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ATIVIDADE ANESTÉSICA E SEDATIVA DE PRODUTOS NATURAIS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

TESE DE DOUTORADO

Alexssandro Geferson Becker

Santa Maria, RS, Brasil

2011

ATIVIDADE ANESTÉSICA E SEDATIVA DE PRODUTOS NATURAIS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

Alexssandro Geferson Becker

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Doutor em Zootecnia.

Orientador: Prof. Dr. Bernardo Baldisserotto

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ATIVIDADE ANESTÉSICA E SEDATIVA DE PRODUTOS NATURAIS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

elaborada por
Alexssandro Geferson Becker

como requisito parcial para obtenção do grau de
Doutor em Zootecnia

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RESUMO

Tese de Doutorado

Programa de Pós-Graduação em Zootecnia

Universidade Federal de Santa Maria, RS, Brasil.

ATIVIDADE ANESTÉSICA E SEDATIVA DE PRODUTOS NATURAIS NO TRANSPORTE DE JUNDIÁ (*Rhamdia quelen*)

AUTOR: Alexssandro Geferson Becker

ORIENTADOR: Bernardo Baldisserotto

Data e Local da Defesa: Santa Maria, 14 de dezembro de 2011.

O transporte de peixes vivos é uma das principais atividades desenvolvidas em pisciculturas, ocasionando muitas vezes alterações fisiológicas, bioquímicas e comportamentais que podem ser prejudiciais a esses animais, contribuindo assim para uma redução na ingestão alimentar, no crescimento, na defesa contra patógenos e, consequentemente, levando a uma maior taxa de mortalidade. Em vista disso, objetivou-se, primeiramente, avaliar os extratos de *Condalia buxifolia* como anestésico em jundiá (*Rhamdia quelen*) e, também, verificar a eficácia da utilização do eugenol, do óleo essencial (OE) de *Lippia alba* e do extrato metanólico (EM) de *C. buxifolia* durante o transporte de jundiá, considerando-se os seguintes indicadores: parâmetros da água, do sangue e bioquímicos, sobrevivência e balanço ionorregulatório. Ao final dos experimentos para verificação da capacidade anestésica, percebeu-se que o EM de *C. buxifolia* na faixa de concentração entre 0,5 – 120 µL L^{-1} possui a capacidade de manter os peixes levemente sedados. Já nos experimentos de transporte, as concentrações de eugenol (1; 1,5; 2,5 ou 3 µL L^{-1}), de OE de *L. alba* (10; 20; 30 ou 40 µL L^{-1}) e de EM de *C. buxifolia* (5; 10; 25 ou 50 µL L^{-1}), independentemente da densidade de carga (169,2; 186,7 ou 275,1 g L^{-1}) e do tempo de transporte (4; 12 ou 6 h) foram eficazes na diminuição do fluxo iônico, da excreção de amônia e, também da mortalidade pós-transporte. Por outro lado, 30 µL L^{-1} de OE de *L. alba* causou uma elevação dos níveis plasmáticos de cortisol e, também induziu ao estresse oxidativo, através do aumento dos níveis de peroxidação lipídica e proteína carbonil e diminuição das defesas antioxidantes. Desta forma, dois novos parâmetros (peroxidação lipídica e carbonilação protéica) podem ser considerados como indicadores de estresse oxidativo induzido por anestésicos. Além disso, é aconselhável a utilização de anestésicos e sedativos no transporte de jundiá, em função dos consistentes resultados obtidos nesta tese.

Palavras-chave: Anestesia. Eugenol. Extrato de plantas. Jundiá. Óleo essencial. Transporte de peixes.

ABSTRACT
PhD Thesis
Graduate Program in Animal Husbandry
Universidade Federal de Santa Maria, RS, Brazil.

ANESTHESIC AND SEDATIVE ACTIVITIES OF NATURAL PRODUCTS IN THE TRANSPORT OF SILVER CATFISH (*Rhamdia quelen*)

AUTHOR: Alexssandro Geferson Becker
ADVISER: Bernardo Baldisserotto
Date and Place of Defense: December 14th, 2011, Santa Maria.

The transport of live fish is one of the main activities developed in fish farms, causing many physiological, biochemical, and behavioral alterations that can be impairment to these animais, contributed to a reduction in the feed ingestion, growth, pathogens defense and, consequently, resulting in the higher mortality rate. In view of this, the objective was, firstly, assess the extracts of *Condalia buxifolia* as anesthetic in silver catfish (*Rhamdia quelen*) and, also, verify the efficacy of the use of eugenol, the essential oil (EO) of *Lippia alba* and the methanolic extract (ME) of *Condalia buxifolia* during transport of silver catfish, through the following indicators: water, blood and biochemical parameters, survival and ionoregulatory balance. At the end of the experiments, it was observed that the ME of *C. buxifolia* in the concentration 0.5 to 120 µL L^{-1} range has the capacity to lightly sedate of fishes. In addition, in transport experiments, the concentrations of eugenol (1, 1.5, 2.5 or 3 µL L^{-1}), EO of *L. alba* (10, 20, 30 or 40 µL L^{-1}) and ME of *C. buxifolia* (5, 10, 25 or 50 µL L^{-1}), regardless of the loading density (169.2, 186.7 or 275.1 g L^{-1}) and transporting time (4, 12 or 6 h) were efficacy in decreasing ion loss, ammonia excretion and, also, mortality after transport. On the other hand, 30 µL L^{-1} of EO of *L. alba* caused an increase in plasma cortisol levels and, also, induced to the oxidative stress throught the increased levels of lipid peroxidation and protein carbonyl and decrease antioxidant defenses. Therefore, two new parameters (lipid peroxidation and protein carbonilation) can be considered as indicators of oxidative stress induced by anesthetics. Moreover, is recommended the anesthetics and sedatives for silver catfish transporting, because of the consistent results showed in this thesis.

Keywords: Anesthesia. Eugenol. Extracts from plants. Silver catfish. Essential oil. Fish transport.

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1 INTRODUÇÃO

A produção da piscicultura brasileira nos últimos anos tem tido um acentuado aumento se comparada com a de dezesseis anos atrás. No ano de 1992, a produção de todas as espécies de peixes foi de 26.000 toneladas, enquanto que os dados de 2008 apontaram para uma produção de aproximadamente 246.000 toneladas somente de peixes de água doce (IBAMA, 2008). Esse aumento na produção pode estar relacionado ao fato de possuirmos diversas espécies com potencial produtivo, seja por crescimento rápido, boa conversão alimentar, rusticidade ou por demanda do mercado consumidor (CRESCÊNCIO, 2005). O aumento da produção de peixes também está ligado a algumas práticas realizadas na piscicultura tais como: biometria, análises patológicas, implante hormonal, vacinação, manejo, captura e transporte. Esses procedimentos muitas vezes também trazem sérios prejuízos econômicos, tanto para os produtores quanto aos compradores, em função da interferência dessas práticas no desempenho desses peixes e também a mortalidade decorrente das mesmas (BARTON, 2000). Em vista disso, várias alternativas têm sido propostas para evitar ou até mesmo reduzir o estresse decorrente das práticas usuais em pisciculturas. Entre essas alternativas está à utilização de sais, anestésicos ou outras substâncias, tais como probióticos, que possam reduzir os possíveis danos fisiológicos e bioquímicos nos peixes.

Frente a todas as variáveis consideradas e buscando-se diminuir ao máximo os fatores geradores de estresse, a utilização de produtos considerados anestésicos parece ser uma valiosa ferramenta na piscicultura durante os procedimentos de coleta de peixes selvagens e também na criação de peixes, sendo que este último gera dois processos provavelmente causadores de alguns fatores de estresse: o manejo e o transporte de peixes vivos. Sabendo-se que os anestésicos são agentes químicos ou físicos, que com o aumento da exposição ou concentração, primeiro acalmam (sedam) um animal e depois causam perda de mobilidade, equilíbrio, consciência e das reações reflexas por evitarem a condução do impulso nervoso (SUMMERFELT e SMITH, 1990) e, considerando-se que a avaliação da passagem dos diferentes estágios de anestesia é bastante subjetiva (GILDERHUS e MARKING, 1987), deve-se levar em consideração os procedimentos de anestesia e a habilidade do manipulador (BURKA et al., 1997). Baseando-se nesses fatos, o processo de anestesia dos peixes tem sido usado por diminuir o estresse ou o dano fisiológico causado pelas práticas de manejo. No entanto, a concentração correta, o tempo de indução e recuperação, bem como os possíveis

efeitos adversos ainda permanecem desconhecidos para a maioria das espécies (ROSS e ROSS, 2008).

Entre os diversos procedimentos utilizados em pisciculturas, iremos nesta tese abordar o transporte de peixes vivos, considerado por muitos o principal causador de estresse. Primeiramente, pode-se destacar que entre os dois tipos de transporte de peixes (sistema fechado e aberto) o mais utilizado no Brasil é o chamado sistema fechado, o qual é realizado através de sacos plásticos parcialmente preenchidos com água, nos quais é injetado oxigênio comercial puro, e que devem ter uma boa espessura para resistirem ao transporte e, também, aos raios duros presentes nas nadadeiras de algumas espécies de peixes, como por exemplo, o jundiá (BERKA, 1986; GOMES et al., 1999, 2006a, b; GOLOMBIESKI et al., 2003; CARNEIRO et al., 2009). O transporte de peixes é influenciado por muitos fatores, incluindo-se a duração do transporte, densidade de carga (CARNEIRO et al., 2009), temperatura (GOLOMBIESKI et al., 2003), parâmetros físico-químicos da água, tamanho e condição física do peixe, e duração do período de depuração antes do transporte (BERKA, 1986). Entre as limitações desse sistema incluem-se a disponibilidade de oxigênio e os aumentos das produções de amônia e dióxido de carbono durante o período do transporte (GOMES et al., 1999, 2006a, b; GOLOMBIESKI et al., 2003; CARNEIRO et al., 2009). Geralmente observa-se uma alteração em vários parâmetros fisiológicos e bioquímicos, tais como: hematologia (DETHLOFF et al., 1999), osmolaridade e/ou balanço dos eletrólitos (McDONALD e MILLIGAN, 1997; GOMES et al., 2006b; CARNEIRO et al., 2009), liberação de hormônios, metabolismo energético e enzimas oxidativas (BARTON e IWAMA, 1991; CARRAGHER e REES, 1994).

Diante dos prejuízos à piscicultura ocasionados pelos sistemas inadequados de transporte de peixes vivos e também pela grande perda ocasionada ao final desse processo, torna-se fundamental e de extrema importância a utilização de sais, anestésicos ou outras substâncias, tais como, probióticos, na busca de se reduzir os fatores apresentados acima. Além disso, essa redução poderá permitir também um aumento na densidade de peixes e uma redução no volume de água, diminuindo ainda mais os custos desse processo e também melhorando o bem-estar dos peixes. Até o presente momento, os produtos mais utilizados são o cloreto de sódio (sal comum), gesso, zeolitos, benzocaína, eugenol e óleo de cravo, os quais apresentam um preço acessível e são de fácil manuseio e aquisição (GOMES et al., 1999, 2006a, b; SINGH et al., 2004; BRANDÃO et al., 2008). Algumas substâncias, tais como o metanosulfonato de tricaína (MS 222), éter monofenílico do etilenoglicol, hidrocloreto de metomidato, tampão tris [tris-(hidroximetil aminometano)] e 2-fenoxietanol também são

utilizados com o objetivo de contribuir com a diminuição do estresse causado pelo processo de transporte (CARMICHAEL et al., 1984; FERREIRA et al., 1984; TEO et al., 1989; SINGH et al., 2004; PARK et al., 2009). No entanto essas substâncias apresentam algumas inconveniências, tais como a necessidade da utilização de luvas em vista da possibilidade de toxicidade ao produtor, dificuldade de importação e, também, preços altos (PAVLIDIS et al., 2003).

Extratos ou óleos essenciais de plantas tem tornado-se uma alternativa viável como anestésicos para peixes, levando-se em conta os altos custos e dificuldades de obtenção dos produtos químicos e sintéticos, geralmente utilizados para esse propósito (FAÇANHA e GOMES, 2005). Portanto, além dos anestésicos, sais ou substâncias apresentadas anteriormente como alternativas para aplicação durante o transporte de peixes, tem sido também reportado em espécies de peixes brasileiros, a utilização do eugenol ou óleo de cravo (INOUE et al., 2005) e do óleo essencial de *Lippia alba* (AZAMBUJA et al., 2011). Porém, até o presente momento nenhum dado é reportado sobre a utilização dos extratos de *Condalia buxifolia* no transporte de peixes.

Vários estudos com peixes nativos brasileiros reportaram a utilização de um fitofármaco, o eugenol [(2-metoxi-4-(2-propenil) fenol, o principal componente do óleo de cravo (70–90% do peso)] ou óleo de cravo, como anestésico (INOUE et al., 2003, 2005, 2011; ROUBACH et al., 2005; VIDAL et al., 2006, 2007a, b; BARBOSA et al., 2007; GONÇALVES et al., 2008; HONCZARYK e INOUE, 2009; OKAMOTO et al., 2009; PEREIRA-DA-SILVA et al., 2009; CUNHA et al., 2010b; GOMES et al., 2011). Esse anestésico é obtido do caule, flores e folhas das plantas *Eugenia caryophyllata* Thunberg e *Eugenia aromatica* Baill.

Em adição, a *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) é caracterizada como sendo uma planta arbustiva aromática, sendo encontrada desde o sul dos Estados Unidos da América (Flórida) até as Américas Central e do Sul (BIASI e COSTA, 2003; HENNEBELLE et al., 2008), Índia (SINGH et al., 2000) e Austrália (DAY e McANDREW, 2003). É conhecida popularmente, no Brasil, como falsa – melissa ou erva cidreira (MATOS et al., 1996; BIASI e COSTA, 2003). A variabilidade na composição do óleo essencial (OE) obtido dessa planta vai depender das diferentes regiões do ramo vegetal, das estações do ano, dos horários de coleta, da metodologia empregada para a sua extração e, principalmente do quimiotipo presente nesse óleo (ATTI-SERAFINI et al., 2002; CASTRO et al., 2002; STASHENKO et al., 2004; NAGAO et al., 2005; BARROS et al., 2009; JANNUZZI et al., 2010). Alguns estudos têm reportado algumas atividades do OE e dos extratos de *L. alba*,

entre as quais destacam-se: atividade antibacteriana, antifúngica, antiviral, antiprotozoária, neurosedativa, analgésica, anti – inflamatória, cardiovascular e antioxidante (ABAD et al., 1997; VIANA et al., 1998; VALE et al., 1999, 2002; GUERRERO et al., 2002; HOLETZ et al., 2002; PUERTAS-MEJIA et al., 2002; ZÉTOLA et al., 2002; AGUIAR, 2006; CALZADA et al., 2006; HENNEBELLE et al., 2006; BORGES-ARGÁEZ et al., 2007). No entanto, até o presente momento são poucos os estudos reportando a utilização do OE de *L. alba* em peixes, destacando-se os realizados por Cunha et al. (2010a, 2011) e Azambuja et al. (2011).

Já o gênero *Condalia*, família Rhamnaceae, compreende 18 espécies distribuídas da América do Norte até a América do Sul, sendo que destas, cinco espécies são encontradas na América do Sul e uma no Brasil: *Condalia buxifolia* Reissek – é uma árvore de aproximadamente 4 metros de altura, sendo encontrada além do Brasil, na Argentina e no Uruguai. É conhecida popularmente, no Brasil, como coronilha-folha-de-buxo ou espinilho. Os estudos realizados com essa planta concentraram-se na identificação e classificação de constituintes químicos, principalmente os alcalóides ciclopeptídicos, os quais estão presentes nas folhas, flores, cascas da raiz e do caule e nas sementes (MARCHAND et al., 1968; MOREL et al., 1979, 1995, 2002; SHAH et al., 1986). Devido ao caráter quimiotaxonômico da Família Rhamnaceae, esses alcalóides exibem várias atividades biológicas, tais como: inseticida (SUGAWARA et al., 1996), sedativa (HAN et al., 1989), antimicrobiana (TSCHESCHE et al., 1974; JOULLIE e NUTT, 1985; GOURNELIS et al., 1997; MOREL et al., 2005;), antiplasmodiana (SUKSAMRARN et al., 2005), imunoestimulante (LIN et al., 2000), e antinociceptiva (TREVISAN et al., 2009). Por outro lado, não há nenhum dado reportado, até o presente momento, sobre a sua utilização como anestésico para peixes.

Portanto, esta tese teve como principais objetivos, primeiramente, avaliar os extratos de *Condalia buxifolia* como anestésico em jundiá (*Rhamdia quelen*) e avaliar os tempos de indução e recuperação anestésicas. Além disso, verificar a eficácia da utilização do fitofármaco eugenol e dos fitoterápicos OE de *L. alba* e extrato metanólico de *C. buxifolia* durante o transporte de jundiá, através dos seguintes indicadores: parâmetros da água, do sangue e bioquímicos, sobrevivência e balanço ionorregulatório. Com base nisso, propõe-se para essa tese, as seguintes hipóteses: os extratos de *C. buxifolia* induzem a anestesia profunda em jundiá, os anestésicos e sedativos auxiliam no controle dos parâmetros da água durante o transporte, reduzem a mortalidade e o estresse oxidativo decorrente do transporte e, ainda, mantém os parâmetros sanguíneos dentro da faixa de confortabilidade à espécie.

2 DESENVOLVIMENTO

Neste item serão apresentados os artigos publicados e/ou submetidos resultantes desta tese:

Artigo 1. Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and the essential oil of *Lippia alba*. Publicado no periódico ***Fish Physiology and Biochemistry*** **(DOI 10.1007/s10695-011-9562-4).**

Artigo 2. Efficacy of eugenol and the methanolic extract of *Condalia buxifolia* during transportation of silver catfish, *Rhamdia quelen*. Esse manuscrito será submetido ao periódico ***Aquaculture Research***.

Artigo 3. Transportation of silver catfish, *Rhamdia quelen*, in water with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia*. Esse manuscrito será submetido ao periódico ***Aquaculture***.

Artigo 4. Essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* induce biochemical stress in silver catfish, *Rhamdia quelen*, after transportation. Esse manuscrito será submetido ao periódico ***Chemosphere***.

ARTIGO 1

Publicado no periódico Fish Physiology and Biochemistry (DOI 10.1007/s10695-011-9562-4).

Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and the essential oil of *Lippia alba*

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Abstract

This study investigated the effectiveness of eugenol and of the essential oil (EO) of *Lippia alba* when used in the transport of the silver catfish (*Rhamdia quelen*). These investigations involved measurements of blood (pH, $Pv\mathrm{O_2}$, $Pv\mathrm{CO_2}$ and $\mathrm{HCO_3^-}$) and water parameters, survival and ionoregulatory balance. Fish (301.24 ± 21.40 g, 28.90 ± 1.30 cm) were transported at a loading density of 169.2 g $\mathrm{L^{-1}}$ for 4 h in fifteen plastic bags (7 L) divided into five treatments: control, 1.5 or 3.0 µl $\mathrm{L^{-1}}$ of eugenol, and 10 or 20 µl $\mathrm{L^{-1}}$ of EO of *L. alba*. The water parameters were measured before (0 h) and after (4 h) transportation. The net $\mathrm{Na^+}$, $\mathrm{Cl^-}$ and $\mathrm{K^+}$ losses were higher in fish from the control treatment compared to the other treatments. The $Pv\mathrm{O_2}$, $Pv\mathrm{CO_2}$ and $\mathrm{HCO_3^-}$ increased significantly in all of the treatments at the end of the transport period. In conclusion, based on the water (total ammonia nitrogen) and ionoregulatory indicators determined in the present study, our findings indicate that eugenol and the EO of *L. alba* are recommended for use in the transport of this species because these anesthetics apparently reduce stress.

