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ÓLEO ESSENCIAL DE *Aloysia triphylla* (L'Hérit) BRITTON PARA JUNDIÁS: CRESCIMENTO, TRANSPORTE, PARÂMETROS BIOQUÍMICOS, METABÓLICOS E OXIDATIVOS

TESE DE DOUTORADO

Carla Cristina Zeppenfeld

Santa Maria, RS, Brasil

2014

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Carla Cristina Zeppenfeld

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Zootecnia, Área de Concentração em Produção Animal na Subárea de Fisiologia de Peixes da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Zootecnia.

Orientador: Prof. Dr. Bernardo Baldisserotto

Santa Maria, RS, Brasil

2014

Universidade Federal de Santa Maria
Centro de Ciências Rurais
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A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

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Elaborada por
Carla Cristina Zeppenfeld

Como requisito parcial para a obtenção do grau de
Doutor em Zootecnia

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Santa Maria, 20 de janeiro de 2014.

"O saber a gente aprende
Com os mestres e
com os livros.
A sabedoria,
se aprende e com a vida
e com os humildes"

(Cora Coralina)

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RESUMO

ÓLEO ESSENCIAL DE *Aloysia triphylla* (L'Hérit) BRITTON PARA JUNDIÁS: CRESCIMENTO, TRANSPORTE, PARÂMETROS BIOQUÍMICOS, METABÓLICOS E OXIDATIVOS

AUTOR: Carla Cristina Zeppenfeld

ORIENTADOR: Bernardo Baldisserotto

Data e local da defesa: Santa Maria 20 de janeiro de 2014.

A adição de extratos vegetais em rações levaram ao aumento de peso em algumas espécies de peixes. O óleo essencial de *Aloysia triphylla* (OEA) tem ação anestésica e sedativa para peixes e reduz o estresse de manuseio. Assim, o presente trabalho teve por objetivo avaliar o efeito OEA como aditivo em rações e transporte para jundiás, *Rhamdia quelen*, em relação ao crescimento, morfologia intestinal, parâmetros metabólicos e estresse oxidativo. No primeiro experimento os peixes foram divididos em três grupos: 0 (controle), 30 ou 40 µL/L adicionado à água OEA. Os peixes foram transportados em sacos plásticos durante 6 horas e ao final foram avaliados: sobrevivência, qualidade da água e parâmetros ionoregulatórios e bioquímicos. No segundo experimento foi adicionada à ração diferentes concentrações de OEA (0; 0,25; 0,5; 1,0 e 2,0 mL/kg), monitorado o crescimento dos jundiás por 60 dias e ao final foi realizada análise morfológica histoquímica do intestino. A adição de OEA na água de transporte de jundiás reduz o estresse e a concentração recomendada é de 40 µL/L. A adição de 2,0 mL/kg de OEA na ração promoveu crescimento e sua utilização é recomendada como um aditivo em alimentos para jundiá.

Palavras-chave: *Aloysia triphylla*. Transporte. Aditivo alimentar. Estresse oxidativo. Jundiá. óleo essencial.

ABSTRACT

Animal Science PhD Thesis
Postgraduate Program in Animal Science
Universidade Federal de Santa Maria

THE ESSENTIAL OIL OF *Aloysia triphylla* (L'Hérit) FOR SILVER CATFISH: GROWTH,TRASNPORT AND BIOCHEMICAL, METABOLIC AND OXIDATIVE PARAMETERS

AUTHOR: Carla Cristina Zeppenfeld
ADVISER: Dr Bernardo Baldisserotto
Date and place of defense: Santa Maria, January 20th, 2014.

The addition of plant extracts in diet led to weight gain in some species fish. The essential oil of *Aloysia triphylla* (EO) has anesthetic and sedative action for fish and reduces stress handling. Thus, the present study aimed to evaluate the effect of the EO as an additive in animal feed and transportation of silver catfish *Rhamdia quelen* in relation to growth, intestinal morphology, metabolic and oxidative parameters, In the first experiment, the fish were be divided into three groups: 0 (control), 30 or 40 µL/L EO added to water. The fish were transported in plastic bags for 6 hours and at the end, survival, water quality and ionoregulatory and biochemical parameters were evaluated. In the second were added to the food and different concentrations of OE (0, 0.25, 0.5, 1.0 and 2.0 mL/kg) the growth of silver catfish was observed for 60 days and at the end morphological and immunohistochemical analysis of gut was performed. The addition of EO in the water of transport silver catfish and reduces stress and the suggested concentration is 40 µL /L. The addition of 2.0 mL OE per kg of diet increased in growth and its use is recommended as a feed additive for silver catfish.

Keywords: *Aloysia triphylla*. Transportation. Food additive. Oxidative stress. Catfish. Essential oil.

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LISTA DE ABREVIATURAS E SIGLAS

CAT   – Catalase

CCK   – Colecistocinina

CNE   – Células neuroendócrinas

CF   – Fator de condição (*condition factor*)

EO   – Óleo essencial (e*ssential oil*)

EH   – Altura enterócitos *(enterocyte height)*

FH   – Tamanho vilosidades (*folds height*)

GPx   – Glutationa peroxidase

GR   – Glutationa redutase

GSH   – Glutationa reduzida

GSSG   – Glutationa oxidada

GST   – Glutationa-S-transferase

H_2O_2 – Peróxido de hidrogênio

HSI   – Índice hepatossomático (*hepatosomatic index*)

$HO^{\bullet}$ – Radical hidroxila

LOOH   – Hidroperóxidos lipídicos (*lipid hydroperoxide*)

LPO   – Lipoperoxidação (*lipid peroxidation*)

NECs   – Células neuroendrócrinas imunorreativas

NF   – Número de vilosidades (*number of intestinal folds)*

NH_3 – Amônia não ionozada

NPY   – Neuropeptídeo Y

OS   – Estresse oxidativo (*oxidative stress*)

RL   – Radicais livres

ROS   – Espécies reativas de oxigênio (*reactive oxygen species*)

RWG   – Ganho de peso relativo (*relative weight gain*)

SGR   – Taxa de crescimento específico (*specific growth rate*)

SNC   – Sistema nervoso Central

SOD   – Superóxido dismutase

TBARS   – Substâncias que reagem ao ácido tiobarbitúrico

TAN   – Amônia total

TEA   – Área epitelial total

VSI   – Índice viscerossomático (*viscerosomatic index*)

SUMÁRIO

1 INTRODUÇÃO

A aquicultura é uma atividade crescente no cenário mundial e representa uma importante fonte de alimento para a humanidade. Segundo dados da FAO (Food and Agriculture Organization), em 2011 o consumo de pescado atingiu 131 milhões de toneladas. Desse total, 64 milhões de toneladas foram provenientes da aquicultura (FAO, 2012). Paralelamente à expansão dessa prática, a melhoria da saúde dos peixes destinados ao consumo humano tem recebido atenção. O bem-estar dos peixes é importante para a indústria não apenas pela aceitação do produto, mas também em termos de eficiência de produção, quantidade e qualidade (ASHLEY, 2007).

Somado a esses fatos, práticas realizadas na aquicultura como biometria, análises patológicas, implantes hormonais e transporte frequentemente expõem peixes a uma variedade de fatores estressantes que têm o potencial de afetar seu desempenho (BARTON, 2000). 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, principalmente num sistema fechado, com sacos plásticos (BERKA, 1986). Entre as limitações desse sistema incluem-se a disponibilidade de oxigênio e o aumento da produção de amônia e dióxido de carbono durante o período do transporte (GOMES et al., 2006; GOLOMBIESKI et al., 2003; CARNEIRO et al., 2009). Geralmente se observa 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; CARNEIRO et al., 2009), liberação de hormônios, metabolismo energético e enzimas oxidativas (BARTON & IWAMA, 1991; CARRAGHER & REES, 1994).

Diante dos prejuízos à piscicultura ocasionados pelos sistemas inadequados de transporte de peixes vivos e também pela grande perda ao final desse processo, é interessante o uso de anestésicos na busca de se reduzir estas perdas. Sabe-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), baseado nesses fatos, o processo de anestesia dos peixes tem sido usado por diminuir o estresse ou o dano fisiológico causado pelas práticas de manejo. Os anestésicos testados até o momento são benzocaína, eugenol e óleo de cravo, os quais apresentam um preço acessível e são de fácil manuseio e aquisição (GOMES et al., 2006; SINGH et al., 2004). Além disso, extratos ou óleos essenciais de plantas tem se tornado uma alternativa viável como anestésicos para peixes (BECKER et al., 2012).

