher at the end than at the beginning of transport. The gray strain had the lowest values of alkalinity and water hardness in the treatment with 50 μL L^{-1} EO of *A. triphylla*. The levels of TAN and carbon dioxide were lower at the end of transport with 40 μL L^{-1} EO of *A. triphylla*

Table 1 Time required for induction and recovery from anesthesia using the essential oil of *Aloysia triphylla* in gray and albino silver catfish juveniles

Concentration (μL L^{-1})	Strains	Stages of anesthesia				Recovery
		Stage 2	Stage 3a	Stage 3b	Stage 4	
20	Gray	1423.6 ± 32.5	–	–	–	46.6 ± 3.9
	Albino	622.8 ± 22.3*	–	–	–	57.0 ± 1.0*
30	Gray	964.9 ± 35.5	1549.0 ± 26.8	–	–	77.7 ± 2.8
	Albino	341.1 ± 7.5*	1051.0 ± 53.1*	–	–	96.8 ± 7.5*
40	Gray	554.2 ± 14.6	846.2 ± 33.7	–	–	127.6 ± 8.9
	Albino	284.2 ± 13.2*	592.3 ± 17.3*	1238.6 ± 59.5*	–	240.6 ± 16.9*
50	Gray	472.5 ± 24.4	662.2 ± 24.7	1178.5 ± 48.9	–	286.0 ± 14.7
	Albino	176.7 ± 14.8*	356.9 ± 10.9*	1021.5 ± 30.7*	–	382.2 ± 24.4
100	Gray	149.8 ± 6.0	247.2 ± 11.5	540.1 ± 26.9	683.8 ± 39.2	320.0 ± 32.2
	Albino	71.0 ± 1.8*	192.4 ± 4.3*	371.4 ± 6.0*	671.8 ± 33.0	417.2 ± 16.5
200	Gray	72.7 ± 2.0	141.3 ± 6.0	237.5 ± 14.4	612.8 ± 30.1	387.0 ± 32.6
	Albino	55.3 ± 4.6*	94.5 ± 5.1*	202.0 ± 7.1	321.9 ± 9.2*	463.9 ± 13.6*
300	Gray	55.2 ± 2.4	78.2 ± 5.3	130.2 ± 6.5	404.5 ± 10.0	498.0 ± 23.2
	Albino	47.0 ± 2.7	61.8 ± 1.9	123.0 ± 10.1	214.6 ± 7.0*	569.7 ± 23.3*
400	Gray	38.4 ± 1.7	58.0 ± 3.4	104.0 ± 9.1	325.5 ± 15.2	500.0 ± 39.2
	Albino	32.6 ± 0.9	50.3 ± 1.2	88.9 ± 7.1*	146.0 ± 5.5*	554.2 ± 26.6*
600	Gray	34.6 ± 1.7	51.1 ± 1.1	76.5 ± 3.0	237.2 ± 7.8	595.5 ± 23.1
	Albino	24.5 ± 1.6*	43.6 ± 3.5*	63.5 ± 3.4*	95.6 ± 6.3*	1006.3 ± 29.0*
800	Gray	19.1 ± 0.8	39.6 ± 3.0	70.6 ± 2.6	175.7 ± 9.0	704.4 ± 22.1
	Albino	16.3 ± 1.2	35.1 ± 1.3*	56.3 ± 4.5*	74.6 ± 2.2*	1098.2 ± 27.4*
Equations	Gray	$\ln y = 10-1/\ln(x)$ $r^2 = 0.98$	$\ln y = -0 + 25/\ln(x)$ $r^2 = 0.99$	$\ln y = 0+28/\ln(x)$ $r^2 = 0.98$	$\ln y = 7 - 0.08/(x^{0.5})$ $r^2 = 0.95$	$\ln y = 28/\ln(x)$ $r^2 = 0.95$
	Albino	$\ln y = 0.25 + 19/\ln(x)$ $r^2 = 0.97$	$\ln y = 0.10 + 23/\ln(x)$ $r^2 = 0.99$	$\ln y = 0.1 + 26/\ln(x)$ $r^2 = 0.99$	$\ln y = 11-1.1/\ln(x)$ $r^2 = 0.99$	$\ln y = 6 - 54/\ln(x)$ $r^2 = 0.91$

Stages of anesthesia induction are in accordance with Schoettger and Julin (1967). Maximum observation time was 30 min. Time to reach each stage is represented in seconds (s)$N = 10$ fish for each concentration tested

Values are expressed as mean ± SEM. Asterisks indicate significant differences between gray and albino strains ($P < 0.05$). The equations fitted above represent a relationship between the times of anesthesia and concentrations of EO, where y = time to reach the stage and x = concentration of EO of *A. triphylla* (in μL L^{-1})

(and also TAN in those transported with 50 μL L^{-1} EO of *A. triphylla*) in the gray strain. Temperature and nitrite were stable after transport and between treatments when compared to the beginning of the transport (Table 3). No mortality was observed during or 48 h after the end of both transport experiments.

Fish from all treatments presented ion loss through the transport, as demonstrated by net ions fluxes negative values. The addition of EO of *A. triphylla* to the water of transport reduced significantly Na^+ and Cl^- loss in both strains when compared to the control fish. Potassium loss was also reduced, except in gray strain in the concentration of 30 μL L^{-1} EO. Net Ca^{2+} fluxes were not affected by treatments (Figs. 1 and 2).

Whole-body cortisol levels were not affected by the transport in both strains. The addition of EO of *A. triphylla* to the water of transport also did not alter whole-body cortisol levels in the gray strain. However, albinos transported with 40 or 50 EO μL L^{-1} of *A. triphylla* presented significantly higher whole-body cortisol levels (Fig. 3).

Table 2 Water parameters before and after transport (5 h) in gray and albino silver catfish juveniles in the Experiment 1 in plastic bags with essential oil of *Aloysia triphylla* added to the water

Water parameters	Strains	Before transport	After transport (treatments)			
			Control	*A. triphylla* (30 μL L^{-1})	*A. triphylla* (40 μL L^{-1})	*A. triphylla* (50 μL L^{-1})
Dissolved oxygen	Gray	4.94 ± 0.07	1.21 ± 0.05*^{a}	1.01 ± 0.07*^{a}	1.30 ± 0.03*^{a}	1.37 ± 0.10*^{a}
	Albino	4.66 ± 0.12	2.03 ± 0.12*^{a}	2.31 ± 0.53*^{a}	2.32 ± 0.50*^{a}	2.30 ± 0.30*^{a}
Carbon dioxide	Gray	13.91 ± 0.27	53.62 ± 5.23*^{a}	51.21 ± 2.28*^{a}	36.61 ± 3.33*^{c}	45.35 ± 1.53*^{b}
	Albino	6.41 ± 0.06	41.24 ± 0.57*^{c}	51.22 ± 1.52*^{a}	51.37 ± 1.01*^{a}	46.99 ± 0.30*^{b}
Alkalinity	Gray	10.65 ± 0.06	28.83 ± 1.10*^{a}	24.25 ± 1.08*^{a}	19.73 ± 0.62*^{b}	21.00 ± 0.72*^{b}
	Albino	5.75 ± 0.04	28.25 ± 0.25*^{a}	25.33 ± 0.75*^{b}	24.32 ± 0.47*^{b}	21.50 ± 0.76*^{c}
Water hardness	Gray	21.25 ± 0.06	20.00 ± 0.13^{b}	20.00 ± 0.15^{b}	20.00 ± 1.33^{b}	34.00 ± 0.66*^{a}
	Albino	12.00 ± 0.14	21.00 ± 1.73*^{b}	26.00 ± 1.15*^{a}	18.00 ± 1.15*^{b}	18.75 ± 1.33*^{b}
pH	Gray	6.75 ± 0.03	6.26 ± 0.01*^{a}	6.23 ± 0.01*^{a}	6.16 ± 0.03*^{a}	6.18 ± 0.02*^{a}
	Albino	6.92 ± 0.03	6.32 ± 0.01*^{a}	6.23 ± 0.05*^{a}	6.16 ± 0.01*^{a}	6.15 ± 0.04*^{a}
Temperature	Gray	23.79 ± 0.02	23.36 ± 0.07^{a}	24.13 ± 0.33^{a}	24.03 ± 0.13^{a}	23.00 ± 0.18^{a}
	Albino	23.93 ± 0.05	24.56 ± 0.06^{a}	24.70 ± 0.12^{a}	23.90 ± 0.17^{a}	23.90 ± 0.05^{a}
Total ammonia nitrogen	Gray	0.64 ± 0.02	6.70 ± 0.36*^{a}	8.27 ± 0.20*^{a}	7.93 ± 0.25*^{a}	7.34 ± 0.36*^{a}
	Albino	0.58 ± 0.05	7.01 ± 0.63*^{a}	6.91 ± 0.68*^{a}	7.53 ± 0.47*^{a}	8.88 ± 0.88*^{a}
Unionized ammonia	Gray	0.0101	0.00649*^{a}	0.00689*^{a}	0.00599*^{a}	0.00529*^{a}
	Albino	0.0104	0.00579*^{a}	0.00589*^{a}	0.00699*^{a}	0.00649*^{a}
Nitrite	Gray	0.06 ± 0.02	0.07 ± 0.02^{a}	0.09 ± 0.02^{a}	0.11 ± 0.02^{a}	0.07 ± 0.02^{a}
	Albino	0.03 ± 0.01	0.10 ± 0.02*^{a}	0.10 ± 0.02*^{a}	0.10 ± 0.02*^{a}	0.09 ± 0.02*^{a}

Values are expressed as mean ± SEM. Asterisks indicate significant differences when compared to values before transport ($P < 0.05$). Different letters in the rows indicate significant differences between treatments after transport ($P < 0.05$). Dissolved oxygen and carbon dioxide were expressed as mg L^{-1}, and total ammonia nitrogen and unionized ammonia as mg N L^{-1}. Alkalinity and water hardness were expressed as mg $CaCO_3$ L^{-1}. Temperature was expressed as °C

Discussion

Anesthesia induction and recovery

The present study demonstrates that the EO of *A. triphylla* has an anesthetic effect in both silver catfish strains. An anesthetic is effective when it features fasting acting (about 3 min), short recovery time (at most 10 min) ease of use, low risk to animals and humans, and concentrations with a wide safety margin (Keene et al. 1998; Park et al. 2009). The lowest concentration of EO of *A. triphylla* able to induce anesthesia in both silver catfish strains is 100 μL L^{-1} (around 11 min). The concentration of EO of *A. triphylla* recommended for anesthesia with induction times and recovery within of the limits proposed by Keene et al. (1998) and Park et al. (2009) was 200 μL L^{-1} for albino strain while for gray strain, it was necessary 400 μL L^{-1} EO. In silver catfish, MS-222 (100–200 mg L^{-1}) induces the anesthesia within 0.95–2.40 min and recovery occurs between 0.30 and 1.44 min; benzocaine at 50–125 mg L^{-1} can induce deep anesthesia within 0.91–2.10 min with recovery between 0.76 and 2.43 min, and quinaldine at 10–250 mg L^{-1} induces deep anesthesia within 0.3–1.88 min and recovery in 0.25–8.75 min (Seigneur 1984). The EO of *L. alba* in the 100–500 μL L^{-1} concentration range induces anesthesia in silver catfish in 1.25–16.28 min and recovery in 4.5–5.82 min Cunha et al. (2010b), and eugenol at 20–50 mg L^{-1} induces deep anesthesia within 3.43–15.0 min, with recovery in 3.4–4.3 min Cunha et al. (2010a), without mortality.

