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ÓLEOS ESSENCIAIS ALTERAM PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS EM DIFERENTES ESPÉCIES DE CRUSTÁCEOS?

Santa Maria, RS, Brasil
2021

Alessandra Janaina Becker

Óleos essenciais alteram parâmetros comportamentais e bioquímicos em diferentes espécies de crustáceos?

Tese apresentada ao Curso de Doutorado do Programa de Pós-graduação em Biodiversidade Animal, Área de concentração em Ecologia e Conservação, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Ciências Biológicas - Área Biodiversidade Animal**

Orientador: Prof. Dr. Bernardo Baldisserotto
Coorientador: Prof. Dr. Wilson Wasielesky Jr.

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RESUMO

ÓLEOS ESSENCIAIS ALTERAM PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS EM DIFERENTES ESPÉCIES DE CRUSTÁCEOS?

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Crustáceos integram funções ecossistêmicas essenciais na natureza, atuando desde consumidores primários até predadores nas redes tróficas aquáticas. Além disso, muitos crustáceos decápodes são importantes recursos econômicos e sociais. Contudo, esses organismos são continuamente expostos a diferentes estressores no ambiente natural e em cativeiro, envolvendo, sobretudo, variações nos parâmetros de qualidade da água e manejo inadequado. Respostas ao estresse demandam elevado gasto energético e induzem a uma cascata de eventos comportamentais, fatores neuroendócrinos e mecanismos imunológicos. Dessa forma, observações comportamentais e fisiológicas fornecem um entendimento completo das perturbações homeostáticas causadas no organismo pelos estressores. Recentemente, os óleos essenciais (OEs) vêm sendo reportados como alternativas na manipulação e diminuição do estresse em crustáceos. Portanto, faz-se necessário investigar o potencial sedativo e anestésico de diferentes OEs, bem como seus efeitos sobre parâmetros comportamentais e bioquímicos em crustáceos, antes de recomendá-los para uso. No primeiro trabalho verificamos que os OEs de *Lippia alba* (OELA) e *Cymbopogon citratus* (OEC) foram eficazes como anestésicos para os camarões *Farfantepenaeus paulensis* e *Litopenaeus vannamei*, respectivamente. Em contrapartida, os OEs de *Ocimum gratissimum* (OEOG) e *Origanum majorana* (OEO) não foram eficientes para essas espécies. Baseado nos resultados encontrados, avaliou-se os efeitos do OEC sobre a sobre a atividade comportamental e bioquímica de *L. vannamei* durante um período de exposição de 6h. Nesse experimento encontramos que a concentração de 10 μL L^{-1} de EOC melhorou a resistência antioxidante sem comprometer o comportamento de natação. Posteriormente, investigamos o potencial do OELA e do linalol (composto majoritário) na redução do estresse e danos oxidativos em fêmeas e machos de *L. vannamei* durante procedimentos de ablação do pedúnculo ocular e extrusão do espermatóforo, respectivamente. As respostas antioxidantes variaram significativamente entre os sexos. Contudo, os OEs geraram um efeito protetor e evitaram a oxidação de lipídios. Os parâmetros bioquímicos analisados nos dois estudos foram: capacidade antioxidante total contra radicais peroxil, glutationa reduzida, grupos sulfidrila associados com proteínas e peroxidação lipídica nas brânquias, hepatopâncreas e músculo. Os OEs também demostraram sucesso na indução a sedação e anestesia do anfipoda *Hyallela bonariensis*. Entre os OEs e seus compostos majoritarios, o de *Aloysia triphylla* (OEAT) e o linalol obtiveram melhores resultados. Além disso, o OEAT reduziu a atividade de locomoção de *H. bonariensis*, mas sem comprometer seu comportamento natural. Dessa forma, podemos concluir que OEs podem ser indicados como anestésicos para diferentes espécies de crustáceos. Porém, o mesmo OE pode apresentar respostas diferentes entre as espécies, demonstrando a necessidade de estudos prévios para garantir o sucesso na aplicação.

Palavras – chave: Anfípoda. Camarões. Comportamento. Estresse oxidativo. Sedação.

ABSTRACT

ESSENTIAL OILS CHANGE BEHAVIORAL AND BIOCHEMICAL PARAMETERS IN DIFFERENT CRUSTACEAN SPECIES?

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Crustaceans integrate essential ecosystem functions in the enviroment, acting from primary consumers to predators in aquatic trophic networks. Furthermore, many decapod crustaceans are important economic and social resources. However, these organisms are continually exposed to different stressors in the natural environment and captivity, involving, above all, variations in water quality parameters and inadequate management. Stress responses demand high energy expenditure and induce a cascade of behavioral events, neuroendocrine factors, and immunological mechanisms. In this way, behavioral and physiological observations provide a complete understanding of the homeostatic disturbances caused in the body by stressors. Recently, essential oils (EOs) have been reported as alternatives for handling and reducing stress in crustaceans. Therefore, it is necessary to investigate the sedative and anesthetic potential of different EOs, as well as to analyze their effects on behavioral and biochemical parameters in crustacean species, before recommending them as safe anesthetics. We found that EOs from *Lippia alba* (EOLA) and *Cymbopogon citratus* (EOC) were effective as anesthetics for the shrimps *Farfantepenaeus paulensis* and *Litopenaeus vannamei*, respectively. On the other hand, the EOs of *Ocimum gratissimum* (EOOG) and *Origanum majorana* (EOO) were not efficient for these species. Based on these results, we investigated the effects of EOC on the behavioral and biochemical activity of *L. vannamei* during an exposure period of 6h. In this experiment, we found that the concentration of 10 μL L^{-1} of EOC improved the antioxidant response without compromising the swimming behavior. Subsequently, we investigated the potential of EOLA and linalool (major compound) in reducing stress and oxidative damage in female and male *L. vannamei* during ocular peduncle ablation and spermatophore extrusion procedures, respectively. Antioxidant responses varied significantly between the sexes. However, EOs generated a protective effect and prevented lipid oxidation. The biochemical parameters analyzed in the two studies were: total antioxidant capacity against peroxyl radicals, reduced glutathione), sulfhydryl groups associated with proteins, and lipid peroxidation in the gills, hepatopancreas, and muscle. The EOs have also demonstrated success in inducing sedation and anesthesia of the amphipod *Hyallela bonariensis*. Among the EOs and their major compounds, that of *Aloysia triphylla* (EOAT) and linalool obtained better results. Furthermore, EOAT reduced the locomotion activity of *H. bonariensis*, but without compromising its natural behavior. Thus, we can conclude that EOs can be indicated as anesthetics for different species of crustaceans. However, the same EO can present different responses between species, demonstrating the need for previous studies to ensure its successful application.

Keywords: Amphipoda. Behavior. Oxidative stress. Sedation. Shrimp.

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1. INTRODUÇÃO

1.1 PADRÕES COMPORTAMENTAIS EM CRUSTÁCEOS

O Filo Crustacea compreende um grupo diverso (cerca de 67 mil espécies) com variados modos de vida e estruturas corporais, sendo formado principalmente por espécies de ambiente aquático e alguns representantes terrestres (RUPPERT & BARNES, 2005). Dentro do ambiente aquático ocupam diversos nichos e habitats, integrando redes tróficas em diferentes níveis, desde consumidores primários e secundários até presas para espécies de outros grupos. O comportamento dos animais é considerado uma característica fundamental para o sucesso das interações com o meio e na busca por recursos, implicando no desenvolvimento de estratégias para atividades essenciais como reprodução, alimentação, hierarquia social e fuga de predadores (YAMAMOTO & VOLPATO, 2007).

O comportamento dos animais consiste de eventos complexos que garantem a manutenção da capacidade de reprodução e a sobrevivência da espécie. Nesse contexto, a determinação das estratégias comportamentais destes animais em termos bioquímico-funcionais, tanto em ambiente natural como em situações de cultivo experimental, possibilita gerar dados para criar ferramentas padronizadas de diagnóstico de saúde animal, alocação de recursos energéticos e monitoramento ambiental (GONYOU, 1994; LAWRENCE, 2008; COOKE et al. 2013). Em termos comerciais, análises comportamentais ajudam a melhorar as práticas de manejo, diagnosticar períodos de vulnerabilidade e garantir ambientes de cultivo mais adequados (BARDERA et al. 2018). Observações comportamentais e fisiológicas fornecem um entendimento completo das perturbações homeostáticas no organismo em resposta a estímulos estressores.

A locomoção é a principal atividade responsável pelo desempenho das funções vitais dos crustáceos aquáticos, sendo diretamente influenciada por fatores ambientais e metabólicos (DANIEL, 1984; ZHANG et al. 2006). Os padrões de locomoção são definidos a partir da percepção das informações do ambiente por sistemas sensoriais que interceptam e transportam para processamento e integração em sistemas neurais que através de mecanismos de sinalização desencadeiam respostas comportamentais (EDWARDS et al. 1994). Os parâmetros de locomoção característicos em crustáceos são a natação, movimentos de evasão, habilidades de enterramento e escavação, encontros agonísticos e forrageamento (PONTES, 2006; YU, et al. 2009; ROBLES-ROMO et al., 2016).

A natação garante a habilidade dos crustáceos se manterem em suspensão no meio permitindo o deslocamento vertical pela coluna d’água e migração por longas distâncias (BEAMISH, 1978; GORDON et al. 2017). O movimento de evasão é um dos comportamentos

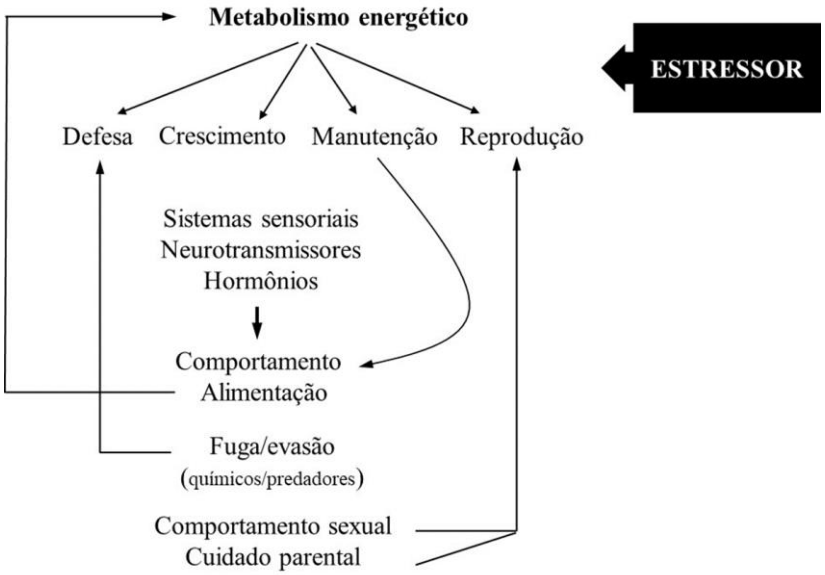
mais definidos, principalmente em camarões, para indicar situações de perigo, e consiste em contrações de curta duração dos músculos abdominais devido a flexões e extensões do abdômen, controlado por gânglios gigantes abdominais e mediado, predominantemente, por metabolismo anaeróbico (NEIL & ANSELL, 1995; ARNOTT et al, 1998; ROBLES-ROMO et al., 2016). A habilidade de escavação e enterramento consistem em uma forma de defesa contra predadores, redução da taxa metabólica e proteção contra mudanças bruscas de temperatura. Através de movimentos dos pleópodos e pereiópodos os animais retiram as partículas de sedimento e empurram o corpo em direção ao substrato, finalizando o processo de enterramento pela atividade das antenas e urópodos (DALL et al. 1990). Em camarões peneídeos, a granulometria do sedimento influencia na escolha do local (SANTOS et al. 2013).

Eventos estressores podem ocasionar um desequilíbrio na homeostase fisiológica do animal, comprometendo o consumo energético além dos níveis basais e gerando alterações comportamentais (ZHANG et al. 2007; YU et al. 2010; DUAN et al. 2014; HASENBEIN et al. 2018; LI et al. 2018, 2019a) (Figura 1). Essas mudanças podem gerar consequências a nível populacional desestabilizando a estrutura e funcionamento das comunidades. Em crustáceos, a principal reserva energética é a glicose, que é armazenada na forma de glicogênio no músculo, hemolinfa, coração e hepatopâncreas (VERRI et al. 2001; ZHU et al. 2016). Durante o aumento da atividade ocorre mobilização das reservas de glicogênio nos tecidos, que pode ser acompanhado pela elevação nos níveis de lactato, para suprir uma maior demanda energética e colaborar no ajuste metabólico (ENGLAND & BALDWIN, 1985; LI et al. 2015; LAGE et al. 2017). Além disso, a capacidade respiratória, osmorregulatória e de filtração também são prejudicadas, pois seu funcionamento é dependente do movimento de apêndices especializados conectados a região da câmara branquial (DE LANGE et al. 2009; LI et al. 2019b). Desse modo, é comum que os crustáceos realizem maior investimento energético em situações mais dispendiosas, tal qual, muda, confrontos, fuga e reprodução, mantendo – se mais inativos em outras situações (SORNOM et al. 2010).

A etologia é uma das principais ferramentas aplicadas para o estudo dos mecanismos comportamentais, baseada em diferentes métodos de observação e experimentação em campo e laboratório (GONYOU, 1994). A construção de um etograma permite a quantificação e qualificação dos comportamentos, classificando por categorias ou sequências, seguido de descrições conforme a espécie estudada e o objetivo proposto. Os registros comportamentais podem ser realizados por meio de filmagens, descrições verbais (escritas ou gravadas), planilhas de registro e registradores automáticos de eventos. Entre os métodos de amostragem mais comum temos: (a) amostragem *ad libitum*, (b) amostragem do animal focal, (c) amostragem por

escaneamento e (d) amostragem por comportamento (ALTMANN, 1974). Em crustáceos decápodes a maioria dos trabalhos comportamentais estão relacionados a avaliações de padrões alimentares, comportamento reprodutivo e densidade populacional (NUNES et al. 2006; MOYLE et al. 2009; CHAVANICH et al. 2016; TREVISAN & SANTOS, 2014; PASCHOAL et al. 2016;). Pesquisas comportamentais com o anfipodes geralmente envolvem testes ecotoxicológicos ou eventos reprodutivos (DUTRA et al. 2009; NEGRO et al. 2013; PEDERSEN et al. 2013; CASTIGLIONI et al. 2018).

Figura 1 - Diferentes níveis de organização biológica do comportamento em resposta a um estressor.



Fonte: Adaptado de Amiard-Triquet (2009).

Avaliação da locomoção em espécies aquáticas pode apresentar limitações, no entanto, o emprego de câmeras digitais e o surgimento de sistemas automatizados com biosensores, microscópios com câmeras acopladas e sistemas de vídeo-tracking configurados com *softwares*, permitem o registro de sequências de imagens, reconstrução de trajetórias e determinação de métricas comportamentais que não seriam possíveis de captar apenas através do olho humano (BIANCO, et al. 2013). São exemplos de *softwares* comportamentais o ANY-maze® video Tracking Software, Ethovision e Biological Early Warning Systems. Dentre as principais métricas que podem ser aplicadas em trabalhos com crustáceos temos: velocidade média e instantânea de natação, distância percorrida, duração dos movimentos, frequência de escape, variações na orientação em um determinado espaço (*turning angle*) e imobilidade (BOWNIK, 2017).

Estudos comportamentais em laboratório possibilitam relacionar variáveis mensuradas sob condições controladas com respostas ecológicas observadas em ambiente natural. Notadamente, o comportamento traduz a forma como os animais se sentem frente a estímulos, estressores ou não, reagindo ou adaptando-se à situação.

1.2 ESTRESSE E BEM-ESTAR EM CRUSTÁCEOS

O estresse é definido como um estado de perturbação do equilíbrio fisiológico ou homeostático causado pela soma de agentes internos e externos sobre o corpo. Respostas ao estresse demandam elevado gasto energético e envolvem um conjunto de ações comportamentais, fatores neuroendócrinos e mecanismos imunológicos, com o intuito de eliminar ou reduzir as ameaças através de ações compensatórias ou adaptativas (LE MOULLAC & HAFFNER, 2000; STONER et al. 2012). Os crustáceos são continuamente expostos a diferentes pressões no ambiente natural e em cativeiro. Por exemplo, estuários são ambientes naturais altamente estressores em razão da ampla variação nas concentrações de oxigênio, salinidade e temperatura (ODUM, 1985). Em laboratório, animais expostos ao ar para captura ou analises biométricas passam por situações de anoxia, resultando na redução do consumo de oxigênio e da atividade locomotora quando retornam aos aquários (GUZMÁN-SÁENZ et al. 2010).

Comportamentos de evasão e posturas de confronto integram respostas iniciais a fatores estressores. Outras alterações comportamentais indicativas de estresse são: letargia, desorientação, movimentos repetitivos de evasão e diminuição da frequência natatória (MAIN & LARAMORE, 1999). A carapaça dos crustáceos representa uma primeira linha de defesa, formando uma barreira externa contra injúrias e invasões de microrganismos (MARTÍNEZ, 2007). A presença de lesões visíveis na carapaça pode sinalizar a ocorrência de um evento estressor. Quando as barreiras físicas e comportamentais não são efetivas no combate ao estressor, mecanismos fisiológicos são ativados para restaurar a homeostase interna. Esses mecanismos integram uma cascata de eventos imunológicos e bioquímicos que podem aparecer em segundos, persistindo por horas ou dias (RODRÍGUEZ & LE MOULLAC, 2000).

O tipo de estressor e o tempo de exposição influenciam na intensidade da resposta metabólica, podendo ser classificado como de origem externa ou interna. Dentre os fatores externos podemos citar a variação de temperatura, exposição ao ar, condições de hipóxia, mudanças de salinidade, diferenças de pH, competição por recursos, ablação do pedúnculo ocular, exposição a contaminantes e manejo inadequado dos organismos. Estressores internos

estão relacionados ao sexo, tamanho corporal, grau de dureza da carapaça e estágio de vida (MANFRIN et al. 2016).

A hemolinfa é responsável pelo controle da resposta imune, sendo composta por uma fração celular, representada pelos hemócitos e uma fração humoral, formada pelo plasma (SODERHÄLL & CERENIUS, 1992). Os fatores celulares e humorais atuam de forma integrada na manutenção da homeostase corpórea e na proteção contra patógenos e parasitas (BARRACO et al. 2008). A resposta imune celular envolve os processos de fagocitose, encapsulação, degradação eliminação de microrganismos e agentes estranhos (RODRÍGUEZ & LE MOULLAC, 2000). Por outro lado, a resposta imune humoral inclui os processos de coagulação da hemolinfa, sistema pró - fenoloxidase (PPO) e liberação de peptídeos antimicrobianos (CERENIUS et al. 2010; CERENIUS & SODERHÄLL, 2013). A diminuição nos níveis de hemócitos circulantes indica redução na resistência imunológica (JOHANSSON et al. 2000).

Indicadores de estresse também incluem o aumento das concentrações de glicose e lactato nos tecidos (APARICIO-SIMÓN et al., 2010; HUBERMAN, 2000; LORENZON et al., 2005). A regulação da glicose é controlada por retroalimentação negativa pelo hormônio hiperglicêmico (CHH) através da quebra do glicogênio intracelular. O CHH é um neuropeptídio membro de uma família de neuropeptídios hormonais que compreende o hormônio inibidor gonadal (GIH), hormônio inibidor do órgão mandibular (MO-IH) e o hormônio inibidor da muda (MIH). O CHH é sintetizado e secretado pelo complexo órgão-X/glândula do seio, localizado no pedúnculo ocular (HARTENSTEIN, 2006). Por um mecanismo de retroalimentação positiva, o lactato regula a liberação de CHH, promovendo a conversão do glicogênio em glicose via metabolismo anaeróbico de carboidratos. Estudos com *Palaemon elegans, Procambarus clarkii* e *Macrobrachium malcolmsonii* demonstraram que as aminas biogênicas, serotonina (5-HT), dopamina e noradrenalina atuam na modulação e liberação de neuropeptídeos e respostas ao estresse (LORENZON et al. 2004; KOMALI et al. 2005; ZOU et al. 2003).

A capacidade osmorregulatória, atividade da fenoloxidase, tempo de coagulação da hemolinfa e os metabólitos da hemolinfa (hemocianina, proteínas totais, lipídios totais, triglicerídeos e colesterol), também compõem variáveis fisiológicas utilizadas como marcadores de estresse (RODRÍGUEZ & MOULLAC, 2000). Além disso, o aumento no requerimento energético pode resultar em elevação na produção de compostos intermediários de oxigênio, desencadeando a ativação de compostos antioxidantes (HERMES-LIMA, 2004). Quando persistente, o estresse pode alcançar níveis crônicos e influenciar negativamente a

capacidade de tolerância ao estressor, inibindo o crescimento, frequência alimentar, parâmetros reprodutivos e supressão das respostas imunológicas (LE MOULLAC & HAFFNER, 2000; BELL & EGGLESTON, 2005). Nesse sentido, o conjunto de respostas ao estresse são classificadas como (i) primária (curto prazo): representada pelo aumento na liberação do HCC; (ii) secundária: consiste da mobilização das reservas de glicogênio e lactato para conversão em glicose; e terciária (longo prazo): caracterizada por prejuízos muitas vezes irreversíveis a saúde do animal (IWANA et al. 1999; FANJUL-MOLES, 2006).

Como observado em estudos conduzidos sob diferentes estímulos estressores a variação de temperatura influenciou na atividade da fenoloxidase e concentração de hemócitos para *Gammarus pulex* (LABAUDE et al. 2017), exposição a hipóxia por 6 h elevou os níveis de glicose e lactato no lagostim de água doce *Paranephrops zealandicus* (BROUGHTON et al. 2017). Por outro lado, fêmeas e machos do anfípoda *Gammarus roeseli* apresentaram diminuição nas atividades de locomoção e ventilação quando expostos a altas salinidades, porém as fêmeas demonstraram maior sensibilidade e perda iônica em relação aos machos (SORNOM et al. 2010). *Litopenaeus vannamei* submetidos a estresse por captura e confinamento por um período de quatro semanas exibiram diminuição nas concentrações de proteína, lipídios e triglicerídeos, mas os níveis de hemócitos e glicose não alteraram significativamente quando comparado aos camarões não estressados, indicando uma resposta de adaptação ao estressor (MERCIER et al. 2006). Portanto, diferentes variáveis metabólicas refletem respostas ao estresse, variando de acordo com a espécie e o estímulo.

Em invertebrados, práticas de manipulação seguindo protocolos de bem-estar animal visando a diminuição do estresse são muitas vezes negligenciadas devido à ausência de legislação específica e por serem considerados seres não sencientes (ELWOOD et al., 2012). A presença de dor nesse grupo ainda é um tema controverso por não haver sinais claros e definidos, como ocorre em mamíferos. A dor é definida como uma experiência traumática associada a danos teciduais que alteram o comportamento e estimulam reações de aprendizado, sendo formada por dois componentes: nocicepção e “dor” (ROSE, 2014; SNEDONN et al., 2015). A nocicepção é a habilidade de detecção de uma lesão corporal pelo animal. Já a “dor” corresponde ao estado emocional ou o “sofrimento” vivenciado (BROOM, 2007; SNEDDON et al., 2014; ELWOOD et al., 2019). No entanto, a capacidade de sentir dor é uma característica evolutiva dos animais, contribuindo para o valor adaptativo e sucesso das espécies, cuja resposta é distinta, variando de acordo com as características morfológicas de cada grupo (DAWKINS, 2012).

Estudos com diferentes espécies desse grupo definem alguns indicadores comportamentais para demonstrar respostas nociceptivas e de desconforto, tais como, comportamentos de “retirada e fuga”, capacidade de aprendizado, evasão, reações motoras de fricção, e autotomia, o que pode indicar potencial de processamento central da resposta pelo sistema nervoso (SN) e capacidade de uso da informação complexa consistentes com a experiência de dor (BATESON, 1991; SHERWIN, 2001; GENTLE, 2011). O SN dos crustáceos é formado por um duplo cordão nervoso ventral com uma série de gânglios dispostos ao longo do corpo, os quais variam em grau de complexidade (RUPPERT e BARNES, 2005). Também já foram identificados peptídeos opioides e seus receptores em alguns invertebrados, os quais são responsáveis pela redução ou eliminação do reflexo nociceptivo e respostas de escape (ELWOOD, 2012).

Recentemente, países como Suécia, Noruega e Nova Zelândia passaram a classificar moluscos cefalópodes e crustáceos decápodes como seres sencientes na legislação de proteção animal (PASSANTINO et al. 2021). No Brasil, existe a Lei Arouca (11794/2008) que regulamenta os procedimentos para o uso científico de animais na pesquisa através do Conselho Nacional de Experimentação Animal (CONCEA) por meio da Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) e da Comissão de Ética no Uso de Animais (CEUA), mas considera apenas vertebrados (CONCEA, 2016). Por outro lado, a Pontifícia Universidade Católica do Paraná (PUCPR) possui protocolos específicos para algumas espécies de invertebrados junto ao CEUA (FISCHER & ALMEIDA, 2012).

O atordoamento elétrico, resfriamento, borbulhamento de CO_2 e adição de cloreto de magnésio ($MgCl_2$) na água são propostos como ferramentas de bem-estar para diminuição do estresse e sofrimento dos crustáceos em procedimentos de manipulação e eutanásia (FREGIN & BICKMEYER, 2016). Entretanto, algumas dessas técnicas parecem não induzir realmente a um estado de anestesia por preservarem a comunicação entre as células nervosas e os centros geradores de ritmicidade, demonstrando processamento normal das informações sensoriais e geração de reflexos nociceptivos (TANG et al. 2010; MARDER, 2011; TANG et al. 2012). Dessa forma, considerando a importância ecologia e econômica dos crustáceos, bem como a responsabilidade cientifica dentro da experimentação animal, o emprego de substancias anestésicas pode dar suporte ao avanço de protocolos que minimizem o estresse e sofrimento nesse grupo.

1.3 PARÂMETROS BIOQUÍMICOS

Em organismos aeróbicos, cerca de 90% do oxigênio molecular (O_2) é consumido pelas mitocôndrias para produção de energia na forma de ATP (HERMES-LIMA, 2004). Em contrapartida, menos de 10% do O_2 é reduzido via cadeia transportadora de elétrons em elementos intermediários altamente reativos, como os radicais ânion superóxido ($O2^{\cdot}$), hidroxila ($^{\cdot}OH$) e peróxido de hidrogênio (H_2O_2) (HERMES-LIMA et al., 2015; SAMET & WAGES, 2018; SARANGARAJAN et al, 2017). Estes compostos reduzidos de O_2 ou espécies reativas de oxigênio (ROS – termo em inglês) são gerados normalmente durante as reações metabólicas, porém um desequilíbrio entre a produção e eliminação das ROS leva a uma perda transitória ou permanente da homeostase, resultando em danos oxidativos em biomoléculas, como proteínas, lipídios, DNA e RNA (DEL RIO et al., 2005; PAMPLONA & COSTANTINI, 2011; SAMET & WAGES, 2018). O aumento nas taxas de biomoléculas oxidadas, bem como, a perda da integridade estrutural e capacidade funcional das células, leva a um estado de estresse oxidativo, estando associado a danos teciduais progressivos e desenvolvimento de doenças.

O conceito de estresse oxidativo também é definido como uma condição de regulação e interrupção da sinalização e controle redox (JONES, 2006). Os animais possuem um sistema de defesa antioxidante complexo responsável por neutralizar, interceptar ou eliminar as ROS (SARANGARAJAN et al., 2017). Esse sistema antioxidante pode ser dividido em dois grupos, o primeiro é constituindo por enzimas de baixo peso molecular como as vitaminas A, C e E, provenientes de fontes exógenas, tal como plantas e alimentos. O segundo grupo é formado por enzimas de alto peso molecular incluindo a catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx) e glutationa reduzida (GSH) (PAMPLONA & COSTANTINI, 2011; HALLIWELL & GUTTERIDGE, 2015). As variações nas concentrações dos antioxidantes são utilizadas como biomarcadores de estresse, sendo divididos em marcadores de exposição, de efeitos ou de suscetibilidade.