Keywords: Anesthesia; Sedation; Ion fluxes; Fish transport

Introduction

The transport of fishes is influenced by many factors, including the duration of transportation, loading density (Carneiro et al. 2009), temperature (Golombieski et al. 2003), water physicochemical parameters, size and physical condition of the fish, and duration of the depuration period before fish transportation (Berka 1986). The transportation of fishes in Brazil involves the use of plastic bags. The limitations of this system include the supply of oxygen and the build-up of ammonia and carbon dioxide produced during transport (Gomes et al. 1999, 2006a, b; Golombieski et al. 2003; Carneiro et al. 2009). Previous studies regarding the transport of silver catfish, *R. quelen*, in plastic bags have evaluated different loading densities (Carneiro et al. 2009), times, temperatures (Golombieski et al. 2003) and salt concentrations (Gomes et al. 1999).

Anesthetics such as MS-222, benzocaine hydrochloride, 2-phenoxyethanol and lidocaine hydrochloride have been used to reduce stress responses during live fish transportation (Carmichael et al. 1984; Ferreira et al. 1984; Teo et al. 1989; Singh et al. 2004; Park et al. 2009). Several studies with native Brazilian fishes reported the use of eugenol [(2-methoxy-4-(2-propenyl) phenol, the major component of clove oil (70-90% of weight)] or clove oil, as an anesthetic (Inoue et al. 2005; Roubach et al. 2005; Vidal et al. 2006; Gonçalves et al. 2008; Cunha et al. 2010b). The essential oil (EO) of *L. alba* (Mill.) N.E. Brown (Verbenaceae), an aromatic shrub with important medicinal properties, is a new anesthetic for fish (Cunha et al. 2010a, 2011). Eugenol and the EO of *L. alba* can be used to anesthetize silver catfish. At concentrations of 50 and 300 µl L^{-1} (equivalent to 50 and 240 mg L^{-1}), respectively, eugenol and the EO of *L. alba* inhibited the increase in plasma cortisol levels after handling (Cunha et al. 2010a, b). However, no studies on the use of these anesthetics in fish transportation have been performed. Therefore, the aim of this study was to investigate the effectiveness of eugenol and of EO of *L. alba* for use during the transport of

silver catfish. The study used the following indicators: blood and water parameters, survival and ionoregulatory balance.

Material and Methods

Experimental procedure

Silver catfish (301.24 ± 21.40 g, 28.90 ± 1.30 cm) were captured from a cage net inside an earth pond at the fish culture sector at the Universidade Federal de Santa Maria campus, Santa Maria, Southern Brazil. Fish did not go through a depuration period because this procedure, although recommended (Amend et al. 1982), is not followed by most fish producers in southern Brazil (Golombieski et al. 2003). Fish were transported at a loading density of 169.2 g L^{-1} for 4 h in fifteen plastic bags with 7 L of water and 8 L of pure oxygen, and they were divided into five treatments (three replicates each). These treatments were as follows: control; 1.5 or 3.0 µL L^{-1} of eugenol (Odontofarma®, Porto Alegre, Brazil, equivalent to 1.5 or 3.0 mg L^{-1}, respectively, because the density of this anesthetic is about 1.06) and 10 or 20 µL L^{-1} of the EO of *L. alba* (equivalent to 8 or 16 mg L^{-1}, respectively, because the density of this EO is about 0.80) (both first diluted in ethanol; 1:10). The transport time was chosen to reduce mortality at this loading density (Golombieski et al. 2003). The concentrations of the EO of *L. alba* in water were within the range that induced only slight sedation in silver catfish within 6 h of exposure (5–20 µL L^{-1}, equivalent to 4–16 mg L^{-1}, respectively) (Cunha et al. 2010a). The eugenol concentrations used in our study were about 10-to 20-fold lower than those causing deep anesthesia in silver catfish within 15 min of exposure (20–50 µL L^{-1}) (Cunha et al. 2010b). A pilot study with 10 fish exposed to 1.5 or 3.0 µL L^{-1} of eugenol demonstrated that they only reached slight sedation within 6 h.

Another experiment evaluated the ventilatory frequency (VF) of the fish exposed to all treatments (n = 6 fish by treatment): control, 1.5 or 3.0 µL L^{-1} of eugenol, and 10 or 20 µL L^{-1} of the EO of *L. alba*. The VF was determined following Alvarenga and Volpato (1995): the VF per minute was quantified by visually counting 20 successive opercular or buccal movements, measuring the elapsed time with a chronometer. The fish (one fish per aquarium) were maintained in aquaria (19.3 x 13.7 x 11 cm) with 1 L of water and the respective anesthetic concentrations. The times chosen to evaluate the VF were 0, 0.5, 1, 2, 3 and 4 h.

The methodology of this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process nº 046/2010).

Plant material

L. alba was cultivated in the experimental area of the Departamento de Fitotecnia, UFSM campus. The aerial parts of the plant were collected in July 2008. The plant material was identified by botanist Dr. Gilberto Dolejal Zanetti, Departamento de Farmácia Industrial, UFSM, and a voucher specimen (SMDB No. 10050) was deposited in the herbarium of the Departamento de Biologia, UFSM.

Essential oil extraction

Essential oil was obtained from the fresh leaves of the plant by steam distillation for 2 h using a Clevenger-type apparatus. In this method, the distillate is collected in a graduated glass tube and the aqueous phase is automatically reused by returning it to the distillation flask (European Pharmacopoeia 2007). The EO samples were stored at -20°C in amber glass bottles.

Water sampling and analyses

Water parameters were measured before and after transportation. Dissolved oxygen (DO) and temperature were measured with a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA). The pH was verified with a DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). Nesslerization verified total ammonia nitrogen (TAN) levels according to the method of Eaton et al. (2005). Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd and Tucker (1992). Carbon dioxide (CO_2) was calculated by the method of Wurts and Durborow (1992).

Ion fluxes

Water samples (5 mL) were collected before and after transportation. Chloride levels were determined according to Zall et al. (1956), and Na^+, K^+ and Ca^{2+} levels were determined with a B262 flame spectrophotometer (Micronal, São Paulo, Brazil). Standard solutions were made with analytical-grade reagents (Vetec or Merck) dissolved in deionized water, and standard curves of each ion to be tested were made for five different concentrations. Net ion fluxes were calculated according to Gonzalez et al. (1998):

$$Jnet = \frac{V([ion]_1 - [ion]_2)}{Mt}$$

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

Blood sampling and analyses

The mixed venous-arterial blood samples (1–1.5 mL) were collected from the caudal vein of each fish using heparinized 3-mL syringes before and after the transporting procedure.

This caudal vein is commonly used for the collection of blood samples in many species of fish, but because of the proximity of the vein to an artery, samples are often mixtures of venous and arterial blood (Sladky et al. 2001; Hanley et al. 2010). The blood samples were kept in ice. The following variables were measured using a clinical analyzer (OMNI C 2413, Roche®, Rio de Janeiro, RJ, Brazil): pH, $Pv\mathrm{O_2}$, $Pv\mathrm{CO_2}$, hematocrit (Hct) and $\mathrm{HCO_3^-}$. The temperature of the clinical analyzer is commonly 37°C, but to determine blood gases, it was corrected to water temperature (20°C) with the assumption that ambient water temperature and individual fish body temperatures were equivalent (Hanley et al. 2010). In addition, Howell et al. (1970) reported that ectoterm vertebrates, including fish, maintain an acid-base balance despite changes in body temperature.

Statistical analyses

All data are expressed as mean ± SEM. Homogeneity of variances among treatments was tested with the Levene test. Data exhibited homogeneous variances, so comparisons between different treatments and times were made using one-way ANOVA and Tukey's test. Analysis was performed using the software Statistica ver. 5.1 (StatSoft, Tulsa, OK), and the minimum significance level was set at $P < 0.05$.

Results

Water parameters and mortality

No mortality was recorded in any treatment following transport. After transport, the highest DO levels and lowest $\mathrm{CO_2}$ levels were found in the control and in the 10 $\mu L\ L^{-1}$ of EO *L. alba* treatment, respectively. Total alkalinity, pH and $\mathrm{NH_3}$ levels in the water did not exhibit any significant differences between the treatments at the end of transport. In addition,

the TAN levels were significantly higher in the control compared with the other groups. Water hardness and temperature did not exhibit any significant differences between treatments after transport (Table 1).

Ion fluxes through transportation

The net Na^+, Cl^- and K^+ effluxes were significantly higher in fish from the control treatment compared with fish in the other treatments. Moreover, the lowest net Cl^- and K^+ effluxes were found for the treatments with 1.5 µL L^{-1} of eugenol and 10 and 20 µL L^{-1} EO of *L. alba*, respectively. The net Ca^{2+} fluxes did not show any significant difference between treatments (Fig. 1).

Blood parameters

The highest $Pv O_2$, $Pv CO_2$ and HCO_3^- values after transport were found in the treatments with 3.0 µL L^{-1} eugenol and 20 µL L^{-1} EO of *L. alba*. Blood pH was not affected by treatments (Table 2).

Ventilatory frequency (VF)

The VF at 0 h was significantly lower in fish from the control treatment compared with the other treatments. The highest VF at 0.5 h was found in the treatments with 3.0 µL L^{-1} of eugenol and 20 µL L^{-1} EO of *L. alba*. After 1 h of exposure, there was no significant difference between treatments, but at 2, 3 and 4 h, the VF was significantly lower in all treatments with anesthetics compared to the control treatment.

In all treatments with anesthetic, there was a significant increase in the VF after 0.5 h of exposure when compared with the other times. However, in the control treatment, there

was a significant decrease in VF in the first half hour. VF remained constant at the other times (Table 3).

Discussion

The lethal concentrations (96 h) of TAN and NH_3 for silver catfish in normoxic conditions (total hardness: 20 mg $CaCO_3$ L^{-1}; 25 °C) are 7.73 and 0.44 mg L^{-1}, respectively, at pH 6.0 (Miron et al. 2008). Total ammonia and NH_3levels were much lower at the end of the transport in the present study than lethal values. Therefore, silver catfish could be transported for a longer period without problems due to ammonia toxicity under the conditions used in these experiments (weight of 300 g, density of 169.2 g L^{-1}, transported by 4 h). The TAN excretion by silver catfish transported in our study was 7.92 mg kg^{-1} fish h^{-1}, about 2.36-fold lower than reported by Carneiro et al. (2009) (18.68 mg kg^{-1} fish h^{-1}) with silver catfish (weight of 20 g, loading density of 150 g L^{-1}, transported by 4 h). This result was expected because ammonia excretion decreases with increasing fish mass in silver catfish (Bolner and Baldisserotto 2007).

In our study, the DO levels after 4 h of transport still remained within a safe range for silver catfish (control group - 7.63 mg L^{-1}) (Braun et al. 2006) because pure oxygen was added to the plastic bags. Oxygen consumption was lower than observed by Golombieski et al. (2003) for the transport of silver catfish for 6 h (weight of 1.0-2.5 g, loading density of 168 g L^{-1}). Silver catfish could reach stage 4 of anesthesia when exposed to concentrations between 20 and 50 µL L^{-1} of eugenol and above 100 µL L^{-1} (equivalent to 80 mg L^{-1}) EO *L. alba* within 15 min (Cunha et al. 2010a, b). This stage is characterized by the loss of reflex activity (i.e., reduction in the opercular movement) and by a lack of reaction to strong external stimuli (Schoettger and Julin 1967). The anesthetic concentrations used in fish transport must induce, at most, stage 2 of anesthesia (stage of deep sedation). Partial loss of equilibrium and

lack of reaction to external stimuli are observed in this stage. Largemouth black bass, *Micropterus salmoides*, exposed to MS-222 (tricaine methanesulfonate) showed enhanced survival and a reduction in stress parameters (plasma glucose and corticosteroids decreased and plasma chloride and osmolality increased) during transport compared to fish transported in water without this anesthetic (Carmichael et al. 1984). Moreover, the use of benzocaine hydrochloride (25 mg L^{-1}) on Mozambique tilapia, *Oreochromis mossambicus*, reduced oxygen consumption at about 1/3 and decreased ammonia and CO_2 excretion (Ferreira et al. 1984). In the fry of the Indian carp *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* (0.09 mg L^{-1}), this treatment also decreased NH_3 excretion (Singh et al. 2004). Park et al. (2009) suggested that lidocaine hydrochloride at concentrations of 5, 10 or 20 mg L^{-1} decreased the metabolic activity of flounder, *Pleuronectes americanus*, because this substance reduced ammonia excretion (about 27.4–30.5%) and oxygen consumption (about 82.7–86%) compared with a control group after 5 h transport time.

Eugenol and EO of *L. alba* in the water used in transport reduced ammonia excretion by silver catfish during transport. These findings are in agreement with those reported by Guo et al. (1995) and Park et al. (2009). These studies found that the overall reduction in ammonia excretion could be directly related to a decrease in the metabolic rate produced by anesthetics.

Stress conditions such as transport and handling increase gill blood flow and paracellular permeability. In freshwater fishes, the result of these changes is ionic loss (Cech Jr. et al. 1996; McDonald et al. 1991). Common salt has been added to the water used in transport to reduce the osmotic gradient between the water and fish plasma. This treatment produces positive results in several species (Barton and Peter 1982; Carneiro and Urbinati 2001) but not in silver catfish (Gomes et al. 1999) or in pirarucu, *Arapaima gigas* (Gomes et al. 2006b). In the present study, eugenol and the EO of *L. alba* in the water of transport reduced ion loss in silver catfish. This effect was probably the result of lower gill blood flow

that occurred because the fish were less agitated. Moreover, Cunha et al. (2010a, b) reported that the cortisol levels did not increase in silver catfish subjected to handling while anesthetized with eugenol or EO of *L. alba*. In addition, other studies (Guo et al. 1995; Singh et al. 2004; Park et al. 2009) also reported that the anesthetics used for fish transport reduce agitation and fish stress.

The blood pH values found in the present study, regardless of treatment, were similar to or slightly lower than those reported for tambaqui exposed to different water pHs (Wood et al. 1998); red pacu (*Piaractus brachypomus*) exposed to MS-222 and eugenol at 50, 100 and 200 mg L^{-1} (Sladky et al. 2001); and yellow perch (*Perca flavescens*), walleye pike and koi (*Cyprinus carpio*) anesthetized with MS-222 (150 mg L^{-1}) and buffered with $NaHCO_3$ (75 mg L^{-1}) (Hanley et al. 2010). The blood gas values ($Pv O_2$, $Pv CO_2$ and HCO_3^-) before transport were similar to or lower than those reported by other studies (Sladky et al. 2001; Souza et al. 2001; Hanley et al. 2010).

Exposure of silver catfish to eugenol or EO of *L. alba* apparently decreased metabolic rate because fish presented significantly lower ammonia excretion, VF (through all transport time) and net ion loss. However, DO and carbon dioxide levels in the water of transport of silver catfish transported with both anesthetics were significantly lower and higher, respectively, than in the water of transport of control fish, indicating the opposite: an increase in metabolic rate through transport. A possible explanation to these conflicting results would be that eugenol and EO of *L. alba* would not reduce metabolic rate but would decrease ammonia excretion. This lower ammonia excretion would induce an increase in plasma ammonia levels. High plasma ammonia levels did not change $Pa O_2$ but increased $Pa CO_2$ and plasma HCO_3^- in rainbow trout (Zhang and Wood 2009), and similarly, silver catfish transported in water with 3 µL L^{-1} eugenol or 20 µL L^{-1} EO of *L. alba* exhibited the highest values of $Pv CO_2$ and HCO_3^- in the blood at the end of transportation. Nevertheless, plasma

ammonia was not measured in the present experiment, and according to Zhang and Wood (2009), high plasma ammonia induced hyper-ventilation in rainbow trout, which was not observed in silver catfish. Additional experiments are necessary to explain these results.

In the present study, the Hct values (26–33%) were similar to those found by Carneiro et al. (2009) in the same species (Hct: 27–30%). The Hct values decreased after transport but showed no significant differences between treatments. Transport procedures are examples of conditions that can produce stress. Such stress could decrease Hct. These considerations suggest a hemodilution caused by osmoregulatory disturbance (Houston et al. 1996; Morgan and Iwama 1997).

In conclusion, on the basis of the findings regarding water (TAN) and osmoregulatory indicators obtained by the present study, our results suggest that the use of eugenol and EO of *L. alba* is advisable for the transport of silver catfish. Additional experiments using higher loading densities would also be of interest in order to assess the importance of these anesthetics in more stressful situations.

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Figure Caption

Fig. 1 Net ion (Na^+, Cl^-, K^+ and Ca^{2+}) fluxes measured for the transport of silver catfish in plastic bags with eugenol and the essential oil of *Lippia alba* added to the water. Values are means ± SEM. *Different letters* indicate significant differences between treatments for the same ion ($P<0.05$).

Table 1 Water parameters before and after transport (4 h) of silver catfish in plastic bags with eugenol and the essential oil of *Lippia alba* added to the water.

Water parameter	Before transport	After transport (treatments)				
		Control	Eugenol (1.5 µl L^{-1})	Eugenol (3.0 µl L^{-1})	*L. alba* (10 µl L^{-1})	*L. alba* (20 µl L^{-1})
Dissolved oxygen	12.27±0.20	7.63±0.46*a	6.58±0.41*b	5.55±0.83*b	7.77±0.52*a	6.12±0.61*b
Carbon dioxide	12.56±0.41	40.51±1.09*c	56.72±1.16*a	57.55±0.53*a	49.63±1.06*b	55.26±0.61*a
Alkalinity	18.60±1.12	27.70±0.90*a	25.50±1.00*a	27.20±0.80*a	25.80±0.90*a	26.20±0.80*a
Water hardness	29.46±1.78	29.60±1.90a	30.70±1.80a	29.80±2.10a	29.30±1.70a	32.00±1.90a
pH	6.78±0.07	5.90±0.08*a	5.77±0.07*a	5.80±0.05*a	5.91±0.07*a	5.83±0.06*a
Temperature	20.10±0.25	20.60±0.32a	20.49±0.27a	20.53±0.34a	20.61±0.36a	20.57±0.26a
Total Ammonia Nitrogen	1.25±0.11	5.36±0.26*a	4.36±0.24*b	4.37±0.25*b	4.40±0.25*b	4.44±0.21*b
Un-ionized ammonia	0.0030	0.0018*a	0.0010*a	0.0011*a	0.0015*a	0.0012*a

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

Table 2 Blood parameters before and after transport of silver catfish in plastic bags with eugenol and the essential oil of *Lippia alba* added to the water

Blood parameter	Before transport	After transport (treatments)				
		Control	Eugenol ($1.5\ \mu l\ L^{-1}$)	Eugenol ($3.0\ \mu l\ L^{-1}$)	*L. alba* ($10\ \mu l\ L^{-1}$)	*L. alba* ($20\ \mu l\ L^{-1}$)
pH	7.33±0.07	7.24±0.03a	7.24±0.06a	7.25±0.05a	7.29±0.05a	7.27±0.03a
PvO_2 (mm Hg)	8.99±0.54	16.47±0.68*b	14.25±0.61*b	22.59±0.89*a	15.24±0.82*b	20.53±1.13*a
$PvCO_2$ (mm Hg)	11.54±1.33	23.81±0.51*b	21.48±0.66*b	27.22±0.73*a	23.07±0.91*b	27.10±0.75*a
Hct (%)	32.64±0.84	26.16±1.25*a	28.09±1.00*a	26.47±1.07*a	27.72±1.00*a	26.56±0.62*a
HCO_3^- ($mmoL\ L^{-1}$)	7.35±0.36	12.05±0.14*b	12.41±0.41*b	14.07±0.16*a	12.87±0.12*b	14.82±0.33*a

Values are means ± SEM. Asterisks indicate significant differences when compared to values before transport ($P < 0.05$). Different letters in the rows indicate significant differences between treatments after transport ($P < 0.05$).

Table 3 Ventilatory frequency (opercular or buccal movements min^{-1}) measured in silver catfish maintained in water with eugenol and the essential oil of *Lippia alba*.