Extratos ou óleos essenciais de plantas tem tornado-se uma alternativa viável como anestésicos para peixes, utilização do eugenol ou óleo de cravo (INOUE et al., 2005) e do óleo essencial de *Lippia alba* (AZAMBUJA et al., 2011). Parodi et al., (2013) concluiram que a adição de 30-50 µL L^{-1} de *A. triphylla* para transporte em juvenis de jundiá, *Rhamdia quelem* promove a liberação de cortisol e a perda de íons, reduzindo o estresse de transporte.

Aloysia triphylla (L'Herit.) Britton, também conhecida pelos sinônimos *Aloysia citriodora* Ortega ex Pers.; *Aloysia citriodora* Paláu; *Aloysia sleumeri* Moldenke; *Lippia citriodora* H.B.K.; *Lippia citriodora* (Lam.) Kunth; *Lippia triphylla* (L'Her.) Kuntze; *Verbena citriodora* Cav.; *Verbena triphylla* L'Hér.; *Zapania citriodora* Lam. (CARNAT et al., 1999; VALENTÃO et al., 1999; PASCUAL et al., 2001; SANTOS-GOMES et al., 2005; DUKE et al., 2008) é um arbusto da família das Verbenáceas de até três metros de altura. As folhas e flores são aromáticas, com odor semelhante ao do limão, usadas tanto para fins medicinais como condimento (MORGAN, 1997). Cresce espontaneamente na América do Sul

(Chile, Argentina, Uruguai a Peru) e foi introduzida na Europa no fim do século XVII. As folhas são largamente usadas em chás por apresentarem propriedades aromáticas, digestivas e antiespasmódicas (SANTOS-GOMES et al., 2005).

Muitos produtores tentam melhorar o rendimento em suas pisciculturas com o uso de promotores de crescimento, antibióticos e agroquímicos (SANTOS et al., 2009). Em função disso, têm crescido a busca por produtos naturais, os quais possuem compostos fenólicos e terpenóides que apresentam atividade antioxidante, funcionando como seqüestradores de radicais e como quelantes de metais (NICIFOROVIC et al., 2010).

Diversos derivados de plantas podem ser utilizados como aditivos promotores de crescimento para peixes, tendo seus efeitos comprovados como: alho, *Allium sativum L.* (DIAB et al., 2002); orégano, *Origanum vulgare* (ZHENG et al., 2009); chá verde, *Camellia sinensis* (CHO et al, 2007), manjericão, *Ocimum basilicum*, noz moscada, *Myristica fragrans* (SIVARAM et al, 2004), yuca, *Yucca verde* (KELLY & KOHLER, 2003), estevia, *Stevia rebaudiana* (SHIOZAKI et al., 2004). Pesquisas têm focado nos efeitos benéficos específicos da inclusão desses micros ingredientes nas rações. Essas substâncias apresentam atividade antioxidante (BOTSOGLOU et al. 2002) de modificação da microbiota intestinal, melhora na digestibilidade e na absorção dos nutrientes, de modificações morfo-histológicas do trato gastrintestinal e melhora da resposta imune (BRUGALLI, 2003).

Em peixes, como em outros vertebrados, os processos digestivos como motilidade, secreção, absorção e imunidade são modulados pelo sistema neuroendócrino (LIN et al., 2000; VOLKOFF et al., 2005). Sinais periféricos para regulação das funções gastrointestinais podem vir de: (1) sistema nervoso autônomo (simpático e parassimpático), (2) sistema nervoso entérico, e (3) o sistema neuroendócrino difuso (SNED) (LE BAIL & BOEUF, 1997; JENSEN et al., 2001; BUDDINGTON & KROGDAHL, 2004). O SNED de peixes mostra semelhanças com o seu homólogo de mamíferos no que diz respeito aos processos

regulatórios (BUDDINGTON & KROGDAHL, 2004). Vários estudos descrevem a distribuição e frequência de células pertencentes ao SNED localizados no trato gastrintestinal (TGI) de peixes utilizando técnicas de imuno-histoquímica (DOMENEGHINI et al., 1999; CINAR & DILER, 2002; BOSI et al., 2004; LEE et al., 2004; BERMÚDEZ et al., 2007; VIGLIANO et al., 2011). Estudo com imuno-histoquímica foi realizado por Hernández et al. (2012) para determinar a distribuição relativa e as freqüências de alguns neuromoduladores do trato digestivo de jundiá (*Rhamdia quelen*), sendo este dividido em seis partes avaliado usando um conjunto de anti-soros específicos. Os resultados mostraram um maior número de células neuroendócrinas imunorreativas no intestino anterior a todos os anti-soros, indicando a função principal destes segmentos com o controle de ingestão de alimentos por meio de sinais periféricos orexígenos e anorexígenos.

Avaliações do número, tamanho e forma dos enterócitos também podem demonstrar uma maior aptidão no controle da ingestão de alimentos e, assim, na modulação do comportamento alimentar dos peixes. As características anatômicas do aparelho digestivo dos peixes estão relacionadas com a natureza e qualidade dos alimentos (SEIXAS FILHO et al., 2001).

A colecistocinina (CCK) é secretada pelas células I presentes especialmente no duodeno e no jejuno, atuando, principalmente, por mecânismo telécrino (POLAK et al.,1993). Ela estimula a contração da vesícula biliar, a secreção pancreática de amilase,a atividade motora intestinal, a secreção de pepsina gástrica e a secreção das glândulas de Brunner, além de inibir a secreção ácida das células parietais, a atividade motora e o esvaziamento gástrico, a contração do esfincter inferior esofágico, a contração do esfíncter de Oddi e a absorção de fluidos e eletrólitos no jejuno e no íleo (GRANNER, 1988; POLAk et al., 1993). A colecistocinina também inibe a ingestão de alimentos, atuando por mecanismo neurócrino no centro da fome e da saciedade no SNC. A liberação de CCK pelas células I é estimulada pela

presença de nutrientes no duodeno, como peptídios, gorduras e carboidratos. A CCK liberada atua sobre o pâncreas exócrino, resultando na liberação das enzimas pancreáticas na luz duodenal (tripsina, amilase e lipase). Essas, por sua vez, inibem a liberação da CCK pelas células I, exercendo um "feedback" negativo e, completando, assim, o ciclo de ação desse hormônio (SWENSON & REECE 1996).

O neuropeptídeo tirosina (NPY), localiza-se nos sistemas nervoso central e periférico, desempenhando a função de neurotransmissor ou neuromodulador, relacionado à noradrenalina (POLAK et al., 1993). O NPY presente no sistema nervoso simpático está freqüentemente, localizado nos neurônios noradrenérgicos e funciona, aparentemente, tanto como vasoconstritor quanto como co-transmissor, juntamente com a noradrenalina. O NPY produz vários efeitos sobre o sistema nervoso central, incluindo aumento na ingestão de alimento (REID, 1998). O NPY encontrado em alguns neurônios secretomotores do sistema nervoso entérico pode inibir a secreção de água e eletrólitos no intestino (KATZUNG, 1998). Também foi encontrado em células L, coexistindo com PYY e glicentina.

Em peixes, como em outros vertebrados, os processos digestivos, tais como motilidade, secreção, absorção e imunidade são modulados pelo sistema neuroendócrino. O hipotálamo desempenha um papel importante na regulação de várias funções gastrointestinais, produzindo fatores que estimulam (orexígenos) ou inibem (anorexígenos) a ingestão integra diversos sinais periféricos (LIN et al., 2000; VOLKOFF et al., 2005). Estes sinais periféricos podem vir de: (1) sistema nervoso autônomo (simpático e parassimpático), (2) sistema nervoso entérico, e (3) sistema neuroendócrino difuso (SNED) (LE BAIL & BOEUF, 1997; JENSEN, 2001; BUDDINGTON & KROGDAHL, 2004). O SNED de peixes mostra semelhanças com o seu homólogo de mamíferos no que diz respeito aos processos regulatórios. No entanto, o SNED apresenta características funcionais únicas relacionadas ao

habitat (de água doce ou salgada), estação, período reprodutivo ou desenvolvimento fase (BUDDINGTON & KROGDAHL, 2004).