Inicialmente, os antioxidantes enzimáticos protegem o organismo através da dismutação do radical hidroxila ($O2^{\cdot -}$) em compostos menos reativos como o H_2O_2, que é posteriormente degradado em O_2 e H_2O. Assim, as defesas antioxidantes dos organismos podem ser quantificadas através da determinação da capacidade antioxidante total contra os radicais peroxil (AMADO et al. 2009). Além disso, o equilíbrio entre GSH e GSSG está associado à manutenção dos mecanismos de sinalização redox nas células (HUBER et al. 2008; BABA & BHATNAGAR, 2018). O tripeptídeo GSH é um tiol não proteico formado por aminoácidos de glicina, glutamato e cisteína, tendo um papel central na redução de peróxidos e manutenção do estado reduzido dos tióis proteicos (HELLOU et al. 2009). O mecanismo de detoxificação pela

GSH envolve sua oxidação em GSSG via doação de uma molécula de hidrogênio ou pela formação de um conjugado catalisado por GST. O GSSG é novamente reduzido a GSH pela enzima glutationa redutase (GR) (SAMET & WAGES, 2018). O equilíbrio entre GSH/GSSG fornece evidências de estresse oxidativo, pois o aumento na disponibilidade de GSH indica proteção contra reações oxidativas, enquanto a geração de GSSG é resultado das reações oxidativas (JONES, 2006).

A peroxidação lipídica ou lipoperoxidação (LPO) é um conjunto de reações geradas pela elevação dos níveis de ROS. Esse processo envolve a desestabilização da camada lipídica das membranas celulares devido à produção de hidroperóxidos a partir da quebra de ácidos graxos poliinsaturados (PUFAS) (OKPALA et al. 2016). Consequentemente, afeta a permeabilidade da membrana e o funcionamento dos receptores e proteínas de membrana envolvidos nas vias de transdução de sinal. Um dos produtos formados durante a LPO e o malondialdeído (MDA) que pode ser mensurado através do pela reação com o ácido tiobarbitúrico (TBA) (HERMES-LIMA et al. 2015). A oxidação das proteínas pelas ROS resulta em modificação de determinados aminoácidos gerando grupamentos carbonil, o qual é responsável pela perda da função fisiológica dessas moléculas.

1.4 ANESTESIA E SEDAÇÃO EM CRUSTÁCEOS

Anestesia é definida como um processo de indução a um estado reversível de analgesia somado a ausência de responsividade, perda dos reflexos musculares e redução do estresse fisiológico (ROSS & ROSS, 2008). A sedação resulta em efeitos calmantes sem alterar a atividade motora e a resposta varia de acordo com o fármaco. O mecanismo de ação dos anestésicos em crustáceos ainda é incerto. Os anestésicos parecem atuar inibindo a atividade neural e motora através do bloqueio dos canais voltagem-dependente de Na^+ e K^+ (BOWNIK, 2015). Outros mecanismos identificados como possivelmente relacionados a regulação de resposta anestésica em crustáceos, semelhante ao observado em vertebrados, são a presença de receptores opioides, neurotransmissores GABA e receptores metabotrópicos (SWANSON et al. 2005, DURANT et al. 2009, PERROT-MINNOT et al. 2017). Respostas anestésicas são variáveis de acordo com a concentração, tempo de exposição, fisiologia da espécie e parâmetros de qualidade d'água.

O aumento do uso de anestésicos em procedimentos com animais aquáticos tem como objetivo facilitar a manipulação, a fim de evitar injúrias e estresse (COYLE, 2004). Isso ocorre porque essas substâncias têm a capacidade de diminuir a atividade metabólica dos organismos, minimizando hiperatividade e respostas secundárias geradas pelo estresse (SNEDDON, 2012).

Assim, podem ser empregados para diminuir o sofrimento dos organismos na realização de biometrias, coleta de hemolinfa, ablação do pedúnculo ocular e no transporte (TAYLOR et al. 2004; AKBARI et al. 2010). Os anestésicos podem ser administrados com fins profiláticos ou terapêuticos, diretamente na água ou como aditivos na ração (NEIFFER & STAMPER, 2009; LEWBART & MOSLEY, 2012).

Um anestésico ideal deve garantir que a indução à anestesia, bem como a recuperação, ocorra de forma rápida, com concentrações que não ultrapassem a margem de segurança para a espécie (SOUZA et al. 2019). O custo, a facilidade na administração e a ausência de toxicidade também são características importantes na escolha do anestésico. A avaliação anestésica em crustáceos envolve a determinação dos tempos de indução aos estágios de (i) sedação (estágio 1), (ii) anestesia (estágio 2) e (iii) recuperação. O estágio de sedação inclui a perda parcial de equilíbrio e redução na atividade de natação, possuindo resposta a estímulos externos; o estágio de anestesia é reconhecido pela perda total do equilíbrio e ausência de reações a estímulos externos; a fase de recuperação é quando o animal retoma o equilíbrio e a posição vertical no aquário (PARODI et al. 2012). Em camarões anestesiados o movimento dos pleópodos e antenas não cessa, apenas reduz.

De modo geral, a maioria dos anestésicos sintéticos apresentam alto custo, baixa eficácia ou efeitos tóxicos em invertebrados (COYLE et al., 2005). MS-222 e 2-fenoxietanol não demonstraram efetividade em induzir anestesia, enquanto Aqui-S^{TM} resultou em significativas taxas de mortalidade para o camarão *Macrobrachium rosenbergii* (COYLE et al., 2005). Por outro lado, os óleos essenciais (OEs) e seus compostos isolados têm demonstrado serem eficazes e seguros em estudos com *Lippia alba* e *Aloysia triphylla* para *L. vannamei, Valeriana officinalis* para *Macrobrachium tenellum* e o óleo de cravo para os camarões *M. rosenbergii* e *Penaeus semisulcatus* (SOLTANI et al., 2004; COYLE et al., 2005; PARODI et al., 2012; PALOMERA et al., 2016).

1.5 ÓLEOS ESSENCIAIS

O uso de plantas com fins terapêuticos ou medicinais é uma prática empregada há milhares de anos pelas populações ao redor do mundo. Atualmente, cerca de 30% dos fármacos são derivados de fontes vegetais (MACIEL et al. 2002). O Brasil é considerado um dos países com a maior biodiversidade de plantas do mundo, apresentando em média 4680 espécies de algas, 1.519 espécies de briófitas, 30 espécies de gimnospermas e 32.715 espécies de angiospermas, entre outros (ZAPPI, 2015; ULLOA et al. 2017). O maior conhecimento sobre a composição, princípios ativos e mecanismos biológicos das plantas intensificou o interesse

na sua utilização em diferentes áreas da indústria e pesquisa animal. Neste contexto, diversos estudos destacam o potencial farmacológico dos OEs na melhora das respostas metabólicas e antioxidantes dos animais aquáticos durante manejo, suplementação alimentar e tratamento profilático (ROSS & ROSS, 2008).

Os OEs são substâncias voláteis derivadas de metabólitos secundários extraídos de diferentes partes das plantas (flores, frutos, raízes, caule e folhas). Os principais grupos de metabólitos secundários são os terpenoides, compostos fenólicos e alcaloides, cuja principal função é a defesa e atração de polinizadores para as plantas (EDRIS, 2007). A distribuição desses compostos nas plantas é influenciada por fatores genéticos e abióticos, como temperatura, luz, água, nutrientes, tipo de solo, entre outros (GOBBO-NETO & LOPES, 2007). Devido suas propriedades aromáticas são amplamente utilizados na produção de perfumes, cosméticos e produtos de limpeza, além do uso como conservante para bebidas e alimentos (MORAIS, 2009).

Assim, essas misturas complexas são formadas por dezenas de compostos em diferentes concentrações que são definidos como: (i) compostos majoritários (20 - 95%), (ii) compostos secundários (1 – 20%) e (iii) elementos traços (menos de 1%) (STICHER, 2015). A ação farmacológica dos OEs ocorre pelas interações entres os diferentes constituintes resultando em efeitos aditivos, sinérgicos ou antagônicos (HELDWEIN et al., 2014). Portanto, OEs podem apresentar propriedades sedativas, anestésicas, antioxidantes, antimicrobianas, imunoestimulantes e anti-inflamatórias, sendo capazes de atuar em diferentes mecanismos de ação nos animais vertebrados e invertebrados (AYDIN & BARBAS, 2020).

Os compostos de origem vegetal têm contribuído para a diminuição do estresse em crustáceos durante a manipulação experimental em laboratório ou em procedimentos aquícolas (BAKKALI et al., 2008; AKBARI et al., 2010). Quando comparado a substâncias sintéticas, os OEs demonstram importantes vantagens por serem biodegradáveis e de baixa toxicidade, podendo ser administrados diretamente na água até mesmo em baixas concentrações (COYLE, 2005; SOUZA et al. 2019). Muitas plantas apresentam antioxidante naturais que ajudam na proteção e manutenção da integridade das suas membranas celulares. A composição química tem influência sobre o potencial antioxidante do OEs. No geral, compostos fenólicos apresentam maior capacidade antioxidante comparado a maioria dos monoterpenos (ZAMORA & HIDALGO, 2016). Entretanto, a interação entre esses constituintes é a responsável pela geração da atividade final do óleo.

1.5.1 Óleo essencial de *Cymbopogon citratus* (DC) Stapf

O *Cymbopogon citratus*, também denominado como “capim-limão” ou “capim-cidreira”, é uma planta herbácea pertencente à família Poaceae. Essa espécie vegetal é originária da Índia, porém se estabelece bem em regiões tropicais e subtropicais, com destaque para cultivos nos estados do Sul e Sudeste do Brasil (MARTINAZZO et al., 2007). É amplamente consumido na forma de chás, em razão das suas ações calmante, espasmolítica e analgésica (TAVARES-DIAS, 2018). Foram observadas propriedades anestésicas deste OE para o camarão *M. rosenbergii* (ADNAN et al. 2021) e duas espécies de peixes ornamentais, *Sciaenochromis fryeri* e *Labidochromis caeruleus* (KIZAK et al., 2018) (Figura 4A).

1.5.2 Óleo essencial de *Ocimum gratissimum* L.

O gênero *Ocimum* (Lamiaceae) compreende um grupo de plantas herbáceas aromáticas que se distribuem em áreas tropicais da África, América e Ásia. A espécie *O. gratissimum* é popularmente conhecida como “alfavaca” ou “manjericão”, sendo tradicionalmente utilizada como condimento e sedativo para o tratamento de dores (LORENZI & MATOS, 2002). Esse OE foi eficiente como anestésico para o matrinxã (*Brycon amazonicus*) (RIBEIRO et al. 2016) e aumentou a proteção antioxidante do pacamã (*Lophiosilurus alexandri)* quando transportado por 4 horas (BOAVENTURA et al. 2021). Além disso, a capacidade antibacteriana e antiparasitária desse OE sugere a sua utilização como substância terapêutica para prevenção e tratamento contra patógenos em sistemas aquícolas (BOIJINK et al. 2016; BANDEIRA JR et al. 2017) (Figura 4B).

1.5.3 Óleo essencial de *Origanum majorana* L.

Origanum majorana (Lamiaceae), conhecida no Brasil pelo nome comum de “manjerona”, é uma espécie rica em compostos bioativos, tal como o linalol, ɣ-terpinol, terpinen-4-ol e sabineno. É uma erva de pequeno porte com folhas aromáticas e perenes (VASUDEVA, 2015). Assim como descrito para outros OEs, essa planta é tradicionalmente utilizada como condimento ou consumida na forma de chás para tratamento de problemas gastrintestinais, tonturas e dores de cabeça (DESHMANE, 2007). Cunha et al. (2017) observaram efeito sedativo e diminuição na perda iônica em jundiás (*Rhamdia quelen*) expostos por 8 h ao OE de *O. majorana* (Figura 4C).

1.5.4 Óleo essencial de *Aloysia triphylla* (L'Hér.) Britton

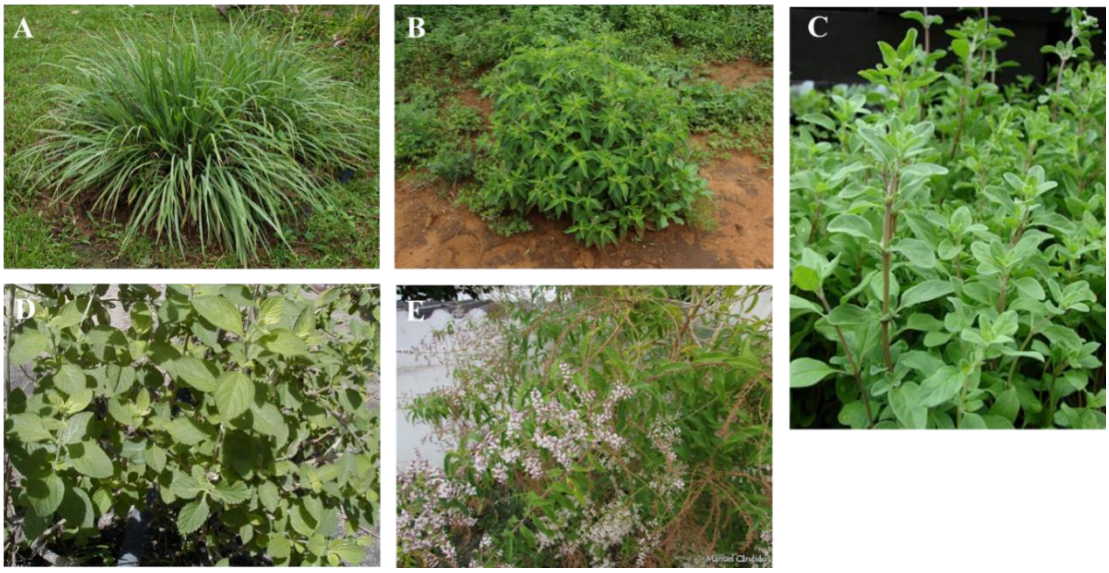
A planta *A. triphylla* (Verbenaceae) ocorre de forma endêmica pela América do Sul, com maior distribuição na região da Argentina. É caracterizada como um arbusto de folhas perenes, bastante ramificado e podendo atingir até 3 metros de altura. Entre os seus nomes populares estão cidrão, cidró, cidró-pessegueiro, cedrina, erva-luisa e cidrozinho. Esse OE já vem sendo bastante utilizado em estudos com peixes devido as suas propriedades analgésicas, anti-inflamatórias, sedativas, antioxidantes e digestivas (PARODI et al. 2016; TEIXEIRA et al. 2016; BECKER et al. 2017; ALMEIDA et al. 2019; DE SOUZA et al. 2020). Recentemente, Parodi et al. (2020) descreveu em seu trabalho que a época do ano tem influência direta sobre o rendimento e o efeito anestésico desse óleo. Pesquisas com os camarões *L. vannamei* e *M. rosenbergii* demonstraram o potencial anestésico e antioxidante da *A. triphylla* and *L. alba* para essas espécies (PARODI et al. 2012*;* CAGOL et al. 2020) (Figura 4D).

1.5.5 Óleo essencial de *Lippia alba* (Mill.) N.E.Brown

A espécie *Lippia alba* (Verbenaceae) é um subarbusto aromático comumente conhecido pelos nomes de "erva cidreira" ou "falsa melissa". Sua distribuição ocorre por todas as regiões do Brasil, assim como ao longo da América Central e do Sul. A composição do OE de *L. alba* é bastante variável e depende do ambiente de cultivo, condições climáticas e período da coleta. Essa espécie vegetal pode apresentar diferentes quimiotipos com atividade farmacológica distinta e que se diferenciam pela predominância de constituintes específicos, entre eles o linalol, citral, carvona, mirceno, limoneno, 8-cineol, cânfora e ɣ-terpinol (TONI et al. 2014; SOUZA et al. 2017).

Estudos anteriores demonstraram o efeito anestésico e sedativo desse OE para o camarão branco *L. vannamei* (PARODI et al. 2014), para o tambaqui *Colossoma macropomum* (DOS SANTOS BATISTA, 2018) e para o jundiá (SUTILLI et al. 2015; BECKER et al. 2012, 2016). Em experimentos de transporte o OE de *L. alba* preveniu o aumento dos níveis de cortisol no tambacu (*Piaractus mesopotamicus* x *Colossoma macropomum*) (SENA et al. 2016) e reduziu a atividade de natação da piranha-preta, *Serrasalmus rhombeus* (ALMEIDA et al. 2018). Quando adicionado à dieta melhorou o estado oxidativo do camarão gigante *M. rosenbergii* (CAGOL et al. 2020) e a resposta imunomoduladora da tilápia do Nilo *Oreochromis niloticus* (RODRIGUES-SOARES et al. 2018) (Figura 4E).

Figura 2- Exemplares de *Lippia alba* (A); *Aloysia triphylla* (B); *Ocimum gratissimum* (C); *Cymbopogon citratus* (D); *e Origanum majorana* (E).

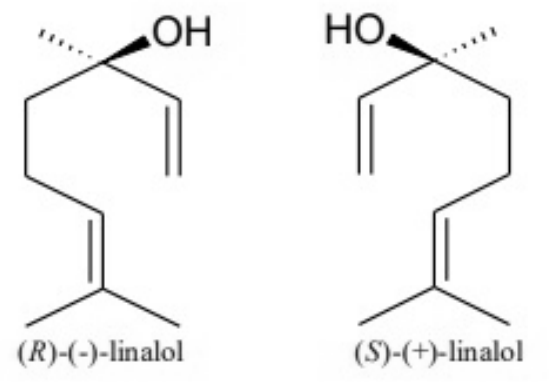


Fonte: Universidade Federal do Ceará - Campus do PICI; Miltra-Nature; PPMAC - Portal de Plantas Medicinais, Aromáticas e Condimentares; Royal Botanic Garden Kew;

1.5.6 Linalol ($C_{10}H_{18}O$)

O linalol é um monoterpeno presente como constituinte majoritário ou secundário de vários OEs, incluindo os de *L. alba* e *A. triphylla* (HELDWEIN et al., 2014). Este composto ocorre naturalmente em duas formas isoméricas ou enantiômeros, de acordo com a quiralidade do terceiro carbono, caracterizando-se na forma levógira (3R - (-) - linalol ou licareol) e na forma dextrógira (3S - (+) - linalol ou coriandrol) (Figura 3) e ainda na sua forma racêmica (SUGAWARA et al. 1998, DE SOUSA et al., 2010). De acordo com estudos, S - (+) - e R - (-) - linalol apresentaram diferenças biológicas (HUTT & O'GRADY, 1996; MITRA & CHOPRA, 2011). Contudo, não foram observadas diferenças nos tempos de indução ao estágio de anestesia para jundiás expostos a ambos os isômeros (SILVA et al., 2017), bem como para o tambaqui e a carpa comum (*Cyprinus carpio*) submetidos a uma mistura racêmica de linalool (MIRGHAED et al., 2016; BALDISSEROTTO et al., 2018). Em adição, o linalol possui mecanismos de ação envolvendo receptores opioides, muscarínicos, dopaminérgicos e canais de K^+ (GUIMARÃES et al. 2013; SOUZA et al. 2018).

Figura 3 - Estruturas enantioméricas do linalol.

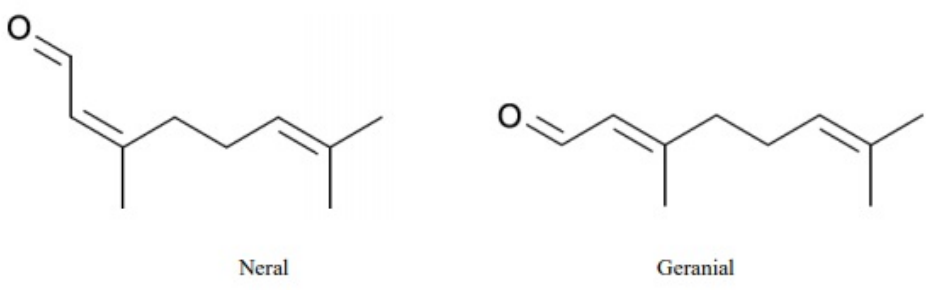


Fonte: Google imagens

1.5.7 Citral ($C_{10}H_{16}O$)

O citral é um monoterpeno volátil encontrado como componente de diversos OEs extraídos de plantas, tal como *C. citratus*, *A. triphylla*, *L. alba*, entre outros. Compreende uma mistura de isômeros neral (*Z*-citral e α-citral) e geranial (*E*-citral e β-citral) (Figura 4) da 3,7-dimetil-2,6-octadienal (MODAK & MUKHOPADHAYA, 2011). Estudos *in vitro* indicaram atividade antioxidante do citral contra radicais peroxila (RABBANNI et al 2006; BASCHIERI et al. 2017). Também tem sido considerado como conservante alimentar devido a sua capacidade de inibição na formação de bactérias (ZHANG et al 2014). Outros estudos avaliaram as propriedades anestésicas e ansiolíticas de OEs compostos majoritariamente pelo citral, encontrando resultados positivos para seu uso em espécies aquáticas (DANIEL et al. 2014; SOUZA et al. 2017; KIZAK et al. 2018; BANDEIRA JR et al. 2018).

Figura 4 - Estrutura química do neral (Z-citral e α-citral) e geranial (E-citral e β-citral), formas isômeras do citral.



Fonte: Google imagens

1.6 CARACTERIZAÇÃO DAS ESPÉCIES ESTUDADAS

1.6.1 *Hyalella bonariensis* Smith, 1874

O gênero *Hyalella* (família Hyalellidae, ordem Amphipoda) inclui cerca de 66 espécies descritas e com distribuição desde a Patagônia até o Canadá (BENTO & BUCKUP, 1999). Compreendem organismos com tamanho corporal entre 0,5 mm a 2 cm. Esse grupo geralmente vive em ambiente dulcícola junto ao substrato e associado a algas ou sedimentos em riachos, lagos ou águas subterrâneas, porém podemos encontrar algumas espécies marinhas (BUENO et al., 2014). Ainda podem ser do tipo endobentônicos, que se enterram no substrato, e os epibentônicos, vivem sobre o substrato. Diferente de outros crustáceos, esse grupo é caracterizado pela ausência de carapaça, corpo comprimido lateralmente e três pares de urópodes (BUENO et al. 2014).

Os espécimes de *H. bonariensis* apresentam dismorfismo sexual caracterizado pelo segundo par de gnatópodos ampliados nos machos e ocorrência de marsúpio nas fêmeas. Os machos geralmente são maiores em tamanho corporal e também é comum a presença de comportamentos pré – copulatórios e confrontos, no qual os machos carregam as fêmeas em sua superfície ventral como forma de proteção até que atinjam seu curto período sexual (CASTIGLIONI, et al. 2016). Os anfípodes são consumidores primários importantes dentro das cadeias tróficas aquáticas, servindo de alimento para aves, peixes e macroinvertebrados.

São organismos considerados excelentes bioindicadores para estudos laboratoriais toxicológicos por possuírem alta sensibilidade a contaminação ambiental, abundância populacional, ciclo de vida curto, desenvolvimento direto, fácil amostragem em campo e simples manutenção em laboratório (DUAN et al., 1997; NEUPARTH, et al., 2002; CASTIGLIONI & BOND-BUCKUP, 2007; DING et al., 2011). Adicionalmente, o conhecimento sobre a biologia e ecologia das espécies é essencial para o desenvolvimento de pesquisas comportamentais em laboratório. Estudos com os anfípodos *Gammarus minus* e *Gammarus pulex* demonstraram o efeito anestésico do óleo de cravo e do MS-222, respectivamente (AHMAD, 1969; VENARSKY, 2006). A utilização de anestésicos em pequenos invertebrados demonstra ser uma promissora ferramenta para estudos *in vivo* que necessitem imobilização durante curtos períodos de tempo, como para analises microscópicas, na aplicação de sensores em avaliações fisiológicas e na manipulação de espécies no ambiente natural. Trabalhos com a *H. bonariensis* ainda são recentes, não havendo pesquisas que envolvendo o emprego de substâncias anestésicas. Dessa forma, estudos com novas espécies contribuem para o maior conhecimento sobre a biologia e o modo de vida.

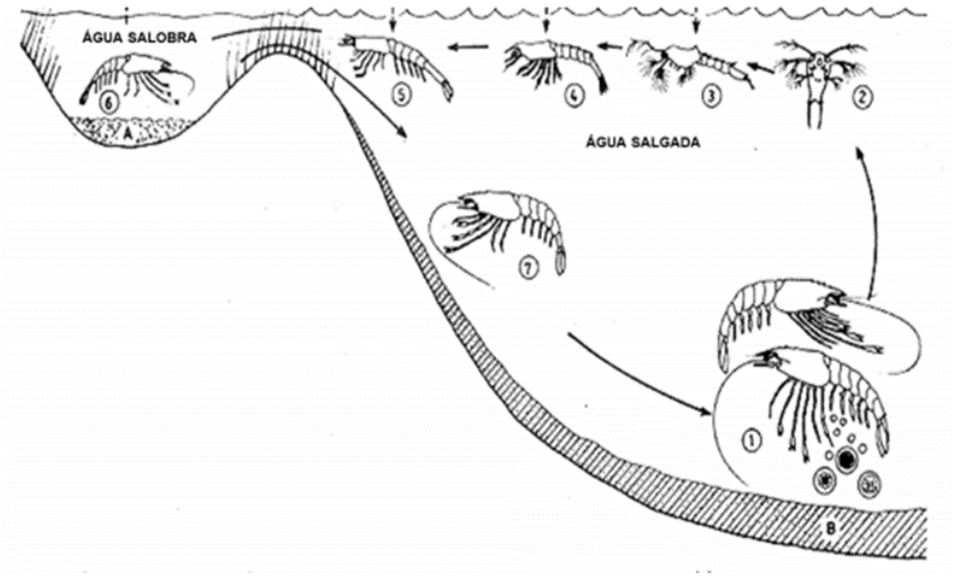
1.6.2 Crustáceos decápodes

A ordem Decapoda contém quase um terço das espécies conhecidas dos crustáceos e inclui animais como lagostas, lagostins, siris caranguejos e camarões. Dentro desse grupo, a família Penaeidae (Rafinesque, 1815) é constituída por 33 gêneros e cerca de 372 espécies e subespécies descritas até o momento. Os camarões peneídeos formam um grupo diversificado de camarões marinhos que se destacam por sua ampla distribuição geográfica, principalmente em áreas tropicais e subtropicais. Esses crustáceos caracterizam – se por possuírem carapaça de quitina, três pares de maxilípedes e cinco pares de pereópodes (BRUSCA e BRUSCA, 2007; RUPPERT e BARNES, 2016).

O ciclo de vida dos camarões peneídeos é marcado por constantes migrações entre os domínios oceânicos de acordo com o estágio de crescimento. As fases larvais envolvem distintas etapas de desenvolvimento morfológico (náuplio, zoea, misis e pós-larvas) (Figura 5), marcado por períodos planctônicos-pelágicos passando para bentônicos-demersais (HOLTHUIS, 1980). Durante a fase de crescimento as pós-larvas migram para regiões de estuário, onde encontram condições de assentamento favoráveis e maior disponibilidade de alimento, até que atinjam a fase juvenil. Os sub-adultos vivem e se reproduzem em mar aberto, completando assim, o ciclo de vida. Por serem animais eurialinos apresentam grande tolerância a amplas variações de salinidade (2-40) (DALL, 1990).