The EO of *A. triphylla* has a wide effective concentration range to induce anesthesia (at least 100–800 μL L^{-1}, since higher concentrations were not tested). That represents an advantage over other anesthetics, for example, silver catfish anesthetized with 60 or 70 mg L^{-1} eugenol, the major component of clove oil, resulted in 20 and 65 % mortality, respectively

Table 3 Water parameters before and after transport (5 h) in gray and albino silver catfish juveniles in the Experiment 2 in plastic bags with essential oil of *Aloysia triphylla* added to the water

Water parameters	Strains	Before transport	After transport (treatments)			
			Control	*A. triphylla* (30 μL L^{-1})	*A. triphylla* (40 μL L^{-1})	*A. triphylla* (50 μL L^{-1})
Dissolved oxygen	Gray	6.08 ± 0.30	1.05 ± 0.12*^{a}	1.07 ± 0.01*^{a}	1.95 ± 0.21*^{a}	1.98 ± 0.41*^{a}
	Albino	5.96 ± 0.23	1.25 ± 0.03*^{a}	2.00 ± 0.31*^{a}	2.05 ± 0.75*^{a}	2.12 ± 0.23*^{a}
Carbon dioxide	Gray	12.73 ± 0.35	48.24 ± 3.05*^{a}	52.48 ± 1.24*^{b}	38.54 ± 1.25*^{c}	43.63 ± 0.08*^{d}
	Albino	8.27 ± 0.17	47.61 ± 0.54*^{a}	49.08 ± 2.02*^{a}	45.09 ± 0.95*^{a}	43.17 ± 1.29*^{a}
Alkalinity	Gray	8.25 ± 0.85	27.55 ± 1.09*^{a}	23.68 ± 1.31*^{a}	24.26 ± 0.43*^{a}	22.15 ± 1.01*^{b}
	Albino	6.25 ± 0.12	25.03 ± 0.34*^{a}	24.26 ± 0.79*^{a}	24.12 ± 0.89*^{a}	22.13 ± 1.92*^{b}
Water hardness	Gray	20.19 ± 0.13	20.12 ± 0.06^{a}	21.12 ± 0.13^{a}	21.72 ± 0.73^{a}	28.15 ± 0.09*^{b}
	Albino	18.95 ± 0.75	20.04 ± 1.11*^{b}	24.00 ± 1.08*^{a}	20.75 ± 1.28*^{b}	19.98 ± 1.25*^{b}
pH	Gray	6.71 ± 0.30	6.32 ± 0.01*^{a}	6.31 ± 0.01*^{a}	6.12 ± 0.01*^{a}	6.15 ± 0.01*^{a}
	Albino	6.29 ± 0.01	6.35 ± 0.01*^{a}	6.25 ± 0.01*^{a}	6.12 ± 0.01*^{a}	6.15 ± 0.02*^{a}
Temperature	Gray	21.42 ± 0.12	22.71 ± 0.10^{a}	22.32 ± 0.07^{a}	23.13 ± 0.27^{a}	22.13 ± 0.06^{a}
	Albino	22.47 ± 0.20	23.58 ± 0.02^{a}	23.12 ± 0.04^{a}	22.74 ± 0.05^{a}	23.07 ± 0.13^{a}
Total ammonia nitrogen	Gray	0.58 ± 0.01	7.81 ± 0.24*^{a}	9.01 ± 0.18*^{a}	5.75 ± 0.79*^{b}	5.89 ± 0.91*^{b}
	Albino	0.77 ± 0.08	6.91 ± 0.53*^{a}	8.91 ± 0.74*^{b}	6.98 ± 0.15*^{a}	7.56 ± 0.76*^{b}
Unionized ammonia	Gray	0.0117	0.00578*^{a}	0.00598*^{a}	0.00612*^{a}	0.00449*^{a}
	Albino	0.0115	0.00683*^{a}	0.00677*^{a}	0.00701*^{a}	0.00529*^{a}
Nitrite	Gray	0.08 ± 0.03	0.06 ± 0.01^{a}	0.08 ± 0.02^{a}	0.10 ± 0.02^{a}	0.08 ± 0.02^{a}
	Albino	0.03 ± 0.02	0.02 ± 0.01^{a}	0.03 ± 0.02^{a}	0.02 ± 0.02^{a}	0.02 ± 0.02^{a}

Values are expressed as mean ± SEM. Asterisks indicate significant differences when compared to values before transport ($P < 0.05$). Different letters in the rows indicate significant differences between treatments after transport ($P < 0.05$). Dissolved oxygen and carbon dioxide were expressed as mg L^{-1}, and total ammonia nitrogen and unionized ammonia as mg N L^{-1}. Alkalinity and water hardness were expressed as mg $CaCO_3$ L^{-1}. Temperature was expressed as °C

Cunha et al. (2010b). On the other hand, Cunha et al. (2010a) reported that the EO of *L. alba* did not cause mortality from anesthesia within the 100–500 mg L^{-1} range. Albino strains showed lower anesthesia induction and longer recovery times when compared to gray strains, in most stages and in the concentrations of EO of *A. triphylla*. Apparently, this difference in sensitivity is anesthetic-dependent because the gray strain is more sensitive to MS-222 and propofol (Gressler et al. 2012). In addition, some studies demonstrating that the effect of the anesthetics can be size-dependent, such as eugenol (Perdikaris et al. 2010; Gomes et al. 2011). In this study, goldfish exposed to different doses of the anesthetic where the induction time decreased with the higher concentration used in large size group, whereas opposite effect was observed for the smaller. According to Woody et al. (2002), this may be because of higher rate of uptake of the anesthetic through the gills in the smaller fish compared to the larger ones. But, to our knowledge, there are no studies analyzing the effect of a given anesthetic in different strains of the same species neither in our work in relation to size.

Transport

Handling and physical illness associated with loading, transportation, and removal of strains have the potential to harm and may even compromise the health of fish transported for long periods. Quality of the water of transport can also influence fish health (King 2009). The ideal level of sedation for fish transport is referred to as deep sedation and includes loss of reactivity to external stimuli decrease in metabolic rate, but maintenance of equilibrium (Summerfelt and Smith 1990; Pirhonen and Schreck 2003). Such level of anesthesia is consistent with stage 2 described by Schoettger and Julin (1967). If fish are too heavily sedated, they lose equilibrium, cease swimming, and may die from suffocation when settling to the bottom, or experiencing mechanical

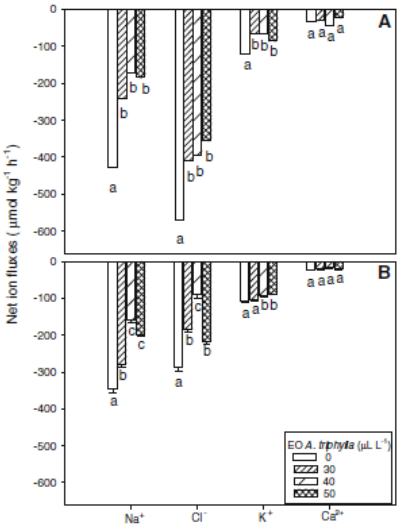

Fig. 1 Net ion (Na^+, Cl^-, K^+, and Ca^{2+}) fluxes measured in the Experiment 1 after the transport of albino (**a**) and gray (**b**) silver catfish in plastic bags with the essential oil of *A. triphylla* added to the water, $N = 3$. Values are expressed as mean ± SEM. *Different lowercase letters* indicate significant differences between treatments for the same ion ($P < 0.05$)

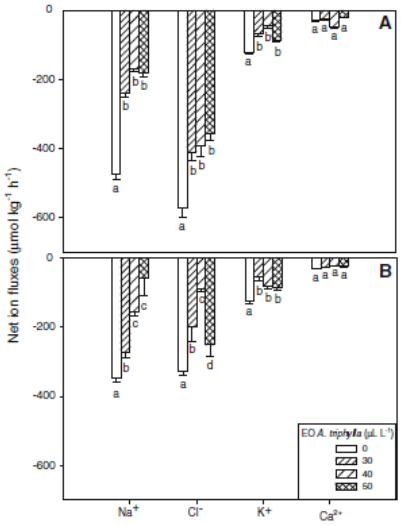

Fig. 2 Net ion (Na^+, Cl^-, K^+, and Ca^{2+}) fluxes measured in the Experiment 2 after the transport of albino (**a**) and gray (**b**) silver catfish in plastic bags with the essential oil of *A. triphylla* added to the water, $N = 3$. Values are expressed as mean ± SEM. *Different lowercase letters* indicate significant differences between treatments for the same ion ($P < 0.05$)

injury from hitting the tank walls when sedation is too light (Cooke et al. 2004).

No transportation was performed using MS-222, quinaldine, and benzocaine in silver catfish, but this species can be exposed for 3 h to 25 or 45 mg L^{-1} MS-222, 30 or 50 mg L^{-1} to quinaldine, and 10 mg L^{-1} benzocaine without mortality. Higher concentrations of MS-222 and quinaldine were not tested but 100, 50, and 30 mg L^{-1} benzocaine led to 100, 60, and 40 % mortality, respectively Seigneur (1984). Clove oil ranging from ~5 to 8.5 mg L^{-1} yielded rapid and stable stage 2 anesthesia in large-mouth bass (*Micropterus salmoides*) during transport. Fish anaesthetized at this level exhibited reduced activity and interaction with conspecifics, but were able to swim Cooke et al. (2004). MS-222 (≤40 mg L^{-1}) and benzocaine (≤20 mg L^{-1}) were optimal in *Puntius filamentosus* to produce light sedation, above which significant loss of equilibrium and mortality resulted after transport (Pramod et al. 2010). Benzocaine at 10 and 20 mg L^{-1} did not reduce the stress caused by transportation in matrinxã, *Brycon amazonicus*, and 20 mg L^{-1} also did not affect survival or glucose levels in transported Nile tilapia, *Oreochromis niloticus*. Therefore, its use is not recommended for such species (Carneiro and Urbinati 2002; Oliveira et al. 2009). The addition of 2-phenoxyethanol to the water of transport (110 and 220 mg L^{-1}) reduced mortality in guppies, *Poecilia reticulata*, during transport in closed systems at high load density for up to 48 h (Teo et al. 1989). Lidocaine hydrochloride (5, 10, or 20 mg L^{-1}) apparently decreased metabolic activity of *Pleuronectes americanus* through transportation because ammonia excretion and oxygen consumption were significantly lower after 5 h of transport than in fish not exposed to the anesthetic Park et al. (2009).

Dissolved oxygen levels and pH were significantly reduced at the end of silver catfish transport, but showed no differences between treatments. When

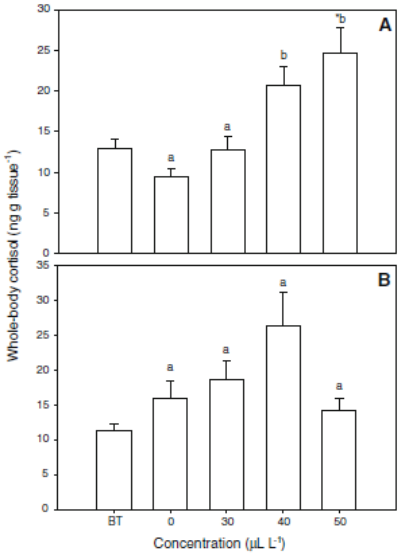

Fig. 3 Whole-body cortisol levels before and after transport of albino (**a**) and gray (**b**) silver catfish with the essential oil of *A. triphylla* added to the water, $N = 3$. Values are expressed as mean ± SEM. *Asterisks* indicate significant differences when compared to values before transport ($P < 0.05$). *Different lowercase letters* indicate significant differences between treatments after transport ($P < 0.05$). *BT* before transport

silver catfish are transported in closed plastic bags, there is an increase in waterborne CO_2 levels Golombieski et al. (2003), which results in lower water pH, as observed in the present experiment. Total and unionized ammonia levels at the end of transport, in the present experiment, were much lower than the lethal values (96 h) determined by Miron et al. (2008) to silver catfish at pH 6.0, and TAN was similar to the values obtained by Carneiro et al. (2009) (around 11 mg L^{-1}) after 4 h of silver catfish transport at the same temperature and with load density of 250 g L^{-1}. Another source of increase in alkalinity could be from the TAN and phosphates excreted by fish (Dickson 1981).