Nos organismos aeróbicos, o metabolismo celular energético e o consumo de oxigênio (O_2) estão envolvidos com a geração das espécies reativas de oxigênio (ROS, do inglês *reactive oxygen species*). Estas espécies tóxicas são intermediários reativos formados na redução parcial do O_2 e podem induzir danos em ácidos nucléicos, proteínas, carboidratos e lipídios, alterando a função dessas macromoléculas nas células, tecidos e órgãos (LUSHCHAK, 2001). Diversos sistemas de proteção existem a fim de protegerem os organismos contra os efeitos deletérios provocados pelas ROS. O sistema de defesa antioxidante consiste de componentes de baixo peso molecular (glutationa, ácido ascórbico, α-tocoferol, etc.), denominado sistema de defesa não enzimático, e das enzimas antioxidantes que compõem o sistema de defesa enzimático. Este último é representado pelas enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR), glutationa-S-transferase (GST), entre outras (BAGNYUKOVA et al., 2006).

Neste estudo, trabalhou-se com o jundiá, *Rhamdia quelen* por ser a espécie nativa mais cultivada no sul do Brasil (BALDISSEROTTO et al., 2009) e ser um peixe que apresenta grande aceitação pelo mercado consumidor devido à sua carne saborosa e ausência de espinhas intramusculares e por ser um bom modelo para anestésicos e transporte (GOLOMBIESKI et al., 2003; BECKER et al., 2012).

2 DESENVOLVIMENTO

Neste item serão apresentados um artigo publicado e um a ser submetido resultante desta tese:

Artigo 1. Physiological and biochemical responses of silver catfish, *Rhamdia quelen*, after transport in water with essential oil of *Aloysia triphylla* (L'Herit) Britton. Publicado no periódico **Aquaculture (DOI:10.1016/j.aquaculture.2013.10.013).**

Manuscrito 2. Essential oil of *Aloysia triphylla* as feed additive promotes growth of silver catfish (*Rhamdia quelen*). Esse manuscrito será submetido ao periódico **Aquaculture Nutrition.**

1   **ARTIGO 1**

2

3 ZEPPENFELD, C. C.; TONI, C.; BECKER, A. G.; MIRON, D. S.; PARODI, T. V.;

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6 silver catfish, *Rhamdia quelen*, after transport in water with essential oil of *Aloysia triphylla*

7 (L'Herit) Britton. Aquaculture**,** 418–419, p.101–107, 2014.

8

Physiological and biochemical parameters of silver catfish, *Rhamdia quelen*, after transport in water with essential oil of *Aloysia triphylla* (L'Herit) Britton

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Abstract

This study investigated the efficacy of the essential oil *Aloysia triphylla* in the transport of silver catfish (*Rhamdia quelen*) considering survival, water, ionoregulatory and biochemical parameters. Fish (262.0 ± 73.5 g) were divided into three groups: 0 (control), 30 or 40 µL/L EO *A. triphylla* added to the water and transported in plastic bags for 6 hours. At the end of transport, dissolved oxygen, alkalinity, pH, temperature, and un-ionized ammonia levels in the water of transport were not significantly different between treatments, but the control group presented the highest total ammonia levels. Fish transported with the EO *A. triphylla* had lower plasma cortisol and ion loss and higher plasma Na^+ and Cl^- levels than control group. Hepatic glycogen, lactate and glucose levels were lower in the liver of fish transported with EO *A. triphylla* when compared to the control. Total free amino acids and protein values in the liver were higher in the treatment with 40 µL/L EO *A. triphylla*. Muscle lactate and glucose levels were higher and lower, respectively, in fish transported with EO *A. triphylla* compared to control. Thiobarbituric acid-reactive substances levels in the liver and muscle were lower in silver catfish transported with EO *A. triphylla* when compared to the control fish. Catalase (CAT) activity was lower in the kidney and higher in the gill of fish transported with EO *A. triphylla* when compared to controls. On the other hand, hepatic CAT activity was lower and higher in fish transported with 30 and 40 µL/L EO *A. triphylla*, respectively, when compared to controls. Protein carbonyl contents of fish transported with EO *A. triphylla* were lower when compared to the control. Based on the improvement of evaluated parameters, the use of EO *A. triphylla* in the water of transport is advisable to fish transportation, being 40 µL L^{-1} the best concentration.

Key words: ion fluxes; water parameters; cortisol; metabolism; oxidative stress

60   **1. Introduction**

61

62   Farmed fish are submitted to several situations as handling and confinement in which

63   a stress response is generated, resulting in physiological and biochemical modifications (Zahl

64   et al., 2012). High stocking densities and frequent net captures are events that provoke

65   chronic stress that, together with subsequent acute transport stress, may cause not only

66   deleterious effects to fish health, but also death (Barcellos et al., 2006). Therefore, the success

67   of fish manipulation depends of the control of adverse conditions in the farmed environment.

68   The transport of fish is stressful in most situations and may provoke several injuries

69   that result in loss of scales and mucus, increasing the outbreak of bacterial and fungal

70   infections (Moyle and Cech, 1998). In addition, in freshwater fish this procedure causes

71   cortisol rise (Barton, 2002), ionoregulatory disturbances and changes in the energy demand

72   and syntheses of metabolites (Gomes et al., 2006a, 2006b; Becker et al., 2012). Therefore,

73   some studies have used anesthetics or sedatives in water of transport, which contributed to

74   reduce ion loss, oxidative damages, mortality and cortisol rise (Barton and Peter, 1982;

75   Azambuja et al., 2011; Becker et al., 2012).

76   Fish anesthesia may be induced by several substances, such as alcohol, ether,

77   barbiturates, quinaldine, tricaine methanesulfonate (MS 222), chlorbutanol, and benzocaine

78   (Gilderhus and Marking, 1987; Gomes et al., 2001; Kiessling et al., 2009; Heo and Shin,

79   2010). However, each of these compounds has been associated with undesirable systemic side

80   effects, as elevated cortisol levels (primary response) and also secondary adverse reactions

81   (acidosis and osmotic stress due to respiratory arrest and insufficient exchange of gas and ions

82   between the blood and the water) (Gilderhus and Marking, 1987; Palić et al., 2006; Zahl et al.,

83   2012). Consequently some less toxic compounds were tested, as AQUI-S™ (contains

84   isoeugenol as active compound) (Meinertz et al., 2006; Kiessling et al., 2009), menthol

85 (Façanha and Gomes, 2005; Simões and Gomes, 2009) and the essential oils (EO) of *Lippia*

86 *alba* (Cunha et al., 2010, 2011; Becker et al., 2012), *Ocimum gratissimum* (Silva et al., 2012)

87 and *Aloysia triphylla* (L'Herit) Britton (Gressler et al., 2012). The exposure to the EO *A.*

88 *triphylla* for 6h improved the antioxidant system against reactive oxygen species (ROS) of the

89 white shrimp (*Litopenaeus vannamei*) (Parodi et al., 2012).

90   The silver catfish, *Rhamdia quelen*, is the native species most raised in southern Brazil

91 (Baldisserotto, 2009). It is recommended the addition of the EO *Lippia alba* and eugenol to

92 the water of transport of this species (Azambuja et al., 2011; Becker et al., 2012). However,

93 there are no studies reporting the effect of the EO *A. triphylla* on fish transport. Thus, the aim

94 of this study was to investigate the effectiveness of this EO in the transport of silver catfish

95 using the following indicators: water and blood parameters, survival, ionoregulatory balance,

96 and biochemical parameters.

97

98   **2.   Materials and methods**

99

100   *2.1.   Experimental procedure*

101   Silver catfish (262.0 ± 73.5 g, 38.5 ± 1.1 cm) were obtained from a fish culture and did

102 not undergo a depuration period because this procedure, although recommended is not

103 followed by most fish producers in southern Brazil (Golombieski et al., 2003). Fish were

104 divided into three treatments (four replicates each) and transported at a loading density of 250

105 g/L for 6h in twelve plastic bags with 15 L of water and pure oxygen. The treatments were as

106 follows: 0 (control), 30 or 40 µL/L EO *A. triphylla* (equivalent to 27 or 36 mg/L, respectively,

107 because the density of this EO is about 0.90) (firstly diluted in ethanol; 1:10). The

108 concentrations of EO *A. triphylla* added to the water of transport were within the range

109 recommended to sedation (Parodi et al., 2012). The methodology of this experiment was

110 approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa

111 Maria (Process nº 046/2010).

112

113   *2.2.*   *Plant material and essential oil extraction*

114   Fresh leaves of *A. triphylla* proceeding from culture in Centro de Educação Superior

115 Norte (CESNORS) – Campus Frederico Westphalen. The aerial parts of the plant were

116 collected in june 2010. The plant material was identified by botanist Dr. Gilberto Dolejal

117 Zanetti, Department of Industry Pharmacy, UFSM, and a voucher specimen (SMDB 11169)

118 was deposited in the herbarium of the Department of Biology. Essential oil was obtained

119 from the fresh leaves of the plant by hydrodistillation for 3 h using a Clevenger-type

120 apparatus (European Pharmacopoeia, 2007) and stored at -4 °C in amber glass bottles.