Time of exposure (h)	Treatments				
	Control	eugenol ($1.5\ \mu l\ L^{-1}$)	eugenol ($3.0\ \mu l\ L^{-1}$)	*L. alba* ($10\ \mu l\ L^{-1}$)	*L. alba* ($20\ \mu l\ L^{-1}$)
0	93.02±1.02Ba	101.61±1.04Ab	100.42±1.34Ab	101.44±0.82Ab	105.26±0.69Ab
0.5	81.24±0.44Cb	111.94±0.58Ba	126.58±0.35Aa	106.10±0.51Ba	121.95±0.21Aa
1	72.16±0.44Ac	76.58±0.60Ac	77.77±0.70Ac	67.30±0.75Ac	71.30±0.75Ac
2	65.25±0.88Ac	53.52±0.78Bd	51.77±0.28Bd	51.06±1.05Bd	53.31±1.05Bd
3	61.60±1.20Ac	47.83±0.57Be	45.37±1.66Be	43.23±1.05Be	42.55±0.86Be
4	68.03±1.17Ac	45.75±0.67Be	42.18±1.76Be	41.72±0.95Be	43.37±0.81Be

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

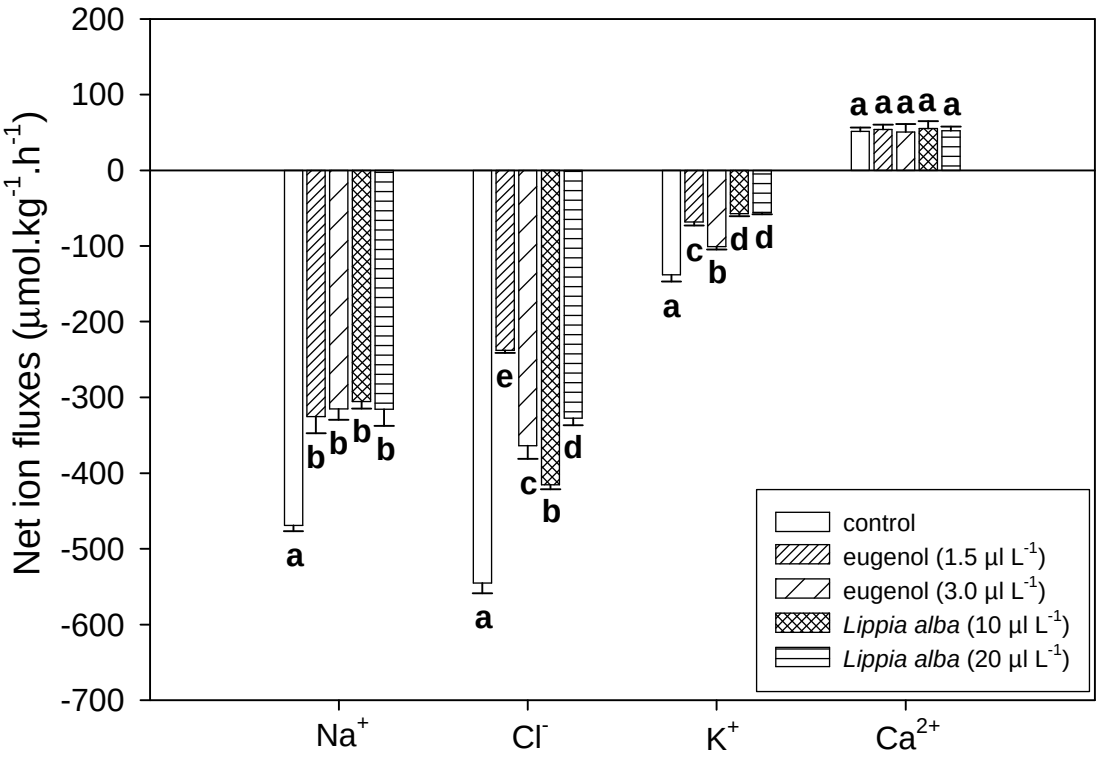
Net ion fluxes (µmol.kg-1.h-1)
200
100
0
-100
-200
-300
-400
-500
-600
-700
a
b b b b
a
e
c b d
a
c b d d
a a a a a
Na+
Cl-
K+
Ca2+
control
eugenol (1.5 µl L-1)
eugenol (3.0 µl L-1)
Lippia alba (10 µl L-1)
Lippia alba (20 µl L-1)

Figure 1.

ARTIGO 2

Efficacy of eugenol and the methanolic extract of *Condalia buxifolia* during transportation of silver catfish, *Rhamdia quelen*

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Abstract

This study investigated four different extracts of *Condalia buxifolia* as silver catfish anesthetic, and also the effectiveness of eugenol and methanolic extract (ME) of *C. buxifolia* for using during transport of this species. In the first experiment, fish of two different weights (1.50 ± 0.02 g and 165.70 ± 22.50 g) were transferred to aquaria containing water with extracts of *C. buxifolia* at concentrations in the 0 – 300 μL L^{-1} range. In the second experiment another group of fish (12.01 ± 1.73 g, 10.27 ± 1.85 cm) was transported for 12 h in fifteen plastic bags divided in five treatments: control, 1 or 2.5 μL L^{-1} of eugenol and 25 or 50 μL L^{-1} of ME of *C. buxifolia*. The ME of *C. buxifolia* at concentrations in the 0.5 – 120 μL L^{-1} range caused only light sedation. The dissolved oxygen levels were lower in the treatments 1 μL L^{-1} of eugenol and 25 μL L^{-1} of ME of *C. buxifolia* and the un-ionized ammonia levels decreased in the treatments with anesthetic. Moreover, the anesthetic and sedative agents decreased of Na^+, Cl^- and K^+ fluxes and, therefore, the addition of both to the water transport is advisable because they reduced fish mortality and ion loss.

Keywords: anaesthesia, ion fluxes, sedative, fish transport

1. Introduction

The use of new substances that allow the induction of some kind of sedation or even deeper levels of anesthesia in fish has been moderately studied (Gilderhus & Marking 1987; Soto & Burhanuddin 1995; Anderson, Mckinley & Colavecchia 1997; Iversen, Finstad, Mckinley & Eliassen 2003; Façanha & Gomes 2005; Cunha, Barros, Garcia, Veeck, Heinzmann, Loro, Emanuelli & Baldisserotto 2010a). In these studies, researchers looked for compounds of easy acquisition and low cost to fish farmers, and that do not present risk to the health of fish and manipulators. This is important because any stimulus presented to a fish can change behavior and physiology, which are reflected in less food intake and consequently, delayed growth and mortality.

Plant extracts or essential oils seem to be a viable alternative as anesthetics for fish, taking into account the high costs and difficulties of obtaining chemical products used for this purpose (Façanha & Gomes 2005). Eugenol [(2-methoxy-4-(2-propenyl) phenol), the major component in clove oil (70-90% of weight)] or clove oil have been used, as anesthetic, in several studies with native Brazilian fishes (Inoue, dos Santos Neto & Moraes 2003; Inoue, Afonso, Iwama & Moraes 2005; Roubach, Gomes, Fonseca & Val 2005; Vidal, Albinati, Albinati & de Mecêdo 2006; Vidal, Albinati, Albinati, de Lira, de Almeida & Santos 2008; Barbosa, Moraes & Inoue 2007; Gonçalves, Santos, Fernandes & Takahashi 2008; Honczaryk & Inoue 2009; Cunha, Garcia, Loro, Fonseca, Emanuelli, Veeck, Copatti & Baldisserotto 2010b). Moreover, this anesthetic is listed in the FDA category of materials “generally regarded as safe” (Ross & Ross 2008). Because of its efficacy, low price, no withdrawal period and lack of negative effects on fish feeding, eugenol, clove oil and iso-eugenol have been considered as “modish anesthetics” of choice in the aquaculture industry (Harper 2003).

However, eugenol impairs the flavor of silver catfish, *Rhamdia quelen*, fillet, and therefore its use is not recommended immediately before slaughter (Cunha *et al.* 2010b).

The tree *Condalia buxifolia* Reissek (Rhamnaceae) is found mainly in South America (Brazil, Uruguay and Argentina) (Bastos 1989) and a study regarding its chemistry identified some peptide alkaloids in its root bark (Morel, Araújo, Silva, Hoelzel, Záchia & Bastos 2002). Peptide alkaloids possess a variety of biological activities, including sedative (El-Seedi, Zahra, Goransson, Verpoorte 2007).

The transport of live fish is a problematic factor in aquaculture. The success of transporting fish depends on many factors including the duration of transportation, water parameters, size, density and physical condition of the fish, and duration of the depuration period before fish transportation (Berka 1986; Golombieski, Silva, Baldisserotto & Silva 2003; Carneiro, Kaiseler, Swarofsky & Baldisserotto 2009; Becker, Parodi, Heldwein, Zeppenfeld, Heinzmann & Baldisserotto *in press*). The most usual system of juveniles transportation in Brazil is the closed system using plastic bags and the limitations of this system are the supply of oxygen and the build-up of ammonia and carbon dioxide produced during transport (Gomes, Golombieski, Chippari-Gomes & Baldisserotto 1999; Golombieski *et al.* 2003; Gomes, Araújo-Lima, Chippari-Gomes & Roubach 2006a; Gomes, Chagas, Brinn, Roubach, Coppati & Baldisserotto 2006b; Carneiro *et al.* 2009; Becker *et al. in press*).

The use of anesthetics during fish transportation has being proposed to reduce stress responses (Guo, Teo & Chen 1995; Inoue *et al.* 2005; Azambuja, Mattiazzi, Riffel, Finamor, Garcia, Heldwein, Heinzmann, Baldisserotto, Pavanato & Llesuy 2011; Cunha, Silva, Delunardo, Benovit, Gomes, Heinzmann & Baldisserotto 2011; Becker *et al. in press*). Monitoring physiological parameters during transportation can provide valuable data for the establishment of adequate management practices, even for situations where there is fish mortality (Sulikowski, Fairchild, Rennels, Howell & Tsang 2005).

Studies of transport of silver catfish, *Rhamdia quelen*, evaluated different times, loading densities and temperatures (Golombieski *et al.* 2003), salt concentrations in the water of transport (Gomes *et al.* 1999); different loading densities (Carneiro *et al.* 2009), and, recently, the effectiveness of eugenol (1.5 or 3.0 μL L^{-1}) and essential oil of *L. alba* during the transport, considering some blood and water parameters, survival and ionoregulatory balance (Becker *et al. in press*).

The aim of this study was to evaluate extracts of *C. buxifolia* as silver catfish anesthetics and to evaluate the time to induction and recovery from anesthesia. Moreover, it investigated the effectiveness of eugenol and of ME of *C. buxifolia* for using during the transport of silver catfish, through the following indicators: water parameters, survival and ionoregulatory balance.

2. Material and Methods

2.1. Animals

Specimens of silver catfish of two different weights (1.50 ± 0.02 g and 165.7 ± 22.5 g) were purchased from the fish farm and transported to the Laboratory of Fish Physiology at the Universidade Federal de Santa Maria, where they were maintained for two weeks in continuously aerated 250 L tanks (temperature 21 ± 1°C; pH 6.8 ± 0.5; dissolved oxygen 6.5 ± 0.8 mg L^{-1}). Juveniles were fasted for 24 h prior to the experiments.

2.2. Plant material

Freeze-dried bark of *Condalia buxifolia* (2.2 kg) was extracted with MeOH in a Soxhlet extractor. The solvent was evaporated under reduced pressure to obtain 430 g of a dark viscous residue (methanolic crude extract – ME). A portion of this extract (100 g) was dissolved in water (500 mL) and extracted successively with n-hexane (3 x 0.5 L), dichloromethane (3 x 0.5 L) and ethyl acetate (3 x 0.5 L), furnishing the following fractions with respective yields: n-hexane (10 g), dichloromethane (7 g) and ethyl acetate (5 g). The identification of the botanical material was performed by comparisons with existing samples in the herbarium of the Departamento de Biologia-UFSM (SMDB3296).

2.3. Experiment I: Anesthesia induction and recovery in fish exposed to extracts *C. buxifolia*

After the adaptation period to laboratory conditions, fish were transferred to aquaria containing 1L of water and ME of *C. buxifolia* in the follow concentrations: 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10, 30, 50, 80, 120 and 300 µL L^{-1} (firstly diluted in ethanol in the proportion 1:10). Previous analysis demonstrated that ethanol at the concentrations tested did not induce sedation or anesthesia in silver catfish (Cunha *et al.* 2010a). The time for anesthesia induction was evaluated according to Schoettger & Julin (1967) (Table 1). The maximum observation time was 30 min. The same procedure was used to test hexane, ethyl acetate and dichloromethane extracts. Fish exposed to the ME of *C. buxifolia* at 1.0 – 50 µL L^{-1}, were observed for 6 h to analyze if sedation would evolve to anesthesia. After induction, juveniles were transferred to anesthetic-free aquaria to measure anesthesia recovery time. Twenty juveniles were used for each concentration tested and each juvenile was used only once.

2.4. Experiment II: Transport

Another group of fish (12.01 ± 1.73 g, 10.27 ± 1.85 cm) was captured from a cage net inside an earth pond at a fish farm near Santa Maria city, Southern Brazil. Fish did not go through a depuration period because this procedure, although recommended (Amend, Croy, Goven, Johnson & McCarthy 1982), is not followed by most fish producers in southern Brazil (Golombieski *et al.* 2003). Fish were transported at a loading density of 186.7g L^{-1} for 12 h in fifteen plastic bags with 1.5 L of water and 3 L of pure oxygen, and they were divided into five treatments (three replicates each). These treatments were as follows: control; 1.0 or 2.5 μL L^{-1} of eugenol (Odontofarma^{TM}, Porto Alegre, Brazil) (equivalent to 1.0 or 2.5 mg L^{-1}, respectively, because the density of this anesthetic is about 1.06) and 25 or 50 μL L^{-1} of the ME of *C. buxifolia* (both first diluted in ethanol; 1:10). The transport time was defined considering that the maximum transport time utilized by producers from Rio Grande do Sul state (Brazil) is 12 hours. The loading density used in this study was higher than the maximum recommended to silver catfish (168 g L^{-1}) (Golombieski *et al.* 2003) to expose fish to a very stressful situation and determine the efficacy of the substances used.

2.5. Water sampling and analyses

Water parameters were measured before and after transportation. Dissolved oxygen (DO) and temperature were measured with an YSI oxygen meter. The pH was verified with DMPH-2 pH meter. Nesslerization verified total ammonia nitrogen (TAN) levels according to the method of Eaton, Clesceri, Rice & Greenberg (2005). Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd & Tucker (1992). Carbon dioxide (CO_2) was calculated by the method of Wurts & Durborow (1992).

2.6. Ion fluxes

Water samples (5 mL) were collected before and after transportation. Chloride levels were determined according to Zall, Fisher & Garner (1956), and Na^+ and K^+ levels were determined with a B262 flame spectrophotometer. Standard solutions were made with analytical-grade reagents dissolved in deionized water, and standard curves of each ion to be tested were made for five different concentrations. Net ion fluxes were calculated according to Gonzalez, Wood, Wilson, Patrick, Bergman, Narahara & Val (1998):

$$Jnet = V([ion]_1 - [ion]_2) \times (M \times t)^{-1},$$

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

2.6. Statistical analyses

All data are expressed as mean ± SEM. Homogeneity of variances between treatments was tested with Levene test. Data exhibited homogeneous variances, so comparisons among different treatments and times were made using one-way ANOVA and Tukey's test. Analysis was performed using the software Statistica ver. 7.0, and the minimum significance level was set at $P < 0.05$. The relationship between the time to reach the stage of sedation and the concentration of the ME of *C. buxifolia* was calculated with the Sigma Plot 11.0 software ($P < 0.05$).

3. Results

3.1. Anesthesia induction and recovery in fish exposed to extracts of *C. buxifolia*

Silver catfish exposed to the hexane, ethyl acetate and dichloromethane extracts of *C. buxifolia* did not present any evidence of sedative or anesthetic effects during the 30-min

198 evaluation period. The ME of *C. buxifolia* at concentrations in the 0.5 – 120 µL L^{-1} range
199 caused only light sedation (stage 1) in silver catfish of both weight tested. Higher
200 concentrations did not alter silver catfish behavior within the 30-min evaluation period, and
201 no difference was observed in the response to this extract between the two weight groups. It
202 was also observed that in fish exposed to concentrations of the ME of *C. buxifolia* higher than
203 10 µL L^{-1}, the time to induce sedation increased as concentration increased (Figure 1). Silver
204 catfish exposed to 1.0 – 50.0 µL L^{-1} ME of *C. buxifolia* for 6 h maintained a uniform depth of
205 sedation, i.e., remained in stage 1.
206
207 **3.2. Water parameters and mortality**
208 After transport, the highest mortality was observed in the control followed by
209 treatments 1 µL L^{-1} of eugenol and 25 µL L^{-1} of ME of *C. buxifolia*. On the other hand, the
210 lowest mortality was observed in the treatments 2.5 µL L^{-1} of eugenol and 50 µL L^{-1} of ME of
211 *C. buxifolia* (Figure 2).
212 The treatments 1 µL L^{-1} of eugenol and 25 µL L^{-1} of ME of *C. buxifolia* exhibited the
213 highest DO levels in the water after transport. In addition, the lowest CO_2 and TAN levels
214 were found in the water of control group. Total alkalinity, water hardness levels and
215 temperature in the water did not exhibit any significant differences among treatments at the
216 end of transport. In addition, pH and NH_3 levels were significantly higher in the control
217 compared to the other groups (Table 2).
218
219 **3.3. Ion fluxes through transportation**
220 The net Na^+, Cl^- and K^+ effluxes were significantly higher in fish from the control
221 treatment compared with fish in the other treatments. Moreover, the lowest net Na^+, Cl^- and

K^+ effluxes were found for the treatments with 1 µL L^{-1} of eugenol and 25 µL L^{-1} of ME of *C. buxifolia* (Figure 3).

4. Discussion

4.1. Anesthesia induction and recovery in fish exposed to ME *C. buxifolia*

Anesthetics are useful to reduce or minimize stress to fish. Several substances and combinations of substances such as alcohol, ether, barbiturics, quinaldine, tricaine methanesulfonate (MS 222), chlorbutanol, and benzocaine have been used to induce anesthesia in fish, presenting undesirable systemic side effects and limited safety margins (Gilderhus & Marking 1987).

Silver catfish were slightly sedated with the ME of *C. buxifolia*, and there was no induction of anesthesia even after six hours. It was not possible to verify the recovery time of the ME of *C. buxifolia*, as there was no anesthetic effect. Consequently, the use of this extract as a sedative rather than an anesthetic is suggested. The best concentration range of the ME of *C. buxifolia* seems to be 0.5 – 10 µL L^{-1}, because higher concentration levels increased the time of sedation for both weight classes of silver catfish. In addition, this extract is very safe because even a concentration 30-fold higher than the maximum concentration recommended did not provoke mortality.