At the end of transport, only the two higher concentrations of the EO of *A. triphylla* had decreased CO_2 levels in the water used for gray strain transport when in comparison with control fish. This result indicates that these concentrations of EO decreased gray strains metabolism, but dissolved oxygen levels were not significantly affected by this EO. Therefore, more specific studies regarding the effect of this EO on metabolism must be performed.

The increase in alkalinity levels after transport may be due to food regurgitation by silver catfish juveniles, as the commercial food fed to them contained calcitic limestone in its composition. Similar results were found by Golombieski et al. (2003) and Becker et al. (2012) at the end of transport of this species. The water of both strains juveniles transport, containing the EO of *A. triphylla,* presented significantly lower alkalinity levels at the end of transport, and consequently, this EO might have reduced regurgitation.

Stress promotes the release of catecholamines that increased blood flow and lamellar recruitment, which also increased gill permeability to water and ions Bonga (1997). The handling of fish through transport also reduces their mucus layer, increasing ion loss, through plasma to water (Flik et al. 1984). The addition of the EO of *A. triphylla* to the water of transport decreased the net loss Na^+, Cl^-, and K^+ for albino and gray strains (except K^+ loss in gray strains exposed to 30 µL L^{-1}). The EO of *Lippia alba* (10 or 20 µL L^{-1}) and eugenol (1.5 or 3.0 µL L^{-1}) also reduced Na^+, Cl^-, and K^+ effluxes in silver catfish after 4-h transportation (Becker et al. 2012). Eugenol (4 mg L^{-1}) was also effective in reducing the Cl^- efflux during and after transport in *Salmo salar* (Iversen et al. 2009). Both benzocaine (15-30 mg L^{-1}) and quinaldine (100 and 250 mL L^{-1}) were efficient in avoiding the decrease of plasma Cl^- in *Labeo rohita* from 1 to 6 h of transport, but for *Hypophthalmichthys molitrix*, this effectiveness was not observed (Hasan and Bart 2007).

Whole-body cortisol levels of silver catfish before transport were similar to basal levels of the cardinal tetra (*Paracheirodon axelrodi*) (Baldisserotto et al. in press). The transport did not increase whole-body cortisol levels in albino and gray silver catfish. This is in agreement with the lack of change in plasma cortisol of gray silver catfish transported for 4 h in a similar load density (Carneiro et al. 2009). As the addition of the 40 and 50 µL L^{-1} EO of *A. triphylla* to the water of transport increased whole-body cortisol levels but reduced ion loss, it is possible that this EO reduced catecholamine liberation through the

transport and consequently the gill blood flow. As the increase in cortisol levels induced Na^+ uptake in zebrafish (*Danio rerio*) (Kumai et al. 2012) and expression levels of tight junction protein (occludin) and paracellular permeability in cultured rainbow trout (*Onchorhynchus mykiss*) gill cells (Kelly and Chasiotis 2011), another possibility is that the whole-body cortisol increase in albino silver catfish might have contribute to reduce net ion loss.

Conclusions

Silver catfish can reach deep anesthesia with EO of *A. triphylla* in the 100–800 μL L^{-1} range without mortality, with 200 μL L^{-1} the best concentration for anesthesia in albino and gray strains, respectively. The addition of 30–50 μL L^{-1} EO to the water of transport decreased ion loss and, probably, regurgitation in both silver catfish strains suggesting that the use of this EO reduces the physiological damage produced by transport. This EO increases whole-body cortisol levels in the albino strain, but as it reduces net ion loss as observed in the gray strain, it can be also recommended for transport.

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Artigo 3

ANESTHETIC INDUCTION AND RECOVERY OF *Centropomus parallelus* EXPOSED TO THE ESSENTIAL OIL OF *Aloysia triphylla* L´HERIT. BRITTON

Artigo em revisão pelos autores

2013

Anesthetic induction and recovery of *Centropomus parallelus* exposed to the essential oil of *Aloysia triphylla* L´Herit. Britton

Thaylise Vey Parodi[1], Celina Alcoforado dos Santos[2], Alexandra Veronez[2], Levy de Carvalho Gomes[2], Berta Maria Heinzmann[3], Bernardo Baldisserotto[1]*

[1]Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil (thayliseparodi@yahoo.com.br)
[2]Laboratório de Ictiologia Aplicada, Universidade de Vila Velha, Vila Velha – ES, Brazil (celina_alcsan@yahoo.com.br; alexandra_veronez@hotmail.com; levy.gomes@uvv.br)
[3]Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil (berta.heinzmann@gmail.com)

* Corresponding author

Bernardo Baldisserotto

Departamento de Fisiologia e Farmacologia

Universidade Federal de Santa Maria

97105.900 – Santa Maria, RS, Brazil

E-mail: bbaldisserotto@hotmail.com

Phone: 55 55 3220-9382 fax: 55 55 3220-8241

Abstract

This study analyzed the efficacy of the essential oil (EO) of *Aloysia triphylla* as an anesthetic and stress reducing agent in the transport of fat snook (*Centropomus parallelus*). Juveniles were placed in 1-L aquaria containing different concentrations of EO (seawater-adapted: 25-300 μL L^{-1}; freshwater-adapted: 200 μL L^{-1}) to identify the times of anesthetic induction and recovery. In an additional experiment, seawater-adapted fat snooks were transported in plastic bags with 10 or 20 μL L^{-1} EO for 6 or 24 h. The increased concentration of EO proportionally decreased the time required for anesthesia induction. All fish recovered from anesthesia at fresh and seawater. Mortality was 30 and 70% in fat snooks transported with 10 and 20 μL L^{-1}, respectively 24 h after transport. The use of 20 μL L^{-1} EO increased blood glucose levels after 12 h of transport, but decreased them after 24 h, lactate levels after 6 h and cortisol levels throughout transportation compared to unexposed fish. The EO reduced total ammonia and dissolved oxygen levels at the end of transport compared to control fish. This EO is recommended for sedation (25-50 μL L^{-1}) and anesthesia (100-300 μL L^{-1}) but not for transport of fat snook because it increased mortality.

Key words: anesthesia, blood parameters, fat snook, natural product, transport

Resumo

Este estudo analisou a eficácia do óleo essencial (OE) de *Aloysia triphylla* como anestésico e redutor de estresse no transporte de robalo-peva (*Centropomus parallelus*). Os juvenis foram colocados em aquários de 1L contendo diferentes concentrações de OE (adaptados à água do mar: 25-300 μL L^{-1}; adaptados à água doce: 200 μL L^{-1}) para identificar o tempo de indução e recuperação anestésica. Numa experiência adicional, robalos adaptados à água do mar foram transportados em sacos plásticos com 10 ou 20 μL L^{-1} OE durante 6 ou 24 h. O aumento da concentração do OE diminuiu proporcionalmente o tempo necessário para a indução da anestesia. Todos os peixes recuperaram da indução à anestesia em água doce e água do mar. Houve mortalidade de 30 e 70% nos robalos transportados com 10 e 20 μL L^{-1} respectivamente 24 h após o transporte. A adição de 20 μL L^{-1} de OE na água de transporte aumentou a glicemia após 12 h de transporte com redução da mesma após 24 h, níveis de lactato após 6h e níveis de cortisol ao longo do transporte comparado aos peixes não expostos. O OE reduziu os níveis de amônia total e oxigênio dissolvido ao final do transporte comparado ao grupo controle. Este OE é recomendado para sedação (25-50 μL L^{-1}) e anestesia (100- 300 μL L^{-1}), mas não para o transporte de robalo-peva por aumentar a mortalidade.

Introduction

The fat snook *Centropomus parallelus* is an inshore tropical euryhaline species from the Western Atlantic, distributed from South Florida (USA) to Florianópolis (Brazil) (Rivas, 1986). Its high flesh quality and market value denote importance for commercial and recreational fisheries as well as a significant aquaculture potential (Bouchereau *et al*., 2000).

Handling is an inevitable precursor to any transportation, and may cause mechanical abrasion and induce some degree of stress (Carneiro & Urbinati, 2002). During transportation, the mechanical stress directly causes a reaction in most animals and is known to induce a cortisol rise in fish (Barton, 2002). Stress may induce the release of epinephrine and norepinephrine by chromaffin tissue in response to stimulation of the sympathetic nervous system, which might increase plasma glucose levels (Reid *et al.*, 1998). The use of anesthetics is common in modern aquaculture and has practical relevance in diverse husbandry manipulations such as selection of fish, their measurement, sampling, tagging, transportation, artificial reproduction and surgery procedures and may also attenuate the physiological response to stress (Weber *et al*., 2009).

The plant *Aloysia triphylla* (L'Herit) Britton grows naturally as a shrub in South America and was introduced in the late seventeenth century in Europe (Carnat *et al*., 1999). The essential oil (EO) of this species presented anesthetic efficacy in the freshwater silver catfish *Rhamdia quelen* (Gressler *et al*., 2012; Parodi *et al*., in press), but no studies in other fish species were performed. Therefore the aim of this study was to investigate the anesthetic induction and recovery times of the EO of *A. triphylla* and its efficacy as a stress-reducing agent during the transport of fat snook. In addition the influence of salinity and size on the effect of this EO was also analyzed.

Materials and methods

Animals

Juvenile *Centropomus paralellus* (2.7±0.6g; 6.4±0.5cm) were acquired from a local supplier near Vitória, ES, Brazil. They were transported to the laboratory and kept for seven days prior to experiments in continuously aerated 100L aquaria with controlled temperature (23.3 ºC), salinity 30.6 ± 0.2 ppt, dissolved oxygen levels 6.0 ± 0.4 mg L^{-1},total ammonia nitrogen 1.17 ± 0.10 mg L^{-1} and conductivity 49.5 ± 0.1 mS. The water was renewed every two days. The animals were fed daily to satiation with commercial carnivorous fish feed with 54% crude protein.

Plant material and essential oil extraction

The plant *A. triphylla* was cultivated in the Centro de Educação Superior Norte (CESNORS)-Campus Frederico Westphalen. A voucher specimen (SMDB No.11169) was identified by botanist Dolejal Gilberto Zanetti, Departamento de Farmácia Industrial, UFSM, and was deposited in the herbarium of the Departamento de Biologia, UFSM. The EO of *A. triphylla* was obtained from fresh leaves of the plants by hydrodistillation according to European Pharmacopoeia (2007) using a Clevenger apparatus for 3h. Essential oil was stored at-20 ºC in amber glass bottles.

Analyses by gas chromatography-mass spectrometry (GC / MS) aimed to identify and quantify the components of the EO and were carried out in a hyphenated system AGILENT 6890, equipped with a mass selective detector 5973 series. The analyses of this EO presented as the major components of *A. triphylla* EO are E-citral (42.30%) and Z-citral (29.92%).

Anesthesia induction and recovery

Juveniles were individually transferred to aquaria that contained 1 L of seawater and EO of *A. triphylla* at 25, 50, 100, 200 or 300 μL L^{-1} (equivalent to 22.5, 45, 90, 180 or 270 mg L^{-1}, respectively because the density of this EO is 0.9), first diluted in ethanol (1:10). To evaluate the time required for anesthesia induction, 20 juveniles were used for each concentration tested.

Each juvenile was used only once, and anesthesia stages were determined according to the method of Schoettger & Julin (1967): stage 1 (sedation, partial loss of reaction of external stimuli); stage 2 (partial loss equilibrium); stage 3 (total loss of equilibrium – a) fish usually turns over but retains swimming ability or b) swimming ability stops but responds to pressure on the caudal peduncle); stage 4 (anesthesia, loss of reflex activity and stage 5 (medullary collapse).

The maximum observation time was 30 min. Control experiments were performed using aquaria that contained only ethanol at the concentration that was equivalent to the highest EO dilution (300 μL L^{-1}). After induction, the juveniles were transferred to anesthetic-free aquaria to measure the anesthesia recovery time.