121

122   *2.3.*   *Water sampling and analyses*

123   Water parameters were measured before and after transportation. Dissolved oxygen

124 (DO) and temperature were measured with an YSI oxygen meter (Model Y5512; YSI Inc.,

125 Yellow Springs, OH, USA). The pH was verified with a DMPH-2 pH meter (Digimed, São

126 Paulo, SP, Brazil). Nesslerization verified total ammonia nitrogen (TAN) levels according to

127 Eaton et al. (2005) and un-ionized ammonia (NH_3) levels were calculated according to Colt

128 (2002).

129   Chloride levels were determined according to Zall et al. (1956) and Na^+ and K^+ levels

130 with a B262 flame spectrophotometer (Micronal, São Paulo, Brazil). Standard solutions were

131 made with analytical-grade reagents (Vetec or Merck) dissolved in deionized water, and

132 standard curves of each ion to be tested were made for five different concentrations. Net ion

133 fluxes were calculated according to Gonzalez et al. (1998):

134
$$Jnet = \frac{V([ion_1] - [ion_2])}{Mt}$$

135

136   where [ion$_1$] and [ion$_2$] are the ion concentrations in the water of transport at the

137   beginning and end of the transport period, respectively, V is the water volume (in L), M is the

138   mass of the fish (in kg) and t is the duration of the transport (in h).

139

140   *2.4.   Blood sampling and analyses*

141   Blood samples (1 to 1.5 mL) were collected from the caudal vein before after tranport

142   (each fish was sampled once) using heparinized syringes, centrifuged 3,000 x *g* to separate the

143   plasma and then kept under frozen. From these samples, cortisol concentration was

144   determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit

145   (EIAgen™ Cortisol Test; BioChem Immuno Systems). The specificity of the test was

146   evaluated by examining the extent of the parallelism between the standard curve for human

147   cortisol concentrations and the curve of a series of dilutions of the plasma samples in PBS

148   (pH 7.4). The plasma ion levels were determined in accordance with the protocols reported to

149   water.

150

151   *2.5.   Biochemical parameters*

152   After transport period, the fish were euthanized by section of the spinal cord to remove

153   the tissues (brain, gills, kidney, liver and muscle) that were kept at -4°C until biochemical

154   analyses.

155

156   *2.5.1. Determination of metabolic parameters*

157   Tissues (liver and muscle) were dissolved in an equal volume of 20% TCA using a

158   Potter-Elvehjem homogenizer. The acid homogenate was centrifuged for 10 min at 10,000 x *g*

159   and the supernatant was used for the metabolic determinations. Glycogen was determined

160 using the method described by DuBois et al. (1956) after KOH and ethanol (1 and 3 mL,

161 respectively) addition for precipitation of glycogen. Homogenate was used to estimate the

162 protein level according to Lowry et al. (1951), lactate by Harrower and Brown (1972),

163 glucose by DuBois et al. (1956), ammonia by Verdouw et al. (1978), and for amino acid

164 quantification, the neutral supernatant homogenates were used for colorimetric amino acid

165 determination according to Spies (1957).

166

167 *2.5.2. Acetylcholinesterase (AChE; E.C. 3.1.1.7) assay*

168 The AChE activity was measured as described by Ellman et al. (1961) and modified by

169 Miron et al. (2005). Tissue samples (brain and muscle) were weighed and homogenized in a

170 Potter-Elvejhem glass/Teflon homogenizer with 150 mM NaCl. The homogenates were

171 centrifuged for 15min at 3,000 x g at 5°C and the supernatant was used as enzyme source.

172 Aliquots of supernatant were incubated at 25 °C for 2 min with 0.1 M phosphate buffer, pH

173 7.5 and 1 mM DTNB as chromogen. After the incubation period, the reaction was initiated by

174 the addition of acetylthiocholine (0.08 mM) as substrate for the reaction mixture..

175 Absorbances were measured spectrophotometric at 412 nm during 2 min.

176

177 *2.5.3. Catalase (CAT; E.C. 1.11.1.6) assay*

178 The catalase activity was assayed by ultraviolet spectrophotometer (Nelson and

179 Kiesow, 1972). Samples of tissues (kidney, gill and liver) were homogenized in a Potter–

180 Elvehjem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.4

181 (with 0.1% Triton X-100 and 150 mM NaCl) (1:20 dilution), centrifuged at 10,000 x g

182 for 10 min at 4 °C. The assay mixture consisted of 2.0 mL potassium phosphate buffer

183 (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M), and 0.01 mL homogenate. Change of H_2O_2

184 absorbance in 60 s was measured at 240 nm.

185   *2.5.4. Protein determination*

186   Protein levels of the homogenate were determined by Comassie blue method

187   using bovine serum albumin as standard (Bradford, 1976).

188

189   *2.5.5. Lipid peroxidation estimation*

190   Lipid peroxidation was estimated in muscle and liver by TBARS (thiobarbituric

191   acid-reactive substances) assay, performed by a malondialdehyde (MDA) reaction with

192   2-thiobarbituric acid (TBA), which was optically measured according to Buege and Aust

193   (1978). Aliquots of supernatants (0.25 mL) were mixed with 10% trichloroacetic acid

194   (TCA) (0.25 mL) and 0.67% thiobarbituric acid (0.5 mL) to adjust to a final volume of

195   1.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 15

196   min at 95 °C. After cooling, it was centrifuged at 5,000 x g for 15 min, and optical

197   density was measured by spectrophotometer at 532 nm.

198

199   *2.5.6. Protein carbonyl assay*

200   The supernatant (0.4 mL) of tissues (muscle, gill, kidney, and liver) was homogenized in

201   10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer. Protein

202   carbonyl content was assayed by the method described by Yan et al. (1995) with some

203   modifications. Soluble protein (1.0 mL) was reacted with 10 mM DNPH in 2N hydrochloric

204   acid (0.2 mL). After incubation at room temperature for 1h in dark, 0.5 mL of denaturing

205   buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3.0%), 2.0 mL of heptane

206   (99.5%), and 2.0 mL of ethanol (99.8%) were added sequentially, vortexed for 40 s, and

207   centrifuged at 10,000 x g for 15 min. Then, the protein isolated from the interface was washed

208   twice by resuspension in ethanol/ethyl acetate (1:1) and suspended in 1 mL of denaturing

209   buffer, and the carbonyl content was measured spectrophotometrically at 370 nm. Assay was

210 performed in duplicate, and two tubes blank incubated with 2N HCl (0.2 mL) without DNPH

211 was included for each sample. The total carbonylation was calculated using a molar extinction

212 coefficient of 22,000 M/cm.

213

214 *2.6.   Statistical analyses*

215   All data are expressed as mean ± SEM. Homogeneity of variances between treatments

216 was tested with the Levene's test. Data exhibited homogeneous variances, so comparisons

217 between different treatments and times were made using one-way ANOVA and Tukey's test.

218 Analysis was performed using the software Statistica ver. 7.0 (StatSoft, Tulsa, OK), and the

219 minimum significance level was set at $P < 0.05$.

220

221 **3. Results**

222

223 *3.1.   Water and plasma parameters*

224   After transport, no mortality was recorded in any treatment. At the end of transport,

225 the levels of dissolved oxygen, carbon dioxide, TAN and NH_3 in the water were significantly

226 higher than at the beginning of the transport. However, there was no significant difference

227 between treatments at the end of transport on dissolved oxygen, carbon dioxide, and NH_3, but

228 TAN levels were significantly lower in both EO *A. triphylla* concentrations compared with

229 the control. In addition, alkalinity, pH and temperature were not affected significantly by

230 transport or treatments (Table 1).

231   The EO *A. triphylla* reduced significantly the net Na^+, Cl^- and K^+ effluxes when

232 compared to control fish (Fig. 1). Moreover, plasma Na^+ and Cl^- levels in the control fish at

233 the end of transport were significantly lower and K^+ significantly higher than those reported

234 before transportation. However, in silver catfish transported with EO *A. triphylla* plasma ion

235 levels were not affected significantly but Na^+ and Cl^- plasma levels after transport were

236 significantly higher compared to control fish (Fig. 2A). Plasma cortisol levels of control fish

237 increased significantly at the end of transport, whereas the groups transported with EO *A.*

238 *triphylla* presented a significant reduction of plasma cortisol levels compared with before

239 transportation and control after transport (Fig. 2B).