Os camarões desempenham importante papel como predadores ou presas dentro da macrofauna bentônica (MANZONI e D'INCAO, 2007). Além da importância ecológica, muitas espécies de camarões possuem elevado potencial econômico e social. Dentre essas espécies podemos citar o *Farfantepenaeus paulensis*, *Litopenaeus schmitti*, *Farfantepenaeus brasiliensis*, *L. vannamei*, *Penaeus monodon* e *Xiphopenaues kroyer*, entre outros (BOOS et al. 2010). No entanto, algumas espécies de peneídeos nativos do litoral brasileiro se encontram sobreexplotadas ou ameaçadas. A diminuição desses estoques naturais tem relação direta com a sobrepesca, ação antropogênica e eventos climáticos que impedem a entrada das larvas em zonas estuarinas (D'INCAO et al., 2002; DIAS_NETO, 2011; IBAMA, 2011; PEREIRA & D'INCAO, 2012). Dessa forma, estudos ecológicos, fisiológicos e comportamentais são grandes aliados na preservação desse grupo e estimulam a produção com fins comerciais ou para repovoamento (BARRON e ROBINSON, 2008).

Figura 5 - Ciclo de vida dos camarões do gênero *Penaeus* (Penaeidae). A: estuários, mangues e lagunas. B: plataforma, oceano. 1- os adultos se reproduzem no oceano, 2- naúplius, 3- protozoea, 4- mysis, 5- os decapoditos entram nos estuários, 6 – juvenis;



Fonte: BOSCHI, (1974).

1.6.2.1 Farfantepenaeus paulensis (Pérez Farfante, 1967)

Popularmente conhecido como camarão rosa, o *F. paulensis* (Figura 6) distribui-se desde o litoral de Ilhéus (Bahia) até Mar del Plata, na Argentina (D'INCAO et al. 2002). Possuem uma ampla faixa de tolerância de temperatura (13-36 °C) (MARCHIORI, 1984). Porém reduzem a atividade de natação e alimentação em períodos com temperaturas abaixo de 15°C, permanecendo mais tempo enterrados no substrato (DALL et al. 1990). Em laboratório, as maiores taxas de crescimento foram observadas em faixas de temperatura entre 29 e 32 °C (SOARES, et al. 2012). O camarão rosa atinge a primeira maturação quando os espécimes chegam a 15,0 cm de comprimento total (D'INCAO et al. 1995). A reprodução é considerada anual, apresentando picos reprodutivos nos meses de outono e primavera ao longo da costa de Santa Catarina em direção ao norte do país (COSTA et al. 2008).

É considerado um importante recurso pesqueiro cuja captura ocorre entre o verão e o início do outono na Lagoa dos Patos, ao sul do Rio Grande do Sul (D'INCAO et al. 1991). O estuário da Lagoa dos Patos constitui um importante ambiente de crescimento para pós-larvas e juvenis dessa espécie. Em vista disso, diversas pesquisas já foram realizadas nesse estuário visando a implementação de sistemas de criação gaiolas e cercados para o camarão *F. paulensis*, principalmente, como forma de renda adicional aos pescadores artesanais e fortalecimento das

tecnologias de produção para essa espécie (WASIELESKY et al. 2001; WASIELESKY et al. 2018; KRUMMENAUER et al. 2006; PRETO et al. 2009). A criação do camarão rosa em cativeiro poderia diminuir a pressão sobre os estoques naturais, estimulando o crescimento das populações, além de permitir maior conhecimento sobre a biologia reprodutiva e comportamental da espécie.

Figura 6 - Camarão rosa, *Farfantepenaeus paulensis*.

Fonte: FAO (2021)

1.6.2.2 Litopenaeus vannamei (Boone, 1931)

O *L. vannamei* (Figura 7) é uma espécie de ambiente marinho e clima tropical com distribuição ao longo da costa do Pacífico, ocorrendo desde o Golfo da Califórnia até o sul do Peru onde as águas apresentam temperaturas acima de 20°C, sendo uma das principais espécies de camarão criada em cativeiro no mundo (FAO, 2020). São comumente chamados de "camarão branco do Pacífico" ou "camarão cinza". De característica bentônica vivem junto ao substrato e apresentam o hábito de enterramento ou escavação. Indivíduos sub-adultos podem ser encontrados em profundidades de até 72 m (DALL, 1990). Estudos demonstram que são ativos tanto durante o dia quanto a noite (PONTES, 2006).

No início da década de 90, o camarão branco foi introduzido no Brasil com fins comerciais devido ao insucesso na criação de outras espécies de peneídeos nativos e exóticos (ROCHA, 2007). Desde então, se consolidou como o principal camarão marinho produzido no país. O sucesso do *L. vannamei* inclui suas características zootécnicas, fácil adaptabilidade, rápido crescimento e pacote tecnológico definido. O Nordeste é a região brasileira com as

melhores condições climáticas e topográficas para o camarão cultivado, com destaque para os estados do Rio Grande do Norte e o Ceará como maiores produtores (IBGE, 2019). A carcinicultura é um setor produtivo mundialmente estabelecido em termos socioeconômicos no suprimento de proteína animal e na capacidade de geração de empregos diretos e indiretos.

Nos últimos anos a carcinicultura mundial vem enfrentando crescentes desafios com o surgimento e disseminação de doenças infecciosas causadas por bactérias e vírus (LIGHTNER, 2011). Os microrganismos ocorrem de forma natural nos ambientes aquáticos estabelecendo relações simbióticas com outros organismos e promovendo a ciclagem de nutrientes. Entretanto, ambientes estressores associados a baixa qualidade d'agua e manejo incorreto propiciam situações favoráveis para patógenos oportunistas, pois diminuem as defesas imunológicas dos camarões. Entre as doenças que acometem os peneídeos destacam – se infecções causadas por bactérias do gênero *Vibrio* spp e o vírus da síndrome da mancha branca (WSSV, White Spot Syndrome Virus) (THITAMADEE et al., 2016). Além da mortalidade e perda de produtividade, a disseminação de patógenos provenientes de cultivos para espécies selvagens é outro problema a ser considerado.

Figura 7 - Camarão branco do Pacífico, *Litopenaeus vannamei.*

Fonte: Da autora.,

Frente a isso, é de extrema importância o estabelecimento de medidas profiláticas que reduzam os impactos causados pelas enfermidades e melhorem a resistência dos animais. Assim, surgem estratégias como a otimização dos sistemas, tal qual o sistema superintensivo em bioflocos (BioFloc Technology ou BFT), aprimoramento de ferramentas biotecnológicas, aplicação de probióticos ou compostos derivados de plantas e protocolos de bem-estar durante a manipulação dos camarões (citação).

1.7 ESTRUTURA DA TESE

O presente trabalho buscou avaliar o potencial anestésico de diferentes OEs em distintas espécies de crustáceos. Em um primeiro momento essa tese aborda o efeito anestésico dos OEs de *L. alba* (EOLA) e *O. gratissimum* (EOOG) para o camarão rosa *F. paulensis*, e EOs de *O. majorana* (EOO) e *C. citratus* (EOC) para o camarão branco *L. vannamei*. Com base nos resultados encontrados no primeiro trabalho foram realizados testes preliminares para definir concentrações ideias para experimentos de longa exposição. Dessa forma foram avaliados os efeitos das concentrações de 5 e 10 μL L^{-1} de OEC sobre parâmetros comportamentais e bioquímicos do *L. vannamei* durante um período de 6 h. Posteriormente, foi investigado o potencial do OE de *L. alba* e do linalol na redução do estresse e danos oxidativos em fêmeas e machos do camarão *L. vannamei* submetidos a procedimentos de ablação do pedúnculo ocular e extrusão do espermatóforo, respectivamente. Por fim, foram realizados trabalhos para investigar a influência dos OEs de *A. triphylla* e *L. alba,* e seus compostos majoritários citral e linalool, na anestesia e comportamento do anfípoda *H. bonariensis* utilizando o software comportamental ANY-maze®. Assim, a presente tese está organizada na forma de manuscritos, os quais estão estruturados de acordo com as normas das revistas a que foram submetidos ou publicados e está dividida em quatro manuscritos, a saber:

Manuscrito I. *Potencial anestésico de diferentes óleos essenciais para duas espécies de camarões, Farfantepenaeus paulensis e Litopenaeus vannamei (Decapoda, Crustacea).*

Manuscrito II. *Respostas comportamentais e bioquímicas do camarão branco do Pacífico, Litopenaeus vannamei, exposto ao óleo essencial de Cymbopogon citratus.*

Manuscrito III. *Determination of the oxidative effect of linalool and the essential oil of Lippia alba during ablation eyestalk and spermatophore extrusion in Litopenaeus vannamei*

Manuscrito IV. *Exposure of Hyalella bonariensis (crustacea, amphipoda) to essential oils: Effects on anesthesia and swimming activity.*

2. OBJETIVOS

2.1 OBJETIVO GERAL

- Avaliar o uso de diferentes óleos essenciais, bem como os seus compostos majoritários, como sedativos e anestésicos para crustáceos e suas implicações na capacidade antioxidante e comportamental desses organismos.

2.2. OBJETIVOS ESPECÍFICOS

- Avaliar a eficácia anestésica na indução e recuperação dos OEs de *L. alba* e *O. gratissimum* para o camarão rosa *F. paulensis*, e OEs de *O.majorana* (EOO) e *C. citratus* (EOC) para o camarão branco *L. vannamei*.
- Verificar o efeito da exposição continua do OE de *C. citratus* em parâmetros comportamentais (natação, evasão, postura e imobilidade) e bioquímicos (ACAP, TBARS, GSH, PSH) para o camarão *L. vannamei*.
- Analisar o potencial antioxidante do OE de *L. alba*, e seu maior composto linalol, em fêmeas e machos do *L. vannamei* anestesiados durante procedimentos de ablação do pedúnculo ocular e extrusão do espermatóforo, respectivamente, através de analises bioquímicas de estresse oxidativo.
- Determinar os tempos de indução a anestesia e recuperação dos OEs de *Aloysia triphylla* e *Lippia alba* e seus compostos majoritários citral e linalool sobre o comportamento da *H. bonariensis*

3. DESENVOLVIMENTO

3.1 MANUSCRITO 1

ANESTHETIC POTENTIAL OF DIFFERENT ESSENTIAL OILS FOR TWO SHRIMP SPECIES, *FARFANTEPENAEUS PAULENSIS* AND *LITOPENAEUS VANNAMEI* (DECAPODA, CRUSTACEA)

POTENCIAL ANESTÉSICO DE DIFERENTES ÓLEOS ESSENCIAIS PARA DUAS ESPÉCIES DE CAMARÕES, *FARFANTEPENAEUS PAULENSIS* E *LITOPENAEUS VANNAMEI* (DECAPODA, CRUSTACEA)

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ANIMAL PRODUCTION

Anesthetic potential of different essential oils for two shrimp species, *Farfantepenaeus paulensis* and *Litopenaeus vannamei* (Decapoda, Crustacea)

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ABSTRACT: *The use of anesthetics in aquaculture ensures better animal welfare and survival during transport and the production cycle. The present study evaluated the anesthetic efficacy of essential oils (EOs) of Lippia alba (EOLA) and Ocimum gratissimum (EOOG) for pink shrimp Farfantepenaeus paulensis, and EOs of Origanum majorana (EOO) and Cymbopogon citratus (EOC) for white shrimp Litopenaeus vannamei. shrimp were exposed to (i) 500, 750 or 1000 µL L^{-1} of EOLA or (ii) 50, 100, 150 or 250 µL L^{-1} of EOOG, and L. vannamei were exposed to (iii) 150, 300 or 500 µL L^{-1} of EOC or (iv) 400 or 800 µL L^{-1} of EOO. The induction times were concentration dependent, with a decrease in induction time with the increase of the EOs concentration, but for EOLA, this pattern was observed only for anesthesia. Induction times for sedation and anesthesia were significantly shorter for shrimp exposed to EOC and EOO. The highest concentration of EOOG (250 µL L^{-1}) resulted in 30% mortality. The recovery time was significantly longer for shrimp exposed to 800 µL L^{-1} of EOO compared to the other EOs. Overall, the action of EOs significantly differed between the two shrimp species. In conclusion, the tested EOs effectively anesthetized F. paulensis and L. vannamei.*

Key words: *anesthesia; crustacean; handling; sedation; natural products.*

Potencial anestésico de diferentes óleos essenciais para duas espécies de camarões, *Farfantepenaeus paulensis* e *Litopenaeus vannamei* (Decapoda, Crustacea)

RESUMO: *O uso de anestésicos em procedimentos aquícolas pode garantir melhor bem-estar e sobrevivência dos animais durante o transporte e ciclo de produção. O presente estudo teve como objetivo avaliar a eficácia anestésica dos óleos essenciais (OEs) de Lippia alba (EOLA) e Ocimum gratissimum (EOOG) para o camarão rosa Farfantepenaeus paulensis, e Origanum majorana (EOO) e Cymbopogon citratus (EOC) para o camarão branco Litopenaeus vannamei. Os camarões foram expostos à: (i) 500, 750 ou 1000 µL L^{-1} EOLA e (ii) 50, 100, 150 ou 250 µL L^{-1} de EOOG para F. paulensis, e (iii) 150, 300 ou 500 µL L^{-1} EOC, e (iv) 400 ou 800 µL L^{-1} de EOO para L. vannamei. Os tempos de indução foram dependentes da concentração. Houve diminuição do tempo de indução, mas para EOLA esse padrão foi observado apenas na anestesia. Os tempos de indução para sedação e anestesia foram significativamente mais rápidos para os grupos EOC e EOO. A concentração de 250 µL L^{-1} de EOOG resultou em 30% de mortalidade. O tempo de recuperação foi significativamente maior a 800 µL L^{-1} de EOO em comparação aos outros OEs. No geral, a ação dos OEs foi significativamente diferente entre as duas espécies de camarões. Em conclusão, ambos os OEs anestesiaram efetivamente o F. paulensis e L. vannamei.*

Palavras-chave: *anestesia; crustáceos; manejo; sedação; produtos naturais; bem-estar.*

INTRODUCTION

Shrimp represent the main group of farmed crustaceans (FAO, 2020). However, the intensification of shrimp farming faces various challenges, such as the increased spread of disease due to incorrect management, which may result in stress and injury (LIGHTNER, 2011). These events lead to the overuse of a variety of antibiotics and antimicrobials, which can accumulate in the cultured shrimp and negatively affect the aquatic environment (ALDERMAN et al., 1998).

Stressful conditions are known to alter the behavior and physiology of crustaceans, compromising their immunity and producing damage at the cellular level (MERCIER et al., 2006). Anesthetics are used in shrimp farming to facilitate handling during certain practices, such as weighing, measuring, tagging, eye ablation, collection of tissue samples and transportation of post-larvae or adults

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to spawning sites or cultured ponds (TAYLOR et al., 2004; AKBARI et al., 2010). Essential oils (EOs) are natural compounds derived from secondary plant metabolites, some of which exhibit sedative and anesthetic properties (SOUZA et al., 2019). Furthermore, because they are natural products, the application of EOs may be more environmentally friendly and cost-effective (REVERTER et al., 2014). Essential oils have been demonstrated to be safe and effective in studies on *Litopenaeus vannamei* (PARODI et al., 2012), *Macrobrachium rosenbergii* (SAYDMOHAMED & PAL, 2009), *Palaemonetes sinensis* (LI et al., 2018a) and *Penaeus monodon* (JIANG et al., 2020).

The Pacific white shrimp, *L. vannamei*, is distributed along the Pacific coast from western Mexico to northern Peru and is considered one of the primary shrimp species produced worldwide (FAO, 2020). In contrast, *Farfantepenaeus paulensis*, known as pink shrimp, is a native marine species distributed from Ilhéus (Brazil) to Mar del Plata (Argentina), which is considered an alternative species for aquaculture in southern and southeastern Brazil (PRETO et al., 2009). To our knowledge, the effect of EOs on pink shrimp has not yet been investigated. Previous studies have demonstrated an anesthetic effect of the EO of *Lippia alba* (EOLA) on *L. vannamei* shrimp (PARODI et al., 2012). Therefore, this study evaluated the potential anesthetic effects of different EOs on two species of penaeid shrimp. We compared the anesthetic and sedative efficacy of EOLA and the EO of *Ocimum gratissimum* (EOOG) on pink shrimp (*F. paulensis*), as well as the efficacy of the EOs of *Origanum majorana* (EOO) and *Cymbopogon citratus* (EOC) on white shrimp (*L. vannamei*).

MATERIALS AND METHODS

Shrimp collection, maintenance and water quality analyses

Farfantepenaeus paulensis (5.96 ± 0.13 g, 9.18 ± 0.32 cm) and *L. vannamei* (22.6 ± 1.2 g, 21.7 ± 0.3 cm) shrimp were obtained from the Marine Station of Aquaculture, Universidade Federal do Rio Grande (FURG), Southern Brazil. The animals were maintained in continuously aerated 250-L tanks filled with seawater, with controlled water parameters. Water temperature (22.1 ± 0.02 °C) was measured using a mercury thermometer (precision ±0.5 °C) and dissolved oxygen (6.14 ± 0.01 mg L^{-1}) with a YSI oxygen meter (Handylab/OXI/set; Schott®, Cambridge, UK). Salinity (28.0 ± 0.2 ppt) was measured with an optical refractometer (RTS – 101; Atago® US, Bellevue, WA, USA) and pH (7.97 ± 0.03) with digital pH meter (Handylab 2 BNC; Schott®, Mainz, Germany). The alkalinity (146.2 ± 5.8 mg $CaCO_3$ L^{-1}) was determined according to BAUMGARTEN et al. (1996), and total ammonia nitrogen (TAN) levels (0.13 ± 0.01 mg L^{-1}) were calculated using the method published by UNESCO (1983).

Plant material and essential oil analysis

The EOs were obtained by steam distillation in a Clevenger apparatus according to the guidelines of the European Pharmacopoeia (2010). The extraction was performed for 2 h for EOLA and 3 h for EOOG. The samples were stored in amber glass vials at -4 °C. Essential oil analyses were carried out using gas chromatography coupled to mass spectrometry, according to SILVA et al. (2012). The EOs of *C. citratus* (EOC) and *O. majorana* (EOO) were purchased from Vimonitti – São Caetano Agroindustry (Santa Maria, Rio Grande do Sul, Brazil). The major components identified for EOLA were linalool (58.37%), 1,8-cineole (6.33%), germacrene D (4.47%) and β-caryophyllene (3.64%). The major compounds of EOOG were eugenol (73.6%), β-bisabolene (18.3%), β-caryophyllene oxide (4.8%) and spathulenol (1.4%). The EOC was mainly composed of citral (74.56%), mircene (11.85%) geraniol (3.08%), pulegone (2.37%) and linalool (1.56%), and EOO contained terpinen-4-ol (20.44%), cis-terpinene, (13.14%), cis-terpineol (12.67%), 2-carene (7.67%) and sabinene (6.96%).

Experimental design

Shrimp were randomly divided into groups and placed in 1-L aquaria for the evaluation of anesthetic activity. Each shrimp was assessed individually and used only once in each replicate at a defined concentration (n = 10 animals per concentration and per anesthetic). Shrimp were exposed to EOs at the following concentrations: (i) 500, 750 or 1000 µL L^{-1} of EOLA or (ii) 50, 100, 150, or 250 µL L^{-1} of EOOG for *F. paulensis*; and (iii) 150, 300 or 500 µL L^{-1} of EOC or (iv) 400 or 800 µL L^{-1} of EOO for *L. vannamei*. After exposure to the EOs, animals were transferred to anesthetic-free aquaria to determine their recovery time. EOs were dissolved in 100% ethanol (1:10). Shrimp of both species were exposed to the control treatment, i.e., aquaria containing anesthetic-free seawater. In addition, shrimp of both species were exposed to ethanol at the highest concentration used to dilute

the EOs (9000 and 7200 µL L^{-1} for *F. paulensis* and *L. vannamei*, respectively). The concentrations of EOs chosen for the biological assays were based on studies by PARODI et al. (2012) and SILVA et al. (2012). Furthermore, the behavioral patterns used to determine the induction time of sedation and anesthesia, as well as the recovery time from anesthesia, were based on descriptions by PARODI et al. (2012) (Table 1). The shrimp were considered recovered when they regained their balance and were able to maintain a vertical position in the aquarium. The maximum observation time to evaluate sedation or anesthesia and the recovery time was 30 min. The response to external stimuli was through the use of a glass rod. Light touches were made on the shrimp's abdomen during the loss of balance or reduced swimming. Sedated shrimp presented a normal escape response to tactile stimuli, and anesthetized shrimp did not react to tactile stimuli.

Statistical analyses

Data were expressed as mean ± SEM. The homogeneity of variance between groups was tested with Levene's test. Anesthetic activity was evaluated by regression analysis (concentration vs. time of anesthesia induction; concentration vs. time of recovery from anesthesia) using Sigma Plot 11.0 software. If no relationship was present, a comparison of the effects of different EO concentrations on anesthesia and recovery times was performed using one-way ANOVA followed by Tukey's test. Analyses were performed using STATISTICA software (version 7.0, StatSoft, Tulsa, OK, USA), with the minimum significance level set at $P < 0.05$.

RESULTS

As the concentrations of EOLA and EOOG increased, the time required for the induction of sedation and anesthesia in pink shrimp decreased. However, for both EOs, the recovery times did not significantly differ between the different concentrations. The time required inducing the sedation and anesthesia with EOLA at 1000 µL L^{-1} was shorter than that for 500 and 750 µL L^{-1} EOLA (Figure 1A). Most shrimp exposed to 50 µL L^{-1} EOOG were not anesthetized within the 30-min evaluation period, while others showed signs of anesthesia close to the maximum time. There were no significant differences to sedation and anesthesia induction between 100 and 150 µL L^{-1} concentrations of EOOG, which induced anesthesia within about 10 min. At a concentration of 250 µL L^{-1}, EOOG quickly induced anesthesia (Figure 1B) but caused 30.0 ± 1.5% mortality. The recovery time was significantly longer for shrimp anesthetized with EOLA than with EOOG.

There were no differences in the time required to induce sedation and anesthesia stages between 150 and 300 µL L^{-1} of EOC. The EOC concentration of 500 µL L^{-1} presented the shortest time to induce sedation and anesthesia stages. Recovery from anesthesia was significantly longer when shrimp were exposed to 300 and 500 µL L^{-1} of EOC (Figure 1C). The lowest concentrations of EOO (50, 100 and 200 µL L^{-1}) failed to induce sedative or anesthetic effects, whereas the highest concentrations (400 and 800 µL L^{-1}) produced sedation and anesthesia in white shrimp. There were no significant differences in the anesthesia induction between 400 and 800 µL L^{-1} EOO. However, the recovery time was significantly longer in shrimp exposed to 800 µL L^{-1} EOO (Figure 1D). Exposure to EOC or EOO presented the shortest times for the induction of anesthesia. Ethanol did not induce sedation or anesthesia in either of the shrimp species tested.

DISCUSSION

Essential oils exhibit various compositions which are dependent on several factors, such as plant-specific genetic characteristics (chemotype), local conditions and extraction methods (EDRIS, 2007).

Table 1 – Stages of anesthesia induction and recovery used for *Litopenaeus vannamei* in this study (adapted from Parodi et al. 2012).

Stage	Description	Behavioural Response
1	Sedation	Partial loss of balance and presence of response to external stimuli
2	Anesthesia	Total loss of balance, but no response to external stimuli
3	Recovery	The shrimps were considered recovered when they returned the normal posture, behavior and swimming in the aquaria.

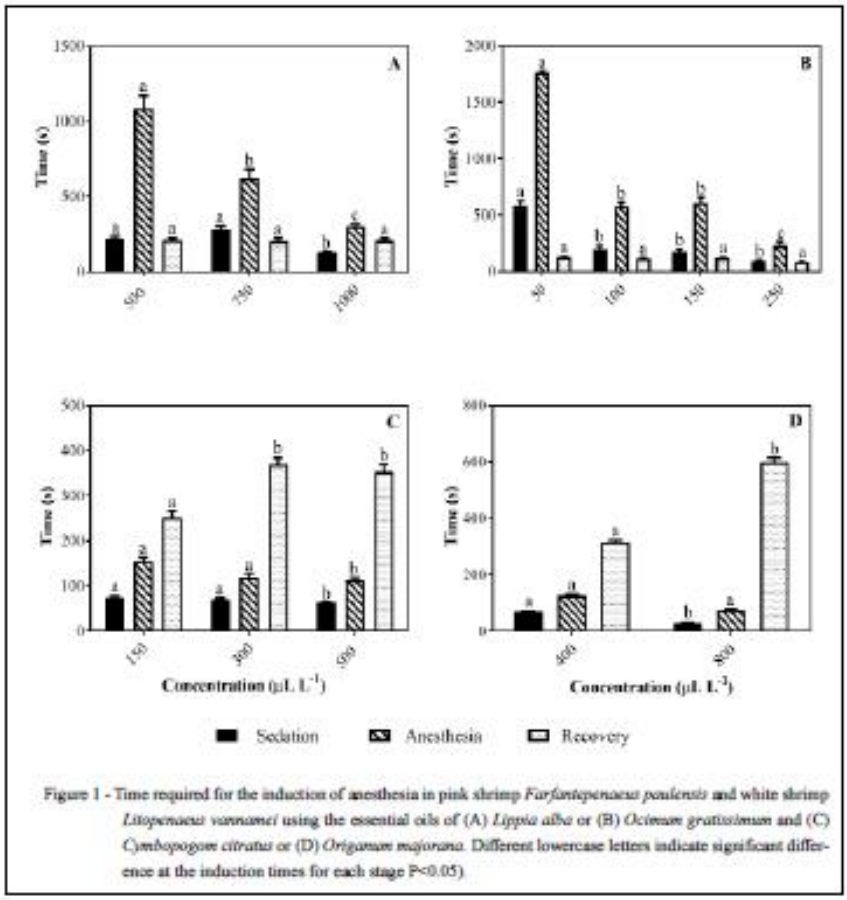

Figure 1 - Time required for the induction of anesthesia in pink shrimp *Farfantepenaeus paulensis* and white shrimp *Litopenaeus vannamei* using the essential oils of (A) *Lippia alba* or (B) *Ocimum gratissimum* and (C) *Cymbopogon citratus* or (D) *Origanum majorana*. Different lowercase letters indicate significant difference at the induction times for each stage P<0.05).

In the present study, chromatographic analysis identified linalool, eugenol, citral and terpinen-4-ol as the major constituents of EOLA, EOOG, EOC and EOO, respectively. These isolated compounds have demonstrated anesthetic activity in several crustaceans, including *L. vannamei*, *M. rosenbergii*, *P. sinensis*, *Neohelice granulata* and *Nephrops norvegicus* (PARODI et al., 2012; COWING et al., 2015; LI et al., 2018b; SOUZA et al., 2018).

In the current study, we reported that the concentration of EOLA directly influenced the time required to induce anesthesia in *F. paulensis*. Similar responses were observed in *L. vannamei* anesthetized with either eugenol or *Aloysia triphylla* (PARODI et al., 2012). Additionally, the anesthesia induction and recovery times of *F. paulensis* exposed to EOLA in our study were faster than those reported by PARODI et al. (2012) for *L. vannamei*. This may be explained by differences in the body size of the shrimp species, as the body weight of pink shrimp is 3-fold lower than white shrimp. Smaller shrimp have higher rate of anesthetic absorption due to the greater gill surface area in relation to body size (BOWNIK, 2015). Studies on *P. sinensis* anesthetized with menthol demonstrated a direct relationship between body size and sensitivity to anesthesia (LI et al., 2018a). However, pink shrimp are likely to be more sensitive to EOLA anesthesia than *L.*

vannamei because even the post-larvae forms take a longer time to reach anesthesia (PARODI et al., 2012).

Pink shrimp exposed to 250 µL L^{-1} of EOOG presented muscle spasms, escape responses and mortality. Similar results were reported for *M. rosenbergii* exposed to quinaldine and Aqui-S™ (COYLE et al., 2005). These side effects occur due to inhibition of the enzyme acetylcholinesterase (AchE) in response to the accumulation of acetylcholine in muscle, which causes excessive muscular stimulation and hyperactivity (NILSSON et al., 1990). However, AchE activity was unchanged in *M. rosenbergii* anesthetized with an anesthetic mixture of menthol and eugenol (SAYDMOHAMED & PAL, 2009). Therefore, the inhibition of this enzyme is not likely to be responsible for behavioral variations. Thus, further studies are needed to investigate the possible toxic effects of EOOG, especially on metabolic parameters in *F. paulensis*.