As the fractions of ME of *C. buxifolia* did not present any sedative or anesthetic effects when tested separately, apparently the effect of ME is not due to a specific compound (that would have been separated in at least one of the fractions), but to the synergism of its compounds. There are no studies regarding the synergism of compounds to anesthetize fishes, but the same principle can be found in some isolated components from the essential oil of two species of *Ocimum* that exhibited either low or no insecticidal activity, and became potently

247 toxic when blended together (Bekele & Hassanali 2001). The antimicrobial nature of
248 *Filipendula vulgaris* essential oil can also be attributed to the synergistic interactions of the
249 compounds constituting the oil rather than to the presence of a single inhibitory agent
250 (Radulović, Mišić, Aleksić, Đoković, Palić & Stojanović 2007). Another interesting effect is
251 that the time to reach the stage of slight sedation increases in silver catfish exposed to the
252 higher concentrations of the ME of *C. buxifolia*. Again, there is no similar results regarding
253 fish anesthetics, but some interactions between plants compounds revealed a clear
254 concentration-dependent interaction. For example, if the minimal inhibitory concentrations
255 were applied, the combination of essential oils of cinnamon and clove exerted an antagonistic
256 effect on the growth of some Gram-negative bacteria. On the other hand, when the
257 concentrations of maximal inhibition were used a synergistic effect could be observed for the
258 Gram-positive bacteria and, therefore, this result revealed a concentration-dependent
259 interaction (Goñi, López, Sánchez, Gómez-Lus, Becerril & Nerín 2009). It is possible that a
260 concentration-dependent interaction occurs with the compounds of the ME of *C. buxifolia*
261 regarding its sedative effect in silver catfish.
262
263 **4.2. Transport experiment: water parameters, survival and ionoregulatory balance**
264 In this study, at the end of the transport, there was significantly higher mortality in the
265 control when compared to the other treatments. Therefore, these results confirm that the
266 anesthetics added in the water transport reduced mortality of silver catfish maintained in the
267 experimental conditions reported in this study.
268 The lethal concentrations (96 h) of TAN and NH_3 for silver catfish in normoxic
269 conditions (total hardness: 20 mg $CaCO_3$ L^{-1}; 25°C) are 7.73 and 0.44 mg L^{-1}, respectively, at
270 pH 6.0 (Miron, Moraes, Becker, Crestani, Spanevello, Loro & Baldisserotto 2008). Total
271 ammonia and NH_3 levels were much lower at the end of the transport in the present study than

lethal values. In addition, exposure to high waterborne NH_3 (0.1 mg L^{-1}) and low DO (3.5 mg L^{-1}) levels for 6 and 24 h caused ionoregulatory changes in this species (Becker, Garcia, Kochhann, Gonçalves, Loro & Baldisserotto 2009). Therefore, low DO levels and high NH_3 levels found in the control treatment could probably explain the increase of mortality in this group.

In the present study DO levels after transport were very low and near the lethal concentration for silver catfish (Braun *et al.* 2006). In addition, oxygen consumption was lower and higher than observed by Golombieski *et al.* (2003; weight of 1.0–2.5 g, loading density of 168 g L^{-1}; transported for 6 h) and Becker *et al.* (*in press*; weight 301.24 g, loading density of 169.2 g L^{-1}; transported for 4 h), respectively, with the same species. This pattern was expected because there is a decline in metabolic rate per unit of body mass with the increase of total body (Bolner & Baldisserotto 2007).

The specimens of silver catfish exposed to concentrations between 20 and 70 µl L^{-1} of eugenol could reach stage 2 of anesthesia within a few minutes (Cunha *et al.* 2010b). On the other hand, silver catfish exposed for 6 h to concentrations between 1 and 50 µL L^{-1} of ME of *C. buxifolia* maintained a uniform sedation – remained in stage 1. During fish transporting, the anesthetic concentrations must induce, at most, stage 2 of anesthesia (stage of deep sedation, which are observed a partial loss of equilibrium and lack of reaction to external stimuli). Carmichael, Tomasso, Simco & Davis (1984) reported an enhanced of survival and a reduction of stress parameters (plasma glucose and corticosteroids decreased and plasma Cl^- and osmolality increased) during transport of largemouth black bass, *Micropterus salmoides*, in water with MS-222. Moreover, the use of benzocaine-hydrochloride (25 mg L^{-1}) on Mozambique tilapia, *Oreochromis mossambicus*, reduced oxygen consumption at about 1/3 and decreased ammonia and CO_2 excretion (Ferreira, Schoonbee & Smith 1984). The addition of 2-phenoxyethanol to the water of transport (110 and 220 mg L^{-1}) was effective in avoiding

297 mortality in guppies, *Poecilia reticulata*, transported at higher loading densities and for long
298 periods of time (Teo, Chen & Lee 1989). In addition, fry of the Indian carp *Catla catla*, *Labeo*
299 *rohita* and *Cirrhinus mrigala* also exposed to 2-phenoxyethanol (0.09 mg L^{-1}) exhibited a
300 decreased NH_3 excretion (Singh, Vartak, Balange & Ghughuskar 2004). Park, Park, Hur,
301 Kim, Chang, Kim, Park & Johnson (2009) suggested that lidocaine hydrochloride at
302 concentrations of 5, 10 or 20 mg L^{-1} decreased the metabolic activity of flounder,
303 *Pleuronectes americanus*, because this substance reduced ammonia excretion (about 27.4 to
304 30.5%) and oxygen consumption (about 82.7 to 86%) compared with a control group after 5 h
305 transport time.

306 The increase of CO_2 levels observed in all treatments at the end of silver catfish
307 transport probably was responsible for the decrease in water pH, as observed in Golombieski
308 *et al.* (2003) and Becker *et al.* (*in press*). Alkalinity levels, regardless of the treatment,
309 increased after transport probably due to regurgitated food, because the fish did not go
310 through a depuration period and the commercial food given to the fish had calcitic limestone
311 ($CaCO_3$) in its composition. Similar results were found by Golombieski *et al.* (2003) and
312 Becker *et al.* (*in press*).

313 Transport and handling operations are stressful situations that can increase ion loss in
314 freshwater fishes by the increase of gill blood flow and paracellular permeability (Cech Jr.,
315 Bartholow, Young & Hopkins 1996; McDonald, Cavdek & Ellis 1991). In the present study,
316 eugenol and ME of *C. buxifolia* in the water of transport reduced ion loss in silver catfish.
317 These results were similar to those found by our research group with the same species with
318 1.5 or 3.0 µL L^{-1} of eugenol and 10 or 20 µL L^{-1} of the essential oil of *L. alba* added to the
319 transport water during 4 h (Becker *et al. in press*). In addition, other studies reported that
320 anesthetics used for fish transport reduced agitation and fish stress (Guo *et al.* 1995; Singh *et*

al. 2004; Park *et al.* 2009). Therefore, eugenol and ME of *C. buxifolia* might have sedated silver catfish during the transport and reducing ion loss.

The obtained results allow concluding that the best concentration range of ME of *C. buxifolia* is 0.5 – 10 μL L^{-1}, because higher concentration levels increased time of sedation. Moreover, the addition of the anesthetic and sedative agents to the water transport is advisable because they reduced fish mortality and ion loss.

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Table 1. Stages of anesthesia in fish (from Schoettger & Julin 1967).

Stage	Description	Behavioural response
1	Light sedation	Partial loss of reaction to external stimuli
2	Deep sedation	Partial loss of equilibrium, no reaction to external stimuli
3a	Total loss of equilibrium	Fish usually turn over but retain swimming ability
3b	Total loss of equilibrium	Swimming ability stops but responds to pressure on the caudal peduncle
4	Anesthesia	Loss of reflex activity, no reaction to strong external stimuli
5	Medullary collapse (death)	Respiratory movement ceases (death)

Table 2. Water parameters before and after transport (12 h) of silver catfish in plastic bags with eugenol and the methanolic extract of *Condalia buxifolia* added to the water.

Water parameter	Before transport	After transport (treatments)				
		Control	eugenol (1 μL L^{-1})	eugenol (2.5 μL L^{-1})	*C. buxifolia* (25 μL L^{-1})	*C. buxifolia* (50 μL L^{-1})
Dissolved oxygen	5.60±0.06	1.46±0.06*d	2.22±0.09*a	1.73±0.04*c	2.22±0.04*a	1.97±0.04*b
Carbon dioxide	4.86±0.11	51.04±1.33*c	85.07±1.12*a	78.48±1.27*b	79.04±1.77*b	76.60±2.13*b
Alkalinity	24.7±0.5	43.0±0.5*a	45.2±1.3*a	41.7±0.8*a	42.0±0.5*a	40.7±2.7*a
Water hardness	21.6±0.5	26.5±0.5*a	28.0±0.5*a	28.0±0.5*a	26.5±0.5*a	26.5±0.5*a
pH	6.98±0.09	6.21±0.05*a	6.04±0.04*b	6.05±0.04*b	6.03±0.05*b	6.03±0.06*b
Temperature	23.1±0.2	28.1±0.3*a	28.1±0.2*a	28.1±0.2*a	28.1±0.2*a	28.1±0.3*a
Total Ammonia Nitrogen	0.10±0.02	5.25±0.12*c	6.12±0.12*a	5.73±0.09*b	5.58±0.09*b	5.66±0.10*b
Un-ionized ammonia	0.0005	0.0060*a	0.0047*b	0.0045*b	0.0042*b	0.0043*b

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

Figures captions

Figure 1. Time to reach the stage of light sedation in silver catfish juveniles of two different weight classes exposed to the methanolic extract of *Condalia buxifolia*. The following equations were fitted to the data:

For fish weighing 1.50 ± 0.02 g

$y = 209.629\ e^{0.015\ x}$

$r^2 = 0.996$

For fish weighing 165.7 ± 22.5 g

$y = 2039.020\ e^{0.017\ x}$

$r^2 = 0.999$

where x = concentration of the methanolic extract of *C. buxifolia* ($\mu L\ L^{-1}$) and y = time for sedation(s).

Figure 2. Mortality after transport of silver catfish in plastic bags with eugenol and the methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different letters indicate difference significant between the treatments ($P < 0.05$).

Figure 3. Net ion (Na^+, Cl^- and K^+) fluxes measured for the transport of silver catfish in plastic bags with eugenol and the methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different letters indicate significant differences between treatments for the same ion ($P < 0.05$).

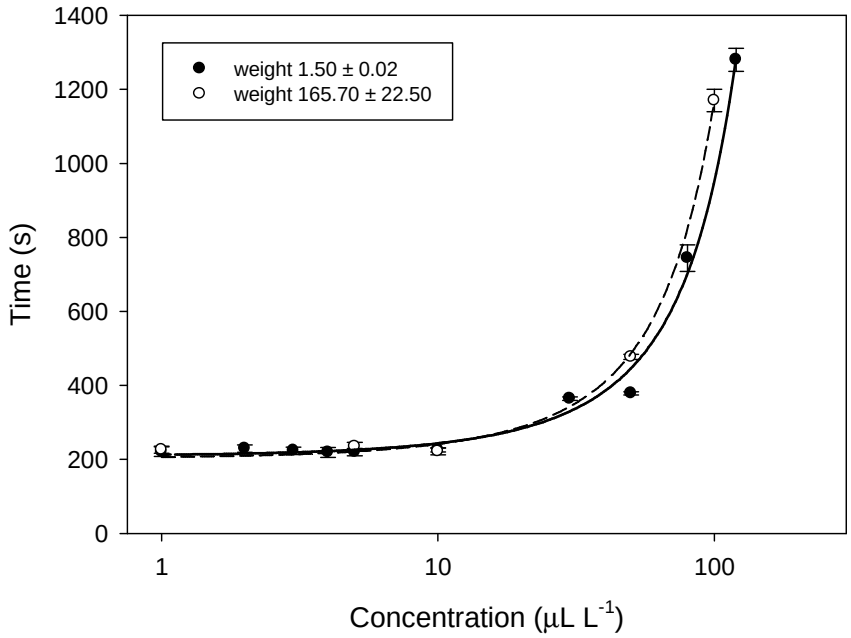
weight 1.50 ± 0.02
weight 165.70 ± 22.50
Time (s)
0
200
400
600
800
1000
1200
1400
1
10
100
Concentration (µL L-1)

Figure 1.

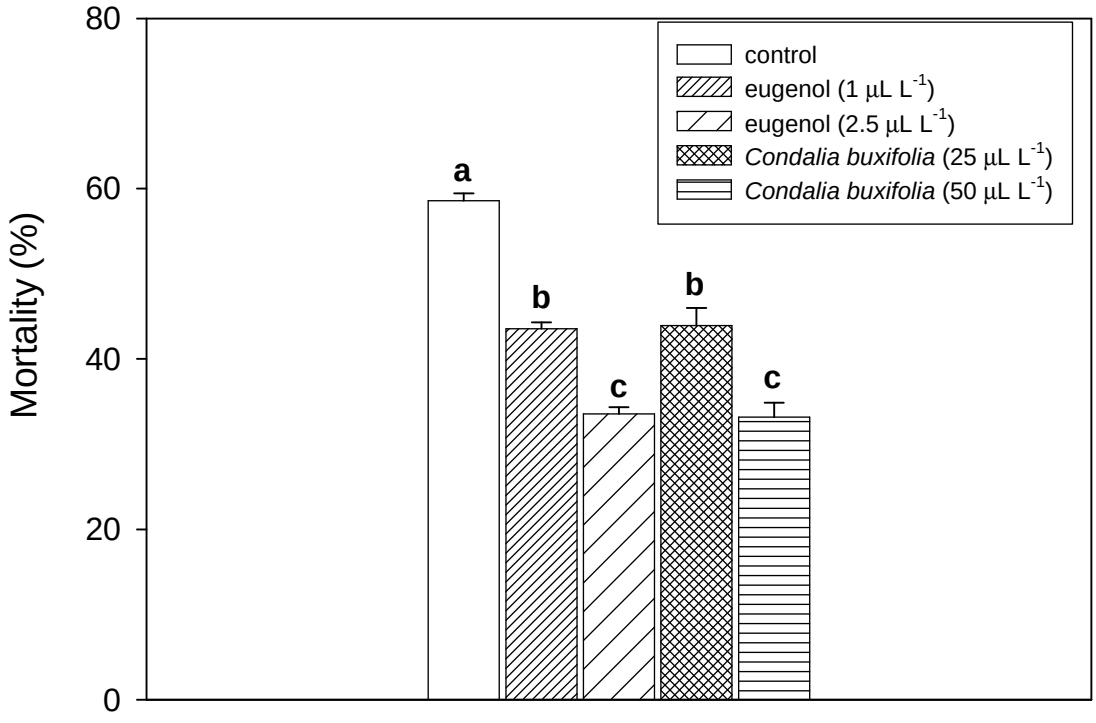
Mortality (%)
80
60
40
20
0
a
b
c
b
c
control
eugenol (1 µL L-1)
eugenol (2.5 µL L-1)
Condalia buxifolia (25 µL L-1)
Condalia buxifolia (50 µL L-1)

Figure 2.

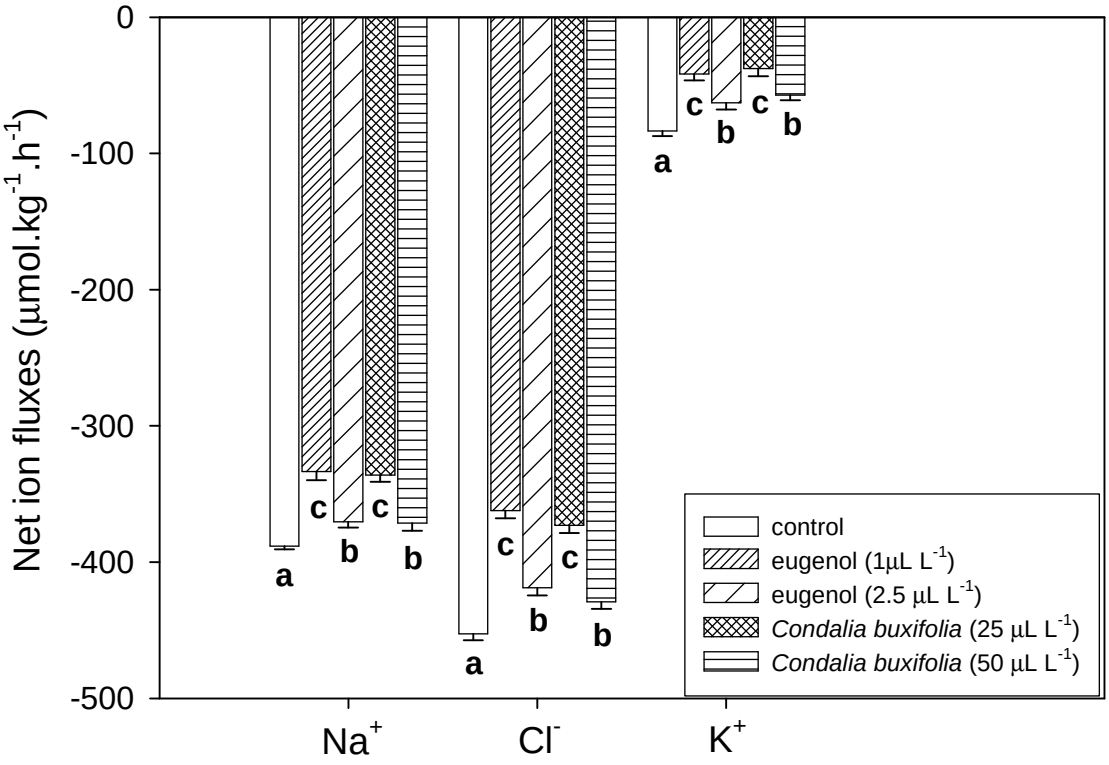
0
-100
-200
-300
-400
-500
Net ion fluxes (µmol.kg-1.h-1)
a
c
b
c
b
a
c
b
c
b
a
c
b
c
b
Na+
Cl-
K+
control
eugenol (1µL L-1)
eugenol (2.5 µL L-1)
Condalia buxifolia (25 µL L-1)
Condalia buxifolia (50 µL L-1)

Figure 3.

ARTIGO 3

Transportation of silver catfish, *Rhamdia quelen*, in water with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia*

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Abstract

This study investigated the effectiveness of the essential oil (EO) of *Lippia alba* and of the methanolic extract (ME) of *Condalia buxifolia* for use during the transport of silver catfish, through the following indicators: blood and water parameters, survival and ionoregulatory balance. Silver catfish, *Rhamdia quelen*, (420.1 ± 8.8 g, 21.2 ± 2.3 cm) were transported at a loading density of 275.1 g L^{-1} for 6 h in fifteen plastic bags with 7 L of water and 8 L of pure oxygen, and divided into five treatments (three replicates each): control (no compound added to the water); 30 or 40 µL L^{-1} of the EO of *L. alba* and 5 or 10 µL L^{-1} of ME of *C. buxifolia*. Before transportation fish were exposed to the EO of *L. alba* (200 µL L^{-1} for three minutes) or the ME of *C. buxifolia* (10 µL L^{-1} for five minutes). Water and blood parameters were measured before and after transportation. Waterborne total ammonia nitrogen levels and net Na^+, Cl^- and K^+ effluxes were highest in the control treatment and lowest in fish transported with ME of *C. buxifolia* added to the water. The highest Pv_{O_2}, Pv_{CO_2} and HCO_3^- values after transport were found in fish transported with 5 µL L^{-1} ME of *C. buxifolia*, followed by these transported with 40 µL L^{-1} EO of *L. alba*. Moreover, plasma cortisol levels were significantly higher in fish transported with 30 µL L^{-1} EO of *L. alba* added in the water than control fish. In conclusion, the concentration of 30 µL L^{-1} of EO of *L. alba* is not advisable for transporting silver catfish, because this concentration enhanced plasma cortisol levels. However, 40 µL L^{-1} of EO of *L. alba* and both concentrations of ME *C. buxifolia* tested were effective in reducing waterborne total ammonia levels and ion loss and are recommended for transporting this fish specie.

Keywords: anaesthetics, blood gases, cortisol, ion fluxes, transport of fish

1. Introduction

The closed system using plastic bags is the main system of freshwater fish transportation, in Brazil, but has some limitations, such as the supply of oxygen and the build-up of ammonia and carbon dioxide produced during transport (Gomes *et al.* 1999; Golombieski *et al.* 2003; Gomes *et al.* 2006a, b; Carneiro *et al.* 2009; Becker *et al. in press*). Several factors, such as duration of transportation, water parameters, size, density and physical condition of the fish and duration of the depuration period before fish transportation can be determinant to success of this procedure (Berka 1986; Golombieski *et al.* 2003; Carneiro *et al.* 2009; Becker *et al. in press*). Some alternatives have been proposed to minimize these problems, as the use of anesthetics, because they may reduce ion loss, plasma cortisol levels, mortality and improve water parameters (Barton & Peter 1982; Becker *et al.* 2011 *in preparation*).