As the fat snook is an euryhaline species that supports a wide salinity range, the anesthetic effect of the EO of *A. triphylla* was also verified in freshwater-adapted juveniles. In this experiment juveniles were kept in a tank which the salinity was gradually reduced by daily addition of 20% freshwater to complete 100% on the fifth day. Fish were then maintained for additional two days in freshwater.The same procedure described for anesthetic induction and recovery to seawater-adapted fish was carried out in this group of animals at 200 μL L^{-1} EO (n=20). This concentration was chosen because it induced a fast anesthesia and recovery in seawater-adapted fat snooks (see results).

Transport

Fat snooks juveniles were placed in 45 plastic bags (volume 2 L). Two juvenile were added per bag containing 0.5 L of seawater with 0, 10 or 20 μL L^{-1} EO that had previously been diluted in ethanol (1:10) and transported for 6, 12 and 24 h. There were five replicates for each combination of treatment and time. Concentrations of 10 and 20 μL L^{-1} were equivalent to 10 and 20% of the lowest concentration able to induce stage 4 of anesthesia in this species (100 μL L^{-1}). The control group (0 μL L^{-1} EO) was subjected to the same procedures but no anesthetic was added to the water. Bags were then inflated with oxygen, tied with rubber strings and packed in plastic boxes, as described by Gomes et al. (2006).

Water samples were collected before the plastic bags were closed and after transport for determination of dissolved oxygen (DO), temperature, conductivity and salinity with an oxygen meter YSI (model Y5512 Yellow Springs, USA). Total ammonia nitrogen (TAN) levels were verified by indophenol method according to APHA (1992).

The animals were removed from the plastic bags and placed individually on a flat surface exposed to air. Blood samples (n= 20 for each treatment, four fish per replicate) were collected from the caudal vein of each fish using heparinized 1 mL syringes (not exceeding 1 min) before and after the transporting procedure (each fish was sampled only once) and the samples were kept in ice. Glucose determination was performed with a digital Accu-Check™ apparatus. Lactate was determined by ultraviolet (UV) enzymatic using kit laboratory Bioclin™. Cortisol levels were measured using the method by competition ELISA Kit (EIA, Kit 55050, Human®). The specificity of the test was evaluated by examining the extent of the parallelism between the standard curve (coefficient of variation=13%) for human cortisol concentrations and the curve of a series of dilutions of the plasma samples in PBS (pH 7.4).

Statistical analyses

All data are expressed as mean ± S.E.M. Homogeneity of variances between treatments was tested with the Levene test. Most data exhibited homogeneous variances (those not homocedastic were log transformed), so comparisons among different treatments and times were made using two-way ANOVA and Tukey's test. Comparison between fresh- and seawater-adapted juveniles exposed to EO 200 μL L^{-1} was done using Student´s test. Analysis was performed using the software Statistica ver. 7.0 (StatSoft, Tulsa, OK). Evaluation of anesthetic activity was performed by regression analysis (concentration x time of anesthesia induction; concentration x time of anesthesia recovery) using the program Slide Write Plus (Advanced Graphics Software) version 4.0 (1996). The minimum significance level was set at $P < 0.05$.

Results

Anesthesia induction and recovery

The sole application of ethanol did not produce an anesthetic effect even at a concentration equivalent to the greater dilution of the EO. No mortality resulted from anesthesia induction within the range tested for both groups. The increasing concentration of EO proportionally decreased the time required for sedation and anesthesia induction but increased proportionally recovery time for seawater-adapted juveniles. When exposed up to 50 μL L^{-1} EO of *A. triphylla* they did not reach stage 4 (deep anesthesia) within the 30 min evaluation period (Table 1).

Freshwater-adapted juveniles exposed to 200 μL L^{-1} EO induced and recovered faster from deep anesthesia (both 3.6 min) than seawater-adapted juveniles at the same EO concentration (5.0 and 7.9 min respectively).

Transport

Temperature, salinity and conductivity of the water transport were not significantly different from before transport and between treatments at the end of transport. The levels of DO and TAN of the water of fat snooks transported with EO of *A. triphylla* were significantly lower than control after 6, 12 and 24 h of transport (except 10 μL L^{-1} after 12 h) (Table 2).

No mortality was observed in the control group at end of the transport. Mortality after 24 h of transport was 30 and 70% in fat snooks transported with 10 and 20 μL L^{-1} EO respectively. Plasma cortisol values increased after all times of transport compared to before transport, and addition of 20 μL L^{-1} EO significantly reduced plasma cortisol levels (Fig. 2A). Blood glucose and lactate levels were not affected significantly by transport in the control group, but fat snooks transported for 6 and 12 h with 20 μL L^{-1} EO (and 12 h with 10 μL L^{-1} EO) presented significantly higher blood glucose levels than control fish at the same time. However, after 6 h blood lactate levels and after 24 h blood glucose levels were significantly lower in fat snooks transported with 20 μL L^{-1} EO compared to control fish (Fig. 2B, C).

Discussion

Anesthetics are necessary for many procedures in aquaculture, but the response to anesthetics may differ widely in each species. An anesthetic is effective when features fast acting (about 3 min), short recovery time (at most 10 min), ease of use, low risk to animals and humans and concentrations contained in a wide safety margin (Keene *et al.*, 2008; Park *et al.*, 2009).

The lowest concentration of EO from *A. triphylla* able to induce deep anesthesia (stage 4) in seawater-adapted fat snooks was 100 μL L^{-1}, but it takes around 10 min to recover. The optimal concentration EO *A. triphylla* for deep anesthesia with induction time

and recovery from anesthesia within the limits proposed by Keene *et al*. (2008) and Park *et al*. (2009) is 300 μL L^{-1} (induction stage 4 in 3.3 min and recovery in 9 min). These are similar to those observed for grey and albino strains of silver catfish: both anesthesia induction and recovery with 300 μL L^{-1} EO of *A. triphylla* took 3.6-6.7 min and 8.3-9.5 min, respectively (Parodi *et al.,* in press). The EO of *Lippia alba* in the 100-500 μL L^{-1} concentration range induces deep anesthesia in silver catfish in 16.28-1.25 min and recovery in 4.5-5.82 min (Cunha *et al*., 2010) and in slender seahorse (*Hippocampus reidi*) 150-300 μL L^{-1} was required to obtain rapid (approximately 3-4 min) deep anesthesia and recovery (about 6 min) (Cunha *et al*., 2011). Menthol at 100 - 200 mg L^{-1} can provoke deep anesthesia in tambaqui (*Colossoma macropomum*) after 1 - 2 min, and recovery lasts 5-12 min (Façanha & Gomes, 2005). Therefore, the optimal anesthetic concentration of the EO *A. triphylla* is within the concentration range of other plant-originated anesthetics.

Freshwater-adapted fat snooks at 200 μL L^{-1} induced anesthesia and recovered faster than seawater-adapted ones. This difference is not related to changes in metabolism due to salinity because this parameter does not affect metabolic demand and growth of fat snooks (Rocha *et al*., 2005; Tsuzuki *et al*., 2007). Increased induction times with salinity increase were also observed in Caspian salmon (*Salmo trutta caspius*) exposed to clove oil (Ghazilou *et al*., 2010).

The use of 10 or 20 μL L^{-1} EO of *A. triphylla* in the water of transport for 24 h provoked mortality in fat snooks. This mortality cannot be explained by the increase of stress or impairment of water quality, because neither plasma cortisol nor dissolved oxygen and ammonia levels were related to mortality rates. The transport of *Haplochromis obliquidens* with 20 μL L^{-1} clove oil for up to 48 h did not induce mortality, but 30 μL L^{-1} clove oil for 12 and 21 h caused mortality (Kaiser *et al*., 2006). The transport of blue tilapia (*Oreochromis aureus*) with clove oil 1, 2 or 3 mg L^{-1} for 24h did not reduce mortality (Akar,

2011). Apparently the concentration range tested for the EO of *A. triphylla* was not appropriate, and lower concentrations may reduce mortality.

Blood glucose levels of fat snook transported with EO of *A. triphylla* increased in the first 12 h of transport while plasma cortisol decreased. Consequently, it is possible that the higher blood glucose levels were due to catecholamine liberation or an increase of metabolism, because dissolved oxygen levels decreased in the water of fat snooks transported with EO of *A. triphylla* were lower than in control fish. The transport of three-spot gourami (*Trichogaster trichopterus*) with 0.4 mg L^{-1} metomidate for 12 h also increased significantly plasma glucose levels (Crosby *et al*., 2012) but in common carp (*Cyprinus carpio*) the same conditions decreased these levels (Crosby *et al*., 2010). The transport for 24 h with MS-222 (125 mg L^{-1}) or eugenol (75 µL L^{-1}) did not modify glucose levels in Siberian sturgeon (*Acipenser baerii*) (Gomulka *et al*., 2008). The EO of *A. triphylla* at 20 µL L^{-1} was able to reduce plasma cortisol levels throughout the transport compared to control group. Cortisol is thought to be one of the mediators of the increase in plasma glucose levels seen in stressful events (Barton *et al*., 2002). Therefore, an adequate concentration of a given anesthetic may reduce stress and mortality through transport.

The transport probably did not lead to an increased muscular activity and, as expected, no differences in lactate concentration were observed during the transport with EO of *A. triphylla*. During heightened activity fish white muscle mainly generates energy by means of an anaerobic metabolism with increased production and accumulation of lactic acid, which must be transported to the liver for metabolizing (Huss, 1995). Lactate concentration has been shown to increase significantly in several species following severe exercise or as a result of hypoxia (Acerete *et al*., 2004). The concentration of lactate reflects the increased levels of physical activity as a response to the different stages of transport (Grutter & Pankhurst, 2000). The low plasma lactate concentration seen in this

study is in accordance with the restriction of movement and thus muscular activity, due to the transport.

In the present study the dissolved oxygen (DO) and total ammonia (TAN) levels in the water of fat snooks transported with EO *A. triphylla* were lower than control group throughout the transport. Up to 12 h of transport (most treatments) the DO levels were higher than at the beginning of the transport because of the direct addition of pure oxygen in the plastic bags. The same results were observed in silver catfish transported with EO *L.alba* and eugenol (Becker *et al*., 2012). The water of transport of *Culter mongolicus* transported with MS 222 (10,20 or 40mg L^{-1}) in the first 12–24h had significantly higher DO and TAN than the control but failed to decrease un-ionized ammonia content. In contrast clove oil (2, 5 or 10 mg L^{-1}) significantly reduced the un-ionized ammonia but failed to improve DO and pH at 12–24h (Lin et al. 2012). Park *et al*. (2009) suggested that lidocaine hydrochloride at 5, 10 or 20 mg L^{-1} decreased the metabolic activity of flounder (*Pleuronectes americanus*) because this substance reduced ammonia excretion (about 27.4–30.5%) and oxygen consumption (about 82.7–86%) compared with a control group after 5 h transport time. These studies found that the overall reduction in ammonia excretion could be directly related to a decrease in the metabolic rate produced by anesthetic.

In conclusion, EO was effective in inducing slight sedation in the fat snooks at 25-50 µL L^{-1} and deep anesthesia at 100–300 µL L^{-1}. The concentration recommended for rapid deep anesthesia is 200 µL L^{-1}. Our results suggest that the use of EO of *A. triphylla* (at least in the concentrations tested) is not advisable for the transport of fat snooks due to the mortality provoked.

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Table 1. Time required for induction and recovery from anesthesia using the essential oil of *Aloysia triphylla* in seawater-adapted *Centropomus paralellus.* Stages according to Schoettger and Julin (1967). Maximum observation time was 30 min. Time to reach each stage in seconds (s). N = 20 for each concentration tested. Values represent means ± SEM. Equations: y = time to reach the stage and x = concentration of the EO of *A. triphylla* (µL L-1).

Table 2. Water parameters before and after different times of transport of *Centropomus paralellus* in plastic bags with different concentrations (in µL L-1) of essential oil of *Aloysia triphylla* added to the seawater. TAN – total ammonia nitrogen.Values represent mean ± SEM. Different lowercase letters indicate significant differences between treatments at the same time of transport. Different capital letters indicate significant differences between the times the same treatment. Temperature (oC); salinity (ppt); conductivity (mS). Dissolved oxygen, total and un-ionized ammonia were expressed as mg L-1. Level of significance for all data $p < 0.05$. * indicates significant difference from the value before transport. There was no statistical comparison of un-ionized ammonia levels because they were very low.