The rationale behind the addition of EOs to water is to induce calming effects in shrimp, with rapid induction (within 3–5 min) and recovery times (10 min or less), without causing harm to the animals (SOUZA et al., 2019). Anesthesia was achieved with EOC within the established time frame, but EOO only induced anesthesia at the highest concentrations. Exposure of *L. vannamei* to 500 and 1000 µL L^{-1} of EOLA led to anesthesia within 30 and 10 min, respectively (PARODI et al., 2012). *Macrobrachium tenellum* prawns required 17 and 8 min, respectively, to reach deep anesthesia at clove oil concentrations of 300 and 900 µL L^{-1} (PALOMERA et al., 2016). Finally, the use of 300 and 900 µL L^{-1} of eugenol induced full anesthesia in *N. norvegicus* in 14 and 4 min, respectively (COWING et al., 2015). Furthermore, increasing concentrations of EOC proportionally decreased anesthesia induction times for two ornamental fish species, *Sciaenochromis fryeri* and *Labidochromis caeruleus* (KIZAK et al., 2018).

Anesthesia in shrimp does not induce total immobility as the pleopods and antennae remain in continuous but reduced movement. The movement of pleopods is associated with the entry of water into the branchial chamber towards the scaphognathite. Consequently, the total loss of activity of the motor appendages would impair the respiratory and osmoregulatory processes (HENRY et al., 2012). In this research, there was no relationship between concentration and recovery time for EOLA and EOOG. In contrast, increased EOLA concentrations have previously been reported to decrease the recovery time of *L. vannamei* (PARODI et al., 2012). Conversely, the time taken by white shrimp to recover from anesthesia increases as the anesthetic concentration increases for EOC and EOO, as observed in the current study. Additionally, the recovery time of *M. rosenbergii* exposed to 100 and 800 µL L^{-1} of eugenol was 17.6 and 55 min, respectively (SAYDMOHAMED & PAL, 2009), and recovery of *Litopenaeus schimitti* and *Farfantepenaeus brasiliensis* from anesthesia with mint oil (*Mentha piperita*) was longer than 10 min (MATULOVIC & OSHIRO, 2016).

The action of EOs significantly differed between the two shrimp species. Shorter induction times were observed for both EOC and EOO when compared to EOLA or EOOG. In addition, the recovery times of *L. vannamei* were longer than those of *F. paulensis*. Shrimp show behavioral and physiological variations resulting from biological factors such as species, stage of life, sex, body size and lipid content, and environmental factors such as temperature or salinity may significantly affect the action of the anesthetic (LI et al., 2018a,b; JIANG et al., 2020). The metabolism and excretion of anesthetics, which are also influenced by the factors described above, may influence the quality of shrimp for consumption. Eugenol residues were undetectable in *M. rosenbergii* tissues within 24 h of exposure (SAYDMOHAMMED et al., 2009). Regarding the other main compounds of the EOs tested in the current study, no studies have assessed the elimination of these compounds in crustaceans; however, linalool could not be detected in tissues of the silver catfish *Rhamdia quelen* after 24 h (BIANCHINI et al., 2020). Consequently, these studies indicate that a withdrawal time of a few days may be required before human consumption of shrimp anesthetized with these EOs. Additional studies must be performed to confirm this hypothesis.

CONCLUSION

In conclusion, all EOs showed anesthetic effects in the tested shrimp species. The results suggested that concentrations of 750 µL L^{-1} of EOLA and 100 µL L^{-1} of EOOG may be indicated for *F. paulensis*, while 150, 300 or 500 µL L^{-1} of EOC and 200 µL L^{-1} of EOO can be considered effective for *L. vannamei*. Thus, these EOs are indicated for use in procedures that require a prolonged anesthesia time and to avoid stress during handling of the crustaceans.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

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3.2 MANUSCRITO 2

BEHAVIORAL AND BIOCHEMICAL RESPONSES IN ADULT PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*, EXPOSED TO THE ESSENTIAL OIL OF *CYMBOPOGON CITRATUS*

COMPORTAMENTO E RESPOSTAS BIOQUÍMICAS DO CAMARÃO BRANCO DO PACÍFICO, *LITOPENAEUS VANNAMEI*, EXPOSTO AO ÓLEO ESSENCIAL DE *CYMBOPOGON CITRATUS*

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Behavioral and biochemical responses in adult Pacific white shrimp, *Litopenaeus vannamei*, exposed to the essential oil of *Cymbopogon citratus*

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Abstract

This study evaluated the effects of continuous exposure to the essential oil of *Cymbopogon citratus* (EOC) on behavioral and biochemical parameters in shrimp *Litopenaeus vannamei.* Adult shrimp were randomly placed in aquaria and divided into the following groups: control (pure seawater), ethanol (360 μL L^{-1} of ethanol), and 5 or 10 μL L^{-1} EOC for 6 h. Shrimp movements were recorded using a camera for 5 min at the following timepoints: 0, 1, 2, 4 and 6 h of exposure. Light sedation and behavioral changes were observed in shrimp in the 10 μL L^{-1} EOC group. The total antioxidant capacity against peroxyl radicals (ACAP) in the gills and hepatopancreas of shrimp exposed to 10 μL L^{-1} EOC was higher than control, whereas in the muscle it was lower in those exposed to 5 μL L^{-1} EOC. In the gills and hepatopancreas, reduced glutathione (GSH) was increased in the control group. Sulfhydryl groups associated with protein (P-SH) were decreased in the gills of shrimp exposed to 10 μL L^{-1} EOC. Thiobarbituric acid reactive substance levels, indicative of lipid peroxidation, were higher in the gills and hepatopancreas of shrimp exposed to ethanol, which indicates ROS formation. It is concluded that EOC reduced the swimming behavior of *L. vannamei* and improved their tolerance with up to 6 h of exposure.

KEYWORDS

Behavior, biochemical parameters, continuous exposure, essential oils, swimming, white shrimp

1 | INTRODUCTION

Essential oils (EOs) are natural compounds derived from secondary plant metabolites with sedative and antioxidant properties (Ross & Ross, 2008). In this context, some studies have reported the use of EOs for reducing stress in crustaceans, such as *Lippia alba* and *Aloysia triphylla* for *Litopenaeus vannamei*, *Valeriana officinalis* for *Macrobrachium tenellum*, and clove oil for *Macrobrachium rosenbergii* and *Penaeus semisulcatus* (Soltani et al., 2004; Coyle et al., 2005; Parodi et al., 2012; Palomera et al., 2016). The mechanism of action of anesthetics in invertebrates seems to be related to the presence of opioid receptors, which are responsible for the process of endogenous analgesia, reduction of nociceptive reflex and escape responses (Gentle, 2011; Elwood, 2012; Robles-Romo et al., 2016).

Penaeid shrimp are benthic species with higher swimming activity and displacement overnight (Santos et al., 2016). Their behavioral patterns are species-specific and vary according to the environment and endogenous rhythms (Herrnkind, 1983; Katz & Harris-Warrick, 1999). Behavioral changes are usually one of the first responses in stressful situations. Thus, behavior is a parameter widely used to assess species welfare (Barr et al., 2008; Elwood, 2012; Fossat et al., 2014). In crustaceans, the escape response is the principal behavior involved in reactions to dangerous situations or toxicity (Araújo et al., 2016). The escape response is characterized by contractions of the abdominal muscles by large axons and involves a high level of energy consumption, mainly via anaerobic metabolism. This response may result in rapid exhaustion, an increase in oxygen consumption, depletion of energy reserves and the accumulation of reactive oxygen species (ROS) (Herberholz et al., 2004; Domenici et al., 2011).

ROS formation occurs due to high rates of oxygen utilization by tissues to meet the energy demands generated during metabolic processes or stressful events, such as catching or transportation (Akbari et al., 2010; Halliwell & Gutteridge, 2015). ROS are the products of the

reduction of molecular oxygen into highly reactive intermediate elements, such as superoxide anion radicals (O2˙-), hydroxyl radicals (˙OH), and hydrogen peroxide (H_2O_2) (Hermes-Lima et al.,2015; Samet & Wages, 2018; Sarangarajan et al., 2017). These prooxidants are produced naturally in aerobic organisms. However, an imbalance between the production and scavenging of antioxidants and oxidizing compounds leads to ROS accumulation and loss of homeostasis, resulting in protein damage, lipid oxidation and DNA strand breaks (Del Rio et al., 2005; Li et al., 2014).

The concept of oxidative stress is also defined as a condition that interrupts signaling and redox control (Jones, 2006). Animals have a complex antioxidant defense system classified as either enzymatic and non-enzymatic, capable of neutralizing, intercepting or scavenging oxidative reactions (Sarangarajan et al., 2017). The enzymatic antioxidant system consists of endogenous enzymes in the organisms, for example catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Pamplona & Costantini, 2011; Halliwell & Gutteridge, 2015). Non-enzymatic antioxidant defenses originate from exogenous sources, for example from plants or other foods, or endogenous of low molecular weight, which include reduced glutathione (GSH). The balance between GSH and GSSG is associated with the maintenance of redox signaling mechanisms in cells (Huber et al., 2008; Baba & Bhatnagar, 2018).

Several antioxidant compounds and bioactive molecules from plants have been used as dietary supplements or in immersion baths as they improve immune and antioxidant defense systems in fish and crustaceans (Parodi et al., 2012; Reverter et al., 2014; Saccol et al., 2016; He et al., 2017; Sarangarajan et al., 2017; Souza et al., 2017; Colombo et al., 2020). The use of EOs as sedatives or anesthetics can improve the antioxidant capacity of shrimp (Akbari et al., 2010, Parodi et al., 2012). The Pacific white shrimp *Litopenaeus vannamei* showed higher total antioxidant capacity against peroxyl radicals (ACAP) and catalase (CAT) activity when

exposed to *Aloysia triphylla* EO for 6 h (Parodi et al., 2012). Anesthesia of *Macrobrachium rosenbergii* with a mixture of eugenol and menthol resulted in lower glucose values (Saydmohammed & Pal, 2009). On the other hand, eugenol decreased the antioxidant capacity but increased GSH concentration in white shrimp (Parodi et al., 2012).

Due to its tranquilizing effect, *Cymbopogon citratus* EO (EOC) can be used as an anesthetic in fish (Kizak et al., 2018; Tavares-Dias, 2018). However, this EO, which has citral as its main compound, is yet to be tested in crustaceans. However, EOs of *L. alba* and *A. triphylla*, which also have citral as their main compound, were efficacious as anesthetics in silver catfish *Rhamdia quelen* (Becker et al., 2018; Souza et al., 2018) and tambaqui *Colossoma macropomum* (Silva et al., 2019). The use of EOs during capture, aerial exposure and transportation of crustaceans reduces mortality and disease occurrence resulting from handling (Coyle et al., 2004; Saydmohammed & Pal, 2009; Cowing et al. 2015). Therefore, this study aimed to evaluate the influence of continuous exposure to EOC on behavioral and biochemical parameters in white shrimp.

2 | MATERIAL AND METHODS

2.1 | Essential oil of *Cymbopogon citratus* (EOC)

The EOC was purchased from Vimontti – São Caetano Agroindustry (Santa Maria, Rio Grande do Sul state, Brazil). The plant *C. citratus* was grown in Santa Maria city, southern Brazil. The EOC extraction was performed by steam distillation. The major compounds of EOC are citral (74.56%), mircene (11.85%), geraniol (3.08%), pulegone (2.37%) and linalool (1.56%).

2.2 | Animals

Adult *L. vannamei* shrimp (25 ± 1g; 21.8 ± 0.2 cm) were obtained from the Marine Station of Aquaculture (Estação Marinha de Aquicultura), Universidade Federal do Rio Grande (Rio

Grande, Brazil), and transferred into two continuously aerated rectangular plastic tanks (0.32 x 0.65 x 0.40 m), with a temperature of 25-27°C and salinity 30 ppt, where they were maintained for seven days prior to the start of the experiments.

2.3 | Preliminary tests: determination of EOC concentrations to be used

To evaluate the appropriate anesthetic concentrations for continuous exposure, the shrimp were maintained in continuously aerated 6 L aquaria (containing 4 L of seawater) and exposed to the following concentrations of EOC: 2.5, 5, 10, 15, 25, 50, 75 and 100 µL L^{-1} (n = 10 animals per concentration) for 12 h. Mortality rates and partial loss of equilibrium were observed over time, as described for anesthesia stages by Coyle et al. (2005). The EOC was dissolved in ethanol 100% (1:10) before being added to the aquaria with water. The higher concentrations of EOC (15, 25, 50, 75, 100 L^{-1}) resulted in mortality within 1 h of exposure, while the lowest (2.5 µL L^{-1} EOC) failed to induce a sedative effect. From these preliminary results, EOC concentrations of 5 and 10 µL L^{-1} EOC were chosen for subsequent testing.

2.4 | Experimental design

A total of 60 shrimp were randomly chosen, placed individually into 6 L tanks (containing 4 L of water), and divided into the following treatments: control (pure seawater), positive control (90 µL L^{-1} ethanol), and EOC concentrations of 5 or 10 µL L^{-1} (n = 15 animals per treatment). The aquaria were maintained for 6 h under daytime conditions with natural light in order to evaluate the effects, compared to control, of continuous exposure to EOC, at both concentrations, as well as ethanol. The ethanol concentration used was equivalent to the highest concentration used to dilute the EOC. The water quality parameters were analyzed at 0 and 6 h.

2.5 | Water quality parameters

The dissolved oxygen and temperature were measured with a YSI oximeter (Model 20 YSI Inc., Yellow Springs, OH, USA). The pH was verified using a pH meter (DMPH-2 pH, Toledo, São Paulo, SP, Brazil) and salinity was measured with an optical refractometer (RTS model-101, Atago® from USA, Bellevue, WA, USA, precision: ± 1 g). The alkalinity was determined according to the method of Baumgarten et al. (1996). Total ammonia was analyzed according to the methodology of the Intergovernmental Oceanographic Commission (1983).

2.4 | Behavioral analysis

Each aquarium was divided into four different virtual zones (A - anterior side/B – posterior side/C – right side/D – left side) to delimit the study area and better monitor the crossings and preferential position of the shrimp within the aquarium (Figure 1a). During the exposure period, animals were constantly monitored through filming to evaluate their swimming behavior, evasion and presence of the reflex response, following the behavioral response criteria methodology for stressed shrimp described by Stoner (2012) and behavior responses by Pontes et al. (2006), with some modifications: (1) swimming: constant swimming or the occurrence of episodes of immobility; (2) number of crossings: frequency of crossings by the shrimp between the marked zones of the aquarium; (3) number of entries into each zone: preference in location of the shrimp to the anterior, posterior, right or left zones; (4) dwell time: the residence time (in minutes) of shrimp in each aquarium zone (anterior, posterior, right, left); (5) movement of the motor appendages: decreased, increased or the absence of pleopod and pereopod movements; (6) posture: maintenance of body balance in the vertical position or loss of equilibrium with the cephalothorax in a horizontal and descending position; and (7) occurrence of evasion movements: contractions of the abdominal muscles followed by jumps.

The behavioral activity and location of the shrimp were registered with a digital camera (Cyber-shot DSC-H300, Sony, Japan) positioned directly above each aquarium (Figure 1b). The video recording was performed for 5 min in each aquarium following 0, 1, 2, 4, and 6 h of exposure (the final recording was taken shortly before tissue sampling). Ethograms were elaborated by observations of the focal animal type. The video recordings for each time point and treatment were analyzed separately.

2.5 | Biochemical measurement

Sampling of gills, muscle and hepatopancreas was performed at 1 and 6 h for protein quantification and antioxidant enzyme assays (n = 5 shrimp per time point each treatment group). Sampled tissues were placed into 1.5 mL polyethylene tubes and stored at -80°C in an ultra-freezer. Hepatopancreas (1.480 g), gills (0.303 g) and muscle (2.061 g) were weighed and homogenized (1:5 W/V) in buffer solution for crustaceans containing Tris-base (20 mM), EDTA (1 mM), $MgCl_2$ (0.05 mM), sucrose (5 mM), and KCl (1 mM), all dissolved in distilled water, adjusted to pH 7.2, and centrifuged at 20,000 × g for 20 min. The supernatants were aliquoted in 1.5 mL polyethylene tubes and stored at -80°C in an ultra-freezer. Subsequently, the total protein content of the homogenized tissues was quantified by the Biuret method using the Doles Total Proteins Kit (λ = 550 nm) on a plate fluorimeter (Synergy HT, Biotek, USA).

2.5.1 | Total antioxidant capacity against peroxyl radicals (ACAP)

The total antioxidant capacity against peroxyl radicals (ACAP) was performed by the method described by Amado et al. (2009). The total ACAP was analyzed through the generation of peroxyl radicals by thermolysis of ABAP (2,2-azobis-2-methylpropionamidinediamine hydrochloride; Sigma) at 37°C, which reacts with H_2DCFDA (2', 7' dichlorofluorescein diacetate) in the presence of ROS and generates a fluorescent compound (DCF). For this, 10 μL of tissue (six wells per sample), previously diluted to 2 mg protein mL^{-1} with

homogenization buffer, 127.5 μL of reaction buffer (pH 7.2, 30 mM HEPES, 200 mM KCl and 1 mM $MgCl_2$), 7.5 μL of ABAP solution at 20 μM (in three of the six wells) and 10 μL H_2DCF-DA at 40 μM (in all wells) were added in a white microplate. The reaction was detected at 485/525 nm for excitation/emission in the microplate reader and ROS formation was monitored for 30 min, with readings every 5 min (Synergy HT, Biotek, USA). The results are expressed as a relative area (the difference between the area with and without ABAP, divided by the area without ABAP).

2.5.2 | Measurement of reduced glutathione (GSH) and concentration of sulfhydryl groups associated with protein (P-SH)

The concentration of reduced glutathione (GSH) and sulfhydryl groups associated with protein (P-SH) was measured using the DTNB (5,5-dithiobis-2-nitrobenzoic acid; Sigma-Aldrich) method according to Sedlak and Lindsay (1968), with modifications. The samples were precipitated with trichloroacetic acid (50% W/V) and centrifuged at 20,000 × g during 10 min at 4°C. An aliquot of 100 μL of the supernatant with 200 μL of 0.4 Tris-base at pH 8.9 was transferred to transparent microplates for fluorescence detection by a fluorometer. The pellet, containing protein, was re-suspended with 240 μL of homogenization buffer for crustaceans for P-SH determination. Then, 100 μL of extract and 160 μL of 0.2 Tris-base were added to the microplate and incubated for 15 min. The absorbance (405 nm) was read in 96-well transparent microplates (Synergy HT, Biotek, USA). The concentrations are expressed in μmoles of GSH per mg of protein and in μmoles of P-SH equivalent per mg of protein to GSH and P-SH, respectively.

2..5.3 | Lipid peroxidation

The lipid peroxidation (LPO) was determined through the concentration of thiobarbituric acid reactive substances (TBARS) (Oakes & Van der Kraak, 2003). This methodology consists of the fluorometric quantification (emission/excitation 553/515 nm) of a reaction between

malondialdehyde (MDA) and thiobarbituric acid (TBA). In test tubes, 20 μL of butylated hydroxytoluene solution (BHT, 67 μM) and 150 μL of 20% acetic acid solution were added to the samples (muscle: 100 μL, gills: 50 μL, hepatopancreas: 30 μL), 150 μL of TBA solution (0.8%), 50 μL of distilled water and 20 μL of sodium dodecyl sulfate (SDS, 8.1%). The samples were heated at 95°C for 30 minutes. Then, 100 μL of distilled water and 500 μL of n-butanol were added to the final solution, which was centrifuged at 3,000 × g for 10 min at 15°C. Next, 150 μL of the supernatant was placed in a microplate reader (Synergy HT, Biotek, USA). The results are expressed in nmol of tetramethoxypropane (TMP) (used as standard) per mg of wet tissue.

2.6 | Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). The homoscedasticity of variances was verified with the Levene's test and normality by Barlett's test. Differences between the treatments were analyzed through one-way ANOVA, followed by Tukey's test. The analyses were performed using Statistica software (version 7.0, StatSoft, Tulsa, OK) with a minimum significance level of 95% ($p < 0.05$).

3 | RESULTS

3.1 | Water quality parameters

Mortality was not observed for any of the experimental treatments throughout the exposure periods. Water quality parameters did not vary significantly between treatments groups over the exposure time, with the exception of the lowest alkalinity levels in the 10 μL L^{-1} EOC at 6 h of exposure and ammonia in control group at 1 h (Table 1).

3.2 | Behavioral analysis

In our experiment, *L. vannamei* remained inactive in the aquarium for most of the time in all groups. The shrimp in the control and ethanol treatments demonstrated higher swimming activity and jumps during the initial recordings (0 and 1 h). A greater frequency of crossings between different zones occurred in the ethanol group at 0 and 1 h compared to the other groups. In the control group (seawater), swimming episodes followed by pauses were observed; the animals were continuously changing position in the aquarium but always holding a vertical posture of cephalothorax without losing balance. Additionally, in the control group, there was an increase in crossings at 1 h of exposure that decreased within 2 h. The white shrimp exposed to 5 and 10 µL L^{-1} EOC exhibited significantly higher swimming activity and frequency of crossings during the initial recording, which decreased over time. The frequency of crossings in the EOC groups was lower than in the control group during the recordings from 4 to 6 h (Figure 2).

In the ethanol group, a slight loss of balance in posture during swimming was observed at the initial time point after exposure; however, this balance was rapidly reestablished. Following the initial exposure, vertical swimming was maintained during the entire exposure period in the control and ethanol groups. The shrimp showed a loss of balance, the occurrence of avoidance movements and horizontal swimming during the initial stages of exposure to the highest concentration of EOC. Episodes of total immobility occurred before each evasion movement in the 5 and 10 µL L^{-1} EOC groups. In both of these treatment groups, balance maintenance was partially recovered at 2 h and totally a recovered after 4 h. The locomotion of the motor appendages was constant in all groups, but slower in the EOC treatment groups. Compared to the control, the shrimp exposed to ethanol had lower residence times in the anterior zone at 6 h and in the left zone between 2-6 h. Meanwhile, the shrimp in both EOC treatment groups had higher residence time in the anterior zone at 1 h, lower residence times in the anterior zone at 6 h, higher residence times in the right zone at 1 h and 6 h (6h only in the

10 µL L^{-1} EOC group) and lower residence times in the right zone at 4 h (only in the 5 µL L^{-1} EOC group) (Table 2).

3.2 | Measurement of biochemical variables

3.2.1 | Total antioxidant capacity against peroxyl radicals (ACAP)

The ACAP in the gills of shrimp exposed to 10 µL L^{-1} EOC was significantly higher (lower relative area) than that of shrimp in the ethanol group, but it was not significantly different from that of the control group at the 6 h timepoint. After 6 h of exposure, reductions in ACAP in the gills were observed only in the shrimp exposed to 5 µL L^{-1} EOC. There was a significant decrease in ACAP in the hepatopancreas of shrimp in the control, ethanol, and 5 µL L^{-1} EOC groups at the 6 h timepoint. In contrast, in the 10 µL L^{-1} EOC group, the lowest and highest ACAP values were observed at 1 and 6 h, respectively. In the muscle samples, the lowest ACAP values were demonstrated in shrimp exposed to 5 µL L^{-1} EOC at 1 and 6 h, respectively (Figure 3).

3.2.2 | Measurement of reduced glutathione (GSH) and concentration of sulfhydryl groups associated with protein (P-SH)

GSH levels in the gills of shrimp in the control group decreased after 6 h. Compared to control group, shrimp exposed to ethanol or either concentration of EOC demonstrated lower GSH levels in their gills at both time points. In the hepatopancreas, GSH concentrations were significantly lower in shrimp exposed to 10 µL L^{-1} EOC after 1 h of exposure and in shrimp exposed to either concentration of EOC for 6 h when compared to either the control or ethanol group. Furthermore, GSH levels decreased in all treatment groups at 6 h of exposure when compared to 1 h. In the ethanol and 5 µL L^{-1} EOC groups, GSH levels increased in the muscle after exposure for 6 h compared to 1 h. Shrimp exposed to either concentration of EOC had

increased muscle GSH (only at 1 h for shrimp exposed to 10 µL L^{-1} EOC) compared to the control groups (Figure 4).

Shrimp in the 5 µL L^{-1} EOC group had a higher concentration of P-SH in their gills compared to the other treatments. After 6 h of exposure, the level of P-SH in the gills decreased in shrimp in the 10 µL L^{-1} EOC group. In the hepatopancreas samples, the concentration of P-SH in the ethanol group decreased compared to the control group after 6 h of exposure. Shrimp exposed to ethanol for 1 h presented lower concentrations of P-SH in their muscle samples than the control group. Shrimp exposed to 5 µL L^{-1} EOC for 6 h had lower concentrations of P-SH compared to similar shrimp exposed for 1 h (Figure 5).

3.2.3 | Lipid peroxidation

The TBARS levels in the gills and hepatopancreas of the ethanol group were significantly higher than that of shrimp in the control group, and both the 5 and 10 µL L^{-1} EOC groups after 1 h of exposure. However, after 6 h of exposure, these differences were reduced and the values were similar to those of the control group. No significant differences in the levels of TBARS were shown in muscle samples after 1 h of exposure, but increased TBARS values were observed in shrimp exposed to 10 µL L^{-1} EOC compared to 5 µL L^{-1} EOC for 6 h (Figure 6).

4 | DISCUSSION

4.1 | Behavioral analysis

Shrimp demonstrate characteristic behaviors in stressful situations, such as changes in their swimming activity, displacement pattern, “withdrawal or escape” capacity and evasion responses (Sneddon et al., 2014; Júnior et al., 2018). The control group showed an increase in the number of crossings after 1 h of exposure, likely indicating some initial stress of acclimation and recognition of the aquarium. Pontes et al. (2006) demonstrated that swimming and exploration are frequent in white shrimp submitted to different photoperiods (12:12/light:dark),

demonstrated by constant activity patterns independent of the period of the day. In the current study, the photoperiod was not expected to directly influence the behavioral pattern of the shrimp because the experiments were performed during the same period of the day. In addition, the residence time in each zone did not indicate any preference for a location in the aquarium. The characteristics of the aquarium or the absence of substrates at the bottom may have influenced this behavior.

Initially, shrimp in the ethanol group demonstrated an increase in swimming activity and agitation, the opposite response to EOC, i.e., agitation, disorientation and a higher number of crossings of the aquarium. Similar to these findings, the injection of morphine has been shown to decrease escape movement responses in *Carcinus mediterraneus* and *Neohelice granulate crabs* immediately, which subsequently promotes habituation of their responses to stimuli, leading to increased locomotor activity (Dyakonova, 2001). Ethanol has depressive effects, and its administration has resulted in intoxication and loss of motor coordination after 35 min of exposure in juvenile crayfish *Procambarus clarkii* (Swierzbinski et al., 2017), as well as disorientation, loss of motility and sedation in zebrafish *Danio rerio* (Araujo-Silva et al., 2018). In other studies, ethanol was not effective as an anesthetic substance in *L. vannamei* (Parodi et al., 2012) and *M. tenellum* (Palomera et al., 2016).