240

241 *3.2.   Biochemical parameters*

242   In the liver, glycogen, lactate and glucose levels were significantly lower in fish

243 transported with EO *A. triphylla* when compared to the control. However, total free amino

244 acids and protein values were significantly higher in the treatment with 40 µL/L EO *A.*

245 *triphylla* in relation to control fish. In addition, muscle lactate and glucose levels were

246 significantly higher and lower, respectively, in fish transported with EO *A. triphylla*.

247 Glycogen, total free amino acids and protein did not show any significant difference when

248 compared to control group (Table 2).

249   Activity of AChE in the brain did not alter significantly by addition of EO *A. triphylla*

250 in the water of transport (in µmol ASCh hidrolized/min/mg protein: control: 0.515±0.07, 30

251 µL/L EO *A. triphylla*: 0.473±0.14 and 40 µL/L EO *A. triphylla*: 0.446±0.07) and the same

252 response was observed in the muscle (in µmol ASCh hidrolized/min/mg protein: control:

253 0.207±0.04, 30 µL/L EO *A. triphylla*: 0.205±0.03 and 40 µL/L EO *A. triphylla*: 0.193±0.03).

254   Levels of TBARS in the liver and muscle were significantly lower in silver catfish

255 transported with EO *A. triphylla* when compared to control fish (Fig. 3A). Activity of CAT

256 was significantly lower in the kidney and significantly higher in the gills of fish transported

257 with EO *A. triphylla* compared to control fish (Fig. 3B). On the other hand, hepatic CAT

258 activity was significantly lower and higher in fish transported with 30 and 40 µL/L EO *A.*

259 *triphylla*, respectively, when compared to control fish. Protein carbonyl contents in liver and

260 kidney of fish transported with EO *A. triphylla* were significantly lower compared to control

261 fish (Fig. 3C). However, this parameter was not affected by treatments in muscle and gills.

262

263 **4. Discussion**

264

265 *4.1.   Water and plasma parameters*

266   The use of substances that can reduce stress responses during transport can be

267 beneficial for fish, especially when these animals are transported for long distances. It is

268 recommended that fish be sedated (loss of reactivity to external stimuli and decrease in

269 metabolic rate) through transport, but the equilibrium must be maintained (Summerfelt and

270 Smith, 1990; Pirhonen and Schreck, 2003). The maintenance of partial equilibrium and

271 swimming capacity is important to avoid physical damage resulting from collision with the

272 plastic bags (Cooke et al., 2004).

273   The problems encountered with the use of plastic bags for the transport of live fish is

274 the supply of oxygen and the build-up of ammonia and carbon dioxide during transport

275 (Golombieski et al., 2003; Gomes et al., 2006; Carneiro et al., 2009).The dissolved oxygen

276 levels were significantly higher at end of silver catfish transport in all treatments, because

277 pure oxygen was added to the plastic bags and water movement inside the bags produced by

278 the displacement of the vehicle during transport increased dissolved oxygen levels. Similar

279 results in silver catfish transported in the same load density for 4h were observed by Carneiro

280 et al. (2009).

281   In the present experiment, TAN values of control group at the end of transport were

282 higher than lethal concentration values (96h) to silver catfish at pH 6.0 determined by Miron

283 et al. (2008), but in the present study no had mortality, However, TAN and NH_3 levels at the

284 end of transport with EO *A. triphylla* added to the water were lower than lethal values

285 Therefore, silver catfish could be transported for a longer period without injury due to

286 ammonia toxicity under the conditions used in this experiment with EO *A. triphylla* added to

287 the water of transport. The addition of this EO in the water of transport decreased ammonia

288 excretion, in agreement with results of Becker et al. (2012) in silver catfish transported with

289 eugenol (1.5 or 3.0 μL/L) and EO *L. alba* (10 or 20 μL/L) and Park et al. (2009) in

290 *Pleuronectes americanus* transported with lidocaine hydrochloride (5, 10 or 20 mg/L).

291 During stressful situations catecholamines and cortisol increase blood flow and gill

292 permeability and this facilitates the transport of oxygen to meet the demand of the tissues. In

293 freshwater fish the change in gill permeability leads to osmoregulatory disturbances, as

294 reduction of ion plasma levels (McDonald and Milligan, 1997).The addition of the EO *A.*

295 *triphylla* to the water of transport decreased the net loss of Na^+, Cl^- and K^+ and avoided

296 changes in the levels of these ions in the plasma. This effect was probably the result of lower

297 gill blood flow that occurred because fish were less stressed, as indicated by the lower plasma

298 cortisol levels. Eugenol (4 mg/L) was also effective in reducing the Cl^- efflux during and after

299 transport of *Salmo salar* (Iversen et al., 2009), benzocaine (15-30 mg/L) and quinaldine (100

300 and 250 ml/L) were efficient in avoiding the decrease of plasma Cl^- in *Labeo rohita* from 1 to

301 6h of transport, but for *Hypophthalmichthys molitrix* this effectiveness was not observed

302 (Hasan and Bart, 2007). The transport with eugenol and EO *L. alba* added to the water

303 decreased ion loss in silver catfish (Becker et al., 2012).

304

305 *4.2.   Biochemical parameters*

306 The addition of the EO *A. triphylla* to the water of transport reduced glucose levels of

307 both liver and muscle, indicating that carbohydrate metabolism was used to provide energy

308 through the transport. Moreover, a higher glycogenolysis and reduced lactate levels in the

309 liver was observed in these groups. This suggests that aerobic metabolism was the main

310 energy supplement required during the transport. In the muscle, lower glucose levels were

311 associated with higher lactate levels, suggesting the occurrence of anaerobic pathway for

312 energy production in fish transported with EO *A. triphylla* added to water. To date no studies

313 investigated changes in the intermediary metabolism in tissues of fish subjected to transport

314 with anesthetics added to water. On the other hand, Barbosa et al. (2007) reported similar

315 results in the plasma of *Brycon amazonicus* anesthetized with eugenol.

316 Stress affects negatively the protein metabolism, inhibiting the synthesis and

317 stimulating protein catabolism (van der Boon et al., 1991; Mommsen et al., 1999). Silver

318 catfish transported in the control treatment and 30 µL/L EO *A. triphylla* presented low levels

319 of protein in the liver compared to fish transported with 40 µL/L EO *A. triphylla.* indicating

320 the protective role of the EO against the stress of transport on protein metabolism. This result

321 favors the use of proteins to other vital functions, since the carbohydrate metabolism was

322 sufficient to meet the energy demand.

323 The detection of alterations in AChE activity is associated with nervous impulse

324 disturbances, cognitive dysfunction, alterations in the processing of sensorial information,

325 cortical organization of the movement and control of brain blood flow (Scremin et al., 1997).

326 The addition of EO *A. triphylla* to water of transportation did not induce changes in the brain

327 and muscle AChE activity of silver catfish. Therefore, sedation observed in silver catfish

328 exposed to EO *A. triphylla* is not due to changes on AChE activity.

329 Oxidative reactions are essential in normal metabolism of aerobic organisms, but ROS

330 are produced during the oxidative metabolism generating free radicals (Boveris and

331 Bermúdez, 1996). In a situation of oxidative stress, fish may present a typical reaction for

332 ROS involving lipoperoxidation (LPO) which can be quantified by the increase of the

333 TBARS levels (Ahmad et al., 2000; Sevgiler et al., 2004). On the other hand, the deleterious

334 effect of ROS can be balanced by the production of antioxidant defences (Ahmad et al.,

335   2000), as CAT (Halliwell and Gutteridge, 1999). In this study, lower TBARS levels in the

336   liver and muscle of silver catfish transported with both concentrations of EO *A. triphylla* in

337   relation to control group were observed. Lower TBARS levels were also observed in the liver

338   of silver catfish transported for 5h or 6h with the EO *Lippia alba* (Azambuja et al., 2011).

339   Furthermore, in the present study the CAT activity was higher in gills and liver of silver

340   catfish transported with 40 µL/L EO   *A. triphylla*. Increase of antioxidant defense in liver of

341   silver catfish also was observed after immersion anaesthesia with same EO used in the present

342   study (Gressler et al., 2012). This increase in antioxidant activity possibly contributed to

343   preventing lipid oxidation in the hepatic tissue.