The essential oil (EO) of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae), an aromatic shrub with important medicinal properties, is a new anesthetic whose action has been established for silver catfish, *Rhamdia quelen*, and slender seahorse, *Hippocampus reidi* (Cunha *et al.* 2010, 2011). On the other hand, the methanolic extract (ME) of *Condalia buxifolia* Reissek (Rhamnaceae), a tree rich in peptide alkaloids that possess a variety of biological activities (El-Seedi *et al.* 2007), caused only slight sedation in silver catfish exposed to concentrations between 1.0-50.0 µL L^{-1} for 6 h (Becker *et al. in preparation*).

Some studies regarding the transport of silver catfish in plastic bags were performed (Gomes *et al.* 1999; Golombieski *et al.* 2003; Carneiro *et al.* 2009), including the use anesthetics added to the water transport (Azambuja *et al.* 2011; Becker et al. *in press, in preparation*). Becker *et al.* (*in press*), evaluated the effectiveness of eugenol (1.5 or 3.0 µL L^{-1}) and of EO of *L. alba* (10 or 20 µL L^{-1}) considering same blood and water parameters,

survival and ionoregulatory balance in silver catfish transported for 4 h. Moreover, in another study, Becker *et al.* (*in preparation*) transported this species for 12 h with eugenol (1.0 or 2.5 μL L^{-1}) or ME of *C. buxifolia* (25 or 50 μL L^{-1}) added to the water of transport and verified the water parameters, survival and ionoregulatory balance. Differently of the early studies, the present study used a different methodology – pre-anesthesia before transport, because, firstly, aimed to avoid any mortality during transport, because the loading density was higher, and second, to verify if the procedure of pre-anesthesia combined with sedation, during transport, would result in improvement of the evaluated parameters.

Therefore, the aim of this study was to investigate the effectiveness of the EO of *L. alba* and of the ME of *C. buxifolia* in transport of silver catfish through of the following indicators: blood and water parameters, survival and ionoregulatory balance.

2. Material and Methods

2.1. Plant materials

L. alba was cultivated in São Luiz Gonzaga, Rio Grande do Sul State, Brazil. The aerial parts of the plant were collected in January 2006. The plant material was identified by the botanist Dr. Gilberto Dolejal Zanetti, Departamento de Farmácia Industrial, UFSM, and a voucher specimen (SMDB No. 10050) was deposited in the herbarium of the Departamento de Biologia, UFSM.

The samples of *C. buxifolia* were collected in the Center-South region of the State of Rio Grande do Sul. The identification of the botanical material was performed by comparisons with existing samples in the herbarium of the Departamento de Biologia-UFSM (SMDB No. 3296).

2.2. Essential oil of Lippia alba and methanolic extract of Condalia buxifolia extraction

Essential oil was obtained from the fresh leaves of *L. alba* by steam distillation for 2 h using a Clevenger-type apparatus. In this method, the distillate is collected in a graduated glass tube and the aqueous phase is automatically reused by returning it to the distillation flask (European Pharmacopoeia 2007). The EO samples were stored at -20°C in amber glass bottles.

Freeze-dried bark of *Condalia buxifolia* (2.2 kg) was extracted with MeOH in a Soxhlet extractor. The solvent was evaporated under reduced pressure to obtain 430 g of a dark viscous residue (methanolic crude extract).

2.3. Experimental procedure

Silver catfish (420.1 ± 8.8 g, 21.2 ± 2.3 cm) were captured from a cage net in a fish farm. Fish did not go through a depuration period because this procedure, although recommended (Amend *et al.* 1982), is not followed by most fish producers in southern Brazil (Golombieski *et al.* 2003). Fish were transported at a loading density of 275.1 g L^{-1} for 6 h in fifteen plastic bags with 7 L of water and 8 L of pure oxygen, and they were divided into five treatments (three replicates each). These treatments were as follows: control; 30 or 40 µL L^{-1} of EO of *L. alba* (equivalent to 24 or 32 mg L^{-1}, respectively, because the density of this EO is about 0.80) and 5 or 10 µL L^{-1} of ME of *C. buxifolia* (both firstly diluted in ethanol; 1:10). Fish that were transported with 30 or 40 µL L^{-1} of EO of *L. alba* were anesthetized with this EO (200 µL L^{-1} for three minutes; as reported by Cunha *et al.* 2010, this concentration induce to the stage 2 of anesthesia within the time proposed above), and those transported with 5 or 10 µL L^{-1} of ME of *C. buxifolia* were sedated with this ME (10 µL L^{-1} for five minutes; as reported by Becker *et al. in preparation*, this concentration induce to the stage 2 of anesthesia within the time proposed above) before placing in the plastic bags. Control fish were placed

directly in the plastic bags. The transport time and concentrations of EO of *L. alba* and of ME of *C. buxifolia* were chosen according to Cunha *et al.* (2010) and Becker *et al.* (*in press, in preparation*). Moreover, the concentrations of EO of *L. alba* and of ME of *C. buxifolia* remained within a sedative safe range for silver catfish (Cunha *et al.* 2010; Becker *et al. in preparation*), in order to avoid any mortality during transport. In addition, the loading density was chosen according to Carneiro *et al.* (2009).

2.4. Water sampling and analyses

Water parameters were measured before and after transportation. Dissolved oxygen (DO) and temperature were measured with an YSI oxygen meter. The pH was verified with DMPH-2 pH meter.

Nesslerization verified total ammonia nitrogen (TAN) levels according to the method of Eaton *et al.* (2005). Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd & Tucker (1992). Carbon dioxide (CO_2) was calculated by the method of Wurts & Durborow (1992).

2.5. Ion fluxes

Water samples (5 mL) were collected before and after transportation. Chloride levels were determined according to Zall *et al.* (1956), and Na^+ and K^+ levels were determined with a B262 flame spectrophotometer. Standard solutions were made with analytical-grade reagents dissolved in deionized water, and standard curves of each ion to be tested were made for five different concentrations. Net ion fluxes were calculated according to Gonzalez *et al.* (1998):

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

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

2.6. Blood sampling and analyses

Blood samples (1–1.5 mL) were collected from the caudal vein of each fish using heparinized 3-mL syringes before and after the transporting procedure. This caudal vein is commonly used for the collection of blood samples in many species of fish, but because of the proximity of the vein to an artery, samples are often mixtures of venous and arterial blood (Sladky *et al.* 2001; Hanley *et al.* 2010). The blood samples were kept in ice. The following variables were measured using a clinical analyzer: pH, PvO_2, $PvCO_2$, hematocrit (Hct) and HCO_3^-. The temperature of the clinical analyzer is commonly 37°C, but to determine blood gases, it was corrected to water temperature (27°C) with the assumption that ambient water temperature and individual fish body temperatures were equivalent (Hanley *et al.* 2010). In addition, Howell *et al.* (1970) reported that ectotherm vertebrates, including fish, maintain an acid–base balance despite changes in body temperature.

Plasma cortisol levels were measured using a commercially available immunoluminometry kit (Immulite 2000) (Diagnostic Products Corporation, Los Angeles CA, USA). The specificity of the test was previously evaluated (Cunha *et al.* 2010).

2.7. Statistical analyses

All data are expressed as mean ± SEM. Homogeneity of variances between treatments was tested with Levene test. Data exhibited homogeneous variances, so comparisons among different treatments and times were made using one-way ANOVA and Tukey's test. Analysis

was performed using the software Statistica ver. 7.0, and the minimum significance level was set at $P < 0.05$.

3. Results

3.1. Water parameters and mortality

No mortality was recorded in any treatment following transport. In all water parameters no significant difference between treatments after transport was observed, except TAN levels, where the highest levels were reported in the control treatment and, lowest levels in the water of fish transported with ME of *C. buxifolia* (Table 1).

3.2. Ion fluxes through transportation

The net Na^+, Cl^- and K^+ effluxes were significantly highest in fish from the control treatment. Moreover, the lowest net Na^+ and Cl^- effluxes were found in fish transported with 5 or 10 µL L^{-1} of ME of *C. buxifolia*. The net Ca^{2+} fluxes did not show any significant difference between treatments (Figure 1).

3.3. Blood parameters

The highest PvO_2, $PvCO_2$ and HCO_3^- values after transport were found in the treatment with 5 µL L^{-1} ME of *C. buxifolia*, followed by the treatment with 40 µL L^{-1} EO of *L. alba*. Blood pH was not affected by treatments. On the other hand, hematocrit (Hct) values decreased after transport, but without significant difference between the treatments after the transportation period (Table 2).

Plasma cortisol levels were significantly higher in fish transported with 30 µL L^{-1} EO of *L. alba* added in the water compared to control fish. Values found to the other treatments were similar (Figure 2).

4. Discussion

The TAN and NH_3 lethal concentrations (96 h) in normoxia (water hardness: 20 mg $CaCO_3$ L^{-1}; 25ºC) of silver catfish are 7.73 and 0.44 mg L^{-1}, respectively, at pH 6.0 (Miron *et al.* 2008). Total ammonia nitrogen and NH_3 levels were much lower at the end of the transport in the present study than lethal values. Therefore, silver catfish could be transported during 6 h without problems due to ammonia toxicity considering the conditions used in our experiments (weight of 420.1 g, loading density of 275.1 g L^{-1}). The TAN excretion by silver catfish transported in our study was 2.36 mg kg^{-1} fish h^{-1}, over 7.91-fold lower than reported by Carneiro *et al.* (2009) (18.68 mg kg^{-1} fish h^{-1}; weight of 20 g, loading density of 150 g L^{-1}, transported for 4 h), and over 3.35-fold lower than reported by Becker *et al.* (*in press*) (7.92 mg kg^{-1} fish h^{-1}) (weight of 301.24 g, loading density of 169.2 g L^{-1}, transported for 4 h), both studies with the same species. The result found in the present study are in accordance with reported by Bolner & Baldisserotto (2007), which affirmed that ammonia excretion decreases with increasing fish mass in silver catfish.

The DO levels found in our study after 6 h of transport still remained within a safe range for silver catfish (control group – 8.68 mg L^{-1}) (Braun *et al.* 2006), because pure oxygen was added to the plastic bags and this addition could be responsible by increasing of the DO levels after the transport. The increase of CO_2 levels observed in all treatments probably was responsible for the decrease of water pH at the end of transport as observed by Golombieski *et*

al. (2003) and Becker *et al.* (*in press*). Alkalinity and water hardness levels, regardless of the treatment, increased after transport and, this increase could be explained by the presence of regurgitated food, because the fish did not go through a depuration period and the commercial food given to the fish had calcitic limestone ($CaCO_3$) in its composition. Similar results were found by Golombieski *et al.* (2003) and Becker *et al.* (*in press*).

Silver catfish could reach stage 4 of anesthesia when exposed to concentrations above 100 µl L^{-1} (equivalent to 80 mg L^{-1}) EO of *L. alba* within 15 min (Cunha *et al.* 2010). On the other hand, when exposed for 6 h to concentrations between 1 and 50 µl L^{-1} ME of *C. buxifolia* this species maintained a uniform sedation – remained in stage 1 (Becker *et al. in preparation*). According to the description of Schoettger & Julin (1967), the stage 1 is characterized by partial loss of reaction to external stimuli and the stage 4 by loss of reflex activity through of the reduction of the opercular movement. The stage 2 of anesthesia is equivalent to deep sedation, with a partial loss of equilibrium and lack of reaction to external stimuli, and is desirable during fish transporting.

Eugenol (1.5 or 3.0 µl L^{-1}) and EO of *L. alba* (10 or 20 µl L^{-1}) added to the water of transport reduced ion loss and ammonia excretion of silver catfish after 4 h of transporting (Becker *et al. in press*). Moreover, with the same species, but transported for 12 h, Becker et al. (*in preparation*) reported reduction of oxygen consumption, NH_3 levels, ion loss and mortality, when eugenol (1.0 or 2.5 µl L^{-1}) was added to the water of transport or ME of *C. buxifolia* (25 or 50 µl L^{-1}). In addition, in specimens of largemouth black bass, *Micropterus salmoides*, transported in water with MS-222 (tricaine methanesulfonate) showed reduction of stress parameters (plasma glucose and corticosteroids) and enhanced survival (Carmichael *et al.* 1984). In Mozambique tilapia, *Oreochromis mossambicus*, oxygen consumption reduced to about 1/3 and ammonia and CO_2 excretion decreased when exposed to benzocaine-hydrochloride (25 mg L^{-1}) (Ferreira *et al.* 1984). The addition of 2-phenoxyethanol to the

water used in transport (110 and 220 mg L^{-1}) was effective in avoiding mortality in guppies, *Poecilia reticulata*, transported at higher loading densities and for long periods of time (Teo *et al.* 1989). In the fry of the Indian carps *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala* (0.09 mg L^{-1}), this treatment also decreased NH_3 excretion (Singh *et al.* 2004). Park *et al.* (2009) suggested that lidocaine hydrochloride at concentrations of 5, 10 or 20 mg L^{-1} decreased the metabolic activity of flounder, *Pleuronectes americanus*, because this substance reduced ammonia excretion (about 27.4 to 30.5%) and oxygen consumption (about 82.7 to 86%) compared with a control group after 5 h transport time.

The blood pH values found in the present study were not altered with compounds added to the transport water. Similar results were found for walleye pike (*Sander vitreus*) and koi (*Cyprinus carpio*) anesthetized with MS-222 (150 mg L^{-1}) and buffered with $NaHCO_3$ (75 mg L^{-1}) (Hanley *et al.* 2010) and silver catfish transported for 4 h with 1.5 or 3.0 µL L^{-1} of eugenol and 10 or 20 µL L^{-1} of EO of *L. alba* added to the transport water (Becker *et al.* 2011). On the other hand, in red pacu (*Piaractus brachypomus*) exposed to MS-222 and eugenol at 50, 100 and 200 mg L^{-1} (Sladky *et al.* 2001) and yellow perch (*Perca flavescens*) anesthetized with MS-222 (150 mg L^{-1}) and buffered with $NaHCO_3$ (75 mg L^{-1}) (Hanley *et al.* 2010) the blood pH values decreased with anesthesia, suggesting that these species could be unable to buffer their blood or due to the process of respiratory or metabolic acidosis. In addition, the other blood gas values ($Pv O_2$, $Pv CO_2$ and HCO_3^-), before transport, were similar or lower than those reported by other studies (Sladky *et al.* 2001; Hanley *et al.* 2010; Becker *et al. in press*).

Silver catfish exposed to EO of *L. alba* and ME of *C. buxifolia* apparently decreased metabolic rate because fish presented significantly lower TAN excretion and net ion loss. However, this lower ammonia excretion could also induce an increase in plasma ammonia levels. In rainbow trout high plasma ammonia levels did not change $Pa O_2$ but increased

PaCO_2 and plasma HCO_3^- (Zhang & Wood 2009), and silver catfish transported in water with 3 µL L^{-1}eugenol or 20 µL L^{-1} EO of *L. alba* also exhibited highest values of *Pv*CO_2 and HCO_3^- in the blood (Becker *et al. in press*). These results are in agreement with findings of the present study: there was an increase in the values of *Pv*CO_2 and HCO_3^- in fish transported with 40 µL L^{-1} EO of *L. alba* and 5 µL L^{-1} ME of *C. buxifolia*. The high plasma ammonia values induced hyperventilation in rainbow trout (Zhang & Wood 2009), but, unfortunately, both plasma ammonia and ventilatory frequency were not measured in the present experiment. Therefore, additional experiments may be important to explain these results.

In the present study, the Hct values (24–30%) were similar to those found by Carneiro *et al.* (2009) (Hct: 27–30%) and Becker *et al.* (*in press*) (Hct: 26–33%) with the same species. After transport Hct values decreased, but without significant difference between treatments. Transport procedures can produce stress and ion loss in freshwater fish through the increase of blood gill flow and paracellular permeability (McDonald *et al.* 1991; Cech Jr. *et al.* 1996). Therefore hemodilution could be caused by osmoregulatory disturbance, probably an influx of water (Houston *et al.* 1996; Morgan & Iwama 1997).

In the present study, EO of *L. alba* and ME of *C. buxifolia* in the water of transport reduced ion loss in silver catfish. These results were similar to those found by our research group with the same species (Becker *et al.* 2011). Other studies reported that anesthetics used for fish transport reduced agitation and fish stress (Guo *et al.* 1995; Singh *et al.* 2004; Park *et al.* 2009). In view of this, EO of *L. alba* and ME of *C. buxifolia* could have reduced gill blood flow because fish were less agitated.

The transport procedures may cause a massive release of hormones, primarily catecholamines (adrenaline and noradrenaline) and corticosteroids (cortisol) (Donaldson 1981; Mazeaud & Mazeaud 1981; Barton & Iwama 1991; Mommsen *et al.* 1999; Reid *et al.* 1998). The plasma cortisol level in control silver catfish at the end of transport (60.63 ng mL^-

^{1}) was higher than those found in non-stressed (around 29 ng mL^{-1}) (Barcellos *et al.* (2001, 2004) or submitted to rapid handling (about 32 ng mL^{-1}) (Cunha *et al.* 2010). On the other hand, Carneiro *et al.* (2009; 56.1 ng mL^{-1}, transport by 4 h; loading density 75 g L^{-1}) found similar results to the reported by our study. The fish transported with EO of *L. alba* at concentration of 30 µl L^{-1} the plasma cortisol level was significantly higher than those transported in the control group. Therefore, this anesthetic concentration is not efficient to reduce this blood parameter. In addition, some studies have reported that the exposure to anesthetics in itself induces increase levels of cortisol (Barton & Peter 1982; Davidson *et al.* 2000; Davis & Griffin 2004; Kiessling *et al.* 2009; Molinero & Gonzalez 1995; Thomas & Robertson 1991). On the other hand, Cunha *et al.* (2010) reported that specimens of silver catfish exposed to the EO of *L. alba* at concentration of 300 µL L^{-1} (equivalent to 240 mg L^{-1}) exhibited lowest plasma cortisol levels 1 and 4 h after handling.

In conclusion, the concentration of 30 µL L^{-1} of EO of *L. alba* is not advisable for transporting silver catfish, because this concentration enhanced plasma cortisol levels. However, 40 µL L^{-1} of EO of *L. alba* and both concentrations of ME *C. buxifolia* tested were effective in reducing waterborne total ammonia levels and ion loss and are recommended for transporting this specie.

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Table 1. Water parameters before and after transport (6 h) of silver catfish in plastic bags with essential oil of *Lippia alba* and methanolic extract of the *Condalia buxifolia* added to the water.

Water parameter	Before transport	After transport (treatments)				
		Control	*Lippia alba* (30 µL L^{-1})	*Lippia alba* (40 µL L^{-1})	*Condalia buxifolia* (5 µL L^{-1})	*Condalia buxifolia* (10 µL L^{-1})
Dissolved oxygen	5.35±0.68	8.68±1.09*a	8.22±0.89*a	7.77±0.78*a	8.74±1.64*a	8.02±0.52*a
Carbon dioxide	6.35±0.47	54.84±2.47*a	58.34±1.93*a	59.28±2.13*a	60.69±3.21*a	57.49±2.79*a
Alkalinity	20.30±1.77	29.14±2.20*a	31.00±1.95*a	31.50±2.29*a	32.25±1.85*a	30.55±2.15*a
Water hardness	15.73±1.72	21.00±1.80*a	22.35±1.50*a	23.12±1.70*a	24.12±1.90*a	23.32±1.30*a
pH	6.74±0.04	6.01±0.05*a	6.03±0.07*a	6.08±0.13*a	6.11±0.05*a	6.13±0.07*a
Temperature	27.53±0.56	27.30±0.62a	26.45±0.48a	25.97±0.95a	26.10±0.91a	25.85±1.07a
Total ammonia nitrogen	1.36±0.12	3.89±0.19*a	3.45±0.14*b	3.24±0.21*b	2.74±0.15*c	2.72±0.13*c
Un-ionized ammonia	0.005	0.0026*a	0.0023*a	0.0024*a	0.0021*a	0.0022*a

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

Table 2. Blood parameters before and after transport (6 h) of silver catfish in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water

Blood parameter	Before transport	After transport (treatments)				
		Control	*Lippia alba* (30 µL L^{-1})	*Lippia alba* (40 µL L^{-1})	*Condalia buxifolia* (5 µL L^{-1})	*Condalia buxifolia* (10 µL L^{-1})
pH	7.32±0.07	7.26±0.04a	7.30±0.04a	7.31±0.04a	7.25±0.04a	7.27±0.05a
PvO_2 (mm Hg)	10.21±1.88	16.08±1.69*c	15.67±1.55*c	21.86±1.31*b	28.23±1.91*a	16.11±1.14*c
$PvCO_2$ (mm Hg)	13.79±0.71	21.57±0.93*c	22.18±1.32*c	27.53±1.01*b	32.28±0.99*a	23.21±1.86*c
Hct (%)	30.46±0.48	24.39±0.85*a	25.67±1.08*a	24.08±0.92*a	25.32±0.94*a	25.15±0.89*a
HCO_3^- (mmol L^{-1})	6.27±0.16	12.45±0.47*c	12.15±0.45*c	13.89±0.50*b	15.58±0.29*a	12.44±0.36*c

Values are means ± SEM. Asterisks indicate significant differences when compared to values before transport ($P<0.05$). Different letters in the rows indicate significant differences between treatments after transport ($P<0.05$)

Figure captions

Figure 1. Net ion (Na^+, Cl^-, K^+ and Ca^{2+}) fluxes measured after the transport of silver catfish in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different letters indicate significant differences between treatments for the same ion ($P<0.05$).