Shrimp exposed to EOC initially presented agitation, which was followed by decreased movements, periods of inactivity, late reactions and a reduction in the number of crossings. These findings demonstrate the potential sedative effect of EOC at low concentrations. Anesthetized shrimp may continue to move their appendages, especially the pleopods and antennas. This response is different from total immobility observed in fish following anesthesia (Ross & Ross, 2008; Coyle et al., 2004). Decreased swimming behavior is a consequence of inhibited neuromuscular transmissions due to EO addition and results in reduced metabolic and respiratory rates (Millar & Atwood, 2004; Bownik, 2015). Similar response patterns have also

observed in *Nephrops norvegicus* anesthetized with eugenol (Cowing, 2015), *Daphnia magna* anesthetized with clove bud (*Eugenia caryophyllus*) (Bownik, 2015) and *L. vannamei* anesthetized with either *L. alba* or *A. triphylla* (Parodi et al., 2012)

At 10 μL L^{-1} EOC, shrimp exhibited episodes of immobility, loss of balance and escape response. This last behavior is related with dangerous situations or toxic effects (García de La Parra, 2006; Robles-Romo et al., 2016). EOC exposure did not demonstrate toxicity over the trial period. The increase in escape responses indicates aversion behavior or irritation of the gills, which signals an initial response to EOC exposure. EOC demonstrates anxiolytic effects via the GABA receptor in mice (de Almeida Costa et al., 2011). Nevertheless, the mechanisms of action of EO in crustaceans are still uncertain. Opioid receptors, GABA neurotransmitters and metabotropic glutamate receptors have been identified in some invertebrates and may be involved in the regulation of the anesthetic response (Ozeky, 1975; Dyakonova, 2001; Hamilton et al., 2016; Perrot-Minnot et al., 2017; Elwood, 2019). Moreover, anesthetics may also act by blocking the voltage-dependent K^+ and Na^+ channels, which inhibit neural and motor activity in these animals (Wycoff et al., 2018).

4.2 | Measurement of biochemical variables

Antioxidant responses in tissues are species-specific and vary according to metabolic rate and oxygen consumption (Narra, 2014). The gills are multi-functional organs that provide an interface with the external environment and are responsible for the absorption and transport of oxygen and ions (Freire et al., 2008; Henry et al., 2012). The hepatopancreas is involved in the carbohydrate and lipid metabolisms, nutrients storage, and detoxification processes (Xu et al., 2018, Chen et al., 2019). This organ is the main place of ROS production (Gibson & Barker, 1979). On the other hand, muscles are organs that present a high glycolytic and gluconeogenic capacity. Therefore, it is hypothesized that a high metabolic rate for maintaining energy

metabolism homeostasis results in a higher susceptibility to oxidative damages in these tissues (Phillips et al., 2012). Thus, these tissues are significant targets in the biochemical and metabolic processes of crustaceans.

Exposure to EOC did not induce higher ACAP in the muscle. On the other hand, the higher ACAP was probably an attempt to minimize the oxidative damage generated in the control group. In addition, the relative area in the muscle was significantly higher compared to the gills and hepatopancreas. Overall, due a high content of polyunsaturated fatty acids (PUFA), crustaceans are highly susceptible to oxidative degradation (Okpala et al., 2016). The increased GSH levels and depletion of sulfhydryl groups in the muscles of shrimp exposed to 5 μL L^{-1} EOC suggests the induction of antioxidant enzymes to compensate for the effects of a lower ACAP. After 6 h of exposure, shrimp sedated with 10 μL L^{-1} EOC showed a higher peroxidation rate in relation to shrimp exposed to 5 μL L^{-1} EOC, but no significant differences were observed when comparing these results with the control group. Therefore, no significant changes in muscle enzymatic activity were caused by exposure of the shrimp to EOC.

Thiol groups are important in maintaining protein structure and function (Baba & Bhatnagar, 2018). GSH is the major non-protein thiol found in all animal tissues where it is used as substrate for GPx, which catalyzes the reduction of H_2O_2 (Baba & Bhatnagar, 2018). Decreased GSH levels lead to changes in the redox state, consequently causing oxidation of lipids and amino acids, such as cysteine and methionine (Jones, 2002). The sulfhydryl groups associated with proteins, P-SH, are protein thiol groups that participate in the protection of catalytic sites on some antioxidant enzymes (Souza et al., 2017). When compared to the control group, decreases in the levels of GSH in the gills and hepatopancreas of shrimp exposed to EOC indicate increased use of GSH as co-factor in the activity of GPx and GST. Salbego et al. (2014) found decreased GSH and GPx activity in the livers of silver catfish *Rhamdia quelen* that were transported in plastic bags containing 30 μL L^{-1} of *L. alba*, when compared to a control group

that was transported in pure water. Thus, variations in GSH levels correspond to different stress scenarios, which resulted in either the attenuation of oxidative damage in our control group or the induction of the antioxidant response in the EOC treatments.

As for P-SH, exposure to 5 μL L^{-1} EOC promoted higher cellular protection against oxidation in the gills compared to control shrimp. In turn, reductions in the content of P-SH groups in shrimp exposed to 10 μL L^{-1} EOC for 6 h implies a pro-oxidant scenario. A study by Colombo et al. (2020) showed that shrimp fed with açai and exposed to non-ionized ammonia (NH_3) had decreased concentrations of P-SH in their gills and muscle. Thus, the presence of P-SH is related to the maintenance and protection of the redox state of proteins, while the depletion of P-SH demonstrates protein carbonylation (Fedorova et al., 2009). Moreover, the regulation of proteins via P-SH results in the reversible carbonylation of proteins, thus providing protection in the form of advanced lipoperoxidation end products (ALEs) (Baba & Bhatnagar, 2018). These results show that both direct and indirect responses to enzymatic activation can be related to the EOC concentration and the duration of exposure.

LPO and antioxidative enzymes are the main biomarkers of oxidative damage in various aquatic animals (Lushchak & Bagnyukova, 2006). An imbalance between the antioxidant system and the generation of ROS provokes the activation of LPO mechanisms, which results in a loss of cell function (Hermes-Lima, 2004). In the present work, shrimp exposed to ethanol demonstrated increased levels of TBARS, a marker of lipid peroxidation, in their gills and hepatopancreas, which indicates ROS formation in response to stress; however, these levels decreased after 6 h of exposure. In previous studies, the addition of ethanol to aquarium water demonstrated no significant effects on the oxidative parameters of tambaqui *Colossoma macropomum* (Saccol et al., 2016), *Brycon amazonicus* (Saccol et al., 2017) or *L. vannamei* (Parodi et al., 2012). There was no significant difference in TBARS levels in the gills and hepatopancreas of shrimp exposed to EOC compared to the control group. Interestingly, *M.*

rosenbergii fed with a diet supplemented with *L. alba* EO exhibited lower TBARS levels in the hepatopancreas (Cagol et al., 2020). However, exposure of *L. vannamei* to 20-40 μL L^{-1} linalool for 8 h increased LPO in the gills and hepatopancreas (Becker et al., 2015).

After 6 h of exposure, the levels of P-SH increased, correlating with decreased levels of ACAP in the gills of the shrimp sedated with 5 μL L^{-1} EOC, indicating compensatory and chemoprotective mechanism. In this case, the addition of EOC initially improved the antioxidant response, providing conditions that benefited ROS production. Similar responses have been observed in *L. vannamei* exposed to 20 μL L^{-1} *A. triphylla* or 20 μL L^{-1} eugenol, where increased and decreased ACAP were observed, respectively (Parodi et al., 2012). EOC at a concentration of 10 μL L^{-1} increased ACAP in the gills and hepatopancreas of shrimp compared to the control group. This observation corroborates with our results concerning GHS levels and TBARS values. In this context, the highest concentration of EOC promoted an improvement in antioxidant status.

5 | CONCLUSION

In conclusion, exposure to EOC influenced the swimming behavior and posture of shrimp, but did not cause mortality, and the exposed shrimp returned to their normal behavior following the initial exposure period. The concentration of 10 μL L^{-1} EOC can improve the physiological and behavioral homeostasis and tolerance of *L. vannamei* shrimp to inadequate handling during long periods of exposure, through participation in the regulation of antioxidant enzymes, including ACAP, GSH and P-SH, thereby preventing the occurrence of lipid damage.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Alessandra J. Becker designed and performed the study, measured biochemical variables, performed the statistical analysis and drafted the paper. Patrícia B. Ramos and José M. Monserrat contributed to the measurement of biochemical variables and manuscript revision. Wilson Wasielesky Jr. and Bernardo Baldisserotto contributed to the design of the study and manuscript revision.

ETHICAL APPROVAL

The use of invertebrate species does not require ethical approval.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Physicochemical water parameters in the treatments control, ethanol, 5.0 and 10 μL L^{-1} of the essential oil of *Cymbopogon citratus* (EOC) of the white shrimp *Litopenaeus vannamei* after 1 and 6 h exposure.

		Water quality parameters				
Treatments	Time (h)	DO (mg L^{-1})	Temperature (°C)	pH	Alcalinity (mg $CaCO_3$ L^{-1})	TAN (mg L^{-1})
Control	1	5.68 ± 0.13	25.7± 0.02	7.74 ± 0.00	141 ± 7.00	0.14 ± 0.04*
	6	6.22 ± 0.08	25.3 ± 0.02	7.81 ± 0.05	146 ± 8.12	0.37 ± 0.08
Ethanol	1	5.84 ± 0.10	24.7 ± 0.09	7.76 ± 0.02	132 ± 8.00	0.32 ± 0.03
	6	5.82 ± 0.12	25.0 ± 0.03	7.81 ± 0.05	146 ± 8.12	0.37 ± 0.14
5.0 μL L^{-1}	1	5.60 ± 0.17	22.0 ± 0.10	7.70 ± 0.02	140 ± 8.36	0.29 ± 0.03
EOC	6	6.10 ± 0.14	22.7 ± 0.02	7.71 ± 0,00	146 ± 8.12	0.33 ± 0.06
10 μL L^{-1}	1	6.60 ± 0.09	22.3 ± 0.06	7.76 ± 0.06	142 ± 1.22	0.29 ± 0.03
EOC	6	6.60 ± 0.10	21.0 ± 0.03	7.76 ± 0.08	115 ± 2.73*	0.30 ± 0.03

DO – dissolved oxygen, TAN – total ammonia nitrogen. *Indicates the treatment that present significant differences from control

Table 2. Residence time of white shrimp, *L. vannamei*, submitted to control treatment, ethanol, 5.0 or 10 μL L^{-1} of the essential oil of *C. citratus*, in each zone of the aquarium. Different lowercase letters in the columns indicate significant differences in the residence time of shrimps between the zones of the aquarium at different times for the same treatment. The different uppercase letters in lines indicate significant differences in the residence time of shrimps between treatments at the same time.

Time in each zone of aquarium (min)					
Time of exposure (h)	Position	Control	Ethanol	5.0 μL L^{-1} EOC	10 μL L^{-1} EOC
0	Anterior	1.132 ± 0.33^{Aa}	1.187 ± 0.40^{Ab}	2.770 ± 0.37^{Aa}	2.125 ± 0.34^{Aa}
1		1.114 ± 0.50^{Aa}	1.010 ± 0.54^{Ab}	3.037 ± 0.50^{Ba}	2.268 ± 1.06^{Ba}
2		1.292 ± 0.20^{Aa}	1.089 ± 0.10^{Aa}	1.920 ± 0.92^{Aa}	1.447 ± 0.46^{Aa}
4		1.400 ± 0.37^{Aa}	1.003 ± 0.70^{Aa}	1.501 ± 0.61^{Aa}	1.801 ± 0.95^{Aa}
6		2.247 ± 1.06^{Aa}	0.973 ± 0.47^{Ba}	0.954 ± 0.70^{Bb}	1.171 ± 0.97^{Ba}
0	Posterior	2.958 ± 0.55^{Aab}	3.287 ± 0.36^{Aa}	2.399 ± 0.60^{Aa}	2.660 ± 0.36^{Aa}
1		3.011 ± 0.87^{Ab}	3.438 ± 0.93^{Aa}	2.293 ± 0.67^{Aa}	2.779 ± 0.94^{Aa}
2		3.524 ± 0.41^{Ab}	2.541 ± 0.20^{Aab}	3.065 ± 0.81^{Aa}	3.514 ± 0.37^{Aa}
4		2.794 ± 0.63^{Aab}	2.803 ± 0.77^{Aab}	3.362 ± 0.87^{Aa}	3.331 ± 0.91^{Aa}
6		3.544 ± 0.51^{Ab}	2.403 ± 0.84^{Aab}	3.379 ± 0.76^{Aa}	3.869 ± 0.97^{Aa}
0	Right	1.001 ± 0.27^{Ba}	2.789 ± 0.30^{Aab}	2.575 ± 0.41^{Aa}	2.446 ± 0.51^{Aa}
1		3.155 ± 0.90^{Ab}	1.973 ± 0.32^{Aab}	2.632 ± 0.37^{Aa}	2.107 ± 0.62^{Aa}
2		2.546 ± 0.60^{Aab}	3.385 ± 0.70^{Aab}	1.794 ± 0.74^{Aa}	1.525 ± 0.44^{Aa}
4		1.925 ± 0.93^{Ba}	3.779 ± 0.40^{Aa}	0.871 ± 0.41^{Cb}	1.659 ± 0.60^{Ba}
6		1.186 ± 0.60^{Ba}	4.156 ± 0.55^{Aa}	3.664 ± 0.56^{Aa}	1.616 ± 0.91^{Ba}
0	Left	3.081 ± 0.63^{Aa}	2.093 ± 0.37^{Aab}	2.166 ± 0.50^{Aa}	2.542 ± 0.40^{Aa}
1		1.933 ± 0.84^{Aa}	2.637 ± 0.31^{Aab}	2.271 ± 0.43^{Aa}	2.549 ± 0.51^{Aa}
2		4.461 ± 1.36^{Ab}	1.481 ± 0.67^{Cab}	2.500 ± 0.91^{Ba}	2.389 ± 0.70^{Ba}
4		2.710 ± 1.00^{Aab}	0.682 ± 0.30^{Bc}	2.216 ± 0.88^{Aa}	2.016 ± 0.64^{Aa}
6		3.890 ± 0.40^{Ab}	0.725 ± 0.50^{Cc}	1.938 ± 0.86^{Ba}	3.707 ± 0.94^{Aa}

EOC - essential oil of *Cymbopogon citratus.*

Figure captions

Figure 1. Schematic representations of the methodological approach used for the evaluation of the behavior of shrimp *Litopenaeus vannamei.* A) Aquarium divided into four different zones: A - anterior side; B - posterior side; C - right side; D - left side, to evaluate swimming, number of crossings, entries and residence time in each aquarium zone. (B) Schematic view of the aquarium in which the behavioral analysis was performed. (C) Schematic drawing of the experimental protocol of exposure to treatments with *C. citratus* (EOC), ethanol and pure seawater (control) during filming for 6 hours. *EtOH: ethanol; EOC: *Cymbopogon citratus*.

Figure 2. The graph shows the frequency of crossings of shrimp *Litopenaeus vannamei* exposed for 6 h to treatments control group (seawater), positive control (ethanol), 5.0 or 10 μL L^{-1} of the essential oil of *Cymbopogon citratus* (EOC). Values are expressed as mean ± standard error (n = 20). The letters (x, y) indicate significant differences between treatments at the same sampling time, and letters (a, b) indicate significant differences between sampling times for the same treatment according to Tukey's test at a significance level of 0.05.

Figure 3. Values of total antioxidant capacity against peroxyl radicals (expressed as the relative area) in the (a) gill, (b) hepatopancreas and (c) muscle in shrimp *Litopenaeus vannamei* exposed to control group (seawater), positive control (ethanol), 5.0 or 10 μL L^{-1} of the essential oil of *C. citratus* (EOC) for 6 h. Values are expressed as mean ± standard error (n = 20). The letters (a, b, c) indicate significant differences between treatments at the same sampling time, and letters (x, y) indicate significant differences between sampling times for the same treatment according to the Tukey's test at a significance level of 0.05.

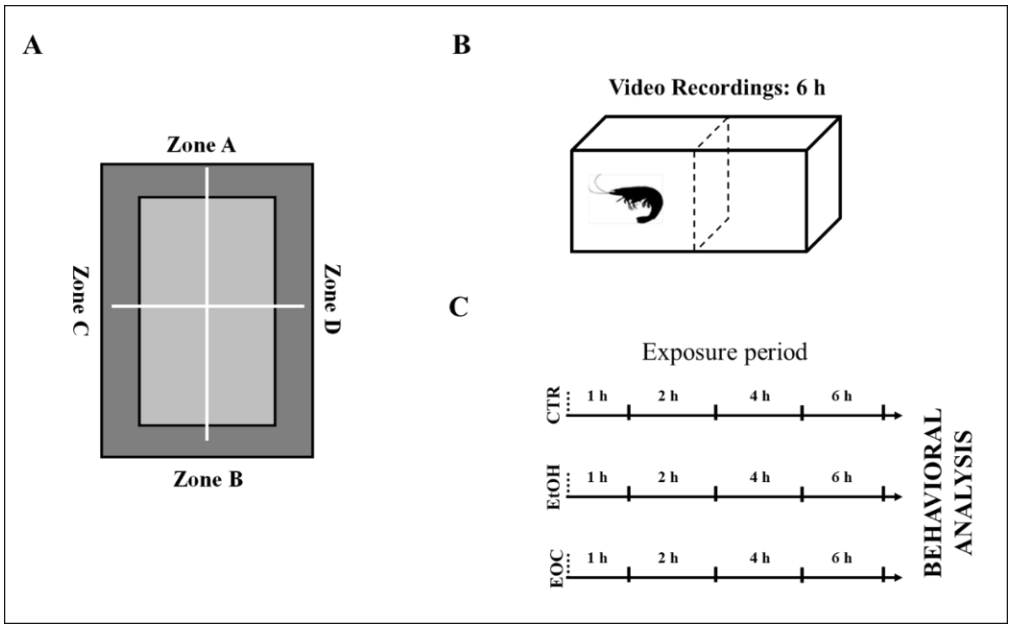
Figure 4. Concentration of reduced glutathione (μmol of GSH mg $protein^{-1}$) in the (a) gill, (b) hepatopancreas and (c) muscle in shrimp *Litopenaeus vannamei* exposed to control group (seawater), positive control (ethanol), 5.0 or 10 μL L^{-1} of the essential oil of *C. citratus* (EOC) for 6 h. Values are expressed as mean ± standard error (n = 20). The letters (a, b, c) indicate significant differences between treatments at the same sampling time, and letters (x, y) indicate significant differences between sampling times for the same treatment according to the Tukey's test at a significance level of 0.05.

Figure 5. Concentration of sulfhydryl groups associated with protein (SH mg $protein^{-1}$) in the (a) gill, (b) hepatopancreas and (c) muscle in shrimp *Litopenaeus vannamei* exposed to control

group (seawater), positive control (ethanol), 5.0 or 10 μL L^{-1} of the essential oil of *C. citratus* (EOC) for 6 h. Values are expressed as mean ± standard error (n = 20). The letters (a, b, c) indicate significant differences between treatments at the same sampling time, and letters (x, y) indicate significant differences between sampling times for the same treatment according to the Tukey's test at a significance level of 0.05.

Figure 6. Content of substances reactive to thiobarbituric acid (nmol of TMP mg per $tissue^{-1}$) in the gill (a) hepatopancreas (b) and muscle (c) in shrimp *Litopenaeus. vannamei* exposed to control group (seawater), positive control (ethanol), 5.0 or 10 μL L^{-1} of the essential oil of *C. citratus* (EOC) for 6 h. Values are expressed as mean ± standard error (n = 20). The letters (a, b, c) indicate significant differences between treatments at the same sampling time, and letters (x, y) indicate significant differences between sampling times for the same treatment according to the Tukey's test at a significance level of 0.05.

Figure 1.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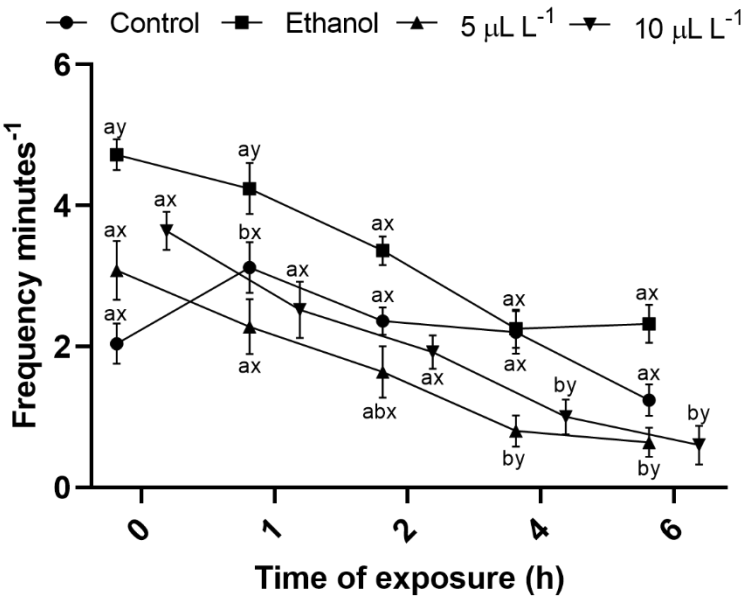
Zone A
Zone C
Zone D
Zone B
B
Video Recordings: 6 h
C
Exposure period
CTR
EtOH
EOC
1 h
2 h
4 h
6 h
BEHAVIORAL ANALYSIS

Figure 2.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Frequency minutes-1
Time of exposure (h)
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2
4
6
0
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2
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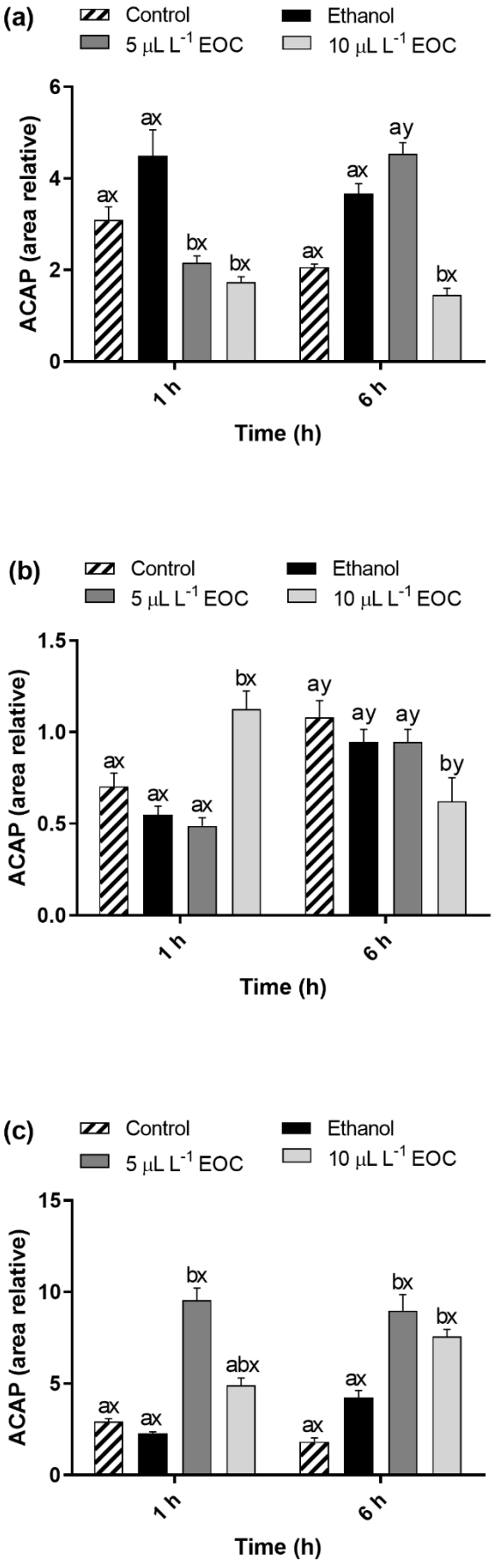
6
ay
ay
ax
ax
bx
ax
ax
ax
ax
ax
ax
ax
ax
ax
abx
by
by
by
by

Figure 3.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10 μL L-1 EOC
ACAP (area relative)
Time (h)
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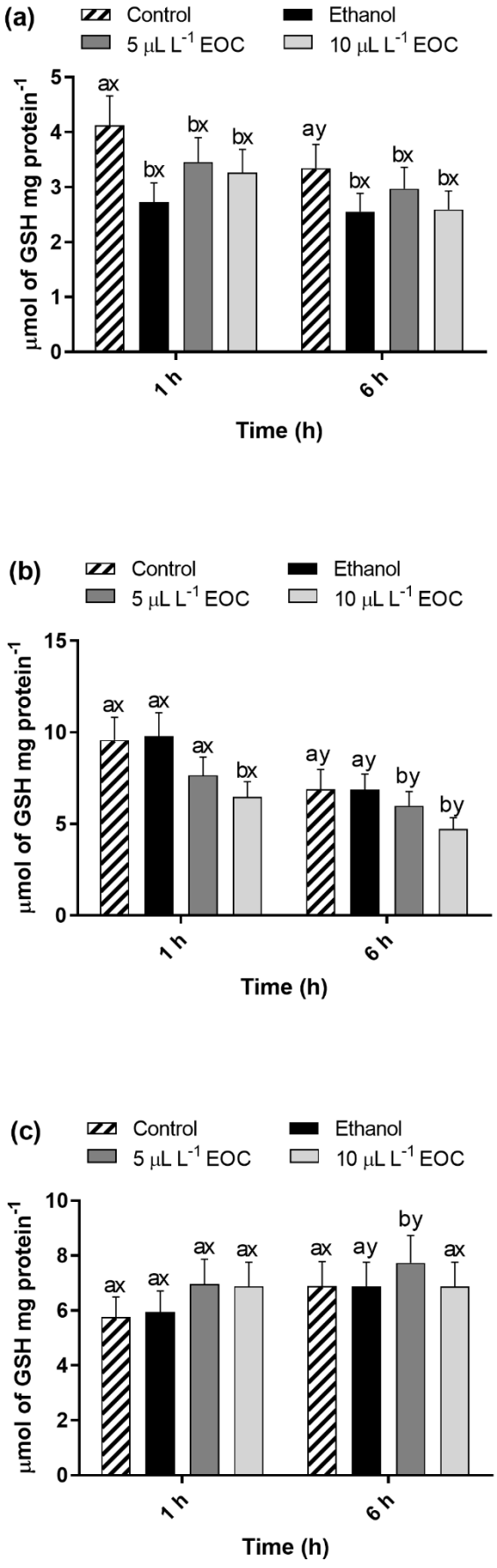
6 h
ax
ax
bx
bx
ax
ax
ay
bx
(b)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
ACAP (area relative)
Time (h)
1 h
6 h
ax
ax
ax
bx
ay
ay
ay
by
(c)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
ACAP (area relative)
Time (h)
1 h
6 h
ax
ax
bx
abx
ax
ax
bx
bx

Figure 4.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Control
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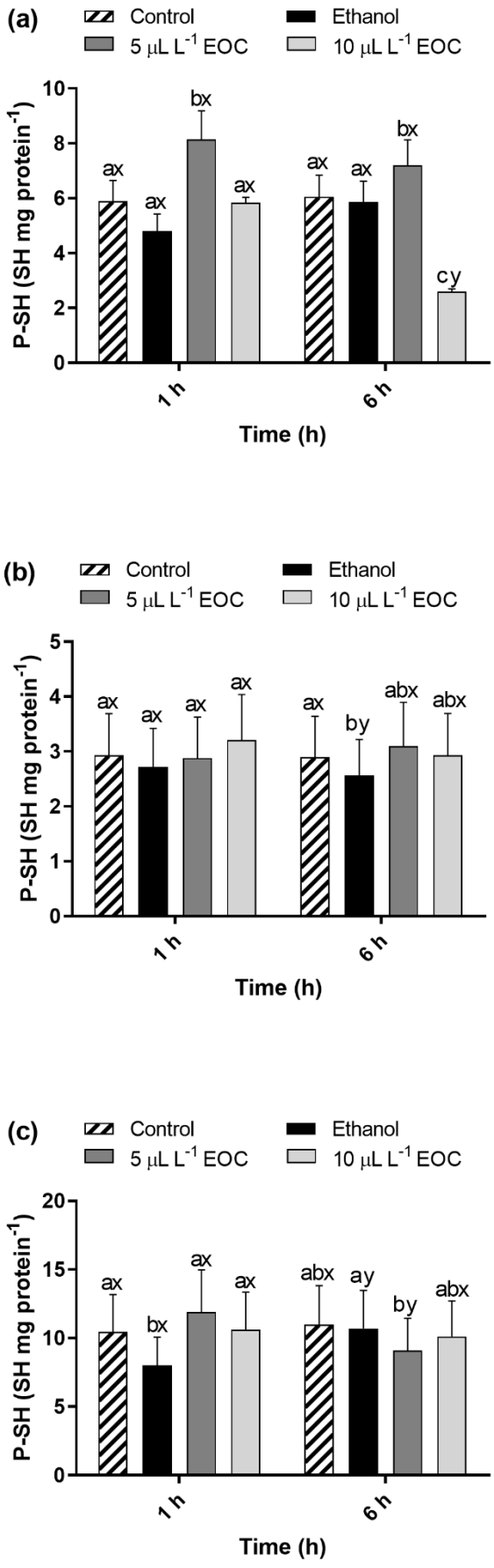
10 μL L-1 EOC
μmol of GSH mg protein-1
ax
bx
bx
bx
ay
bx
bx
bx
1 h
6 h
Time (h)
(b)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
μmol of GSH mg protein-1
ax
ax
ax
bx
ay
ay
by
by
1 h
6 h
Time (h)
(c)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
μmol of GSH mg protein-1
ax
ax
ax
ax
ax
ay
by
ax
1 h
6 h
Time (h)

Figure 5.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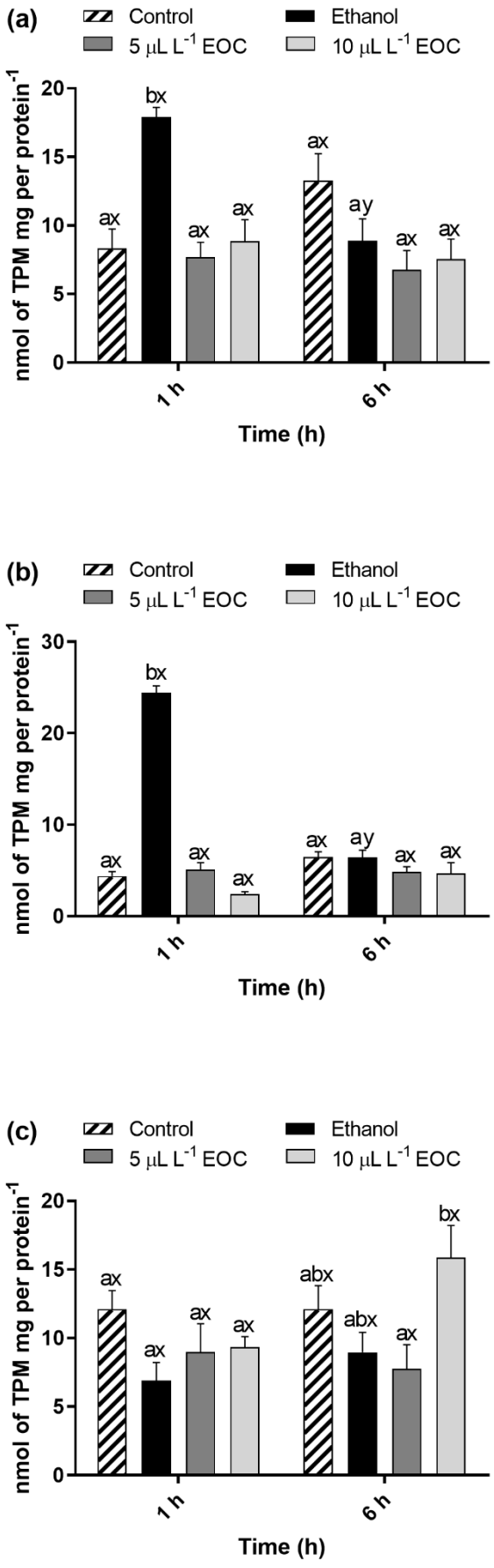
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
P-SH (SH mg protein-1)
ax
ax
bx
ax
ax
ax
bx
cy
1 h
6 h
Time (h)
(b)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
P-SH (SH mg protein-1)
ax
ax
ax
ax
ax
by
abx
abx
1 h
6 h
Time (h)
(c)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
P-SH (SH mg protein-1)
ax
bx
ax
ax
abx
ay
by
abx
1 h
6 h
Time (h)

Figure 6.