344   In addition to lipid and DNA damage, oxidative stress can also promote protein

345   oxidation (Halliwell and Gutteridge, 1999; Morales et al., 2004). The oxidative modification

346   is considered responsible for the formation of carbonyl groups in protein due to the highly

347   reactive hydroxyl radical (OH^+), which is one of the ROS generated in the process leading to

348   oxidative stress (Oliver, 1987). In this study the carbonyl protein content was lower in the

349   kidney and liver of fish transported with EO *A. triphylla* when compared to control fish,

350   demonstrating the protective role of EO *A. triphylla*.

351

352   **5. Conclusions**

353

354   In conclusion, the addition of EO *A. triphylla* to   water of transport of silver catfish

355   reduce the stress of transport, as demonstrated by lower plasma cortisol levels, ammonia

356   excretion and ionoregulatory changes. Furthermore, changes in metabolic and oxidative stress

357   parameters suggest the presence of compensatory mechanisms to improve fish oxidative

358   status. Thus, the use of EO *A. triphylla* in the water is advisable for fish transportation, being

359   40 µL/L the best concentration.

360

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Table 1. Water parameters before and after transport (6h) of silver catfish (*Rhamdia quelen*) in plastic bags with essential oil of *Aloysia triphylla* added to the water.

Water parameter	Before transport	After transport (treatments)		
		Control	*A. triphylla* (30 µL/L)	*A. triphylla* (40 µL/L)
Dissolved oxygen	6.45±0.40	17.95±1.64[*]	15.13±3.09[*]	14.88±4.32[*]
Carbon dioxide	4.88±0.34	14.85±2.85*	17.33±2.23*	18.01±2.53*
Alkalinity	44.83±8.55	35.00±3.24	32.00±4.34	33.00±4.02
pH	7.30±0.15	6.66± 0.14	6.61±0.14	6.66±0.11
Temperature	17.60±0.60	18.57±1.00	18.52±1.00	19.01±0.80
Total ammonia nitrogen	0.83±0.62	10.34±0.65[*a]	6.02±0.16[*b]	6.09±0.19[*b]
Un-ionized ammonia	0.0052±0.04	0.018±0.007[*]	0.013±0.004[*]	0.014±0.003[*]

Values are expressed as mean ± SEM. Asterisks indicate significant differences when compared to values before transport ($P < 0.05$). Different lowercase letters in the rows indicate significant differences between treatments after transport ($P < 0.05$). Dissolved oxygen and carbon dioxide were expressed as mg/L, and total ammonia nitrogen and un-ionized ammonia were expressed as mg N/L. Alkalinity was expressed as mg $CaCO_3$/L, and temperature as ºC.

1 **Table 2.** Metabolites in tissues of silver catfish, *Rhamdia quelen*, after transport (6h) with

2 essential oil of *Aloysia triphylla* added to the water.

Tissue	Treatments		
Metabolites	Control	*A. triphylla* (30 µL/L)	*A. triphylla* (40 µL/L)
Liver			
Glycogen	95.9±1.8	77.8±1.6[*]	78.1±1.2[*]
Lactate	20.5±0.6	14.6±0.6[*]	10.7±0.4[*]
Glucose	12.4±0.6	9.3±0.2[*]	6.8±0.2[*]
Total free amino acid	17.8±1.9	21.4±1.4	30.7±1.7[*]
Protein	110.2±3.6	121.7±3.5	140.5±4.6[*]
Muscle			
Glycogen	27.5±2.2	26.1±1.2	23.3±1.6
Lactate	14.1±0.7	18.9±1.3[*]	20.3±1.6[*]
Glucose	9.7±0.6	5.2±0.5[*]	5.2±0.3[*]
Total free amino acid	11.9±0.8	10.1±0.5	11.6±0.7
Protein	60.7±1.2	57.4±1.4	60.8±0.6

3 Values are expressed as mean ± SEM (n = 9 per treatment). Asterisks indicate significant

4 differences when compared to the control ($P < 0.05$). Glycogen, lactate, glucose and total free

5 amino acid were expressed in µmol/g tissue. Protein was expressed in mg protein/g tissue.

6   **Figure captions**

7

8   **Figure. 1**. Net ion fluxes in silver catfish, *Rhamdia quelen*, transported in plastic bags with

9   the essential oil *Aloysia triphylla* added to the water. Values are expressed as mean ± SEM.

10   Different letters indicate significant differences between treatments for the same ion (P <

11   0.05).

12   **Figure 2.** Effect of the essential oil of *Aloysia triphylla* added to the water on plasma ion (A)

13   and cortisol (B) levels of silver catfish, *Rhamdia quelen*, after transport in plastic bags. Values

14   are expressed as mean ± SEM. Asterisks indicate significant differences when compared to

15   values before transport (P < 0.05). Different lowercase letters indicate significant differences

16   between treatments after transport (P < 0.05).

17   **Figure 3.** Levels of TBARS (A) and catalase (CAT; B) and content of protein carbonyl (C) in

18   tissues of silver catfish, *Rhamdia quelen*, transported in plastic bags with essential oil of

19   *Aloysia triphylla* (EOA) added to the water. Values are expressed as mean ± SEM. Asterisks

20   indicate significant differences when compared to the control in the same tissue (P < 0.05).

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Figure 1.

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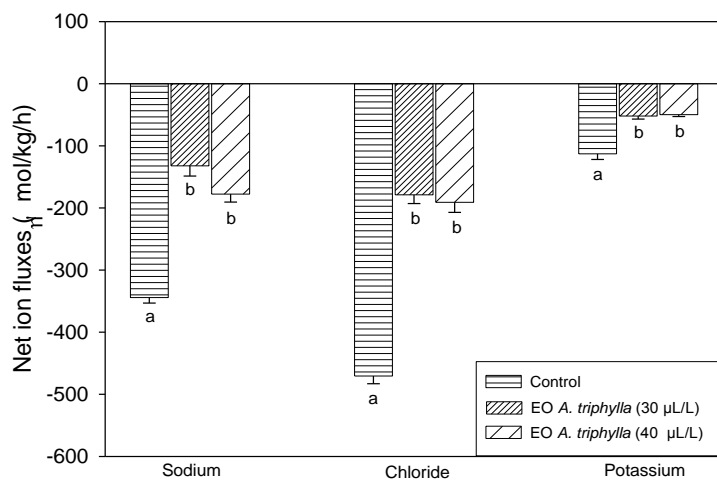
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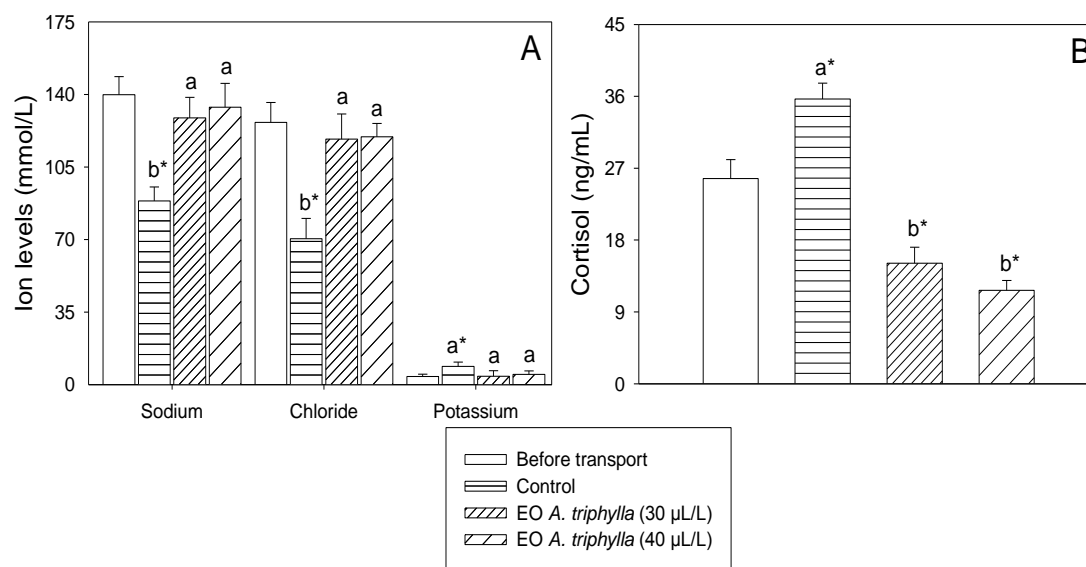
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46 **Figure 2.**