Figure 2. Effect of the essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water on plasma cortisol levels after the transport of silver catfish in plastic bags. Values are means ± SEM. Different letters indicate significant differences between treatments for the same ion ($P<0.05$).

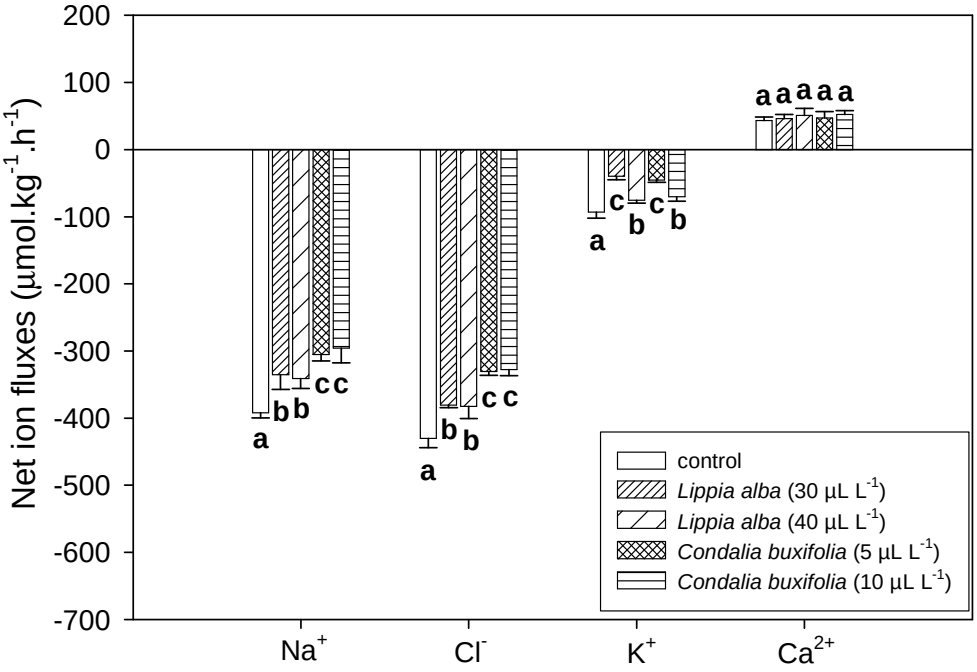
200
100
0
-100
-200
-300
-400
-500
-600
-700
Net ion fluxes (µmol.kg-1.h-1)
a b b c c
a b b c c
a c b c b
a a a a a
Na+
Cl-
K+
Ca2+
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 1.

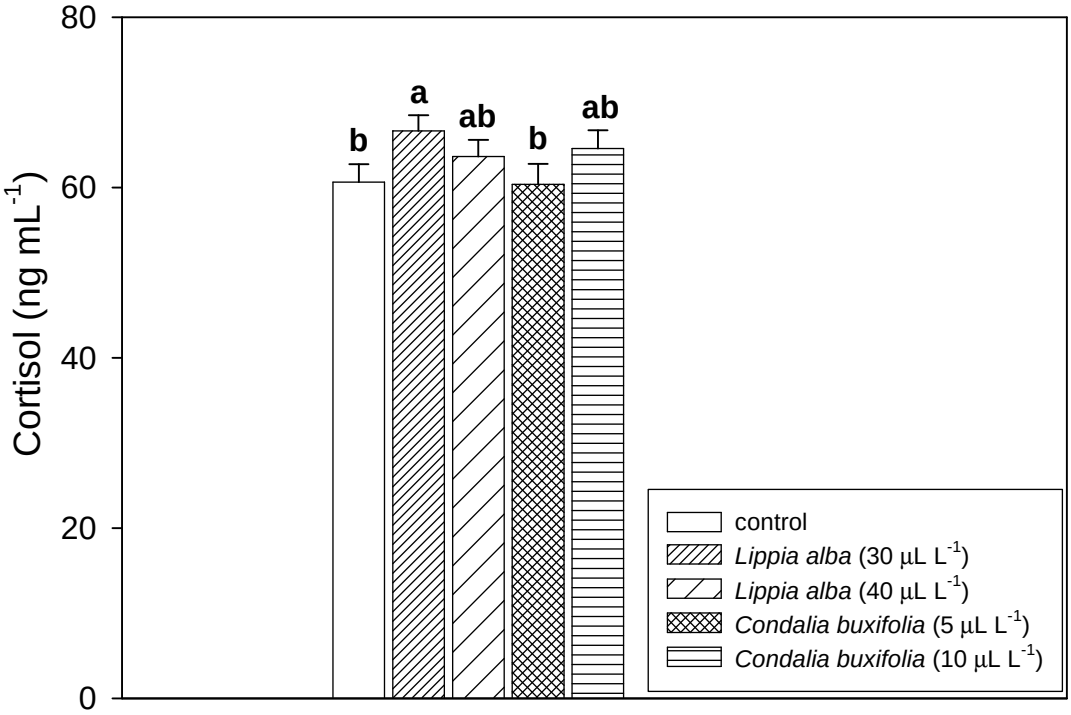
80
60
40
20
0
Cortisol (ng mL-1)
b
a
ab
b
ab
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 2.

ARTIGO 4

Essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* induce biochemical stress in silver catfish, *Rhamdia quelen*, after transportation

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Abstract

The purpose of this study was to investigate the effects of the essential oil (EO) of *Lippia alba* and the methanolic extract (ME) of *Condalia buxifolia* on some biochemical parameters in silver catfish (*Rhamdia quelen*) after transport. Fish (420.1 ± 8.8 g, 21.2 ± 2.3 cm) were transported at a loading density of 275.1 g L^{-1} for 6 h in fifteen plastic bags with 7-L of water, divided in five treatments: control, 30 or 40 µL L^{-1} of EO of *L. alba* and 5 or 10 µL L^{-1} of ME of *C. buxifolia*. Before transportation fish were anesthetized with EO of *L. alba* (200 µL L^{-1} for three minutes) or ME of *C. buxifolia* (10 µL L^{-1} for five minutes). The results showed that in all the treatments with anesthetics added to the water of transport the hepatic catalase (CAT) activity was significantly lower than in the control at the end of transport. On the other hand, in fish transported at 30 µL L^{-1} of EO of *L. alba* hepatic TBARS levels and protein oxidation were significantly higher compared to the control. In the treatments 30 µL L^{-1} of EO of *L. alba* and 5 µL L^{-1} of ME of *C. buxifolia* the glutathione S-transferase (GST) activity in the liver was significantly lower compared to the control. In addition, liver GSH levels were significantly lower in fish transported with both concentrations of the EO of *L. alba.* The LPO/CAT+GPx ratio indicated that the balance between lipoperoxidation and total antioxidant enzymes activities was significantly higher in in fish transported with 30 and 40 µL L^{-1} of EO of *L. alba*. In conclusion, the concentration of 30 µL L^{-1} of EO of *L. alba* is not advisable to transport silver catfish for 6 h with a loading density of 275.1 g L^{-1}, because this concentration induces oxidative stress which can be observed by increased TBARS levels and protein carbonyl and decreased antioxidant defenses. It is concluded that measurement of induction of lipid peroxidation and protein carbonyl may provide useful indicators of exposure to oxidative stress-inducing anesthetic in fish transport.

Keywords: anesthetics, antioxidant defenses, oxidative stress, transport of fish

1. Introduction

The transportation of fish in Brazil involves the use of plastic bags and this system has limitations like the supply of oxygen and the build-up of ammonia total nitrogen and carbon dioxide levels (Gomes et al. 1999, 2006a, b; Golombieski et al. 2003; Carneiro et al. 2009; Becker et al., *in press*). These variations on water parameters provoke stress that could be eliminated or minimized, at least in some species, through the addition of salts, anesthetics and probiotics in the transport water (Carneiro and Urbinati, 2001; Gomes et al., 2003a, b; 2006a, b, 2009; Brandão et al., 2008; Carvalho et al., 2009; Azambuja et al., 2011; Cunha et al., 2011; Becker et al., *in press*).

Fish farmers, generally, added pure oxygen to the plastic bags before transport and this can cause variation on the dissolved oxygen levels and depress metabolic rate, blood flow rearrangement and effective ways of energy production (Nilsson and Renshaw, 2004). In addition, the primary intracellular energy source is the ATP, one of the most important neurotransmitters in the purinergic system, which is responsible for modulating the signaling and biosynthetic processes, such as vascular homeostasis, cell size maintenance, neuronal signaling, immune function, and protein and lipid modification (Fredholm, 1995; Brake and Julius, 1996; Burnstock, 1998; Gayle et al., 1998; Enjyoji et al., 1999; Marcus et al., 2003; Schweibert and Zsembery, 2003; Fields and Burnstock, 2006).

The exposition to hyperoxia, anoxia or hypoxia may result in oxidative changes, because oxygen consumption can determine the levels of reactive oxygen species (ROS) generated and also the antioxidant status (Wilhelm-Filho et al., 2001, 2002; Azambuja et al., 2011). The oxidative metabolism of cells is a continuous source of ROS, resulting from univalent reduction of O_2, that can damage most cellular components, such as carbohydrates, lipids and proteins, and consequently leading to cell death (Miyata et al., 1993; Ahmad et al.,

2000; Morales et al., 2004). To protect from these highly reactive intermediates, living organisms possess a biochemical defense system consisting of enzymatic and non-enzymatic antioxidants that scavenge them. Nevertheless, under several situations, the rate of ROS generation exceeds that of their removal and oxidative stress occurs (Sies, 1986; Di Giulio et al., 1995; Halliwell and Gutteridge, 2000; Livingstone, 2001). The most important antioxidant enzymes of the organisms are superoxide dismutase (SOD), which detoxifies $O_2^{\bullet-}$, catalase (CAT), which reduces H_2O_2, glutathione peroxidase (GPx), which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction, and glutathione reductase (GR) which catalyzes the NADPH-dependent regeneration of glutathione (GSH) from the oxidized form (GSSG) generated by GPx (Halliwell and Gutteridge, 2000).

The essential oil (EO) of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) is a new anesthetic whose action has been established for silver catfish, *Rhamdia quelen*, and slender seahorse, *Hippocampus reidi* (Cunha et al., 2010, 2011). On the other hand, the methanolic extract (ME) of *Condalia buxifolia* Reissek (Rhamnaceae), caused only slight sedation in silver catfish exposed to concentrations between 1.0-50.0 µL L^{-1} for 6 h (Becker et al., *in preparation*).

Increase of plasma cortisol and glucose levels are good indications of stress responses (Iwama et al., 2004; Urbinati and Carneiro, 2004). However, other biochemical parameters like enzymatic activities are also very important to understand stress at a cellular level and how fish respond to the transport and if the addition of an anesthetic to the water could be beneficial or harmful to these animals. To our knowledge just one study evaluated enzyme activities in fish tissues after transport (Azambuja et al., 2011) and only with EO of *L. alba* (concentration of 10 µL L^{-1}). Therefore, the purpose of this study was to investigate the effects of the EO of *L. alba* and the ME of *C. buxifolia* on some biochemical parameters in

silver catfish after transport. Moreover, an attempt has also been made to assess usefulness of these parameters as biomarkers for fish transport.

2. Material and Methods

2.1. Chemical and reagents

The substrates ATP, ADP, AMP, as well as trizma base, sodium azide, HEPES, acetylthiocholine iodide, 5,5′dithiobis-2-nitrobenzoic acid (DTNB), and Coomassie brilliant blue G were obtained from Sigma Chemical Co and bovine serum albumin, K_2HPO_4, from Reagen.

2.2. Plant materials

L. alba was cultivated in São Luiz Gonzaga, Rio Grande do Sul State, Brazil. The aerial parts of the plant were collected in January 2006. The plant material was identified by the botanist Dr. Gilberto Dolejal Zanetti, Departamento de Farmácia Industrial, UFSM, and a voucher specimen (SMDB No. 10050) was deposited in the herbarium of the Departamento de Biologia, UFSM.

The samples of *C. buxifolia* were collected in the Center-South region of the State of Rio Grande do Sul. The identification of the botanical material was performed by comparisons with existing samples in the herbarium of the Departamento de Biologia-UFSM (SMDB3296).

2.3. Essential oil of Lippia alba and methanolic extract Condalia buxifolia extraction

Essential oil was obtained from the fresh leaves of the plant by steam distillation for 2 h using a Clevenger-type apparatus. In this method, the distillate is collected in a graduated glass tube and the aqueous phase is automatically reused by returning it to the distillation flask (European Pharmacopoeia, 2007). The EO samples were stored at -20°C in amber glass bottles.

Freeze-dried bark of *Condalia buxifolia* (2.2 kg) was extracted with MeOH in a Soxhlet extractor. The solvent was evaporated under reduced pressure to obtain 430 g of a dark viscous residue (methanolic crude extract).

2.4. Experimental procedure

Silver catfish (420.1 ± 8.8 g, 21.2 ± 2.3 cm) were captured from a cage net in a fish farm. Fish did not go through a depuration period because this procedure, although recommended (Amend et al. 1982), is not followed by most fish producers in southern Brazil (Golombieski et al. 2003). Fish were transported at a loading density of 275.1 g L^{-1} for 6 h in fifteen plastic bags with 7 L of water and 8 L of pure oxygen, and they were divided into five treatments (three replicates each). These treatments were as follows: control; 30 or 40 µL L^{-1} of EO of *L. alba* (equivalent to 24 or 32 mg L^{-1}, respectively, because the density of this EO is about 0.80) and 5 or 10 µL L^{-1} of ME of *C. buxifolia* (both firstly diluted in ethanol; 1:10). Fish that were transported with 30 or 40 µL L^{-1} of EO of *L. alba* were anesthetized with this EO (200 µL L^{-1} for three minutes; as reported by Cunha et al. 2010, this concentration induce to the stage 2 of anesthesia within the time proposed above), and those transported with 5 or 10 µL L^{-1} of ME of *C. buxifolia* were sedated with this ME (10 µL L^{-1} for five minutes; as reported by Becker et al. *in preparation*, this concentration induce to the stage 2 of anesthesia within the time proposed above) before placing in the plastic bags. Control fish were placed directly into the plastic bags. The transport time and concentrations of EO of *L. alba* and of

ME of *C. buxifolia* were chosen according to Cunha et al. (2010) and Becker et al. (*in press*; *in preparation*). Moreover, the concentrations of EO of *L. alba* and of ME of *C. buxifolia* remained within a sedative safe range for silver catfish (Cunha et al., 2010; Becker et al., *in preparation*), in order to avoid any mortality in the transport. In addition, the loading density was chosen according to Carneiro et al. (2009).

The water parameters were monitored before and after transporting, and, the mean values for these parameters were the following: dissolved oxygen (8.29 mg L^{-1}), carbon dioxide (58.13 mg L^{-1}), alkalinity (30.89 mg $CaCO_3$ L^{-1}), water hardness (22.78 mg $CaCO_3$ L^{-1}), pH (6.07), temperature (26.33 ºC), total ammonia nitrogen (3.21 mg L^{-1}) and un-ionized ammonia (0.0023 mg L^{-1}). Dissolved oxygen and temperature were measured with an YSI oxygen meter. The pH was verified with DMPH-2 pH meter. Nesslerization verified total ammonia nitrogen levels according to the method of Eaton et al. (2005). Un-ionized ammonia levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd and Tucker (1992). Carbon dioxide was calculated by the method of Wurts and Durborow (1992).

After transport, all fish were dipped in ice-slurry (2.4 kg ice: 3.6 L water) for 5 min and killed by spinal cord section, whole brain and liver were carefully removed to determine biochemical parameters.

2.5. NTPDase (ecto-apirase, ecto/CD39; E.C. 3.6.1.5) and 5'-nucleotidase (CD73; E.C. 3.1.3.5) activities assays

The NTPDase enzymatic assay of the whole brain was carried out in a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μL as described by

Schetinger et al. (2000). Twenty microliters of enzyme preparation (8–12 μg of protein) were added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of ATP or ADP as substrate to obtain a final concentration of 1.0 mM and incubation proceed for 20 min.

5′-nucleotidase activity was determined essentially by the method of Heymann et al. (1984) in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 μL. Twenty microliters of enzyme preparation (8–12 μg of protein) were added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of AMP as substrate to a final concentration of 2.0 mM and proceeded for 20 min. In all cases, reaction was stopped by the addition of 200 μL of 10% trichloroacetic acid (TCA) to obtain a final concentration of 5%. Following, the tubes were chilled on ice for 10 min. The released inorganic phosphate (Pi) was assayed by the method of Chan et al. (1986) using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the synaptosomal fraction after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate. Enzyme activities are reported as nmol Pi released min^{-1} mg $protein^{-1}$.

2.6. Antioxidant enzymes

SOD (SOD; E.C. 1.15.1.1) activity was determined in liver as the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine (0.017 mL) and 50 mM glycine–NaOH (pH 10.5) (1 mL). A unit of SOD is defined as the amount of enzyme that inhibits by 50% the speed of detector (epinephrine) reduction. Enzyme activity was expressed in unit mg $protein^{-1}$ using the method described by Misra and Fridovich (1972).

Catalase (CAT; E.C. 1.11.1.6) hepatic activity was assayed by ultraviolet spectrophotometer (Nelson and Kiesow, 1972). Samples of were homogenized in a Potter–Elvehjem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.4 (with 0.1% Triton X-100 and 150 mM NaCl) (1:20 dilution), centrifuged at 10 000*g* for 10 min at 4 °C. The assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M), and 0.01 mL homogenate. Change of H_2O_2 absorbance in 60 s was measured at 240 nm. Catalase activity was calculated and expressed in μmol min^{-1} mg $protein^{-1}$.

2.7. Glutathione S-transferase (GST) assay

GST activity was measured in liver following the method described by Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) (0.15 mL) as a substrate which was added to mixture containing potassium phosphate buffer (20 mM, pH 6.5) (2.5 mL), reduced glutathione (10 mM) (0.3 mL) and homogenate (0.05 mL). The formation of S-2,4-dinitrophenyl glutathione was monitored by the increase in absorbance at 340 nm against blank (buffer and other reagents used to measure the enzyme activity in the absence of sample). The extinction coefficient used for CDNB was 9.6 mM cm^{-1}. The activity was expressed as μmol GS-DNB min^{-1} mg $protein^{-1}$.

2.8. Glutathione peroxidase

The enzyme activity was measured in liver according to Paglia and Valentine (1967). The assay solution contained 100 mM potassium phosphate buffer, pH 7.0, 1 mM GSH, 0.15 mM NADPH, 0.1 U mL^{-1} of glutathione reductase, 100 mM azida and a suitable sample of enzyme solution. After pre incubation, the reaction was started with the addition of peroxides.