Table 1

Concentration ($\mu L\ L^{-1}$)	Induction (s)				Recovery (s)
	Stage 2	Stage 3a	Stage 3b	Stage 4	
25	129.3±5.6	434.2±33.8	-	-	238.3±40.7
50	98.6±9.4	282.4±21.8	993.1±28.8	-	304.4±45.4
100	42.2±4.4	83.7±9.8	218.8±28.6	580.9±41.9	330.2±59.8
200	16.7±5.3	56.9±6.2	114.2±33.8	301.5±27.9	479.1±12.2
300	8.3±1.8	19.6±3.9	77.5±16.5	202.3±43.1	543.5±42.2
Equations	$y= 181.5e^{(-0.01x)}$	$y= 694.7e^{(-0.019x)}$	$y= 4128.9e^{(-0.029x)}$	$y= 1019.6e^{(-0.006x)}$	$y=379.1e^{(-0.0009x)}$
	$r^2=0.99$	$r^2=0.98$	$r^2=0.97$	$r^2=0.98$	$r^2=0.95$

Table 2.

Water quality	Dissolved Oxygen	Temperature	Salinity	Conductivity	TAN
Tank water	5.47±0.03	24.30±0.84	31.25±0.63	45.33±0.94	1.17±0.10
Before Transport (0)	6.19±0.19	24.25±0.07	30.25±0.07	47.66±0.30	0.38±0.10
6 h transport					
Control	9.47±0.77^{*aA}	24.26±0.06^{aA}	31.20±0.6^{aA}	47.46±0.67^{aA}	5.80±1.83^{*aA}
10	7.59±1.71^{*bA}	24.30±0.10^{aA}	31.26±0.06^{aA}	47.24±0.10^{aA}	3.68±0.37^{*bA}
20	8.44±1.00^{*bA}	24.26±0.06^{aA}	31.23±0.11^{aA}	47.26±0.06^{aA}	1.36±0.99^{*cA}
12 h transport					
Control	11.63±1.89^{*aB}	24.30±0.01^{aA}	31.20±0.26^{aA}	47.34±0.18^{aA}	5.91±0.06^{*aA}
10	9.80±1.40^{*bB}	24.30±0.01^{aA}	31.10±0.38^{aA}	47.15±0.42^{aA}	4.90±0.47^{*aB}
20	6.31±2.14^{cB}	24.30±0.01^{aA}	31.36±0.06^{aA}	47.43±0.03^{aA}	3.01±1.31^{*bB}
24 h transport					
Control	8.82±0.82^{*aA}	24.20±0.10^{aA}	31.33±0.06^{aA}	47.16±1.08^{aA}	10.1±0.38^{*aB}
10	4.18±1.83^{*bC}	24.26±0.05^{aA}	31.40±0.01^{aA}	47.47±0.04^{aA}	4.78±0.55^{*bB}
20	3.54±1.14^{*bC}	24.33±0.06^{aA}	31.33±0.05^{aA}	47.43±0.12^{aA}	7.2±0.87^{*cC}

Figure captions

Figure 1. Time required for induction and recovery from anesthesia using the essential oil (EO) of *Aloysia triphylla* in juveniles from *Centropomus paralellus* adapted to fresh water for seven days. Stages according to Schoettger and Julin (1967). Maximum observation time was 30 min. Time to reach each stage in seconds (s). N = 20 for each concentration tested. * indicates significant difference from seawater-adapted fish exposed to EO 200 μL L^{-1}.

Figure 2. Blood parameters before and after transport of *Centropomus paralellus* in plastic bags with essential oil of *Aloysia triphylla* added to seawater. (A) glucose, (B) cortisol and (C) lactate. Control – only water, BT – before transport. Values represent mean ± SEM. Level of significance for all data $p < 0.05$. * indicates significant difference from the value before transport (BT). Different lowercase letters indicate significant differences between treatments at the same time of transport. Different capital letters indicate significant differences between times in the same treatment.

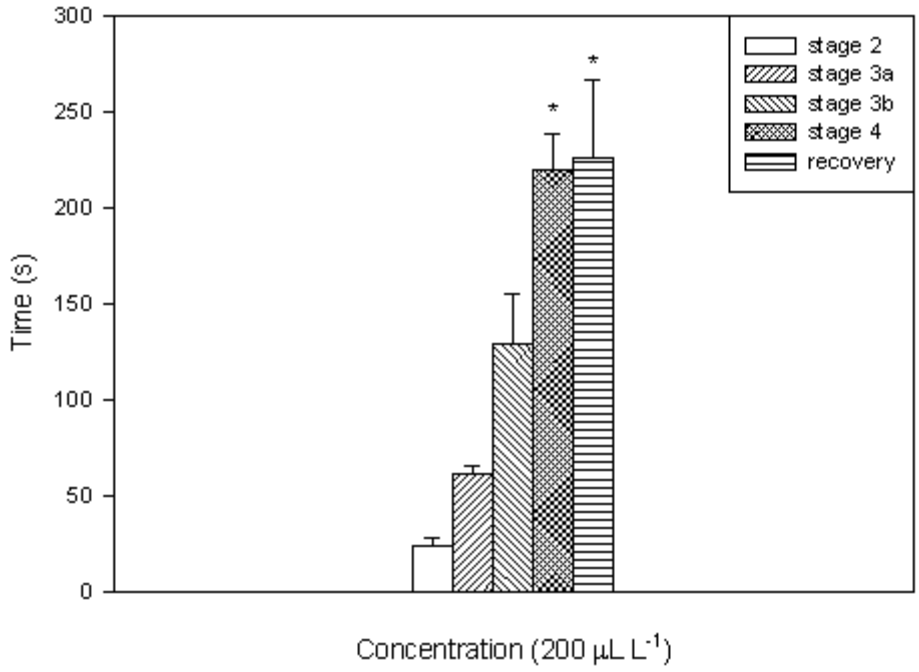
300
250
200
150
100
50
0
Time (s)
stage 2
stage 3a
stage 3b
stage 4
recovery
*
*
Concentration (200 µL L-1)

Fig 1

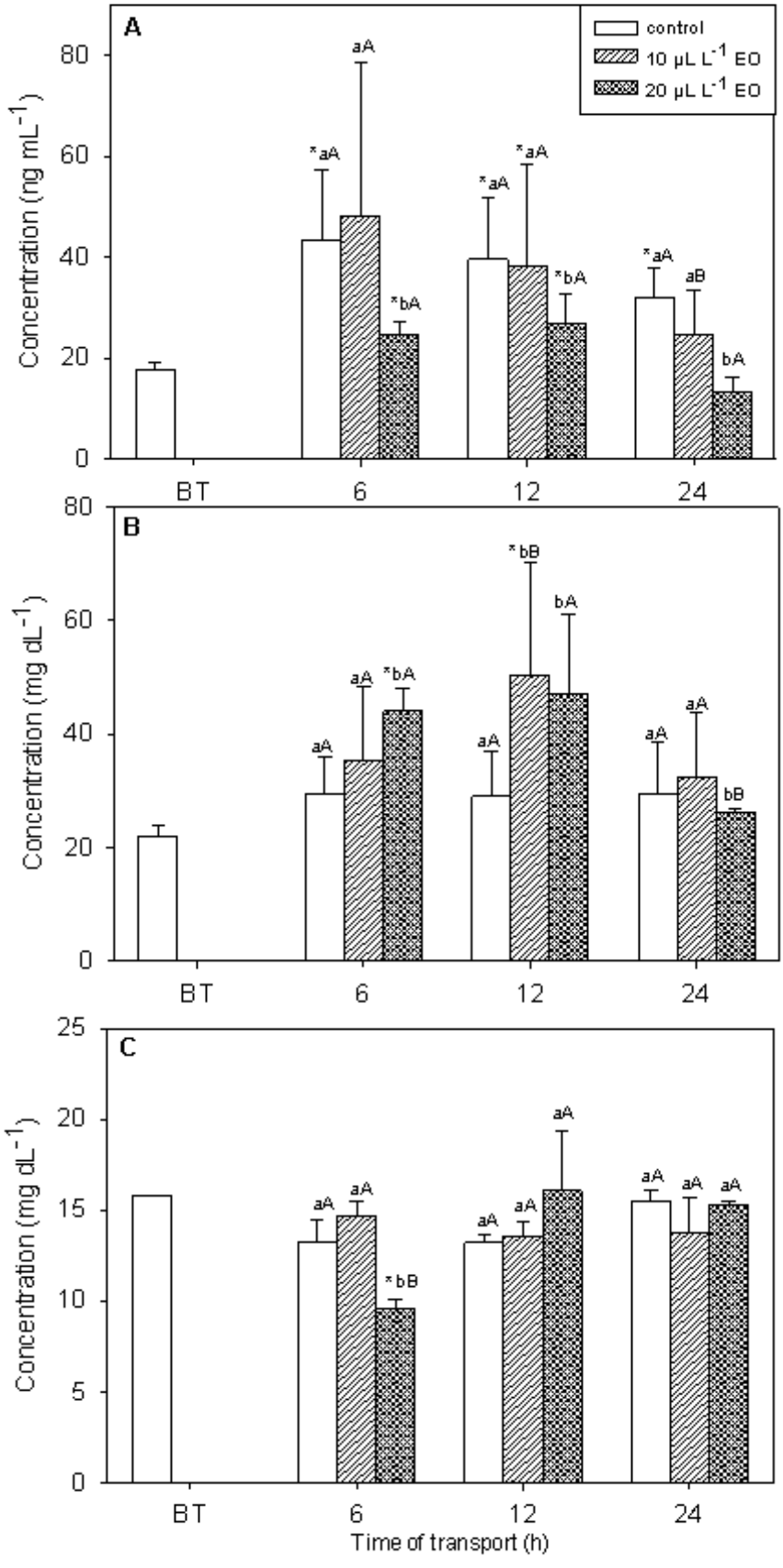
A
control
10 µL L-1 EO
20 µL L-1 EO
Concentration (ng mL-1)
80
60
40
20
0
*aA
aA
*bA
*aA
*aA
*bA
*aA
aB
bA
BT
6
12
24
B
Concentration (mg dL-1)
80
60
40
20
0
aA
aA
*bA
*bB
bA
aA
aA
bB
BT
6
12
24
C
Concentration (mg dL-1)
25
20
15
10
5
0
aA
aA
*bB
aA
aA
aA
aA
aA
aA
BT
6
12
24
Time of transport (h)

Fig.2

Artigo 4

EVALUATION OF THE EFFECTIVENESS OF EUGENOL IN BLOCKING RESPONSES TO A NOXIOUS STIMULUS IN THE TELEOST ZEBRAFISH, *Danio rerio*

Artigo em revisão pelos autores

2013

Evaluation of the effectiveness of eugenol in blocking responses to a noxious stimulus in the teleost zebrafish, *Danio rerio*

Thaylise Vey Parodi[a], Bernardo Baldisserotto[a], E. Don Stevens[b]

[a]Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil

[b]Department of Biomedical Science, UPEI-AVC, Charlottetown, PE, C1A4P3, Canada

* Corresponding author

Bernardo Baldisserotto

Departamento de Fisiologia e Farmacologia

Universidade Federal de Santa Maria

97105.900 – Santa Maria, RS, Brazil

E-mail: bbaldisserotto@hotmail.com

Phone: 55 55 3220-9382 fax: 55 55 3220-8241

ABSTRACT

Fish respond to noxious stimulus changing their behavior. Eugenol has wide use as anesthetic for aquatic organisms because of its low price, availability and safety while handling. The aim of this study was to verify if eugenol could block or reduce the effect of a noxious stimulus (5% acetic acid injection) and to propose a new model of analgesia investigation in fish measuring median velocity, high speed swimming, freeze (immobility time), vertical movement. There was a significant effect for all four parameters to eugenol itself using concentrations 10 and 20 μL L^{-1}, but the lowest concentrations (1, 2 and 5 μL L^{-1}) were sufficiently to block noxious stimuli. The duration of the analgesic effect of eugenol was tested. There was a significant time effect for two parameters: vertical movement and freeze time decreased in fish exposed to eugenol. The results of these tests suggest that the effect does not persist longer than the first observation time of 47 min and it can be a promissory model of the analgesia.