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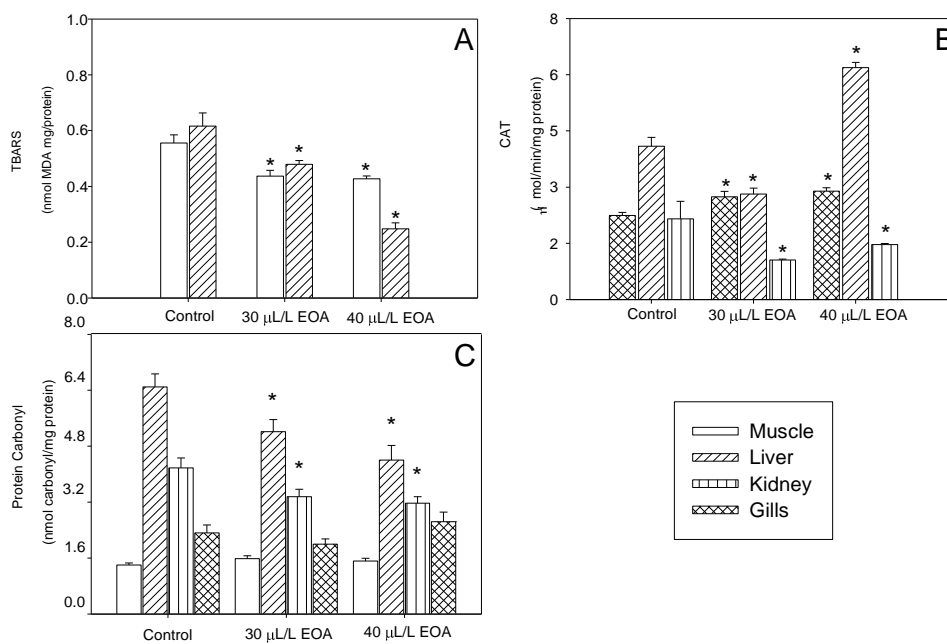
1 **Manuscrito 1**

2

3 **Essential oil of *Aloysia triphylla* as feed additive promotes growth of silver catfish**

4 **(*Rhamdia quelen*).**

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Abstract

This study examined the growth of juvenile silver catfish (*Rhamdia quelen*) fed with different levels of the essential oil (EO) of *Aloysia triphylla* added to the diet (0 - control, 0.25, 0.5, 1.0 and 2.0 mL EO per kg of diet) for 60 days.The group fed 2.0 mL EO per kg of diet showed better results after 60 days on growth, body weight, weight gain and specific growth rate. Histological and immunohistochemical analyzes were performed in the intestine. Height of folds was significantly higher in the control group and 0.25 mL EO per kg of diet compared to those fed 0.5 mL EO per kg of diet. There was a significant increase in the number of folds in 1.0 and 2.0 mL EO per kg of diet compared to the other groups. The enterocyte height was significantly lower in 1.0 mL of EO per kg of diet group compared with 0.25 mL of EO per kg of diet. The number of cells that reacted to CCK and NPY did not differ between groups. The addition of 2.0 mL EO per kg of diet increases length, weight, relative weight gain, specific growth rate and consequently its use is recommended as a feed additive for juvenile silver catfish.

Key words: Digestive tract, Immunohistochemistry, food additive

1. Introduction

Financial success in fish farming is related to advances in biology, nutrition and environmental management of the production cycle (Staykov *et al.* 2005). Many producers seek to improve fish performance with the use of growth promoters, antibiotics and agrochemicals (Santos *et al.* 2009). As a result, there is a growing search for plant-derived growth promoter additives, and good results were found with the use of garlic (Diab *et al.* 2002); oregano (Zheng *et al.* 2009); green tea (Cho *et al*. 2007), basil nutmeg (Sivaram *et al*.

52 2004), yuca (Kelly & Kohler. 2003) and stevia (Shiozaki *et al*. 2004). Phenolic compounds

53 and terpenoids from plant additives have antioxidant activity, acting as radical scavengers

54 (Botsoglou *et al.* 2002; Níciforovíc *et al.* 2010), modifying the intestinal flora, thus improving

55 digestibility and nutrient absorption, resulting in morphological and histological changes in

56 the gastrointestinal tract and improving the immune response (Brugalli 2003).

57   *Aloysia triphylla* (L'Herit.) Britton is a shrub of the Verbenaceae family of up to three

58 meters tall. The leaves and flowers are aromatic, with odor similar to that of lime, used for

59 medicinal purposes either as seasoning (Morgan 1997) and has sedative and anesthetic

60 activity in fish in press (Parodi *et al.* 2012).

61   In this study it was used the silver catfish, *Rhamdia quelen* because is the native

62 species most raised in south Brazil (Baldisserotto *et al*. 2009)

63   In fish, as in other vertebrates, the digestive processes, such as motility, secretion,

64 absorption and immunity are modulated by the neuroendocrine system (Lin *et al.* 2000;

65 Volkoff *et al.* 2005). Peripheral signals for the regulation of gastrointestinal functions may

66 come from: (1) the autonomic nervous system (sympathetic and parasympathetic), (2) the

67 enteric nervous system, and (3) the diffuse neuroendocrine system (DNES) (Jensen, 2001;

68 Buddington & Krogdahl 2004). The DNES fish shows similarities with its mammalian

69 homologous with respect to regulatory processes (Buddington & Krogdahl 2004). Several

70 studies describe the distribution and frequency of cells belonging to DNES located in the

71 gastrointestinal tract (GIT) of fish using immunohistochemical techniques (Domeneghini *et*

72 *al.* 1999; Cinar & Diler 2002; Bosi *et al.* 2004; Lee *et al.* 2004; Bermúdez *et al*. 2007;

73 Vigliano *et al.* 2011, Hernández *et al.*, 2012; Vieira-Lopes et al. 2013). The digestive tract of

74 silver catfish presents a higher number of neuroendocrine cellsin the ascendant and

75 descendant intestine, indicating the primary role of these segments in the modulating feeding

76 behavior of fish by means of the integration of orexigenic and anorexigenic peripheral signals

77 (Hernández *et al.* 2012).

78   The objective of this work was to seek an alternative product low cost, with minimal

79 toxic effects that is inducing growth, increasing fish production.

80

81 **2. Materials and methods**

82 *2.1. Fish and culture conditions*

83   The experiments were conducted in a recirculating aquaculture system in the Fish

84 Physiology Laboratory at the Universidade Federal de Santa Maria (UFSM), Rio Grande do

85 Sul (RS), Brazil. Silver catfish (4.38 ± 0.12 g, 11.62 ± 0.53 cm) were obtained from a

86 producer of the city of Victor Graeff (RS) and acclimated to the laboratory conditions for ten

87 days. After this period, the fish were divided into 50 L (20 fish/tank). Water parameters were

88 checked daily (temperature, pH and dissolved oxygen) or weekly (alkalinity, hardness, total

89 ammonia and nitrite). The experimental protocol was approved by the Ethical and Animal

90 Welfare Committee of the UFSM under registration n° 46/2010.

91

92 *2.2. Water sampling and analyses*

93   Dissolved oxygen (DO) and temperature were measured with an YSI oxygen meter

94 (Model Y5512; YSI Inc., Yellow Springs, OH, USA). The pH was verified with a DMPH-2

95 pH meter (Digimed, São Paulo, SP, Brazil). Total ammonia nitrogen (TAN) levels were

96 measured according to Eaton *et al*. (2005) and un-ionized ammonia (NH_3) levels were

97 calculated according to Colt (2002). Alkalinity was determined according to Boyd & Tucker

98 (1992) Water hardness was analyzed by the EDTA titrimetric method (Eaton *et al*. 2005),

99 nitrite by the method of Boyd & Tucker (1982).

100

101   *2.3. Plant material and essential oil extraction*

102   The plant species *A. triphylla* was cultivated in the city of Frederico Westphalen (RS).

103   The aerial parts of the plant were collected in June 2010, the plant was identified by the

104   botanist Dr. Gilberto Dolejal Zanetti of the Department of Industrial Pharmacy, UFSM, and

105   voucher specimen (SMDB no. 11169) was deposited in the herbarium of the Department of

106   Biology (UFSM).

107   The *A. triphylla* EO was obtained from fresh leaves using hydrodistillation, which was

108   performed with a Clevenger apparatus (3 h) aaccording to guidelines from the European

109   Pharmacopoeia (2007). The EO was stored at −20 °C in amber glass bottles. Its density was

110   approximately 0.9 g mL^{-1}. The major components of *A. triphylla* EO are β-citral (20.78%) and

111   α-citral (29.41%).