(a)
Control
Ethanol
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10 μL L-1 EOC
nmol of TPM mg per protein-1
20
15
10
5
0
bx
ax
ax
ax
ax
ay
ax
ax
1 h
6 h
Time (h)
(b)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
nmol of TPM mg per protein-1
30
20
10
0
bx
ax
ax
ax
ax
ay
ax
ax
1 h
6 h
Time (h)
(c)
Control
Ethanol
5 μL L-1 EOC
10 μL L-1 EOC
nmol of TPM mg per protein-1
20
15
10
5
0
ax
ax
ax
ax
abx
abx
ax
bx
1 h
6 h
Time (h)

3.3 MANUSCRITO III.

DETERMINATION OF THE ANTIOXIDANT EFFECTS OF LINALOOL AND THE ESSENTIAL OIL OF *LIPPIA ALBA* IN THE SHRIMP *LITOPENAEUS VANNAMEI* SUBMITTED TO EYESTALK ABLATION AND SPERMATOPHORE EXTRUSION

DETERMINAÇÃO DOS EFEITOS ANTIOXIDANTES DO LINALOL E DO ÓLEO ESSENCIAL DE LIPPIA ALBA NO CAMARÃO *LITOPENAEUS VANNAMEI* SUBMETIDO A ABLAÇÃO OCULAR E EXTRUSÃO DO ESPEMATÓFORO

O manuscrito será submetido para publicação no periódico *Aquaculture*.

Determination of the antioxidant effects of linalool and the essential oil of *Lippia alba* in the shrimp *Litopenaeus vannamei* submitted to ablation eyestalk and spermatophore extrusion

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Abstract

Essential oils (EOs) minimize the stress effects in the handling of aquatic animals. This study evaluated the anesthetic effect of *Lippia alba* EO (EOLA) and its major compound linalool (a mix of S-(+) and R-(-) isomers) on antioxidant responses of shrimp *Litopenaeus vannamei* after eyestalk ablation and spermatophore extrusion. Female and male shrimps were divided in the following groups: pure seawater (control), ethanol (9000 µL L^{-1} of ethanol), 750 µL L^{-1} of EOLA, 1000 µL L^{-1} of EOLA, 350 µL L^{-1} of linalool, and 500 µL L^{-1} of linalool. Shrimps were sedated for 3 min. Then, females were ablated unilaterally by cauterization (cutting the eyestalk with hot scissors). In males, the spermatophores were removed via manual extrusion. Biochemical variables were analyzed in the gills, muscles, and hepatopancreas at the end of the experiments. Our results showed several effects of EOLA and linalool, which were dependent upon sex. The total antioxidant capacity (ACAP) of gills and muscle increase significantly in both sexes when exposed to control and ethanol groups than EOLA and linalool groups. Concomitantly, thiobarbituric acid reactive substance (TBARS) levels increased in these tissues. Ablated females appear to be more sensitive to oxidative damages. However, the concentration of 500 µL L^{-1} of linalool improved the antioxidant response caused by eyestalk ablation. The protective effect was observed mainly in the hepatopancreas. In males, both concentrations of EOLA or linalool induced a mild antioxidant effect. Thus, the OEs should be indicated to reducing tissue damage in reproductive procedures, being the concentration of 500 µL L^{-1} of linalool more effective to female shrimps.

Keywords: Antioxidant status, vegetable extractives, reproduction, white shrimp, welfare

1. Introduction

The penaeid shrimps are differentiated between males and females by the first and second pair of abdominal pleopods, modified in copulatory organs. (Hobbs and Lodge, 2010). Overall, these animals depend on hormonal control and specific environmental stimuli (salinity, temperature, or feeding,) to begin the process of gonadal maturation (Harlioglu et al., 2018). In laboratory, main techniques used for induction of maturation and spawning are eyestalk ablation in females and spermatophore extrusion in males to artificial fertilization (Primavera, 1985; Ibarra et al., 2007; Rotllant et al., 2018).

The X-organ-sinus gland complex (XO-SG) is located in the eyestalks of shrimps. This neuroendocrine tissue regulates the synthesis and storage of neurohormones involved in several metabolic pathways such as vitellogenesis and molt (Webster, 2015; Alfaro-Montoya et al., 2018). The eyestalk ablation interferes in the release of vitellogenesis inhibiting hormone (VIH), leading in hormonal imbalance and physiological changes. Consequently, the lowest VIH rates in the hemolymph induce an increase of vitellogenin, which stimulates the maturation of the gonads and a higher number of spawnings (Alfaro-Montoya et al., 2018). Manual extrusion of the spermatophore in males has been described in the literature as a simple technique. This activity is employed to obtain the sperm mass from the terminal ampoule for artificial insemination and sperm quality analysis (Parnes et al., 2006; Alfaro-Montoya, 2010; Nakayama et al., 2020)

Both reproductive methods demonstrate success in the spawns, hatching rate, and fecundity (number of eggs and nauplii) in captivity. However, these strategies may cause injuries and stress, reproductive exhaustion, decrease immunological defense, and mortality (Almeida et al., 2004; Mercier et al., 2006; Braga et al., 2018). Application of ethical protocols in the production systems supports more sustainable practices ensuring compliance with the "five freedoms" (i.e. freedom from thirst, hunger, and malnutrition; discomfort; pain, injury,

and disease, to express normal behavior, and from fear and distress) of animal welfare (Little et al., 2018). Therefore, stressful situations are responsible for economic losses and health consequences to shrimp.

The unbalance between production or removal of antioxidant and pro-oxidant agents results in alteration of metabolic homeostasis, increasing the formation and accumulation of reactive oxygen species (ROS) (Hermes-Lima, 2015). These ROS are responsible for oxidative damage in biomolecules. Oxidative stress is also defined as a condition of regulation and interruption of redox signaling and control (HUBER et al., 2008; BABA and BHATNAGAR, 2018). In contrast, the organisms show endogenous and exogenous antioxidants capable of intercept and/or eliminate ROS. The antioxidant responses include antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), and low reduced glutathione (GSH) by the conjugation this antioxidant with GSSG (Hermes- Lima, 2004; Lushchak, 2011; Lesser, 2012).

Currently, natural substances are employed in proceedings with aquatic animals to improve immune and antioxidant defense systems (Coyle et al., 2005; Parodi et al., 2012; Cagol et al., 2020; Colombo et al., 2020; Schmitz et al., 2020; Becker et al., 2021a). The essentials oils (EOs) are natural substances derived from secondary plants metabolites with sedative, anesthetics, and antioxidants properties (Akbari et al., 2010; Hoseini et al., 2018; Li et al., 2018). The EOs of *Lippia alba*, *Aloysia triphylla*, *Origanum majorana*, and *Cymbopogon citratus* have been found to present anesthetic and antioxidant effects to *Macrobrachium rosenbergii* (Cagol et al., 2020), Pacific white shrimp *Litopenaeus vannamei* (Parodi et al., 2012), and pink shrimp *Farfantepenaeus paulensis* (Becker et al., 2021b).

Eyestalk ablation and spermatophore extrusion are traumatic reproductive procedures whose previous anesthesia may improve shrimp welfare and encourage awareness in terms of animal sustainability (Taylor et al., 2004). Based on these experimental findings and the fact

that no study has been published concerning the possible effects of EOs on reproductive methods to white shrimp, we evaluated the efficacy *of Lippia alba* EO (EOLA) and its major compound linalool (a mix of S-(+) and R-(-) isomers) during eyestalk ablation and spermatophore extrusion of *L. vannamei* by quantification of oxidative parameters.

2. Material and methods

2. 1. Animals

Females (n = 90) and males (n = 90) of *L. vannamei* were obtained from the Marine Station of Aquaculture (FURG). Shrimps were stocked in two circular maturation tanks (5000 L) for one-week acclimation, with constant dissolved oxygen levels (6.9 ± 0.12 mg L^{-1}), temperature (28 ± 0.01 °C), salinity (28 ppt) and photoperiod 14:10 h (light: dark). Animals were fed daily with commercial feed containing 38% crude protein (Potimar Active 38 - Guabi) offered ad libitum.

2.2. Essential oils

Leaves of *Lippia alba* (Mill.) N. E. Brown (Verbenaceae) were obtained from fresh plants cultivated at the Universidade Federal de Santa Maria campus, Frederico Westphalen, Southern Brazil. The EO extraction from the leaves of these plants was performed by steam distillation, in a Clevenger apparatus and stored at -20ºC. Synthetic linalool (a mix of S- (+) and R- (-) isomers) was commercially acquired from Sigma-Aldrich.

2.2. Experimental design

The experimental design consisted in the following treatments (n = 15 females and 15 males per treatment): (i) pure seawater (control); (ii) ethanol (9000 µL L^{-1} of ethanol, equivalent to the highest concentration used for dilution of EOLA); (iii) 750 or 1000 µL L^{-1} of EOLA; (iv)

350 or 500 µL L^{-1} of linalool. The anesthetics were diluted in absolute ethanol at a ratio of 1:10 before being added to the aquaria with water.

Previously, animals were divided and placed individually into aquaria (containing 1 L of seawater) with anesthetics (EOLA or linalool), ethanol or pure seawater for 3 min to anesthesia. After, they were weighed. Then, females were ablated unilaterally by cauterization (cutting the eyestalk with hot scissors). In males, the spermatophores were removed via manual extrusion by slight dorsoventral pressure on the 5^{th} pair of pereiopods on the male (Petersen et al., 1996). In the end, females and males (n = 5 each) were euthanized by cryoanesthesia and their organs (gills, muscle, hepatopancreas) collected. Sampled tissues were placed into 1.5 mL polyethylene tubes and stored at -80 °C in an ultra-freezer. The remaining anesthetized shrimp were placed in the anesthetic-free tanks to measure the survival rate (12 h). Shrimp were individually marked by the different cutting of the uropod.

2.3. Biochemical assays

2.3.1. Preparation of sample

Samples were homogenized in buffer solution for crustaceans containing Tris-base (20 mM), EDTA (1 mM), $MgCl_2$ (0.05 mM), sucrose (5 mM), and KCl (1 mM), all dissolved in distilled water, adjusted to pH 7,2, and centrifuged at 20,000 × g for 20 min. The supernatants were aliquoted in 1.5 mL polyethylene tubes and stored at -80 °C in an ultra-freezer. Subsequently, the total protein content of the homogenized tissues was quantified by the Biuret method using the Total Proteins Kit Doles (ʎ = 550 nm) in a plate fluorimeter (Synergy HT, Biotek, USA).

2.3.2. Total antioxidant capacity against peroxyl radical (ACAP)

The total antioxidant competence (ACAP) was performed by the method described by Amado et al. (2009). The total ACAP was analyzed through the generation of peroxyl radicals

by thermolysis of ABAP (2,2-azobis-2-methylpropionamidinediamine hydrochloride; Sigma) at 37°C, which reacts with H_2DCFDA (2', 7' dichlorofluorescein diacetate) in the presence of ROS and generates a fluorescent compound (DCF). For this, 10 μL of tissue (six wells per sample), previously diluted to 2 mg protein mL^{-1} with homogenization buffer, 127.5 μL of reaction buffer (pH 7.2, 30 mM HEPES, 200 mM KCl, and 1 mM $MgCl_2$), 7.5 μL of ABAP solution at 20 μM (in three of the six wells), and 10 μL H_2DCF-DA at 40 μM (in all wells) were added in a white microplate. The reaction was detected at 485/525 nm for excitation/emission in the microplate reader and ROS formation was monitored for 30 min, with readings every 5 min (Synergy HT, Biotek, USA). The results were expressed as a relative area (the difference between the area with and without ABAP, divided by the area without ABAP).

2.3.3. Lipid peroxidation

The lipid peroxidation (LPO) was determined according to the methodology described by Oakes and Van der Kraak (2003), which consists of the fluorimetric quantification (emission/excitation 553/515 nm) of a reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). In test tubes, 20 μL of butylated hydroxytoluene solution (BHT, 67 μM) and 150 μL of 20% acetic acid solution were added to the samples (muscle: 100 μL, gills: 50 μL, hepatopancreas: 30 μL), 150 μL of TBA solution (0.8%), 50 μL of distilled water and 20 μL of sodium dodecyl sulfate (SDS, 8.1%). The samples were heated at 95 °C for 30 minutes. Then, 100 μL of distilled water and 500 μL of n-butanol were added to the final solution, which was centrifuged at 3,000 × g for 10 min at 15 °C. Next, 150 μL of the supernatant was placed in a microplate reader (Synergy HT, Biotek, USA). The results were expressed in nmol of tetramethoxypropane (TMP) (used as standard) per mg of wet tissue.

2.3.4. Measurement of reduced glutathione (GSH) and concentration of sulfhydryl groups associated with protein (P-SH)

The concentration of reduced glutathione (GSH) and sulfhydryl groups associated with protein (PSH) was measured using the DTNB (5,5-dithiobis-2-nitrobenzoic acid; Sigma-Aldrich) method according to Sedlak and Lindsay (1968), with modifications. The samples were precipitated with trichloroacetic acid (50% W/V) and centrifuged at 20,000 × g during 10 min at 4°C. An aliquot of 100 μL of the supernatant with 200 μL of 0.4 Tris-Base at p H 8.9 was transferred to transparent microplates for fluorescence detection by a fluorometer. The pellet, containing protein, was re-suspended with 240 μL of homogenization buffer for crustaceans for P-SH determination. Then, 100 μL of extract and 160 μL of 0.2 Tris-Base were added to the microplate and incubated for 15 min. The absorbance (405 nm) was read in 96-well transparent microplates (Synergy HT, Biotek, USA). Results were expressed in terms of μmoles of GSH equivalents/mg of proteins.

2.4. Statistical analysis

All data were expressed as means ± standard error (S.E.M). The homoscedasticity of variances was verified by the Levene' test.The significant differences between the samples were analyzed through one-way ANOVA, followed by Tukey's test. The Kruskal-Wallis ANOVA test (non-parametric test) followed by mean comparisons by ranks were used when data were not homogenous. The analyzes were performed using Statistica Software (version 7.0, StatSoft, Tulsa, OK), with minimum significance level of 95% ($p < 0.05$).

3. Results

Mortality was not observed during eyestalk ablation and spermatophore extrusion procedures and through recovery time.

3.1. Total antioxidant capacity against peroxyl radical (ACAP)

The ACAP in the gills of male shrimps in all treatments were significantly higher (lower relative area) when compared to the females. Within the females group, there was a significant difference between the control, ethanol, 750 µL L^{-1} of EOLA, and 350 µL L^{-1} of linalool, where females exposed to the concentration of 750 µL L^{-1} of EOLA presented higher antioxidant capacity when compared to control and linalool, respectively (Fig. 1a).

Differences between females and males were observed in the hepatopancreas in 500 µL L^{-1} of linalool, there was a decrease in ACAP for males. The ACAP of hepatopancreas of females was not affected significantly by the treatments. There was a significant increase in ACAP in the hepatopancreas of males in the control group when compared to other treatments (Fig. 1b).

In the muscle, differences between males and females were found to control, ethanol, and 750 µL L^{-1} of EOLA. In these cases, males presented higher antioxidant capacity (lower area relative) than females. For both sexes it was observed a decrease in ACAP with the increase of the anesthetic concentration. Within the control treatment, shrimps showed a higher ACAP than observed in EOLA or linalool groups. The concentration of 500 µL L^{-1} of linalool demonstrated lower ACAP when compared to the control group for both sexes (Fig. 1c).

3.2. Lipid peroxidation

In the gills, differences between males and females occurred in the following treatments: control, ethanol, 750 µL L^{-1} of EOLA, and 350 µL L^{-1} of linalool, where males presented higher lipid peroxidation compared to females. In males shrimp sedated with EOLA and linalool, there was a significant decrease in TBARS levels than the control and ethanol groups. Females exposed to 1000 µL L^{-1} had higher TBARS values compared to the control group (Fig. 2a).

The TBARS content in the hepatopancreas was relatively higher in relation to the other organs. A significant reduction of the TBARS levels in the hepatopancreas was observed in females treated with 500 µL L^{-1} of linalool than males in the same concentration. In males, there was an increase in lipid damage in the groups ethanol, 1000 µL L^{-1} of EOLA, and 500 µL L^{-1} of linalool when compared to the control treatment. The hepatopancreas of females sedated with 500 µL L^{-1} of linalool exhibited similar TBARS content to the control group (Fig. 2b).

TBARS content in muscle differed significantly between males and females exposed to the 1000 µL L^{-1} of EOLA and 350 µL L^{-1} of linalool, where males shrimp presented higher lipid peroxidation. Males sedated with 350 or 500 µL L^{-1} of linalool showed lower TBARS values than the control group. In addition, reductions in TBARS in the muscle were observed only in the females exposed to control and 500 µL L^{-1} of linalool. (Fig. 2c)

3.3. Measurement of reduced glutathione (GSH)

In the gills of females exposed to ethanol, 750 µL L^{-1} of EOLA, and 350 µL L^{-1} of linalool were observed higher levels of GSH compared to males to same concentrations. Concerning the control group, males exposed to 1000 µL L^{-1} of EOLA and 350 µL L^{-1} of linalool exhibited an increase and decrease of GSH activity, respectively. Females anesthetized with both anesthetics showed higher GSH levels in the gills than the control group, except for 500 µL L^{-1} of linalool (Fig. 3a).

The hepatopancreas of both males and females exposed to the control group, 750, and 1000 µL L^{-1} of EOLA presented similar values of GSH. Males of the control group showed lower GSH levels compared to other treatments, except for 750 µL L^{-1} of EOLA. Females anesthetized with 1000 µL L^{-1} of EOLA exhibited higher GSH activity compared to other treatments (Fig. 3b).

In the muscle, the concentration of 1000 µL L^{-1} of EOLA exhibited similar GSH activity for both sexes. However, within the other treatments, there was a significant decrease in GSH levels in males when compared to females. Males anesthetized with 1000 µL L^{-1} of EOLA demonstrated higher GSH activity than the control group. Exposure of females to 750 µL L^{-1} of EOLA and 350 µL L^{-1} of linalool caused a reduction in GSH values compared to the control group (Fig. 3c).

3.4. Concentration of sulfhydryl groups associated with protein (P-SH)

In the gills, the concentration of sulfhydryl groups in the females decreased significantly in all treatments compared to males, indicated oxidation in the proteins. Males exposed to 1000 µL L^{-1} of EOLA and 350 µL L^{-1} of linalool showed reduced P-SH levels compared to the control group. In the gills of females, there was an increase in the P-SH values in the ethanol group and 750 µL L^{-1} of EOLA in relation to the control treatment (Fig. 4a).

In hepatopancreas, there were no differences at 750 µL L^{-1} of EOLA between both sexes. However, females exhibited higher protein damage (lower P-SH levels) in the other treatments. There was an increase in P-SH activity in males at the concentration of 1000 µL L^{-1} of EOLA compared to the control group. Females anesthetized with 1000 µL L^{-1} of EOLA, 350 and 500 µL L^{-1} linalool showed reduced P-SH values than the control treatment (Fig. 4b).

In the muscle of females was observed an increase in the protein damage in ethanol and EOLA concentrations when compared to males. Values of P-SH in the males were significantly lower to control than other treatments, except for 350 µL L^{-1} of linalool. Within females, the ethanol group and EOLA concentrations presented higher P-SH levels than the control (Fig. 4c).

4. Discussion

The reproduction of penaeid shrimp is based on a basic technological package, characterized by genetic selection and induction to mature or reproduce. Advances in reproductive research have the potential for improving the shrimp industry and involve the development of endocrinological studies, commercial protocols, and alternatives to eyestalk ablation (Alfaro-Montoya et al. 2019). Different strategies could be used to enhance the reproductive performance of captive broodstock. Some studies observed differences in metabolic parameters associated with to use of nutrients stores between sexes during reproductive events (Rosa et al. 1997; Perazzolo et al. 2002; Sainz-Hernández et al. 2008). In addition, ablation has been linked to causing homeostasis imbalances, while the repetitive extrusion of spermatophores is associated with to process of deterioration or melanizing (Braga et al., 2018; Zacarias et al., 2019; Nakayama et al., 2020).

Stressful episodes may destabilize steady-state ROS levels of production or elimination processes (Lesser, 2006). The consequences of this enhancement can be inducing acute or chronic oxidative stress, disturbing cellular metabolism and its regulation, and damaging cellular constituents (Le Moullac and Haffner, 2000; Stoner, 2012; Manfrin et al., 2016). The EOs contain compounds that give their antioxidants activity, as phenols and monoterpenes. The antioxidant competence of the *L. alba* EO has been tested in some aquatic species, like *L. vannamei, F. paulensis, M. rosenbergii,* and *R. quelen* (Becker et al., 2012; Parodi et al., 2012; Souza et al., 2017; Cagol et al., 2020; Becker et al., 2021a). Also, linalool has been found to have sedative and antioxidant efficiency (Heldwein et al., 2014; Baldisserotto et al., 2018; Silva et al., 2017). In the present work, linalool was the primary constituent of EOLA, representing, 59.80% of its composition, followed by cineole (10.29%), germacrene D (6.49%), and germacrene B (4.28%). Our results showed that linalool, when used alone, produced a similar result to *L. alba* EO in the antioxidant response.

Analyses of TBARS are commonly used to assess oxidative damages (Hermes-Lima, 2015). LPO generates oxidation of the lipoproteins of cellular membranes, producing peroxides from polyunsaturated fatty acids (PUFAS) (Okpala et al., 2016). The concentration of 500 µL L^{-1} of linalool was able to prevent lipid damage in gills and muscles in females and males. Several biomolecules might reduce or minimize the process of LPO, including phenolic compounds and monoterpenes, known to scavenge malondialdehyde (MDA), the molecule measured in the TBARS assay (Zamora and Hidalgo, 2016). Interestingly, sedation of females with linalool resulted in lower levels of TBARS when compared to those exposed to EOLA. This study also revealed that TBARS values in the gills of the males were significantly higher than in females. However, both EOLA and linalool avoided the formation of peroxides and TBARS in the gills of the sedated males compared to the control group. Additionally, diets supplemented with EOLA promoted increased antioxidant enzymes and decreased LPO for the prawn *M. rosenbergii* (Cagol et al., 2020).

The antioxidant capacity enables the understanding of the defense strategies or adaptation of organisms to stressful conditions (Amado et al., 2009). In the present work, EOLA and linalool no induced improvement in the ACAP response in all concentrations in females and males. In general, non-sedated shrimp (control and ethanol group) had higher ACAP (lower relative area) than the anesthetized groups. Similarly, lower ACAP was found for this same species submitted to long-term anesthesia with EOLA and *C. citratus* EO (exposure time in both experiments was 6 hours) (Parodi et al., 2012; Becker et al., 2021b). In the gills, both sedated and non-sedated males exhibited higher ACAP values than females. Ablation causes an increase in respiratory rate and decreasing metabolism, which explains higher ACAP levels in the gills of females of the control and ethanol groups. Under these circumstances, shrimp spends more metabolic energy (Rosas et al., 1993). Consequently, needs mobilizing resources

from others tissues, such as muscle. However, it was also observed significative lipid damage, decrease GSH and increase ACAP values in the muscles of males.

Reduced glutathione (GSH) is the first line of antioxidant defense against excessive production of ROS, being responsible for the maintenance redox state of cells (Pamplona and Constantini, 2011). In the males, the concentration of 1000 μL L^{-1} of EOLA induced higher GSH activity in the gills, muscles, and hepatopancreas compared to the control. In turn, elevations in the content of P-SH groups in the hepatopancreas and muscle of males were observed at 1000 μL L^{-1} of EOC. The relation between the higher GSH and P-SH activity indicates that EOs should be inducing antioxidant responses against oxidative stress caused by extrusion of the spermatophore. Moreover, EOLA and linalool promoted higher hepatopancreas GSH levels in all males treatments. The balance between GSH production and use via reactions of conjugation by GST or GSSG controls the redox state of cells through feedback mechanisms (Dickinson and Forman, 2002; Hermes-Lima et al., 2015). Therefore, GSH responses are marked by an initial increase followed by depletion and a subsequent increase to equilibrium.