The value for a blank reaction with the enzyme source replaced by buffer was subtracted for each assay. The rate of reaction was recorded at 37°C by following the decrease in absorbance at 340 nm. Enzyme activity was determined at 37°C by measuring the disappearance of NADPH at 340 nm and expressed as nmoles NADPH min^{-1} mg $protein^{-1}$.

2.9. Nonenzymatic antioxidant

Nonprotein thiols groups (GSH) was studied as nonenzymatic antioxidant. An aliquot of the hepatic supernatant (1.0 mL) was mixed with 1.0 mL 10% trichloroacetic acid followed by centrifugation. GSH levels were determined by the method of Ellman (1959). Supernatants (0.25 mL) were used for determination with 5,5′-dithio-bis(2-nitrobenzoic acid) 10 mM (DTNB) (0.05 mL) and phosphate buffer 0.5 mM (pH 6.8) (0.7 mL). The optical density of reaction product was read at 412 nm on a spectrophotometer, and results were expressed as μmol nonprotein thiols g $fish^{-1}$.

2.10. Lipid peroxidation estimation

Lipid peroxidation in liver was estimated by a TBARS (thiobarbituric acid-reactive substances) assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), which was optically measured according to Buege and Aust (1978). Aliquots of supernatants (0.25 mL) were mixed with 10% trichloroacetic acid (TCA) (0.25 mL) and 0.67% thiobarbituric acid (0.5 mL) to adjust to a final volume of 1.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 15 min at 95 °C. After cooling, it was centrifuged at 5000*g* for 15 min, and optical density was measured by spectrophotometer at 532 nm. TBARS levels were expressed as nmol MDA mg $protein^{-1}$.

2.11. Protein carbonyl assay

Hepatic supernatant (0.4 mL) was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer. Protein carbonyl content was assayed by the method described by Yan et al. (1995) with some modifications. Soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2N hydrochloric acid (0.2 mL). After incubation at room temperature for 1 h in dark, 0.5 mL of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL of heptane (99.5%), and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 40 s, and centrifuged at 10 000*g* for 15 min. Then, the protein isolated from the interface was washed twice by resuspension in ethanol/ethyl acetate (1:1) and suspended in 1 mL of denaturing buffer, and the carbonyl content was measured spectrophotometrically at 370 nm. Assay was performed in duplicate, and two tubes blank incubated with 2N HCl (0.2 mL) without DNPH was included for each sample. The total carbonylation was calculated using a molar extinction coefficient of 22 000 M cm^{-1}. The protein carbonyl content was expressed as nmol carbonyl mg $protein^{-1}$.

2.12. Protein determination

Protein was determined by the Coomassie blue method following Bradford (1976), using bovine serum albumin as standard, absorbance of samples was measured at 595 nm.

2.13. Statistical analyses

All data are expressed as mean ± SEM. Homogeneity of variances among treatments was tested with Levene test. Data presented homogeneous variances, so comparisons between different treatments were made by ANOVA and Tukey's test. Analysis was performed using the software Statistica ver. 7.0 (StatSoft, Tulsa, OK), and the minimum significance level was set at $P < 0.05$.

3. Results

The NTPDase and 5' nucleotidase activities in whole brain of silver catfish did not show any significant difference between the treatments (Figure 1). The SOD activity in the liver did not present any significant difference between treatments (Figure 2A), CAT activity was significantly lower in fish transported with EO of *L. alba* or ME of *C. buxifolia* than in control fish (Figure 2B).

The GST activity in the liver was significantly lower in silver catfish transported with 30 µL L^{-1} of EO of *L. alba* or 5 µL L^{-1} of ME of *C. buxifolia* compared to control fish (Figure 3A). The GPx activity in the liver was significantly lower in all treatments with *L. alba* and *C. buxifolia* compared to the control (Figure 3B). Moreover, the lowest activity was observed in fish transported with 40 µL L^{-1} of EO of *L. alba* (Figure 3B). The levels of GSH in liver were significantly lower in fish transported with both concentrations of EO of *L. alba* (30 and 40 µL L^{-1}) compared to the other treatments, but transportation with ME of *C. buxifolia* did not change this parameter (Figure 3C).

The TBARS levels and protein carbonyl in the liver were higher in treatment with 30 µL L^{-1} of EO of *L. alba* added to the water of transport when compared to control, but other treatments did not change TBARS and protein carbonyl in the liver (Figure 4A and 4B).

The LPO/CAT + GPx ratio indicated that the balance between lipoperoxidation and total antioxidant enzymes activities was significantly higher in treatments with 30 or 40 µL L^{-1} of EO of *L. alba* compared to control fish and those transported with 5 µL L^{-1} of the ME of *C. buxifolia*, which were similar (Figure 5).

4. Discussion

Generally, anesthetics cause a depression of the central nervous system (CNS), either by interrupting the action potential of axons, release of neurotransmitters, excitability of the membrane or, yet, a combination of all these actions (Ross and Ross, 2008). In addition, independently of the anesthetic used, uptake occurs by the gills (mainly) and skin and arriving at the circulatory system, when it could block any reflex actions (Summerfelt and Smith, 1990). In view of this, as the EO of *L. alba* and ME of *C. buxifolia* exhibited anesthetic and sedative effects, respectively, in silver catfish (Cunha et al., 2010a; Becker et al., *in preparation*), making it possible to state that these compounds affected the CNS of this species.

The primary intracellular energy source is the ATP, which acts also as an extracellular signaling molecule (Fields and Burnstock, 2006) and is considered to be one of the most important neurotransmitters in the purinergic system (Fredholm, 1995). The purinergic signaling is responsible for modulating the signaling and biosynthetic processes in which nucleotides are involved, such as vascular homeostasis, cell size maintenance, neuronal signaling, immune function, and protein and lipid modification (Brake and Julius, 1996; Burnstock, 1998; Gayle et al., 1998; Enjyoji et al., 1999; Marcus et al., 2003; Schweibert and Zsembery, 2003). However, in our study there was no significant difference between treatments, and therefore, the concentrations of EO of *L. alba* and the ME of *C. buxifolia* used in this study did not exert any effect on enzymes NTPDase and 5'-nucleotidase. Nevertheless, this demonstrates the importance of the neurotransmitters in several biological processes, such as protein and lipid modification, therefore, showing the possible the relationships between purinergic system and oxidative metabolism of cells, which is found through of ROS production.

The oxidative stress is characterized by a disbalance among pro-oxidants and antioxidants, in favor of the pro-oxidants, leading to potential damage (Sies, 1991; Halliwell

and Gutteridge, 2000). In view of this, the antioxidant defense system is constituted by antioxidant enzymes, such as SOD, CAT, GPx, and GST, and by the nonoxidant defense system, like GSH (Storey, 1996; Halliwell and Gutteridge, 2000; Trenzado et al., 2006).

The SOD and CAT are enzymes that protect the organisms of the oxidative damage partially removing oxygen species (Di Giulio et al., 1989). The present study did not find significant difference in the SOD activity between the treatments after transporting silver catfish. Similar results were reported in other species (Karakoc et al., 1997; Lushchak et al., 2001, 2005; Garcia et al., 2008). In addition, Azambuja et al. (2011) reported that silver catfish transported (loading density 140 – 200 g L^{-1}) for 6 h (normoxic conditions: 7.29 – 7.35 mg L^{-1} O_2) with 10 µL L^{-1} EO of *L. alba* added to the water, also did not showed alteration in the antioxidant enzymes SOD and CAT. On the other hand, in our study, there was a significant reduction in hepatic CAT activity in all the treatments with anesthetic or sedative added in the water. In addition, the CAT activity is likely to affect the capacity of liver cells to defend themselves and respond to anesthetics – induced oxidative stress. In addition, impairment in antioxidative enzymes will produce an imbalance between pro – and antioxidant system causing the formation of toxic hydroxyl radicals with direct consequences on cellular integrity and function (Di Giulio et al., 1989; Halliwell and Gutteridge, 2000). A lower level of CAT activity in the liver of silver catfish transported in water with anesthetic could be attributed to an increased production of the superoxide radical (O_2^-), as an excess of this anion is known to inhibit CAT activity (Bainy et al., 1996). Lower hepatic CAT activities also were found in carp (*Cyprinus carpio*) exposed for 4 days to 300 mg L^{-1} of a natural polymer extracted from the exoskeleton of crustaceans – chitosan (Dautremepuits et al., 2004) and juveniles of *Prochilodus lineatus* exposed to different concentrations (2.5, 5.0 and, 7.5 g L^{-1}) of neem extract for 24 h (Winkaler et al., 2007).

The alterations in GPx activity are generally accompanied by changes in the level of GSH, because the GSH is co-substrate for H_2O_2 decomposition by GPx (Sies, 1999). The major cellular thiol that participates in cellular redox reactions – GSH – displayed an important role in the detoxification of electrophilic metabolites catalyzed by glutathione S-transferase (GST) (Sies, 1999; Latha and Pari, 2004). Moreover, GSH may act as a non-enzymatic antioxidant and a cofactor and substrate for GPx and GST enzyme activities (Barata et al., 2005). High GSH levels may protect cellular proteins against oxidation via the GSH redox cycle or by directly detoxifying the ROS generated by exposure to stressors agents (Ruas et al., 2008), but the low GSH content may modulate the activity of GPx and GST enzymes (Brouwer and Brouwer, 1998) as suggested in the present study in silver catfish transported with 30 or 40 µL L^{-1} of EO of *L. alba*.

The lower GST activity in the liver of silver catfish transported with 30 µL L^{-1} of EO of *L. alba* could be explained by a compensation mechanism through the increase of the oxidant levels due to the highest levels of TBARS found in this treatment. On the other hand, the lowest GST activity in silver catfish transported with 5 µL L^{-1} of ME of *C. buxifolia* could be explained by impairment in the detoxifying capacity of the fish in this treatment. Azambuja et al. (2011) reported that silver catfish transported for 6 h in normoxic conditions and with 10 µL L^{-1} EO of *L. alba* added to the water showed lower hepatic TBARS levels when compared to the fish transported without EO in the water, but GST activity did not show any significant alteration.

Under stressful conditions the protective system can be overridden by a rapid production of large amounts of ROS, leading to various modifications in lipids and proteins (Shacter et al., 1994; Stadtman and Berlett, 1998; Zusterzeel et al., 2001). In the present study, TBARS levels and protein carbonyl in the liver of silver catfish were higher upon addition of 30 µL L^{-1} of EO of *L. alba* in the water of transport, indicating higher lipid

peroxidation and protein oxidation, and, therefore, the antioxidant defenses were not totally able to effectively scavenge them.

The cellular injury induced by ROS production could be confirmed by the LPO/CAT+GPx ratio as suggested by Ruas et al. (2008). This ratio establishes the balance between cellular injury expressed by LPO induced by ROS and the main antioxidant enzymes of defense of the organisms (CAT+GPx), which are able to neutralize ROS and/or intermediary metabolites responsible by cellular damage (Ruas et al., 2008). The increased LPO/CAT+GPx ratio allow affirming that large amounts of hydrogen peroxide were produced, overcoming the capacity of CAT and GPx enzymes in neutralize ROS production and resulting in a LPO, mainly in treatment with 30 $\mu L\ L^{-1}$ of EO of *L. alba*.

In conclusion, the use of 30 $\mu L\ L^{-1}$ of EO of *L. alba* is not advisable to transport silver catfish, because this concentration produced lipid peroxidation and protein oxidation. It is concluded that measurement of induction of lipid peroxidation and protein carbonyls may be used as an indicator of exposure to oxidative stress-inducing anesthetics in fish. Moreover, future studies should be done to investigate if lower concentrations to than those tested in this study could show better results.

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Figures captions

Figure 1. NTPDase and 5' nucleotidase activities in whole brain of silver catfish transported in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water using ATP (A), ADP (B) and AMP (C) as substrate. Values are means ± SEM. Different lowercase letters indicate difference significant between the treatments ($P<0.05$).

Figure 2. Superoxide dismutase (SOD) and catalase (CAT) activities (A and B, respectively) in liver of silver catfish transported in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different lowercase letters indicate difference significant between the treatments ($P<0.05$).

Figure 3. Glutathione S-transferase (GST) (A) and glutathione peroxidase (GPx) (B) activities and Nonprotein thiols groups (GSH) (C) levels in liver of silver catfish transported in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different lowercase letters indicate difference significant between the treatments ($P<0.05$).

Figure 4. TBARS levels (A) and protein oxidation (B) in liver of silver catfish transported in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different lowercase letters indicate difference significant between the treatments ($P<0.05$).

Figure 5. LPO/CAT+GPx ratio in liver of silver catfish transported in plastic bags with essential oil of *Lippia alba* and methanolic extract of *Condalia buxifolia* added to the water. Values are means ± SEM. Different lowercase letters indicate difference significant between the treatments ($P<0.05$).

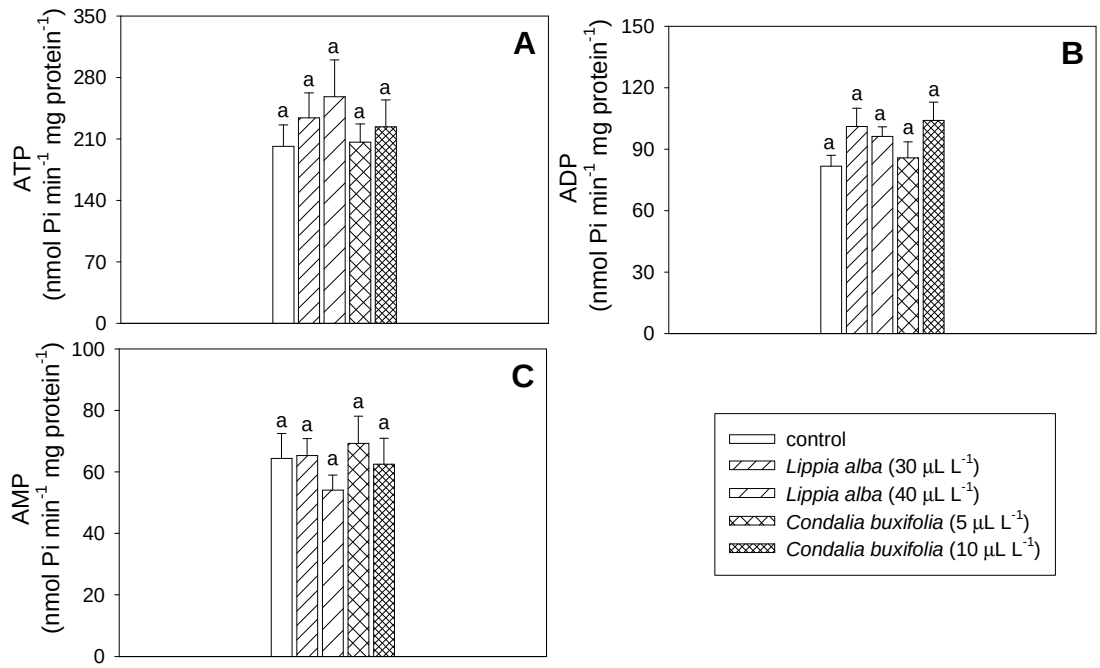
A
ATP
(nmol Pi min-1 mg protein-1)
350
280
210
140
70
0
a
B
ADP
(nmol Pi min-1 mg protein-1)
150
120
90
60
30
0
C
AMP
(nmol Pi min-1 mg protein-1)
100
80
60
40
20
0
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 1.

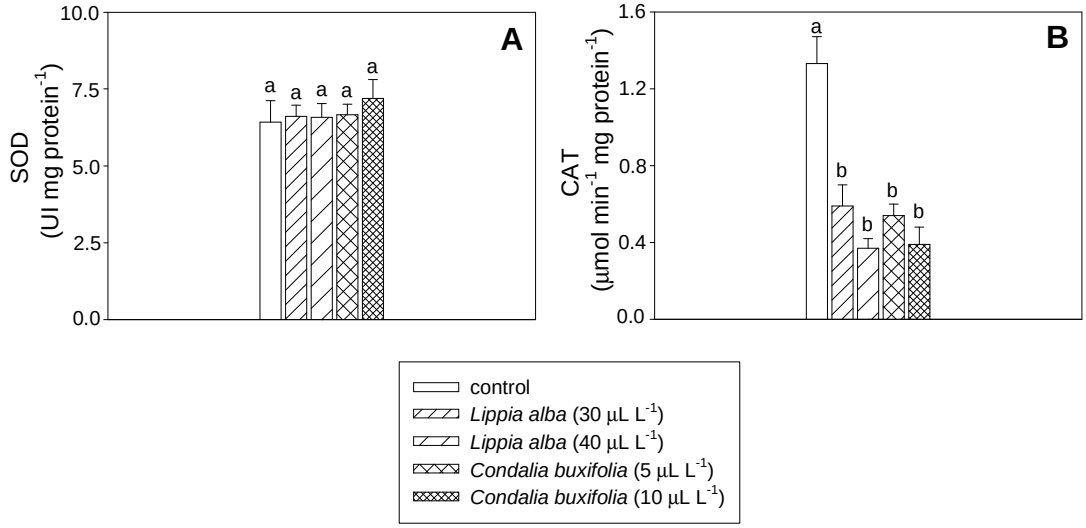
A
B
SOD
(UI mg protein-1)
CAT
(µmol min-1 mg protein-1)
10.0
7.5
5.0
2.5
0.0
1.6
1.2
0.8
0.4
0.0
a
a
a
a
a
a
b
b
b
b
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 2.

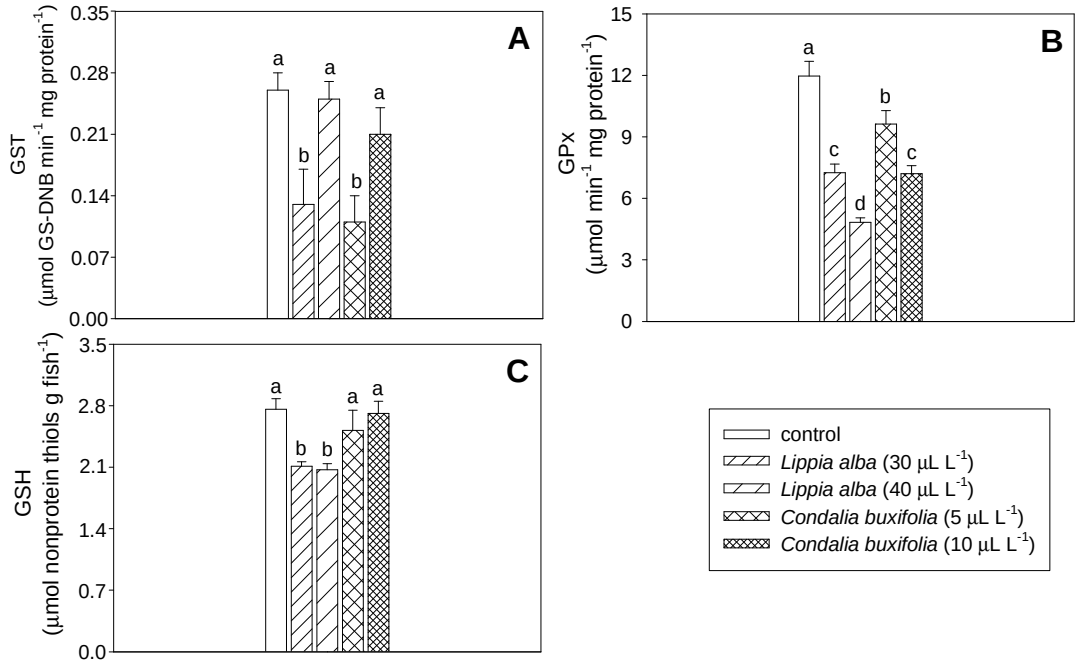
A
GST
(µmol GS-DNB min-1 mg protein-1)
0.35
0.28
0.21
0.14
0.07
0.00
a
b
a
b
a
B
GPx
(µmol min-1 mg protein-1)
15
12
9
6
3
0
a
c
d
b
c
C
GSH
(µmol nonprotein thiols g fish-1)
3.5
2.8
2.1
1.4
0.7
0.0
a
b
b
a
a
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 3.