Introduction

Fish have nociceptors that are electrophysiologically similar in the way they respond to noxious stimuli to those found in mammals (Ashley, 2009). Similarly, fish respond to a noxious stimulus with a change in behavior, sometimes called a nocifensive response (Rose et al. 2012). There is some evidence that these nocifensive responses can be modified by pretreatment of the fish with pharmacological agents (Stevens et al. 2004). The question of whether or not a nocifensive behavioral response indicates that fish "feel" pain is controversial. Arguments for the case have been detailed by Braithwaite (2010) and those against by Rose (2012). Independent of the outcome of the argument concerning fish 'pain', all sides agree that fish welfare must be given the utmost consideration. It is with this in mind that we carried out the present experiments to test the effectiveness of eugenol in blocking responses to a noxious stimulus in fish.

A number of reviews have argued that zebrafish (*Danio rerio*) is a good model in the search for new drugs and for the study of the effects of drugs (Correia et al. 2011) by identifying stable mutations with phenotypes, overexpression of pathogenic proteins in specific cell types (Oates et al. 2000; Draper et al. 2001) and the experiments include investigation dose–response relationship (Stevens 2009).

Eugenol (2 methoxy-4-(2-propenyl) phenol) is the principal active ingredient (85 – 95%) of clove oil, which is derived from leaves, buds and stems of the clove tree (*Eugenia caryophyllata*) and has wide use as anesthetic for aquatic organisms because of its low price, availability and safety while handling (Sladky et al. 2001; Iversen et al., 2012; Hoseini et al., 2013). However, neither clove oil nor any individual active ingredient of clove oil (including eugenol, isoeugenol, or methyleugenol) is the subject of an FDA approved animal drug application (FDA,

2002). Concerns regarding this class of chemical compounds led to the nomination of eugenol, isoeugenol, and methyleugenol for investigation under the National Toxicology Program (NTP, 1983). NTP determined that eugenol is an equivocal carcinogen and methyleugenol is carcinogenic to rodents. It is considering the use the eugenol only alone (FDA, 2007).

Several studies have evaluated the use of eugenol to reduce hyperactivity during handling, especially during routine procedures such as weighing, vaccination, blood sampling, tagging, experimental surgery and veterinary procedures, thus reducing fish stress (Keene et al. 1998; Sladky et al. 2001; Roubach et al. 2005; Cunha et al. 2010;. However, stress is not pain; they involve different receptors, different pathways, and different parts of the central nervous system. Our focus in the present experiment was response to a noxious stimulus, not stress. The aim of the present study was to test the efficacy of eugenol in fish. The initial experiment was designed to test a wide range of concentrations and test a wide range of times to resolve the appropriate concentrations and the duration of its effect. Then these results were used in the second experiment to test if eugenol could block or reduce the effect of a noxious stimulus and when possible utilize it like a new model for analgesia investigation in fish.

Materials and Methods

Animals and housing

A total of 120 large adult (3-4 months old, 0.47 ± 0.29 g and 3.0 ± 0.51 cm) “wild type” zebrafish were obtained from a commercial distributor (Pets Unlimited, Charlottetown, PEI, CA). All fish were given at least seven days to acclimate to the laboratory environment and housed in groups of 20-30 animals per 32 L tank.

Tanks were filled with filtered system water and maintained at 25–27°C. Illumination was provided by fluorescent lights on a 24-h cycle (12:12). All fish used in this study were fed flake food with automatic feeders twice a day. Food was not provided 24 h before or during the experiments. Maintenance and treatment protocols were approved by University of Prince Edward Island (UPEI) animal care commute (AUP 09-004).

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solutions of acetic acid (5%) were dissolved in deionised water. Eugenol was purchased commercially (Odontofarma®, Porto Alegre, Brazil) and the analysis of this product by gas chromatography/mass spectrometry (GC-MS) (Varian Saturn 2200, Agilent Inc., Santa Clara, CA, USA) revealed 99.03 ± 0.15% eugenol (n=3) (Gomes et al., 2011).

Experiment 1 – Effect of eugenol in and of itself (no noxious stimulus)

Behavioral testing was performed between 11.00 h and 15.00 h. Each fish was placed individually in a 1.6 L test arena (11 x 5.4 x 16 cm) supplied with tank water at 25°C and constant aeration with change of water each 45 min time.

To diminish influence of external stimuli the top and sides of the test arena were covered. After a 5 min settling period, swimming activity was recorded for 35 s (time p0). Then each fish was transferred to a 100 mL beaker containing a specific eugenol concentration and kept for 45 min. After eugenol exposure, the fish was returned to the test arena and after a 5 min settling period, swimming activity was recorded for 35 s (time p1, i.e., 5 min after eugenol exposure). Then the animal was

placed in a recovery tank (with no eugenol) for 45 min, returned to the test arena and after a 5 min settling period, swimming activity was recorded for 35 s (time p2, i.e., 51 min after eugenol exposure). Then the animal was placed in a recovery tank (with no eugenol) for a second 45 min, and then returned to the test arena and after a 5 min settling period, swimming activity was recorded for 35 s (time p3, i.e., 97 min after eugenol exposure).

The eugenol concentrations (0, 1, 2, 5, 10 and 20 μL L^{-1}) and exposure times were chosen based on previous tests using concentrations below those which induce anesthesia in fish. Eight zebrafish were used for each concentration tested and each fish was tested only once.

Experiment 2 – effect of eugenol on response to a noxious stimulus

Based on the results of experiment 1, only eugenol concentrations lower than 10 (i.e., 0, 1, 2 and 5 μL L^{-1}) were tested and only in the first time period (i.e., 5 min after the eugenol exposure). The noxious stimulus was an injection of 5 μL of 5 % acetic acid solution into the face between the nares, using a Hamilton syringe (33 gauge needle). The syringe was mounted on micro syringe pump controller (WPI, model UMC4) to control the injection.

Video analysis and parameters measured

The parameters used to analyze behaviour were based on the experimental models proposed by Cachat et al. (2010) and Mathuru et al. (2012). Videos (640 x 480 at 29.9 Hz) were captured using LoliTrack (Loligo® Systems), Tjele, Denmark version 1.0.0. The camera faced the front of the tank. Position of the fish was calculated later using LoliTrack (ver 4.0). Raw position data (two dimensions) was

imported into Excel and Visual Basic programs were used for the analysis. A median filter was applied to the raw data to reduce pixel noise then position and velocity were calculated every four frames (i.e., at 0.13 s intervals). Velocity was binned into 17 bins. Four parameters were used: median velocity of the 35 s trial, freeze (duration of no movement - velocity values in the lowest bin), high speed swimming (velocity values in the highest bin), and vertical movement (total distance moved in the y direction only during the 35 s trial).

Statistical Analysis

The difference between pre-exposure and each post-exposure was used for statistical analysis. ANOVA and Dunnet´s test were used to compare each concentration with control (no eugenol). Effect of treatment (noxious stimulus or control) and effect of concentration were analyzed using two-way ANOVA and Tukey test. Statistical analyses were performed using Statistica Academic version 10.0 or Minitab ver 16 with a minimum significance level of $p < 0.05$. Data were expressed as mean ± SEM.

Results

Initial tests were carried out to establish the lowest concentration of eugenol that did not have an effect in and of itself and to test duration of its effect. There was a significant concentration effect for all four parameters: median velocity (0.002); freeze time (0.005); time at high velocity (0.036); vertical movement (0.036) (Fig 1) at the first post exposure trial (5 min). Post-hoc tests comparing results of different

concentrations with control (without eugenol) showed that the highest concentrations (10 and 20 μL L^{-1}) increased freeze time and decreased all others parameters. However, the lowest concentrations (1, 2 and 5 μL L^{-1}) did not change any of the measured parameters.

The duration of eugenol effect was tested using only the highest concentrations (10 and 20 μL L^{-1}). One-way ANOVA revealed that there was a significant time effect for two parameters: median velocity ($P = 0.016$); freeze time ($P = 0.001$) which increased and decreased respectively but not for the other two (time at high velocity ($P = 0.149$); vertical movement ($P = 0.108$) (Fig 2). The effect did not last longer than the first observation time of 47 min.

The freeze time and vertical movement reduced after acetic acid injection when the fish were previously exposed to eugenol 1 or 2 μL L^{-1} (Fig. 3C). The other parameters did not alter significantly.

Discussion

The present experiment tested a new protocol to study analgesic effect in fish, investigating parameters that modified the behavior of the animal during noxious stimuli. The utility of non-mammalian models in the study of pain was presented (Stevens 2007). The particular advantages of using zebrafish as a model in chemical screens was reviewed by Bowman and Zon (2010), who argued that there is no better vertebrate suited to high-throughput phenotyping. The main advantages are its cost effectiveness, large number of offspring and abundance of molecular and genomic tools.

Analgesic protocols are available for a variety of animals (Harms et al. 2005; Rang et al. 2007). Research on teleost fish demonstrates that potentially painful events impair their normal behavior and may be indicative of discomfort. These results suggest that the nociception the fish were experiencing might have taken up the majority of their attention, and they either ignored the novel object or could not divert attention to a fear response (Harms et al. 2005).

Therefore, it appears as if attention to the noxious event the fish was more important than performance of fear behavior in this experimental paradigm. Although the animals experiencing noxious stimulation did not show fear-related avoidance behavior, they did show reduced nociception-related behaviors. The study of Ehrensing et al. (1982) showed that goldfish learned to avoid an electric shock. The aversive nature of the stimulus was important for learning and memory consolidation. Similarly, rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), and zebrafish (*Danio rerio*) that experienced a noxious stimulation exhibited rapid changes in physiology and behavior that persisted for up to 6 hours (Reilly et al. 2008) and thus were not simple reflexes.

The literature showed, in few data, that fish are not only capable of sensing pain stimuli, but display behavioral responses to pain similar to those displayed by mammals (Chervova *et al*. 1997). In experimental studies of higher vertebrates, the stimuli include alerting, starting, avoidance, etc. (Charpentier 1968). Swimming is the principal functional parameter of fish, and in many species it is done mainly by lateral movements of the body. In the present method, discrete behavioral acts were selected as the estimating measure: velocity, freeze, swimming and movement. In the present study, it was tested ways for demonstrate pain stimulation. No anesthetics were used in the course of the experiments because they could distort

the responses to the analgesic tested. After eugenol injection – and for the least 47 min - the hyperactivity of the tested: fish were observed using 1 and 2 μL L^{-1}. This observation was confirmed for reduction immobility of the fish that explored the ambient after induction pain when treated with eugenol.

In other studies, rainbow trout that received an injection of dilute acetic acid to the upper and lower frontal lips rubbed the affected area against the available substrates (Ashley et al. 2009). In contrast, saline-injected fish did not demonstrate this anomalous behavior, which may have had the function of reducing the intensity of the noxious sensation, as has been described in humans and mammals that rub an affected area to reduce pain (Roveroni et al. 2001).

In zebrafish the acetic acid provoked a stimuli fear. This analysis was supported because the fish spent more time on freeze time without movement. Rainbow trout suspended normal feeding behavior after the injection of acid in their lips and resumed feeding only when the adverse changes in physiology subsided (Sneddon 2003). Fish that experience a noxious stimulus also display a dramatic rise in gill ventilation rate. Studies in goldfish and trout showed that they can learn to avoid a noxious electric shock, as they avoided entering the area where the shock was given even when food was present (Millsopp and Laming 2008).

Most behavioral changes in fish after noxious stimulation occur in the period immediately after the treatment for up to 120 minutes, peaking between 60 and 90 minutes. Such changes include anomalous behaviors such as rubbing the affected area, rocking on the substrate to and fro on both pectoral fins, and swimming less. These behaviors are conspicuous and easily observable if fish are in glass tanks; observation is more difficult in opaque tanks and in large numbers of fish (Sneddon, 2009).