Thiol groups have fundamental roles in antioxidant protection, regulation of enzymatic activity, cellular signaling mechanisms, and others functions. (Dickinson and Forman, 2002). Under conditions of oxidative stress, these groups can undergo oxidation forming reversible disulfide bonds between protein thiol groups and low-molecular-mass thiols (Baba and Bhatnagar, 2018). GSH prevents the oxidation of the protein thiols by the formation of mixed disulfides protein resulted in glutathionylated proteins (Jones, 2006). Thus, PSH values quantify reversible protein oxidative (Erel, and Neselioglu, 2014). In females, the lower P-SH levels could suggest amino acid oxidation lipids and amino acids. These changes could be associated with higher metabolism and mobilization of nutrients due to an increase in energy requirements to gonad development.

The same concentrations induced different responses between females and males, which seems to be related to metabolic differences, as well as the procedure performed in the animals. Overall, linalool showed a protective effect against lipoperoxidation, in the females hepatopancreas. Studies reported that ablated females could be more susceptible to stress than males. A study by Almeida et al. (2014) with ablated *F. paulensis* demonstrated an increase in the antioxidant response after 45 days, while Becker et al. (2021b) observed a decrease in GSH levels after 6h of exposure to the EO of *C. citratus*. Therefore, more studies would be needed to assess the time of the response of GSH. Eyestalk ablation induces a higher release of CHH neuropeptides. In this way, spermatophore extrusion activates the prophenoloxidase system (proPo). These processes involve immunosuppression, antioxidant responses, and considerable energetic mobilization. Thus, shrimp welfare is necessary to ensure better reproductive results.

5. Conclusion

Our results showed that several effects of EOLA and linalool were dependent upon sex. Differences between males and females in biochemical activity indicated that metabolic efforts in shrimps were sex-related. Ablated females appear to be more sensitive to oxidative damages. However, the concentration of 500 μL L^{-1} of linalool improved the antioxidant response caused by eyestalk ablation. The protective effect was observed mainly in the hepatopancreas. In males, both concentrations of EOLA or linalool induced a mild antioxidant effect. Thus, the both sedatives tested have a role in protecting in reducing tissue damage in reproductive procedures. However, additional studies are needed to determine the antioxidative response of the females and males at different periods after the eyestalk ablation or spermatophore extrusion and the effects of anesthesia/sedation on reproductive parameters,

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Figures.

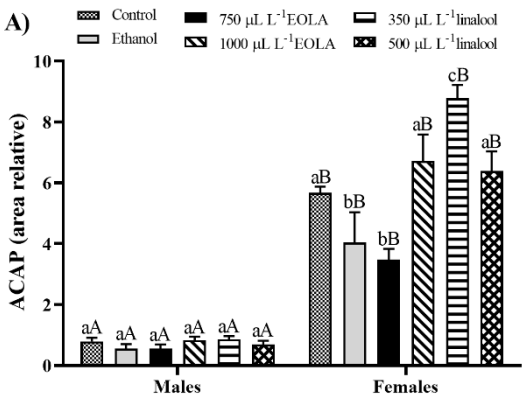
Figure 1. Total antioxidant capacity against peroxyl radicals (expressed as the relative area) in gill, hepatopancreas and muscle of males and females of *Litopenaeus vannamei.* Total antioxidant capacity against peroxyl radicals (ACAP) in gills (A), hepatopancreas (B), and muscle (C) in males and females of white shrimp *L. vannamei* sedated with sweater (control), ethanol, EOLA or linalool during extrusion of spermatophore and eyestalk ablation, respectively. Data were expressed as means ± S.E.M (n = 120). The letters (a, b, c) indicate significant differences between treatments for same-sex shrimp, and letters (A, B, C) indicate significant differences between females and males for the same treatment according to the Tukey's test at a significance level of 0.05.

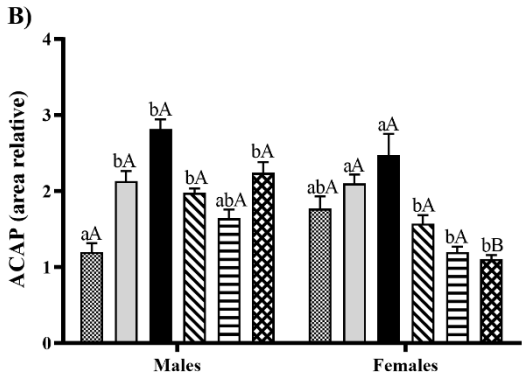
Figure 2. Content of substances reactive to thiobarbituric acid (nmol of TMP mg per tissue^{-1}) in gill, hepatopancreas and muscle of males and females of *Litopenaeus vannamei*. T Content of substances reactive to thiobarbituric acid (nmol of TMP mg per tissue^{-1}) gills (A), hepatopancreas (B), and muscle (C) in males and females of white shrimp *L. vannamei* sedated with sweater (control), ethanol, EOLA or linalool during extrusion of spermatophore and eyestalk ablation, respectively. Data were expressed as means ± S.E.M (n = 120). The letters (a, b, c) indicate significant differences between treatments for same-sex shrimp, and letters (A, B, C) indicate significant differences between females and males for the same treatment according to the Tukey's test at a significance level of 0.05.

Figure 3. Concentration of reduced glutathione (µmol of GSH mg protein^{-1}) in gill, hepatopancreas and muscle of males and females of *Litopenaeus vannamei.* Reduced (GSH) (µmol of GSH mg protein^{-1}) in gills (A), hepatopancreas (B), and muscle (C) in males and females of white shrimp *L. vannamei* sedated with sweater (control), ethanol, EOLA or linalool during extrusion of spermatophore and eyestalk ablation, respectively. Data were expressed as means ± S.E.M (n = 120). The letters (a, b, c) indicate significant differences between treatments for same-sex shrimp, and letters (A, B, C) indicate significant differences between females and males for the same treatment according to the Tukey's test at a significance level of 0.05.

Figure 4. Concentration of sulfhydryl groups associated with protein (SH mg protein^{-1}) in gill, hepatopancreas and muscle of males and females of *Litopenaeus vannamei.* Concentration of sulfhydryl groups associated with protein (SH mg protein^{-1}) in gills (A), hepatopancreas (B), and muscle (C) in males and females of white shrimp *L. vannamei* sedated with sweater (control), ethanol, EOLA or linalool during extrusion of spermatophore and eyestalk ablation, respectively. Data were expressed as means ± S.E.M (n = 120). The letters (a, b, c) indicate significant differences between treatments for same-sex shrimp, and letters (A, B, C) indicate significant differences between females and males for the same treatment according to the Tukey's test at a significance level of 0.05.

Fig. 1


A)
Control
Ethanol
750 µL L-1EOLA
1000 µL L-1EOLA
350 µL L-1linalool
500 µL L-1linalool
ACAP (area relative)
Males
Females


B)
ACAP (area relative)
Males
Females

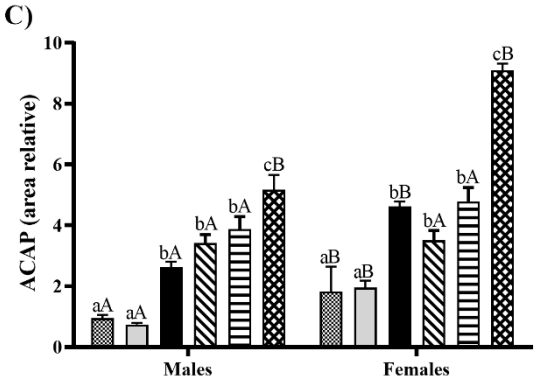
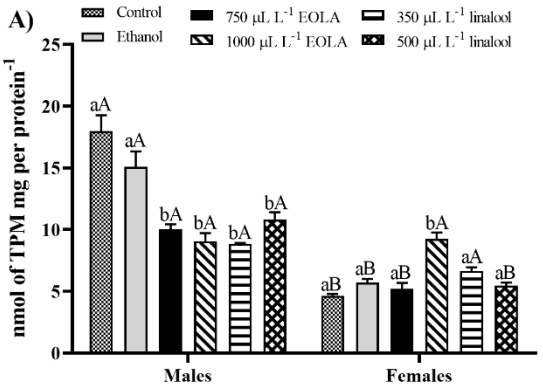
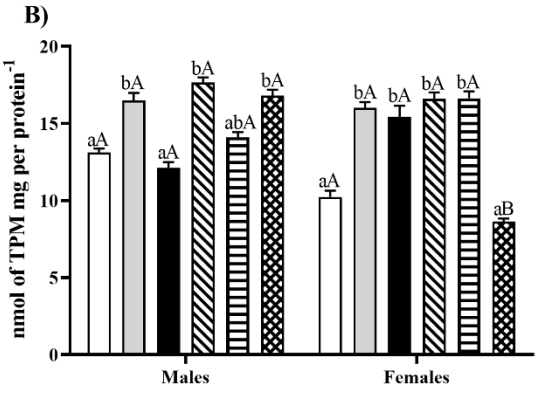

C)
ACAP (area relative)
Males
Females

Fig 2.


A)
Control
Ethanol
750 µL L-1 EOLA
1000 µL L-1 EOLA
350 µL L-1 linalool
500 µL L-1 linalool
nmol of TPM mg per protein-1
Males
Females


B)
nmol of TPM mg per protein-1
Males
Females

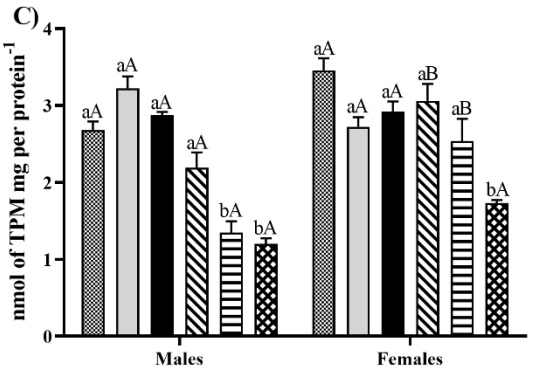

C)
nmol of TPM mg per protein-1
Males
Females

Fig 3.

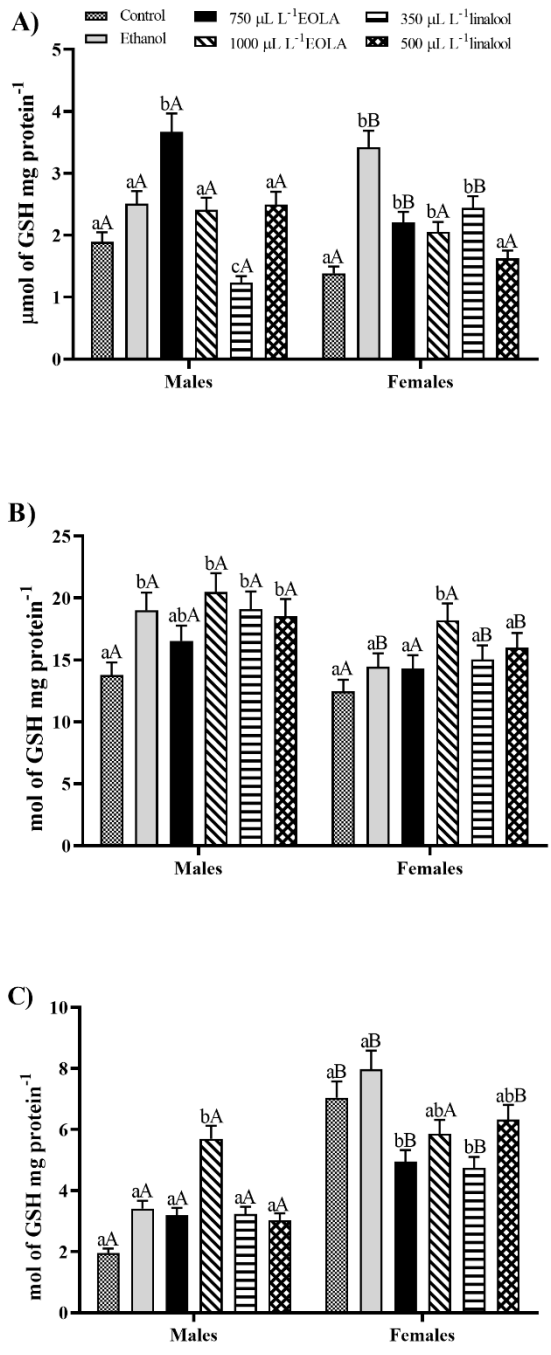
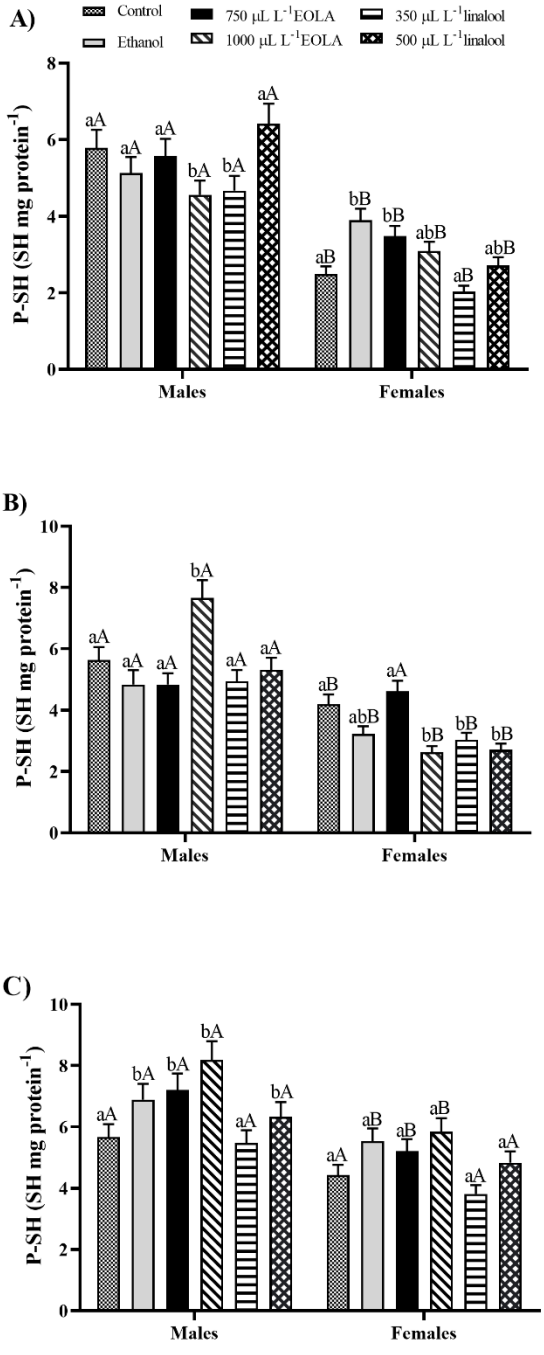

A)
Control
Ethanol
750 µL L-1EOLA
1000 µL L-1EOLA
350 µL L-1linalool
500 µL L-1linalool
µmol of GSH mg protein-1
Males
Females
B)
mol of GSH mg protein-1
Males
Females
C)
mol of GSH mg protein-1
Males
Females

Fig. 4


A)
Control
Ethanol
750 µL L-1EOLA
1000 µL L-1EOLA
350 µL L-1linalool
500 µL L-1linalool
P-SH (SH mg protein-1)
Males
Females
B)
P-SH (SH mg protein-1)
Males
Females
C)
P-SH (SH mg protein-1)
Males
Females

3.4 MANUSCRITO IV.

EXPOSURE OF *HYALELLA BONARIENSIS* (CRUSTACEA, AMPHIPODA) TO ESSENTIAL OILS: EFFECTS ON ANESTHESIA AND SWIMMING ACTIVITY

EXPOSIÇÃO DA *HYALELLA BONARIENSIS* (CRUSTACEA, AMPHIPODA) EM ÓLEOS ESSENCIAIS: EFEITOS DE ANESTESIA E NA ATIVIDADE DE NATAÇÃO

O manuscrito será submetido para publicação no periódico *Fishes.*

Exposure of *Hyalella Bonariensis* (Crustacea, Amphipoda) to Essential Oils: Effects on Anesthesia and Swimming Activity

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ABSTRACT

Amphipods are frequently used as bioindicators of water quality in experimental or behavior trials. Thus, it is a group considered suitable for use as a model organism in tests with essential oils (EOs). This study evaluated the time required for anesthesia induction and recovery of the amphipod crustacean *H. bonariensis* exposed to the essential oils of *Aloysia triphylla* (EOAT) and *Lippia alba* (EOLA), and their major compounds citral and linalool, respectively. In addition, we evaluated the locomotor activity of amphipods using ANY-maze® software. Mortalities were observed at concentrations of 100 and 200 µL/L of citral (50.0 ± 0.39%) and 750 µL/L of EOLA (66.7 ± 0.33%). Except for linalool, increased concentrations of the compounds of the essential oils decreased the time for sedation and anesthesia induction. There were differences for the induction of anesthesia ($p < 0.05$) and recovery ($p < 0.05$) between EOLA and linalool treatments, but not between that for EOAT and citral. Reduced locomotor activity and longer time and episodes of freezing were observed in animals exposed to EOAT. The EOs and their major compounds induced anesthesia and affected the locomotor activity of *H. bonariensis*, Therefore, EOAT and linalool are recommended for anesthesia of this species. EOAT can also be utilized in long-term exposure.

KEYWORDS

Amphipod, behavior, citral, linalool,locomotor activity, natural anesthetics

1. Introduction

The genus *Hyalella* Smith, 1874 (family Hyalellidae, order Amphipoda) includes about 91 described species distributed from Patagonia to Canada (Bento & Buckup 1999). These amphipods consist of freshwater and some marine species that live in the benthic environment, which is usually associated with algae or sediment (Bueno *et al*., 2014; Castiglioni *et al*., 2016). Amphipods are important species in the trophic chain and are used as bioindicators of water quality in ecotoxicological trials due to their high sensitivity to environmental impacts and contamination, short life cycles, easy sampling, and simple laboratory maintenance (Duan *et al*., 1997; Neuparth, *et al*., 2002; Castiglioni and Bond-Buckup, 2007; Ding *et al*., 2011).

Behavioral changes provide important tools for the ecological and health status of animals (Barr *et al*., 2008, Elwood, 2011). For example, changes in locomotion, swimming speed, feeding, and ventilation frequency may indicate neurotoxic actions or interference in the neuromuscular transmission by various substances in experimental assays (De Lange *et al*., 2014; Bownik, 2015). Stress affects the locomotor capacity (Mark and Nesse, 1994), social interactions, and escape from predators (Fossat *et al*., 2014), as observed in experiments with amphipod *Gammarus fossarum* (Perrot-Minnot *et al*., 2017) and crayfish (*Procambarus clarkii*) (Bacqué-Cazenave *et al*., 2017). Observations of individuals of a species can contribute to understanding the relationship between environmental factors and populations, which would support management, either for conservation at the environmental level or for standardization of laboratory protocols (Boyd *et al*., 2002).

A variety of natural and synthetic anesthetic substances have been investigated to reduce metabolism and stress in crustacean species (Valente, 2022). Previous studies have reported the use of tricaine methanesulfonate (MS-222), Aqui-STM (Coyle *et al*., 2005), 2-phenoxyethanol (Jensen *et al*., 2013), and quinaldine (Guzmán-Sáenz *et al*., 2010) on crustaceans. However, these compounds were found to be neither sufficiently safe nor effective for crustaceans.

Essential oils (EOs) have been widely employed with a diversity of invertebrates, including amphipods and shrimp, mainly because they have therapeutic properties, are easily accessible, and biodegrade in the environment. Clove oil (*Eugenia caryophyllata*); the EOs of *Lippia alba* (EOLA), *Aloysia triphylla* (EOAT) and *Melaleuca alternifolia*; and some major EO compounds, such as eugenol, terpinen-4-ol, linalool, and citral, have been found to present sedative, anesthetic, and antioxidant properties in some species, including *Daphnia magna* (Bownik 2015), *Gammarus minus* (Venarsky and Wilhelm, 2006), *Litopenaeus vannamei* (Parodi *et al*., 2012), *Macrobrachium rosenbergii* (Saydmohammed and Pal 2009), and *Neohelice granulata* (Souza *et al*., 2018).

In small invertebrates, anesthetics can be used for short-term immobilization, such as for in vivo studies, microscopic analysis, the application of sensors in physiological assessments, and the manipulation of species in thewild. We hypothesized that *H. bonariensis* (Santos *et al*., 2008) may be suitable for use as a behavioral model in EOs tests. Therefore, the aim of this study was to determine the time required for anesthesia induction and recovery of *H. bonariensis* exposed to EOAT and EOLA and their major compounds citral (mix of neral and geranial) and linalool (mix of S-(+) and R-(-) isomers) and evaluated their effects on the locomotor activity of amphipods using the ANY-maze® video monitoring software. Our hypothesis is that the EOs and compounds tested will induce anesthesia and reduce locomotor activity of H. bonariensis.

2. Material and methods

2.1. Animals

Specimens of *H. bonariensis* (5 mm) were collected in Santa Maria municipality in the central region of Rio Grande do Sul, South Brazil. The crustaceanswere collected with a hand net (250 μm mesh) and transported to the laboratory in 150 mL plastic bottles with a maximum

of five individuals each. In laboratory maintained at a temperature of 20°C and photoperiod-controlled room (12L:12D), the animals were acclimatized in continuously aerated 5 L aquaria with leaves and sediment in the bottom. They were left in acclimatization conditions for at least one week before study.

2.2. Essential oils and major constituents

L. alba (Mill.) N. E. Brown (Verbenaceae) (EOLA) and *A. triphylla* (L'Herit) Britton (Verbenaceae) (EOAT) were obtained from plants cultivated at the Universidade Federal de Santa Maria campus at Frederico Westphalen, Rio Grande do Sul. The EOs extraction was performed via hydrodistillation as described by Parodi *et al*. (2012). Linalool and citral were acquired from the company Sigma-Aldrich. The major components identified for EOLA were linalool (59.8%), cineole (10.29%), germacrene D (6.49%); germacrene B (4.78%) and β-caryophyllene (3.64%) and [1R-(1R*,4E,9S*)]-Bicyclo [7.2.0] undec-4-ene (4.78%). The EOAT was mainly composed of β-citral (45.59%), 1-methyl-4-(1-methylethenyl)-cyclohexene (20.26%), caryophyllene (6.02%), [1R-(1R*,4R*,6R*,10S*)]-5-Oxatricyclo [8.2.0.0(4,6)-] dodecane (4.3%).

2.3. Anesthesia induction and recovery

For the determination of the anesthetic activity, 112 amphipods (n = 8 animals per concentration and anesthetic) were randomly divided and placed into 50 mL beakers (n = 2 amphipods per beaker). Animals were exposed to the following concentrations: clean dechlorinated tap water (control) or solutions containing ethanol (6750 µL/L, equivalent to the highest concentration used to dilute EOLA) with either (i) 250, 500, or 750 µL/L of EOLA, (ii) 150, 300 or 500 µL/L of EOAT, (iii) 100, 200 or 400 µL/L of linalool, or (iv) 100, 200 or 400 µL/L of citral. After the exposure, animals were transferred to containers free of anesthetics to determine recovery time. The anesthetics were diluted in absolute ethanol at a ratio of 1:10 before being added to the test beakers. Anesthetic induction and recovery were evaluated

according to Coyle et al. (2005): partial loss of equilibrium (stage 1 - sedation), total loss of equilibrium and no reaction to external stimuli (stage 2 - anesthesia), and recovery of equilibrium and body movement (stage 3 - recovery). Each amphipod was tested only once. The maximum observation time for sedation or anesthesia induction was 30 min. Induction time and recovery time were recorded using a digital stopwatch (expressed in seconds). The studied concentrations were selected according to preliminary tests to observe if the EOs and compounds could induce sedation and/or anesthesia using n = 3 for each concentration, from 25 to 750 µL/L of each EO and compound.

2.4. Locomotor activity

Forty amphipods (n = 5 animals per concentration and anesthetic) were transferred individually to transparent aquaria containing 40 mL aerated freshwater (± 24°C). The following treatments were tested: (i) clean dechlorinated tap water (control), (ii) 1800 µL/L of ethanol, (iii) 75 µL/L of EOAT, (iv) 100 and 200 µL/L of EOLA, or (v) 50 and 75 µL/L of linalool. The animals were exposed to anesthetic baths in each treatment for 5 min. In this second experiment, concentrations were chosen based on the results of the anesthesia experiments. The concentrations used were the lowest concentrations required for anesthesia induction. The aquarium test was divided into four different virtual zones (A—upper side; B—bottom side; C—right side; D—left side) to delimit the locomotor activity and location of the animals.

Animal movements were recorded for 5 min with a digital camera (Sony Cyber-shot DSC-H300). Digital analysis of the videos was performed using ANY-maze® software (Stoelting CO, USA) with the aim scoring the following behavioral parameters: total distance traveled (m); mean speed (m/s), maximum speed (m/s); absolute turning angle; freezing episodes, time freezing (s); number of crossings between the tank zones; number of entries in

each virtual zone (upper/bottom and right/left) and dwelling time in each zone (upper/bottom and right/left) (Table 1). The videos for each anesthetic were analyzed separately**.**

[TABLE 1]

2.5. Statistical analysis

The data were expressed as the mean ± SE. The homoscedasticity of variances was verified with the Levene's test and normality was assessed using the Kolmogorov-Smirnov test. The significant difference between the time needed for anesthesia induction and the concentration of the anesthetic were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The total distance traveled, mean speed, maximum speed, absolute turning angle, freezing episodes, number of crossings between the tank zones, number of entries in each virtual zone and dwelling time in each zone data were compared using one-way ANOVA followed by Tukey's test, or the Kruskal-Wallis test, followed by Dunn's post hoc test. Analyses were performed using the Statistical version 7.0 (StatSoft, Tulsa, OK) software, and the minimum significance level was set at $p < 0.05$.

The graphics of total distance traveled, mean speed, maximum speed, absolute turning angle, freezing episodes and time freezing were performed using Graph Pad Prism 6 (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Anesthesia induction and recovery with EOLA and linalool

Mortality occurred at 66.7 ± 0.33% at 750 µL/L of EOLA, mainly during the recovery time. Increasing concentrations of EOLA decreased the time required for the induction of sedation and anesthesia stages, but this relationship was not observed for linalool (Figure 1a, b). The EOLA concentration of 750 µL/L shortened the time for the induction of stages 1 (less than 1 min) ($F = 11.883$; $p = 0.0026$) and 2 of anesthesia ($F = 12.039$; $p = 0.0024$) compared

with 250 or 500 µL/L of EOLA. The time for the induction of stage 2 of anesthesia to 250 or 500 µL/L of EOLA was 3.27 ± 0.08 min and 2. 15 ± 0.04 min, respectively. However, the recovery time was significantly shorter following exposure to the lower EOLA concentrations (F = 13.802; p = 0.001) (Figure 1a). There were no significant differences with stages 1 and 2 of anesthesia between 100 and 200 µL/L of linalool. The concentration of 400 µL/L of linalool reduced the time required for the induction of anesthesia (stage 2) (F = 8.431; p = 0.015) and increased recovery time (F = 2.795; p = 0.010) (Figure 1b). Amphipods anesthetized with 500 and 750 µL/L of EOLA showed a lower time for the induction of anesthesia (stage 2) than those anesthetized with 100 and 200 µL/L of linalool. Recovery from anesthesia was significantly longer at 750 µL/L of EOLA compared with all EOLA and linalool treatments.