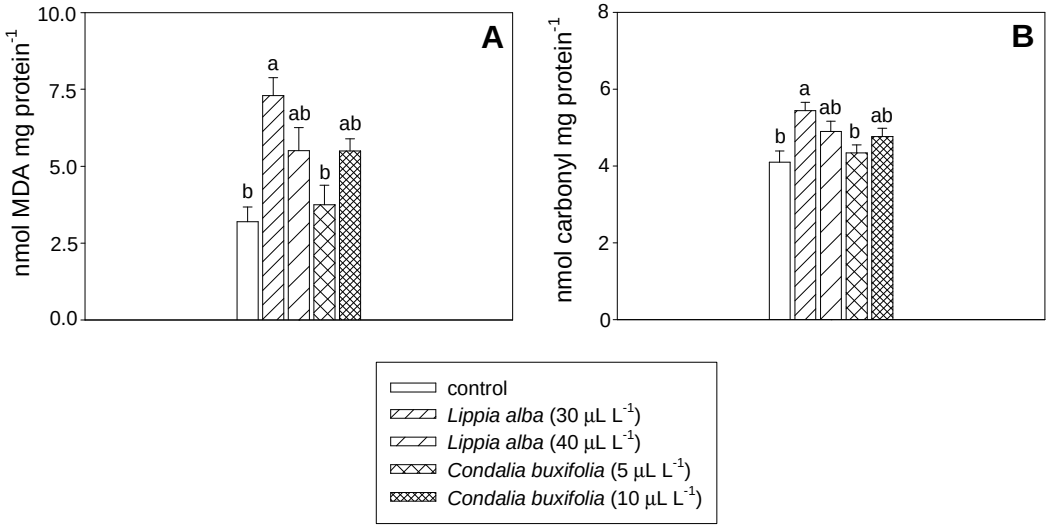
A
B
nmol MDA mg protein-1
nmol carbonyl mg protein-1
10.0
7.5
5.0
2.5
0.0
8
6
4
2
0
a
ab
b
ab
b
a
ab
b
ab
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 4.

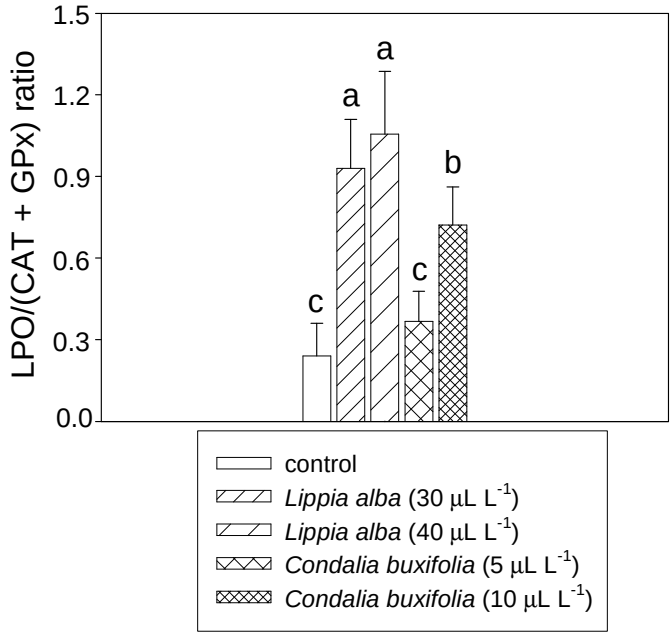
LPO/(CAT + GPx) ratio
1.5
1.2
0.9
0.6
0.3
0.0
a
a
b
c
c
control
Lippia alba (30 µL L-1)
Lippia alba (40 µL L-1)
Condalia buxifolia (5 µL L-1)
Condalia buxifolia (10 µL L-1)

Figure 5.

3 DISCUSSÃO GERAL

Como reportado anteriormente, a utilização de anestésicos no transporte de peixes vivos vem crescendo muito nos últimos anos em nosso país. Sendo assim, procurou-se investigar as possíveis eficiências de anestésicos e sedativos de origem natural no transporte de jundiá, sendo eles os seguintes: eugenol, óleo essencial (OE) de *Lippia alba* e extrato metanólico (EM) de *Condalia buxifolia*.

Primeiramente, constatou-se que o EM de *C. buxifolia* exerce capacidade de sedação em espécimes de jundiá. Concentrações entre 0,5 – 120 µL L^{-1} causam uma sedação leve. Em adição, quando os animais foram expostos a concentrações na faixa de 1,0 – 50,0 µL L^{-1} de EM de *C. buxifolia* constatou-se que eles mantiveram-se durante um período de 6 h em uma profundidade de sedação constante, ou seja, permaneceram no estágio 1. Além disso, o aumento das concentrações de EM de *C. buxifolia* refletiram em um aumento do tempo de sedação. Para aplicação de anestésicos no transporte de peixes vivos sugere-se que os mesmos mantenham os animais no máximo no estágio 2 de anestesia, o qual de acordo com os achados de SCHOETTGER e JULIN (1967) caracteriza-se pela perda parcial do equilíbrio e nenhuma reação aos estímulos externos.

Entre os vários parâmetros avaliados, os relacionados à água apresentaram resultados bastante interessantes. As concentrações letais de amônia total nitrogenada (TAN) e amônia não-ionizada (NH_3) para jundiá mantido em condições de normóxia, são, em pH 6,0, iguais a 7,73 e 0,44 mg L^{-1} e, em pH 7,5, iguais a 2,31 e 1,45 mg L^{-1}, respectivamente (MIRON et al., 2008). Os valores de NH_3 reportados após os períodos de transporte, em nossos experimentos, foram significativamente menores aos encontrados pelos autores acima, portanto, espécimes de jundiá transportados nas mesmas condições das utilizadas nos experimentos podem suportar longos períodos de transporte sem problemas de intoxicação por metabólitos nitrogenados. Os níveis de oxigênio dissolvido (OD) na água, após o transporte, permaneceram dentro da faixa de tolerância recomendada para a espécie (BRAUN et al., 2006), com exceção para o experimento no qual os animais foram transportados com eugenol (1 ou 2,5 µL L^{-1}) ou EM de *C. buxifolia* (25 ou 50 µL L^{-1}) adicionados à água por um período de 12 horas, no qual observou-se valores de OD extremamente baixos e muito próximos do nível letal para a espécie. Os baixos níveis de OD reportados nesse experimento podem ter sido em função do período de transporte, visto que o consumo de oxigênio tem um aumento acentuado com o passar do tempo.

Além disso, em comparação aos outros dois experimentos, independentemente da concentração e do anestésico ou sedativo utilizado, nesse último tivemos uma maior taxa metabólica (representada pelo consumo de oxigênio), pois como reportado por Bolner e Baldisserotto (2007) há um declínio da taxa metabólica por unidade de massa corpórea com o aumento do peso total em jundiá.

O aumento dos níveis de gás carbônico reportados em nossos experimentos, em todas as concentrações, ao final do transporte, foram, provavelmente, responsáveis pela diminuição do pH da água, como reportado por Golombieski et al. (2003), com a mesma espécie. Os níveis de alcalinidade, independentemente do tratamento, aumentaram após o transporte, provavelmente devido à regurgitação de alimento, pois os peixes não passaram por um período de depuração antes do transporte e a ração comercial dada aos animais possui carbonato de cálcio ($CaCO_3$) em sua composição.

A verificação do fluxo de íons é um importante parâmetro de avaliação do transporte de peixes vivos, pois através dele é possível perceber se o procedimento causou algum distúrbio osmorregulatório nos animais. O transporte e o manejo de peixes são clássicas condições geradoras de estresse, pois elas podem causar um aumento do fluxo sanguíneo nas brânquias e da permeabilidade paracelular. Em peixes de água doce, o resultado dessas mudanças é a perda iônica (McDONALD et al., 1991; CECH Jr. et al., 1996). A utilização de sal comum adicionado à água de transporte tem apresentado resultados positivos para algumas espécies, porém para jundiá e pirarucu não se observou essa mesma resposta. Nesta tese, todos os compostos utilizados (eugenol, OE de *L. alba* e EM de *C. buxifolia*), independentemente da concentração utilizada foram eficientes na redução da perda de íons em espécimes de jundiá. Portanto, esse efeito pode estar relacionado, provavelmente, a um menor fluxo sanguíneo branquial pelo fato dos peixes estarem menos agitados devido à presença dos anestésicos.

Os parâmetros relacionados ao sangue, foram avaliados por meio da medição dos gases sanguíneos (pH, $Pv O_2$, $Pv CO_2$ e HCO_3^-) e do hematócrito (Hct). Os valores dos gases sanguíneos, apesar de algumas alterações, não indicaram efeitos fisiológicos prejudiciais após o transporte. Além disso, a redução dos valores de pH sanguíneo podem estar relacionados a uma incapacidade de tamponamento do sangue ou, ainda, devido a algum processo de acidose metabólica ou respiratória. Em adição, os valores de Hct reportados nos experimentos com eugenol (1,5 ou 3,0 µL L^{-1}), OE de *L. alba* (10, 20, 30 ou 40 µL L^{-1}) e EM de *C. buxifolia* (5 ou 10 µL L^{-1}) (24–33%) foram similares aos encontrados por Carneiro et al. (2009), com a mesma espécie (27–30%). Em nossos

experimentos observou-se uma diminuição dos valores de Hct após os períodos de transporte, mas sem nenhuma diferença significativa entre os tratamentos. Entre as respostas de estresse ocasionadas pelos procedimentos de transporte, em peixes de água doce, temos o aumento do fluxo sanguíneo branquial e da permeabilidade paracelular, resultando na perda iônica (McDONALD et al., 1991; CECH Jr. et al., 1996), como já reportada anteriormente. Portanto, a possível hemodiluição observada nesses experimentos pode ter sido causada por algum distúrbio osmorregulatório, provavelmente um influxo de água (HOUSTON et al., 1996; MORGAN e IWAMA, 1997).

Alguns autores têm reportado que os aumentos dos níveis de cortisol e glicose plasmáticos são respostas típicas ao estresse geradas pelos procedimentos de transporte (IWAMA et al., 2004; URBINATI e CARNEIRO, 2004). Entretanto, outros parâmetros bioquímicos, tais como atividades enzimáticas, também são muito importantes para compreendermos como os peixes respondem ao transporte e, se a adição de anestésicos a água pode ser benéfica ou prejudicial a esses animais. Até o presente momento, somente um trabalho havia avaliado a atividade de enzimas em tecidos de peixes após o transporte (AZAMBUJA et al., 2011) e somente com o OE de *L. alba* (concentração de 10 $\mu L\ L^{-1}$).

Os anestésicos, em geral, podem causar depressão do sistema nervoso central (SNC), através da sua ação sobre os axônios, liberação de neurotransmissores, excitabilidade da membrana, ou, ainda pela combinação de todas essas ações (ROSS e ROSS, 2008). Além disso, independentemente do anestésico utilizado, a captação ou absorção ocorre principalmente pelas brânquias e pele, chegando, em seguida, ao sistema circulatório, onde irá bloquear as ações reflexas (SUMMERFELT e SMITH, 1990). Em adição, como já reportado por Cunha et al. (2010a) e por resultados obtidos em nosso trabalho, o OE de *L. alba* e o EM de *C. buxifolia* apresentaram efeitos anestésico e sedativo, respectivamente, em espécimes de jundiá. Portanto, é possível estabelecer que esses compostos afetam o SNC dessa espécie. A fonte de energia intracelular primária é o ATP, o qual atua como uma molécula de sinalização extracelular (FIELDS e BURNSTOCK, 2006), sendo considerado um dos mais importantes neurotransmissores no sistema purinérgico (FREDHOLM, 1995), o qual é responsável pela modulação dos processos de sinalização e biossintéticos, nos quais estão envolvidos os nucleotídeos, tais como a homeostase vascular, manutenção do tamanho celular, sinalização neuronal, função imune e modificações de proteínas e lipídios (BRAKE e JULIUS, 1996; BURNSTOCK, 1998; GAYLE et al., 1998; ENJYOJI et al., 1999; MARCUS et al., 2003; SCHWEIBERT e ZSEMBERY, 2003). No entanto, no experimento com concentrações de OE de *L. alba* e

EM de *C. buxifolia* não foram encontradas diferenças significativas nas enzimas principais do sistema purinérgico (NTPDase e 5'-nucleotidase). Independentemente disso, e como reportado anteriormente, os neurotransmissores atuam em vários processos biológicos e, portanto, mesmo não tendo sido encontrado diferenças nos parâmetros do sistema purinérgico, pode-se estabelecer alguma relação entre esse sistema e o metabolismo oxidativo das células, através da verificação de espécies reativas de oxigênio (EROs).

O estresse oxidativo é caracterizado pelo desbalanço entre os pró-oxidantes e os antioxidantes, resultando em potenciais danos celulares (SIES, 1991; HALLIWELL e GUTTERIDGE, 2000). Em vista disso, o sistema de defesa é constituído por enzimas antioxidantes, tais como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa S-transferase (GST), e pelo sistema de defesa não-oxidante, constituído, por exemplo, pelos grupos tióis não-protéicos, sendo em sua maior parte representados pela glutationa (GSH) (STOREY, 1996; HALLIWELL e GUTTERIDGE, 2000; TRENZADO et al., 2006).

As enzimas SOD e CAT têm como uma de suas principais funções protegerem os organismos de danos oxidativos através da remoção parcial de espécies de oxigênio (DI GIULIO et al., 1989). Como reportado por outros estudos (KARAKOC et al., 1997; LUSHCHAK et al., 2001, 2005; GARCIA et al., 2008; AZAMBUJA et al., 2011) em nossos experimentos não foi observada qualquer alteração na atividade da SOD. Por outro lado, a atividade da CAT hepática diminuiu consideravelmente. Portanto, uma menor atividade da CAT em jundiás transportados com anestésicos pode ser atribuída a um aumento da produção do radical superóxido (O_2^-), como reportado também em outras espécies (BAINY et al., 1996; DAUTREMEPUITS et al., 2004; WINKALER et al., 2007).

Alterações na atividade da GPx são, geralmente, acompanhadas por mudanças nos níveis de GSH, pois o GSH é co-substrato para a decomposição do peróxido de hidrogênio (H_2O_2) através da GPx (SIES, 1999). O principal grupo tiol que participa nas reações de redução nas células – GSH – desempenha um importante papel na detoxificação dos metabólitos eletrofílicos catalisados pela GST (SIES, 1999; LATHA e PARI, 2004). Altos níveis de GSH podem proteger as proteínas celulares contra a oxidação via ciclo redox da GSH ou diretamente pela detoxificação das EROs geradas pela exposição a agentes estressores (RUAS et al., 2008), mas o baixo conteúdo de GSH pode modular a atividade das enzimas GPx e GST (BROUWER e BROUWER, 1998) como é sugerido para os resultados

obtidos em nossos experimentos, quando jundiás foram transportados em água com 30 ou 40 µL L^{-1} de EO de *L. alba*.

A produção de EROs pode induzir danos à célula, os quais podem ser confirmados pela taxa entre a peroxidação lipídica (POL) e as enzimas antioxidantes CAT e GPx, como é sugerido por Ruas et al. (2008). Essa taxa estabelece o balanço entre a injúria celular expressa pela POL induzida pelas EROs e as principais enzimas antioxidantes de defesa dos organismos (CAT+GPx), as quais são capazes de neutralizar as EROs e os intermediários metabólicos responsáveis pelos danos celulares (RUAS et al., 2008). Em nossos experimentos, observou-se um aumento das quantidades de H_2O_2, através dessa taxa, nos tratamentos com 30 e 40 µL L^{-1} de OE de *L. alba*. Por outro lado, somente o tratamento 30 µL L^{-1} de OE de *L. alba* exibiu aumento dos níveis de TBARS e proteína carbonil, indicando altos níveis de peroxidação lipídica e oxidação de proteínas e, portanto, há uma possível modificação na estrutura de proteínas e lipídios e, ainda, as defesas antioxidantes, pelo menos, nessa concentração, não estão sendo eficazes no controle desses parâmetros.

De modo geral, os resultados do presente trabalho mostraram que os anestésicos utilizados nos experimentos melhoraram os parâmetros da água, principalmente com relação aos níveis de excreção de amônia total. Além disso, os níveis de oxigênio dissolvido permaneceram dentro de uma faixa confortável para a espécie e a perda de íons foi substancialmente controlada pelos anestésicos durante o transporte. Por fim, ainda há muitos aspectos a serem explorados em relação aos anestésicos e às concentrações anestésicas e sedativas a serem utilizadas durante o transporte de jundiá, bem como diferentes densidades de carga e tempos de transporte e, é claro, a verificação de outros parâmetros fisiológicos, bioquímicos, comportamentais e moleculares que possam permitir a elaboração de um pacote tecnológico para essa espécie.

4 CONCLUSÕES

Considerando-se os objetivos propostos nesta tese de doutoramento, de que os produtos fitoterápicos (óleo essencial de *Lippia alba* e extratos de *Condalia buxifolia*) e o fitofármaco (eugenol) pudessem reduzir os parâmetros de estresse decorrentes do transporte de peixes vivos, em nosso caso, do jundiá, e de que um dos extratos de *C. buxifolia* fosse capaz de exercer alguma atividade anestésica ou sedativa nessa espécie, esse estudo teve como principais conclusões os seguintes itens:

1. O extrato metanólico de *Condalia buxifolia* possui a capacidade de sedação em jundiá, portanto é favorável a sua utilização em procedimentos de transporte, mas não em procedimentos nos quais seja necessária uma anestesia profunda dos animais.

2. Todas as concentrações utilizadas de eugenol (1; 1,5; 2,5 ou 3 µL L^{-1}), de óleo essencial de *Lippia alba* (10; 20; 30 ou 40 µL L^{-1}) e de extrato metanólico de *Condalia buxifolia* (5; 10; 25 ou 50 µL L^{-1}), independentemente da densidade de carga e do tempo de transporte foram eficientes na diminuição do fluxo iônico, ou seja, da perda de íons.

3. As concentrações de eugenol (1 e 2,5 µL L^{-1}) e de extrato metanólico de *Condalia buxifolia* (25 e 50 µL L^{-1}) foram eficientes na redução da mortalidade de jundiás transportados durante 12 h em uma densidade de carga de 186,7 g L^{-1}. Portanto, aconselha-se a utilização dessas concentrações em situações semelhantes às utilizadas nesse estudo.

4. Os anestésicos utilizados nesse estudo mostraram-se capazes de reduzir a excreção de amônia durante o procedimento de transporte, portanto, há um menor risco de jundiás intoxicarem-se com altos níveis de metabólitos nitrogenados.

5. A utilização da concentração de 30 µL L^{-1} de OE de *L. alba* não é aconselhável para o transporte de jundiá, pois essa concentração aumentou os níveis plasmáticos de cortisol e, induziu ao estresse oxidativo através da peroxidação lipídica e carbonilação de proteínas.

6. Os parâmetros de peroxidação lipídica e carbonilação protéica podem ser considerados novos indicadores de exposição ao estresse oxidativo induzido por anestésicos em peixes.

7. Por fim, propõe-se a realização de mais experimentos com os agentes anestésicos e sedativos utilizados nessa tese de doutorado, principalmente no que se refere à busca do entendimento dos mecanismos de ação desses agentes e, também, estudos a nível molecular.

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ANEXO A – Frasco de eugenol adquirido comercialmente e utilizado nos experimentos (FONTE: Arquivo pessoal).

ANEXO B – Partes aéreas da espécie *Lippia alba* (FONTE: http://naturezaquecuida.blogspot.com/2011_05_01_archive.html).

ANEXO C – Partes aéreas da espécie *Condalia buxifolia* (FONTE: http://www6.ufrgs.br/fitoecologia/florars/open_sp.php?img=2856).

ANEXO D – Espécime de jundiá (*Rhamdia quelen*) utilizado nos experimentos (FONTE: Arquivo pessoal).