A reduction in activity is easily measurable, but requires an existing measure of normal pretreatment behavior; most published studies record baseline observations for 15 to 30 minutes (Sneddon, 2003), so this is not usually too costly in terms of time, although an assessment of high numbers of fish would require more time. To zebrafish in immersion bath with eugenol the time for blocking the noxious stimulis was observed for no more than 47 min.

The results of the present study are similar to those of previous studies and are suggestive of a complex behavioural response to the noxious stimulus (Sneddon 2003; Reilly et al. 2008). Reductions in activity could be viewed as a protective, vegetative response to prevent further injury and aid recovery. However, changes in activity level suggest a complex behavior rather than a reflex reaction (Gentle and Hill, 1987), indicating higher brain processing that may control decision making.

Conclusions

A change in behavioral responses of zebra fish to acetic acid is a reasonable model to test analgesics. The response scales with stimulus intensity after treatment with acid was attenuated by eugenol, but not in a concentration-dependent manner, and the analgesic effect of eugenol was not maintained more than 47 min. The change in behavior of zebrafish associated with the noxious stimulus can be monitored and suggest a novel model for study analgesia in fish.

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Figure captions

Figure 1. Effect of eugenol in and of itself on behavioral parameters of zebrafish at the first post-exposure test.

Figure 2.Duration of effect of eugenol in and of itself on behavioral parameters of zebrafish. The effect of eugenol immersion was only evident at the first observation period (47 min) but behavior returned to normal for the later observation periods. The values at the two highest concentrations (10 and 20) were pooled for this figure.

Figure 3. Effect of eugenol concentrations on modifying the response after a noxious stimulus (acetic acid injection) and control (no noxious stimulus) pain at time p1. * indicate significant difference from no pain group at the same eugenol concentration.

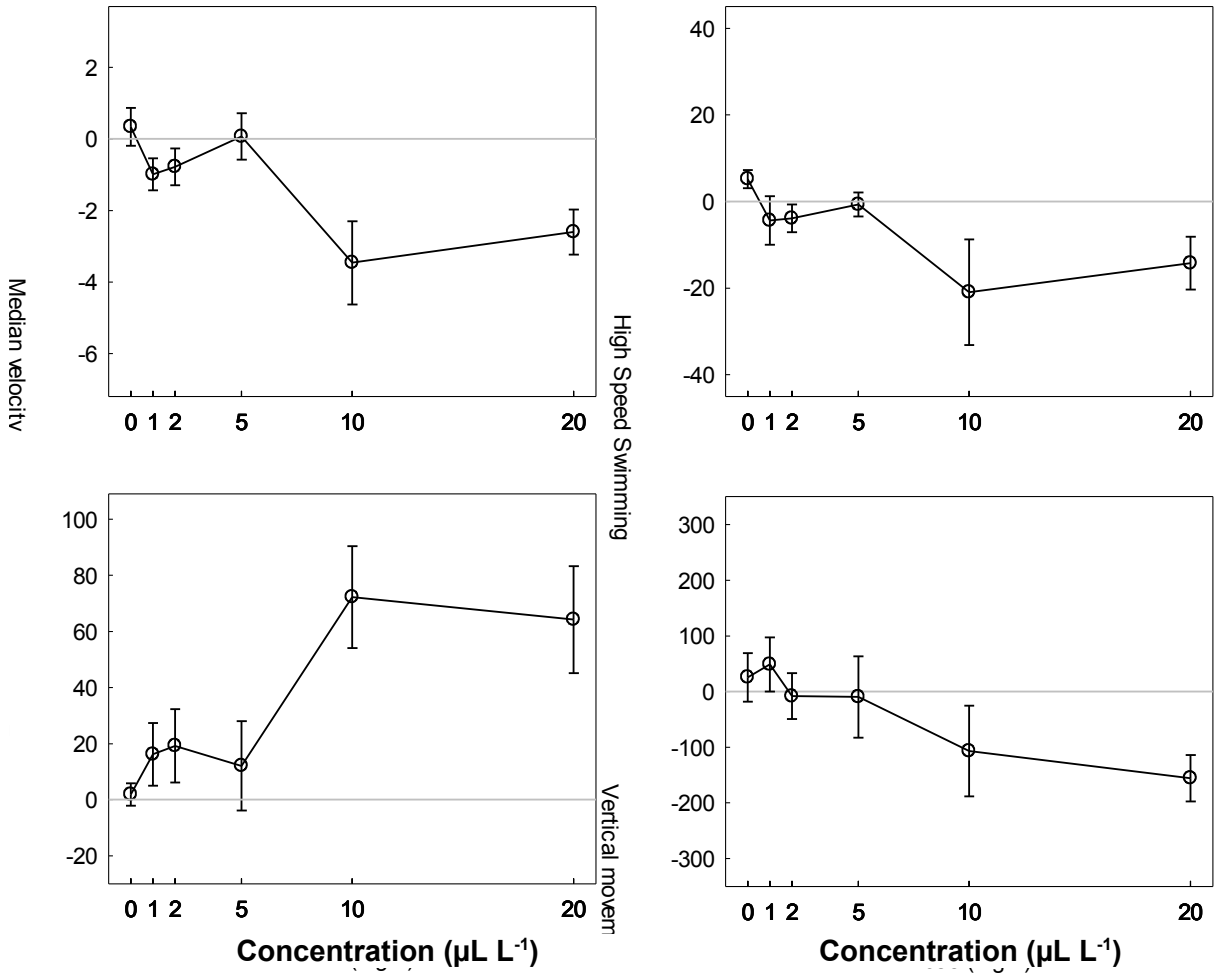
Median velocity
High Speed Swimming
Vertical movem
Concentration (µL L-1)
Concentration (µL L-1)
0 1 2 5 10 20

Fig. 1

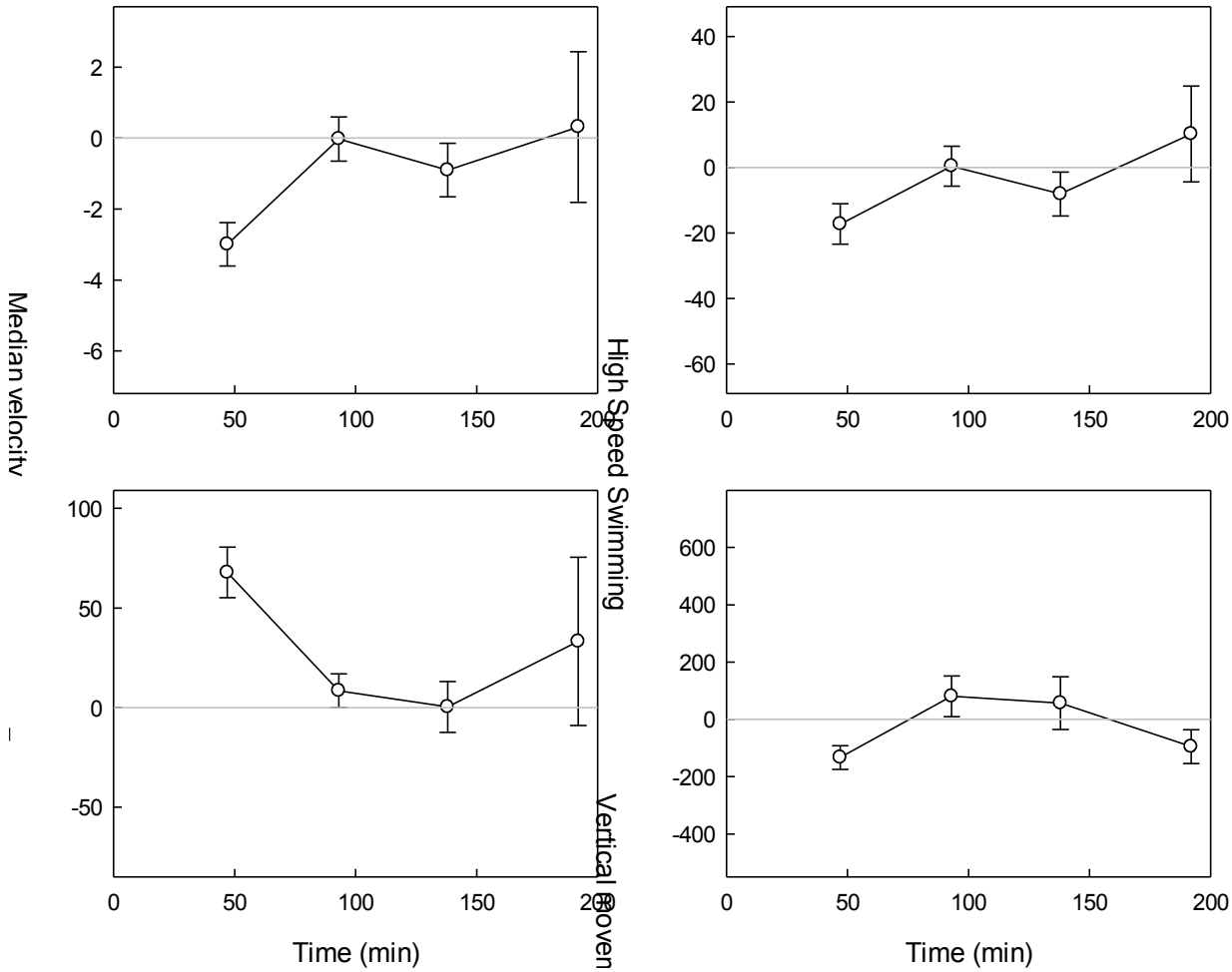
Median velocity
High Speed Swimming
Vertical Movement
Time (min)
Time (min)

Fig 2

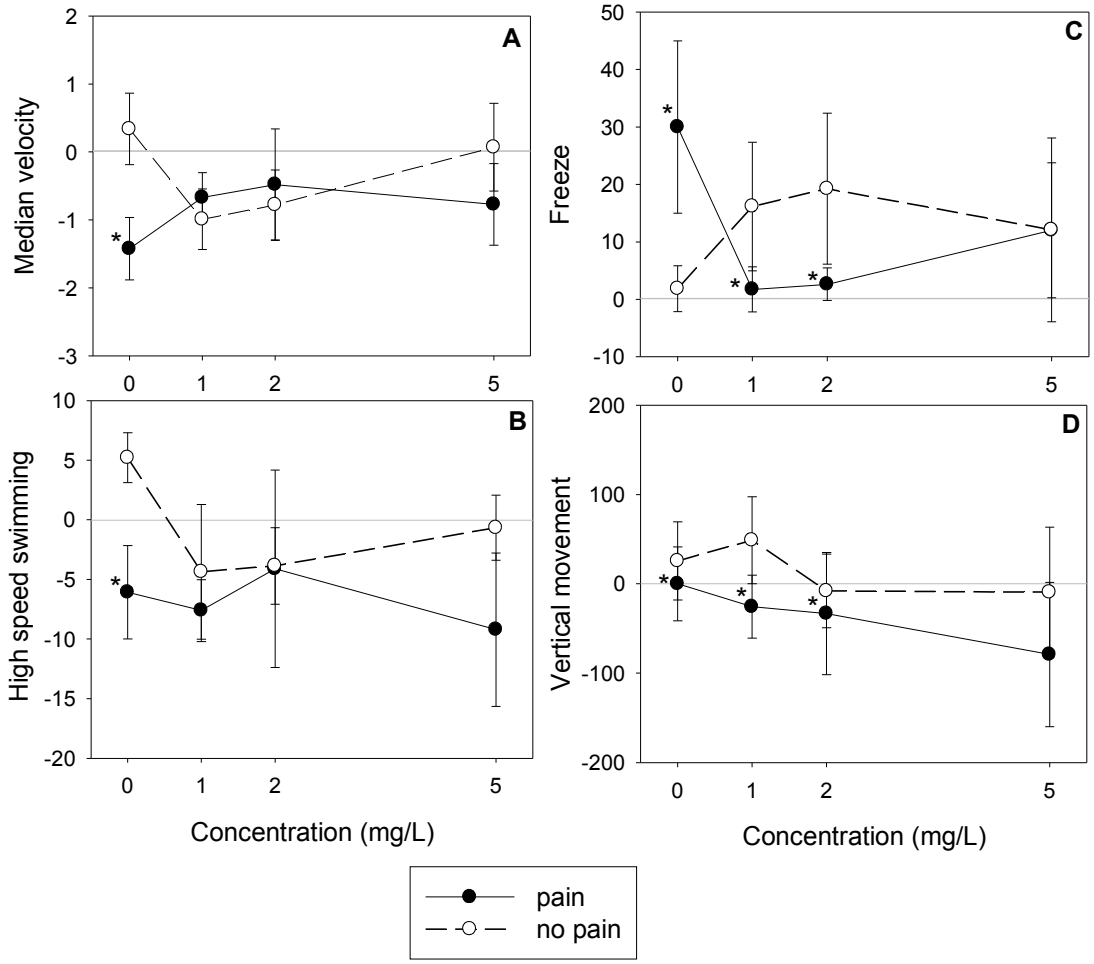
A
B
C
D
Median velocity
High speed swimming
Freeze
Vertical movement
Concentration (mg/L)
Concentration (mg/L)
pain
no pain