3.2. Anesthesia induction and recovery with EOAT and citral

Mortality was 50.0 ± 0.39% at the concentrations of 100 or 200 µL/L of citral. Increasing concentrations of EOAT and citral decreased the time required for the induction of sedation and anesthesia stages. Amphipods anesthetized with EOAT demonstrated a reduced time for the induction of stages 1 (F = 11.925; p = 0.0001) and 2 (F = 15.358; p = 0.008) with higher concentrations. The EOAT concentration of 500 L/L induced fast sedation (1.15 ± 0.01 min) and anesthesia (1.87 ± 0.11 min). However, the recovery time was significantly lower for 150 and 300 L/L EOAT (F = 12.194; p = 0.002) (Figure 1c). The concentration of 100 µL/L of citral promoted the longest time required for the induction of anesthesia stages 1 (F = 18.925; p = 0.0008) and 2 (F = 15.358; p = 0.008) (3.26 ± 0.03 min and 7.00 ± 0.18 min, respectively). The shortest times for the induction of anesthesia were observed at 400 µL/L of citral. The recovery times were significantly higher for 200 µL/L of citral compared to 100 µL/L of citral (7.33 ± 0.14 min and 5.11 ± 0.60 min, respectively) (F = 11.794; p = 0.003) (Figure 1d). The concentration of 150 µL/L of EOAT and 100 µL/L of citral induced longer sedation (stage 1) and anesthesia (stage 2) compared to the other EOAT and citral treatments. No differences were

found for the induction of anesthesia stage 1 and stage 2 between the EOAT and citral. The recovery times were longer at 200 µL/L of citral and 500 µL/L EOAT.

[FIGURE 1]

3.3. Locomotor activity

EOAT resulted in the most distinct behavioral parameters in relation to swimming speed, line crossings, distance traveled, and freezing compared to the control groups. EOLA and linalool treatments resulted in locomotor activity that was similar to that of the control group, with the exception of the maximum speed observed at 200 µL/L. Greater agitation and slight loss of equilibrium were observed for the ethanol group at the initial time. Linalool caused agitation of the animals throughout the time of exposure.

The concentration of 75 µL/L of EOAT resulted in a lower mean speed compared to the control group ($F = 1.1583$; $p = 0.006$) (Figure 2a). The values of maximum speed at 75 µL/L of EOAT and 200 µL/L of EOLA were significantly lower than those of the control ($F = 6.096$; $p = 0.001$) (Figure 2b). There were no significant differences in absolute turn angle between concentrations of EOs and major compounds in relation to the control and ethanol groups ($F = 0.504$; $p = 0.070$) (Figure 2c). The concentration of 75 µL/L of EOAT resulted in a higher time of freezing compared to all other samples evaluated ($F = 2.899$; $p = 0.0007$) (Figure 2d), while freezing episodes showed no differences between treatments with EOs and major compounds compared to the control group. (Figure 2e).

[FIGURE 2]

The total distance that the amphipods traveled between each zone of the aquarium was significantly lower for those exposed at 150 µL/L of EOAT when compared to the control groups ($F = 1.161$; $p = 0.011$) (Figure 3a). The number of crossings observed between the different tank zones was similar for the treatments with the addition of EOs or major compounds compared to the control group (Figure 3b).

[FIGURE 3]

Overall, there were no differences in the number of entries between zones in each treatment. The amphipods submitted to both concentrations of linalool showed a higher number of entries for the treatments with EOLA and ethanol when compared to the control group. Amphipods exposed to EOAT exhibited a decreased number of entries to all zones compared with those of the control (Figure 4). There were no preferences for different zones observed between all EOs and major compounds. The animals exposed to 50 µL/L of linalool, 75 µL/L of EOAT, 100 and 200 µL/L of EOLA remained for a longer time at the bottom of the aquarium (zone B) than the control group did. In contrast, amphipods of the control and ethanol groups stayed for longer periods on the right side (zone C) but without differences between zones A and B for the ethanol group. In the control group, amphipods spent most of the time swimming in the upper zone (zone A) compared with the bottom zone (zone B).

[FIGURE 4]

4. DISCUSSION

4.1 Anesthesia induction and recovery

Different compositions of EOs may result in distinct pharmacological effects during anesthesia. These differences in composition are influenced by environmental conditions, soil cultivation, collection season, genotypic variations, and extraction method (Gobbo-Neto and Lopes, 2007). In the present study, linalool and β-citral were identified as the primary constituents of EOLA and EOAT, representing 59.80% and 45.59%, respectively. The results demonstrated that linalool and citral alone were effective on the amphipods, *H. bonariensis*, as sedative and anesthetic substances. Additionally, EOAT and citral were equally successful in anesthesia induction, but EOLA was less efficient than linalool.

In this research, we found that the concentration of EOLA directly influenced the time required to induce anesthesia in *H. bonariensis*. The shrimp *L. vannamei* anesthetized with EOLA presented similar responses (Parodi *et al*., 2012). However, the time taken by the amphipod to recover from anesthesia was longer with the increased EOLA concentrations. Moreover, the higher EOLA concentration induced anesthesia faster but resulted in a toxic effect. The EOLA was more effective in terms of speed of anesthesia induction and recovery times for *H. bonariensis* than has been observed for *L. vannamei* and *F. paulensis* exposed to a similar concentration of 500 µL/L of EOLA (16 and 30 min, respectively) (Parodi *et al*., 2012, Becker *et al*., 2021a). In general, small crustaceans have a higher sensitivity to anesthesia due to greater gill surface area in relation to body size (Bownik, 2015; Li *et al*., 2018).

Linalool occurs naturally in two isomeric forms, which differ according to carbon 3 chirality, characterized by the levorotatory form (*3R*-(-)-linalool or licareol) and the dextrorotatory form (*3S*-(+)-linalool or coriandrol) (Sugawara *et al*., 1998). According to some studies, *S*-(+)- and *R*-(-)- linalool presented biological differences (Mitra and Chopra, 2011). Silva et al. (2017) did not observe differences in the induction times for stage 2 of anesthesia for silver catfish *Rhamdia quelen* exposed to both isomers (Silva *et al*., 2017). In comparison with EOLA, linalool induces anesthesia and recovery with a longer time at a concentration of 200 µL/L. On the other hand, the concentration of 400 µL/L of linalool led to a faster recovery without causing mortality. Both linalool and EOLA induced anesthesia within the indicated time frame for crustaceans (3 – 5 min) (Souza *et al*., 2019a). However, linalool has been shown to be safer for use in studies with *H. bonariensis*.

The pharmacological action of EOs could be a direct effect of major compounds, interactions among active substances, or the synergistic activity between constituents (Heldwein *et al*., 2014). Our results indicated that there was no significant difference in time to induce anesthesia between EOAT and citral. The concentrations of 150 µL/L and 100 µL/L of

EOAT induced anesthesia at above to time range recommended (higher than 5 min). The mortality of the amphipods anesthetized with 100 or 200 µL/L of citral can be related to the longer time of exposure compared to those exposed at 400 µL/L of citral. Anesthesia with the citral chemotypes of *L. alba* is not recommended for *R. quelen* because it caused a stressful condition (Souza *et al*., 2019b). These results support the hypothesis that the final effect of the EOAT is the result of the synergism of its different components.

4.2. Locomotor activity

Anesthetic substances may result in behavioral alterations, including locomotor performance, swimming velocity, reduction of complex or aggressive movements, and stimulation of the frequency of stationary behaviors (Ozeki 1975; Bownik 2015, 2016; Cowing *et al*., 2015). The different methods that were used for the behavior assessment indicated that EOLA and linalool concentrations used in the locomotor experiment did not result in significant behavioral changes. However, parameters of speed, distance traveled, freezing behavior, and number of entries in the different zones of the aquarium showed changes in response to EOAT, mainly compared to the control group.

The reduction in mean velocity, maximum velocity, number of crossings, and distance traveled in the aquarium by amphipods exposed to EOAT was related to the increase in time without moving. The decrease in maximum velocity at 100 µL/L of EOLA and 75 µL/L of EOAT may be explained by the interaction on the γ-aminobutyric acid (GABA) receptor complex (Heldwein *et al*., 2012) or inhibition of locomotor activity due to depressive action on neuromuscular synapses, respectively (Bownik, 2015). Furthermore, recent studies have revealed the involvement between a GABA neurotransmitter and metabotropic glutamate receptors in the regulation of anxiolytic effects in invertebrates (Swanson *et al*., 2005; Durant et al., 2009; Perrot-Minnot et al., 2017). A decrease in the swimming velocity was also observed

in *D. magna* exposed to clove oil (Bownik, 2015) and *L. vannamei* anesthetized with *Cymbopogon citratus* EO (Becker *et al*., 2021b).

The *Hyallela* genus is an essential part of the benthic macrofauna in aquatic environments (Kruschwitz, 1978; Wellborn, 1995; Muskó, 1990). Its population distribution is regulated mainly by the presence or absence of aquatic macrophytes in rivers or lakes (Jacobucci and Leite, 2008; Castiglioni *et al*., 2016). Light stimuli in the eyes of amphipods are associated with escape behavior to avoid stressful or dangerous situations (Araújo *et al*., 2015). Thus, the presence of substrates or refuges structures determines the behavioral pattern of *H. bonariensis* in both natural and artificial environments. We observed that the addition of EOs can help prevent negative effecs caused by the absence of a substrate in the laboratory or transport this species to other places Moreover, this hypothesis is confirmed by the higher activity of the control group near the upper zone of the aquarium. The results suggest that the EOs prevent alterations in the natural tendency of the location or distribution of amphipods in the bottom of the aquarium.

In crustaceans, stress behavior results in increased aggressiveness and locomotor activity as a primary response. Chronic stress provokes a increase in metabolic consumption of by organisms, compromising health status, reproduction, foraging behavior, and the sociability of the animals (Fossat *et al*., 2015; Hamilton *et al*., 2016; Crook *et al*., 2014). Some amphipod species increase investment in essential behavior, such as mating behavior or food-intake rates, as a form of reducing the state of vulnerability and utilize a protective or defensive response to stressful experiences (Dunn *et al*., 2008; Dianne *et al*. 2014; Mohammad *et al*., 2016; Perrot-Minnot *et al*., 2017). This type of behavior variation in amphipods influences other behaviors, such as swimming patterns and the response of escape from predators. These events can trigger consequences at the individual or population level, endangering energetic transference within

important aquatic food webs in stream ecosystems (MacNeil *et al*., 1999; Bossus *et al*., 2014; Worischka *et al*., 2015).

The EOAT and EOLA induced an anxiolytic behavior in zebrafish *Danio rerio* and *R. quelen* through an increase in swimming activity in the upper section of the tank, without altering locomotion (Bandeira Junior *et al*., 2018). Consistently, EOLA and 50 µL/L of linalool did not influence the swimming pattern of *H. bonariensis,* but EOAT was responsible for the reduced exploratory activity of amphipods in the present experiment. These results do not demonstrate the anxiolytic action of EOs to *H. bonariensis,* but further studies are needed to demonstrate if this effect is related to the protection of stress.

The evaluation of the effects of natural products on aquatic animals are important for reducing the impact of chemical products in aquaculture. As demonstrated in the current study, some of the products tested provoked mortality at some of the concentrations tested. The amphipod *H. bonariensis* proved to be an interesting model for testing natural products, complementing other aquatic organisms traditionally used in toxicological tests, such as zebrafish embryos (Capatina *et al.* 2020; Capparuci *et al.* 2022).

5. Conclusions

In conclusion, our study provides information regarding the anesthetic action of EOs and two major EO compounds on the behavioral activity of the amphipod *H. bonariensis*. The concentrations of 250 or 500 µL/L of EOLA; 100, 200, or 400 µL/L of linalool; 150, 300, or 500 µL/L of EOAT; and 400 µL/L of citral were effective in the induction of sedation and anesthesia in *H. bonariensis*. Due to their toxic natures, EOA concentrations higher than 500 µL/L and citral are not recommended as an anesthetic for this species. EOAT is recommended for behavioral and long-exposure tests and could also potentially be utilized in the transport, immobilization into laboratory analyses, or collection of these amphipods.

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Table 1. Description of the behavioral features analyzed by ANY-maze® software.

Behavioral Category	Behavior	Description
Speed	Mean speed	Average animal speed as a function of the distance traveled in the aquarium.
	Maximum speed	Maximum speed of the animal in the aquarium.
Maneuvering	Absolute turning angle	The sum of the absolute angle between each movement vector of the animal.
Immobility	Freezing episodes	Number of times the animal froze during the test.
	Time freezing	Total amount of time during the test that the animal was freezing.
Tank exploration	Total distance	Sum of the total distance that the animal travelled between each point during the test.
	Crossings between the tank zones	Numbers of crossings of the animal between the different zones of the aquarium.
	Entries in each virtual zone	Counts the number of times the animal entered in each zone.
	Dwelling time in each zone	Total amount of time the animal spent in the zone.

Figures

Figure 1. Time required for anesthesia induction and recovery of the amphipod *H. bonariensis* exposed to *L. alba* EO (a), linalool (b), *A. triphylla* EO (c), and citral (d). Different lowercase letters above the bars indicate significant differences in the time required for anesthesia induction or recovery between concentrations for the same anesthetics or major compounds. Different uppercase letters indicate significant differences in the time required for anesthesia induction or recovery between the anesthetics and their major compounds (*L. alba* X linalool; A. *triphylla* X citral) ($p < 0.05$). Data are presented as mean ± SEM ($n = 8$).

Figure 2. Locomotor parameters and comparison between immobility periods observed to *H. bonariensis* groups during behavioral trial. Mean speed (a). Maximum speed (b). Absolute turning angle (c). Time of freezing (d). Freezing episodes (e). Different letters on the right side of the bars indicate significant differences between anesthetic concentrations ($p < 0.05$; by one-way ANOVA followed by Tukey's or Kruskal-Wallis tests; $n = 5$ per group).

Figure 3. Tank exploration of *H. bonariensis* exposed to control, ethanol, 75 µL/L of *A. triphylla* EO (EOAT). 50 and 75 µL/L of linalool, 100 and 200 µL/L of *L. alba* EO (EOLA). Distance (a). Line crossings (b). Different letters indicate significant difference in stay at the aquarium zones between the different treatments ($p < 0.05$; by one-way ANOVA followed by Tukey's or Kruskal-Wallis tests; $n = 5$ per group).

Figure 4. Comparative analysis of the time spent in each zone of aquarium for the amphipod *H. bonariensis* during exposure to control, ethanol, 75 µL/L of *A. triphylla* EO (EOAT). 50 and 75 µL/L of linalool, 100 and 200 µL/L of *L. alba* EO (EOLA).

Fig 1.

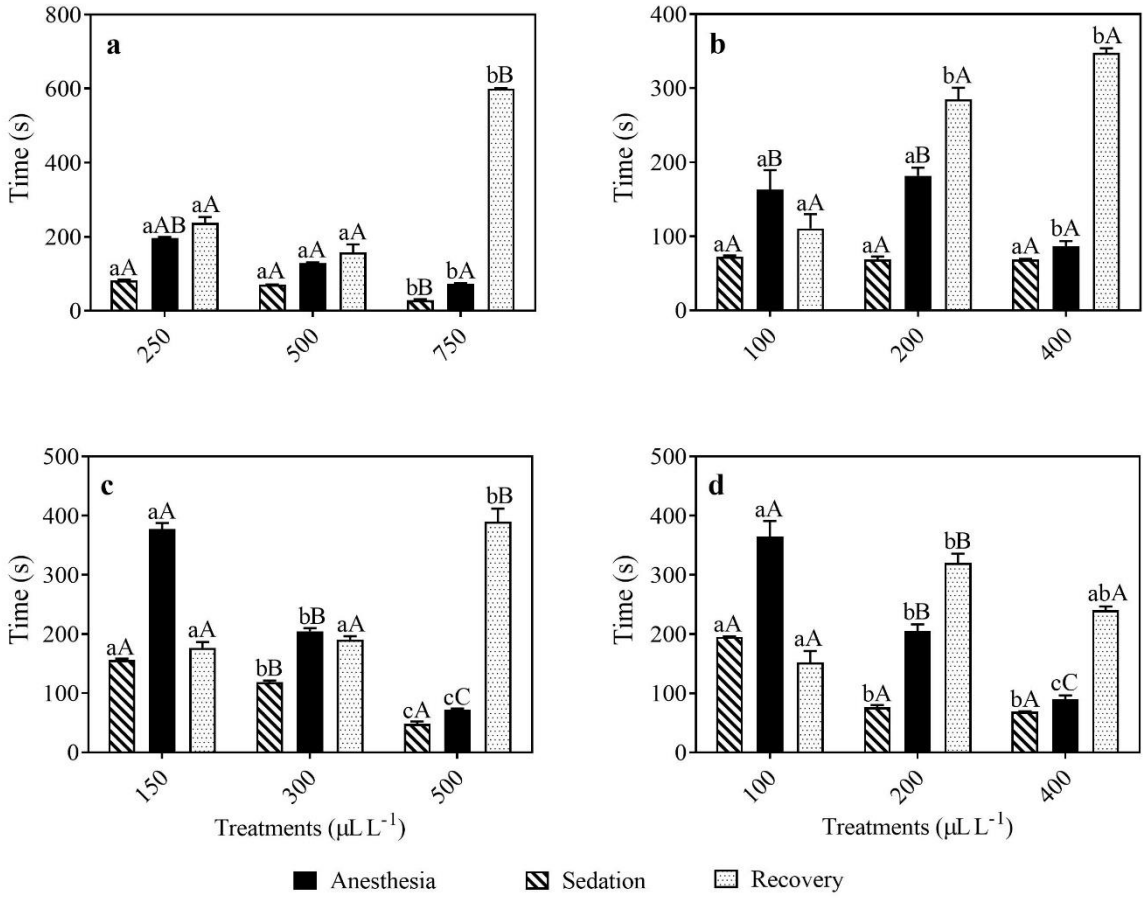


Fig. 2.

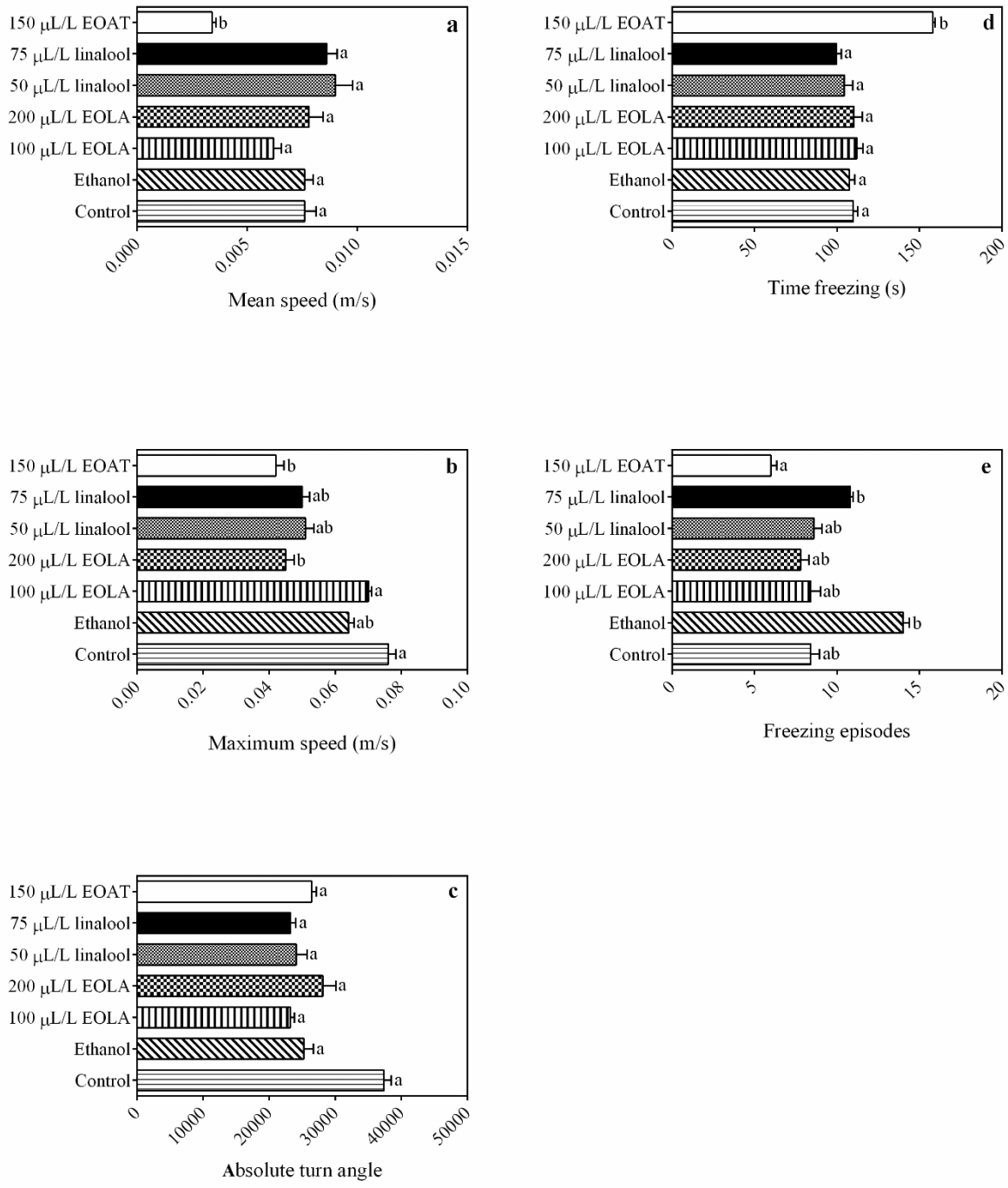

a
150 µL/L EOAT
75 µL/L linalool
50 µL/L linalool
200 µL/L EOLA
100 µL/L EOLA
Ethanol
Control
b a a a a a a
0.000 0.005 0.010 0.015
Mean speed (m/s)
d
b a a a a a a
0 50 100 150 200
Time freezing (s)
b
b ab ab b a ab a
0.00 0.02 0.04 0.06 0.08 0.10
Maximum speed (m/s)
e
a b ab ab ab b ab
0 5 10 15 20
Freezing episodes
c
a a a a a a a
0 10000 20000 30000 40000 50000
Absolute turn angle

Fig 3.

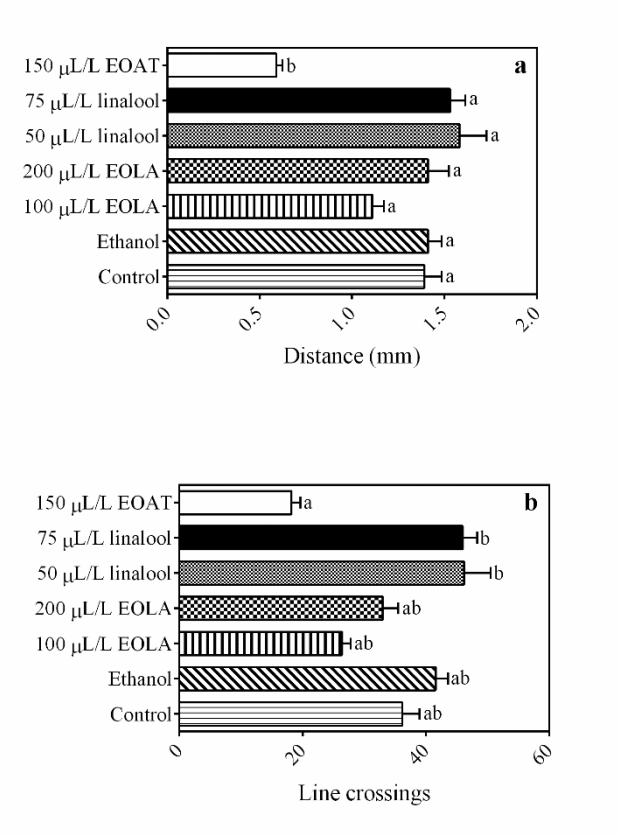
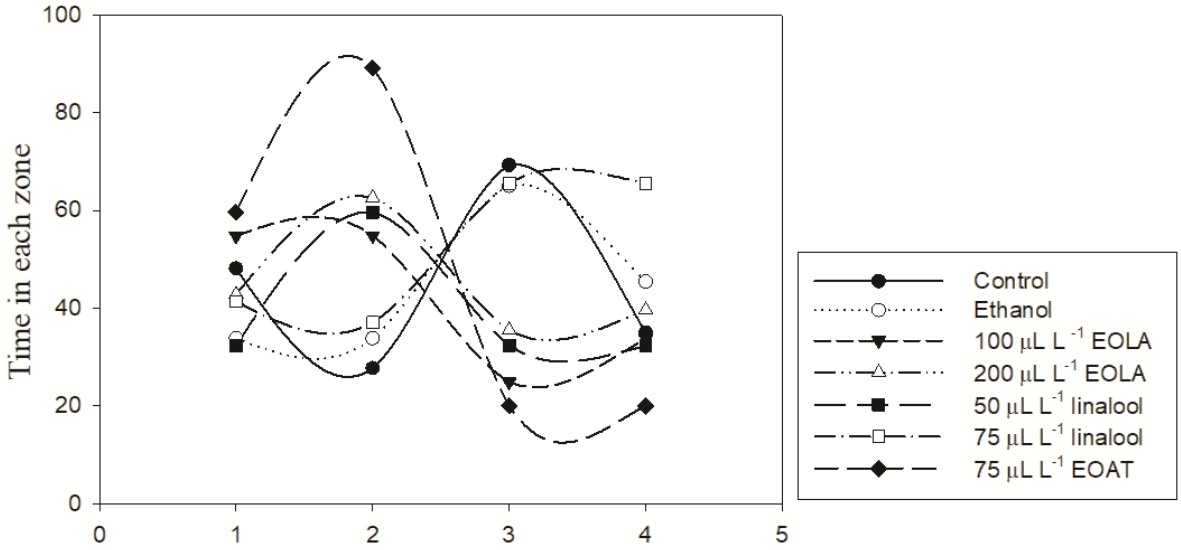

a
150 μL/L EOAT
75 μL/L linalool
50 μL/L linalool
200 μL/L EOLA
100 μL/L EOLA
Ethanol
Control
b
a
a
a
a
a
a
0.0
0.5
1.0
1.5
2.0
Distance (mm)
b
150 μL/L EOAT
75 μL/L linalool
50 μL/L linalool
200 μL/L EOLA
100 μL/L EOLA
Ethanol
Control
a
b
b
ab
ab
ab
ab
0
20
40
60
Line crossings

Fig 4.


Time in each zone
100
80
60
40
20
0
0
1
2
3
4
5
Control
Ethanol
100 µL L -1 EOLA
200 µL L-1 EOLA
50 µL L-1 linalool
75 µL L-1 linalool
75 µL L-1 EOAT

4. CONCLUSÃO

A partir dos resultados encontrados durante a condução dos experimentos da presente tese, apresentamos as seguintes conclusões:

- Os óleos essenciais, bem como os seus compostos majoritários, possuem potencial efeito anestésico nos crustáceos *L. vannamei, F. paulensis e H. bonariensis.*
- As respostas anestésicas são espécie-especificas, sendo que uma mesma concentração pode induzir respostas diferentes entre as espécies.
- OE de *L. alba* demostrou ser efetivo nos tempos de indução à anestesia e recuperação para o camarão *F. paulensis*, enquanto o OE de *C. citratu*s apresentou os melhores resultados para o camarão *L. vannamei*,
- A exposição prolongada ao OE de *C. citratus* resultou em efeitos positivos sobre o comportamento dos camarões *L. vannamei*, não prejudicando a atividade de natação, equilíbrio e exploração, induzindo respostas antioxidantes na maior concentração.
- O linalol isolado demonstrou capacidade de proteção a danos oxidativos em fêmeas abladas. Contudo, a variação nas respostas sugere diferenças na alocação dos recursos nutricionais entre machos e fêmeas relacionados ao maior esforço no investimento energético para maturação das gônadas, com provavelmente maior mobilização de nutrientes em fêmeas.
- As concentrações de citral testadas não são indicadas para uso em anfipodas, contudo o OE de *A. tryphilla* pode ser utilizado para anestesia de curta ou longa exposição para esses organismos, sem alterar o padrão comportamental natural da espécie.

Dessa forma, podemos concluir que a utilização de OEs é uma alternativa promissora para redução do estresse em procedimentos com crustáceos, proporcionando maior bem estar aos animais e o desenvolvimento sustentável da atividade de aquicultura.

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Ato de entrega de dissertação/tese

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COMPONENTE

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