Fig.3

DISCUSSÃO

Sabe-se que a imobilização química na aquicultura ajuda os animais a minimizar os efeitos causados por manipulações. Assim, espera-se que peixes submetidos ao processo de anestesia não sofram distúrbios bioquímicos e fisiológicos. O estresse também afeta a atividade dos leucócitos, facilitando a infestação por parasitas e a mobilização de energia causada pelo estresse afeta negativamente os mecanismos de resistência do peixe, uma vez que estes mecanismos também demandam energia (SCHRECK,1996).

A espécie vegetal *Aloysia triphylla* apresenta relatos entre os usos na medicina popular como agente sedativo (CARNAT et al., 1999), nos casos de insônia e ansiedade (PASCUAL et al., 2001). Não havia relatos na literatura que comprovassem realmente sua eficácia. Diante da grande importância econômica das espécies aquáticas, a preocupação com a qualidade do produto ofertado e a preocupação ambiental surgiu a possibilidade de utilização do óleo essencial de *A. triphylla* na imobilização ou mesmo redução do estresse durante manuseio e transporte de algumas espécies aquáticas de importância comercial.

O anestésico é considerado ideal quando reúne algumas características, tais como atuação rápida (cerca de três minutos); curto tempo de recuperação (cerca de cinco minutos); facilidade de aplicação e baixo risco para animais e ser humano (KEENE *et al*., 1998). Porém, a ação dos anestésicos pode ser influenciada por fatores como a temperatura da água, nível de oxigênio disponível, estado fisiológico, concentrações/doses, tamanho, espécie em experimentação, entre outros (WOOD, NELSON, RAMSTAD et al., 2002). Diferentes estudos realizados tem a finalidade de definir procedimentos de anestesia assim como a substância e a concentração mais indicada para cada espécie.

Nos experimentos de anestesia realizados com jundiás (*Rhamdia quelen*) com óleo essencial (OE) de *A. triphylla* observamos alterações nos tempos de resposta e indução em relação à variedade (albina ou cinza) em altas concentrações (200 - 800 μL L^{-1}) com rápida indução e lenta recuperação para albinos (Artigo 1). A hipótese para esta variabilidade encontra-se na rusticidade e ampla distribuição natural dos jundiás cinzas, conferindo a estes supostamente maior resistência aos fatores externos (BALDISSEROTTO, 2003).

A área branquial maior em relação ao corpo aumenta a superfície de absorção nos peixes menores. Desta forma, o peixe menor absorve mais anestésicos do que os peixes maiores e consequentemente seu tempo de indução à anestesia é mais rápido e sua recuperação mais lenta (ROUBACH et al., 2002). Os óleos essenciais são lipofílicos e tem maior afinidade pelos tecidos adiposos e maior facilidade de atravessar a barreira hematoencefálica, geralmente diminuindo o tempo de indução e aumentando a recuperação em peixes maiores (ZAHL et al., 2010). A menor concentração de óleo essencial em nosso experimento utilizada para anestesiar peixes foi verificada em juvenis de jundiá (cinzas e albinos) (± 4g e 10 cm) e *Centropomus paralellus* (robalo peva) (±3g e 8cm) em 100 μL L^{-1}, com tempos de indução e recuperação similares entre as espécies (Artigo 1 e 3). Embora as espécies tenham sido diferentes, o tamanho e peso foram similares. GRESSLER et al. (2012) utilizando o mesmo OE de *A. triphylla* em concentração aproximada (135 mg L^{-1}) em jundiás maiores (±95g e 22cm) evidenciou aproximado tempo de indução e recuperação ao de nosso estudo, independente do tamanho e peso dos exemplares. Portanto, pode-se recomendar o uso de OE de *A. triphylla* pelo menos para jundiás de diferentes tamanhos e juvenis de robalo-peva.

Anestesia em camarões também apresenta literatura limitada. Comparando a atividade anestésica do OE de *A. triphylla* para camarões da espécie *Litopenaeus vannamei* (Artigo 2) com jundiás (Artigo 1) e robalos-peva (Artigo 3) verificamos que camarões sub-adultos anestesiam na mesma concentração de OE que peixes (100 μL L^{-1}) com tempos de indução e recuperação aproximados entre si e superiores que em peixes, enquanto que as pós-larvas anestesiam somente em concentrações superiores (300 μL L^{-1}) (Artigo 2).

Quando os animais são submetidos a situações estressores quando manipulados, diversas alterações fisiológicas e bioquímicas são observadas (FREDERICKS et al., 1993; MORGAN & IWANA, 1997), como o aumento dos níveis de cortisol, hiperglicemia e imunossupressão (WENDELAAR BONGA, 1997). Para jundiás o OE foi eficaz em prevenir estas alterações, evidenciado pela ausência de alteração nos valores de cortisol durante 4 horas de transporte (Artigo 2). Para robalos a exposição ao OE foi eficaz em 24 horas na redução de estresse, marcados pela redução nos níveis de glicose e cortisol sanguíneo (Artigo 3), o qual justificou a maior imobilidade do animal, detectada pela não alteração do lactato ao longo do período, correspondendo a um menor gasto aeróbico com exercício.

Em peixes, a lenta respiração durante a anestesia pode induzir hipóxia, estimulando as defesas antioxidantes tanto enzimáticas quanto não enzimáticas (DI MARCO et al., 2008). A eficácia na prevenção da produção de espécies reativas de oxigênio em camarões foi verificada tanto para o eugenol como os OEs de *A. triphylla* e *L. alba*, aumentando a expressão de enzimas detoxificantes como glutationa S-transferase e catalase (Artigo 1). Essa eficácia já havia sido comprovada com o uso de OE de *A. triphylla* em jundiás (GRESSLER et al., 2012). Possivelmente estes constituintes atuam compensando os danos do estresse oxidativo assim que o animal se recupera da anestesia (BUZADZIC et al., 1992).

Entretanto, CHO & HEATH (2000), em seus estudos com salmão chinook, *O. tshawytsch*, verificaram que os níveis de depressão do sistema nervoso central atingidos pelos animais submetidos ao MS-222 ou ao eugenol não necessariamente mitigaram respostas fisiológicas, especialmente de secreção do cortisol e SOIVIO & NIKINMAA (1981) relataram o mesmo em truta arco-íris (*Oncorhyncus mikyss*). Portanto, o próprio anestésico pode induzir efeitos indesejáveis, alterando as variáveis bioquímicas (SLADKY *et al*., 2001; WOODY *et al*., 2002) ou mesmo a morte pelo tempo de exposição prolongado, mesmo em baixas concentrações, como visto para larvas de *L. vannamei* (Artigo1).

Por outro lado, HUNN e GREER (1991) reportaram que não houve alteração nos parâmetros sanguíneos (cortisol e glicose) do salmão do Atlântico, *Salmo salar*, após ser anestesiado com MS-222 e benzocaína; enquanto ROBERTSON *et al*. (1988) verificaram que peixes transportados com MS-222 não apresentam mudanças nos parâmetros fisiológicos, devido à redução de estresse obtido pelo fármaco.

Nos parâmetros hematológicos, alterações na taxa de hemoglobina, no hematócrito ou no número de eritrócitos após estresse podem sugerir hemoconcentração ou hemodiluição por disfunção osmorregulatória (HOUSTON *et al*., 1996). Já as respostas fisiológicas de estresse variam de acordo com a intensidade do agente estressor, sendo pouco expressivas quando os peixes são expostos a estímulos mais brandos e por rápido período de tempo (ROBERTSON *et al*., 1988).

A eficácia anestésica e sedativa do eugenol e dos OEs de *A. triphylla* e *L. alba* em peixes são bem relatados na literatura e para camarões verificadas no Artigo 1, inclusive o eugenol já é utilizado na aquicultura, mas ainda não de forma

regulamentada. A não regulamentação do uso do eugenol foi devido a um estudo mostrar haver incidência ambígua de carcinogenicidade e adenomas no fígado quando o óleo de cravo foi acrescentado à dieta de ratos fêmea (CAS n°223, 1992). Porém, a FAO (2007) permitiu o uso de eugenol para anestesia em peixes, pois um estudo posterior atribuiu o efeito carcinogênico a um outro constituinte do óleo de cravo, o metileugenol. Hume (1983) provou que o eugenol, tanto *in vivo* como *in vitro*, apresenta diferentes tipos de toxicidades, podendo causar ao organismo: dermatites, reações alérgicas, disfunção hepática, coagulação intravascular disseminada e hipoglicemia severa. Entretanto esses efeitos só são observados em doses maiores que 10^{-3}mol/L.

Não só no intuito de preservar a integridade do pescado como produto final e preocupação com bem-estar, mas também no intuito em investigar a percepção da dor em animais aquáticos, os estudos deram continuidade com a investigação da atividade analgésica (Artigo 4).

O efeito sedativo e anestésico do eugenol já foi demonstrado para várias espécies de peixes, dentre elas: tambaqui, *Colossoma macropomum* (ROUBACH et al, 2005), pintado, *Pseudoplatystoma corruscans* (VIDAL et al, 2006), jundiá, *Rhamdia quelen* (CUNHA et al., 2010) e robalo-flecha, *Centropomus undecimalis* (SOUZA-JUNIOR; ALVES-JUNIOR, 2006), bem como a capacidade do eugenol em reduzir o estresse no transporte e manuseio (CUNHA et al., 2010).

O eugenol em baixas concentrações inibe a atividade nervosa, pois penetra com rapidez na bainha de mielina de uma fibra ou feixe nervoso, bloqueando a geração e a condução do impulso nervoso, promovendo um efeito analgésico e anestésico local. A remoção do eugenol do local de ação confere retorno à normalidade (ESCOBAR, 2002). Substâncias anestésicas são frequentemente utilizadas para reduzir a hipermotilidade dos peixes, que é uma fonte considerável de danos teciduais durante procedimentos de manejo e /ou transporte (INOUE et al., 2003; VIDAL et al., 2006).

CONCLUSÃO

- O eugenol (50 µL L^{-1}) e os óleos essenciais de *A. triphylla* (100 µL L^{-1}) e *L. alba* (500 µL L^{-1}) promoveram anestesia e sedação durante o transporte de sub-adultos e larvas de camarões *L. vannamei*, além de serem eficazes na atividade antioxidante;

- O OE de *A. triphylla* anestesiou juvenis cinzas e albinos de jundiá, *Rhamdia quelen* e robalo-peva, *Centropomus parallelus* (100 µL L^{-1}) além de reduzi o estresse durante transporte.

- Resultados do estudo com eugenol (1, 2 e 5 µL L^{-1}) em peixe-zebra, *Danio rerio* indicam uma nova proposta de observação comportamental durante experimentos de dor em peixes

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