112   Essential oil GC–MS TIC analysis was performed using an Agilent-6890 gas

113   chromatograph coupled with an Agilent 5973 mass selective detector under the following

114   conditions:HP-5MS column (5%-phenyl–95%-methylsiloxane, 30 m × 0.25 mm × 0.25 μm);

115   EI–MS: 70 eV; operating conditions: split inlet 1:100; temperature program, 40–260 °C; 40

116   °C for 4 min; ramp rate, 4°C min^{-1}; carrier gas, He; flow rate, 1 mL min^{-1}; injector and

117   detector temperature, 220°C; interface temperature 250°C; Databank NIST 2002. The

118   constituents of the EO were identified by comparing their mass spectra with a mass spectral

119   library (NIST, 2002) and by comparison of the Kovats retention index with literature data

120   (Adams 2001).

121

122   *2.4. Diets and experimental design*

123   Five diets were formulated based on the study of Lazzari *et al*. (2007). All

124   ingredients were finely ground, weighed and mixed by kneading until homogeneous.

125   Different concentrations of the *A. triphylla* EO (0-control, 0.25, 0.5, 1.0 or 2.0 mL EO per kg

126   of diet) were added to the mixture together with canola oil (Table 1). Water was then added to

127   the diets, and a drying process was performed in a forced air circulation oven for 24 h (35°C).

128   Finally, the pellets were broken, sieved and stored in a freezer until use. The fish received the

129   experimental diets until apparent satiation once a day (9 am) for 60 days. The experimental

130   design resulted in five groups (performed in four replicates each).The cleaning of the tanks

131   was performed 30 minutes after feeding through siphoning for removal of waste (remains of

132   food and feces).

133

134   2.5. Sample collection and analytical methods

135   *2.5.1. Growth performance detection*

136   The fish were weighed and measured on days 0, 30 and 60 of the experiment; however, the

137   biomass of each replicate was determined weekly to adjust the food supply. Growth

138   performance was calculated as follows:

139   Relative weight gain (RW) = $100 \times$ (final body weight − initial body weight) / initial body

140   weight; Specific growth rate (SGR) = $100 \times$ (ln final weight − ln initial weight) / days of

141   experiment; Hepatosomatic index (HSI) = $100 \times$ (liver weight) / (whole body weight);

142   Viscerosomatic index (VSI) = $100 \times$ (visceral weight) / (whole body weight); Condition

143   factor (CF) = $100 \times$ (body weight) / (body length)^{3}

144

145   *2.5.2. Histological and immunohistochemical procedure*

146   At the end of the experiment three juveniles per treatment were collected. Samples

147   were fixed in Bouin's fluid (12 h) and embedded in paraffin wax after processing in a graded

148   ethanol series. Microtome sections (1-3 μm thick) were collected on slides pretreated with

149   silane (3-amino-propyltriethoxysilane; Sigma Chemical, St Louis, MO, USA), allowed to dry

150   overnight and then de-waxed and hydrated. Samples were stained with routine staining (H&E)

151 and immunostaining with anti-cholecystokinin 8-Azide free (CCK, Abcam™ Labs, ab27441)

152 and anti-neuropeptide Y (NPY, Abcam™ Labs, ab30914).

153   For immunostaining, all incubations described here were performed at room

154 temperature in a humid chamber less the primary antibodies, and all washing procedures

155 consisted of three successive 5 min immersions in 0.1 M phosphate-buffered saline (PBS; 8

156 mM Na_2HPO_4, 3 mM NaH_2PO_4, 150 mM NaCl). Endogenous peroxidase activity was

157 blocked by incubation in peroxidase blocking solution (3% H_2O_2 in PBS) for 30 min, and

158 after a rinse in PBS, the sections were treated with 3% skim milk powder for 15 min to block

159 non-specific antibody binding. After rinsing in PBS tissue sections were incubated with CCK

160 (1:500) and NPY (1:500) in a humidified chamber overnight at 4°C, washed with PBS.

161 Followed by 30 min incubation at room temperature with super enhancer (Super Sensitive™

162 Link Detection System, BioGenex, CA), and another 30 min incubation with polymer-HRP

163 (Super Sensitive™ Label HRP Detection System, BioGenex, CA) label. Immunostaining was

164 finally developed with DAB (3-3' diaminobenzidine tetrahydrochloride), immersed in

165 deionised water to stop the reaction, counterstained with haematoxylin, dehydrated, and

166 coverslipped. In each series of stained sections, positive and negative controls were included

167 to assess the specificity of the assay. Sections of pig were used as positive controls. Negative

168 control slides were sections in which the primary antibody was replaced by PBS.

169   Histological analyses were carried out to evaluate the following morphological

170 features of the anterior intestine: EH, enterocyte height; FH, folds height; NF, number of

171 intestinal folds over 100μm distance; TEA, total epithelial area mm^2 over 100μm distance.

172   Data are means of independent measurements cross-sectional of the proximal intestine

173 for 12 fish per diet, except for EH for which data are means of 30 measurements per fish,

174 while for every antibody the total number of immunopositive each 1000μm of epithelium was

175 determined. Cells measurements were done using a Leica DM500 microscope and a Leica

176   ICC50 digital camera. The values per treatment were taken with an image analysis system:

177   Leica Application Suite 3.4.1.

178

179   *2.6. Statistical analysis*

180   The results are expressed as the mean ± standard error of the mean (S.E.M). The

181   Levene´s test was performed to evaluate the homogeneity of variances of the data. Data

182   showed homogeneous variances, so comparisons between different treatments were made

183   using a one-way analysis of variance (ANOVA) followed by Tukey test. All analyses were

184   performed using Statistica Software 7.0 (Stat Soft, Tulsa, OK) and differences were

185   considered significant at $P<0.05$.

186

187

188   **3. Results**

189   *3.1. Water quality parameters*

190   The water parameters remained stable throughout the experimental period. The

191   temperature was maintained at 24.2 ± 0.22°C, pH at 7.03 ± 0.02 and dissolved oxygen at 6.68

192   ± 0.4 mg L^{-1}. Hardness (26 ± 1.7 mg L^{-1} $CaCO_3$), alkalinity (42.0 ± 0.9 mg L^{-1} $CaCO_3$), nitrite

193   (0.06 ± 0.01 mg L^{-1}), total ammonia (0.83 ± 0.62mg L^{-1}) and non-ionized ammonia (0.0052 ±

194   0.04 mg L^{-1}) were kept in the desired range.

195

196   *3.2. Growth performance*

197   The highest final weight, relative weight gain and specific growth rate were obtained

198   in fish fed EO of *A. triphylla* 2.0 mL EO per kg of diet ($P<0.05$). There was no significant

199   difference for hepatosomatic index, viscerosomatic index and condition factor between the

200   groups ($P>0.05$) (Table 2).

201 *3.3. Histological and immunohistochemical analyzes*

202   The folds height was significantly higher in the control and 0.25 mL EO per kg of diet

203   groups compared with those fed with 0.5 mL EO per kg of diet ($P<0.05$). There was a marked

204   increase in the number of folds in 1.0 and 2.0 mL EO per kg of diet compared to other groups

205   ($P<0.05$). There was no significant difference in the absorptive area between the different

206   groups ($P>0.05$). The enterocytes height was significantly lower in the group EO 1.0 mL EO

207   per kg of diet compared to the group EO 0.25 mL EO per kg of diet, but there was no

208   significant difference between these groups and the others ($P>0.05$). The number of cels that

209   reacted to CCK and NPY did not differ between the groups ($P>0.05$) (Table 3).

210

211

212   **4. Discussion**

213   The physico-chemical parameters of the water were within acceptable levels for silver

214   catfish (Baldisserotto & Silva 2004).

215   The addition of 2.0 mL EO per kg of diet *A. triphylla* in the diet increased silver

216   catfish length, final weight, relative weight gain and specific growth rate when compared to

217   the control grouop. The use of EO of oregano (*Origanum heracleoticum L.*) in diets for

218   channel catfish (*Ictalurus punctatus*) significantly increased weight gain and feed conversion

219   (Zheng *et al*. 2009). In *Oncorhynchus mykiss* the addition of thymol-carvacrol originated from

220   *Origanum vulgare* in the diet resulted in increased final growth, body weight and feed

221   conversion (Ahmadifar *et al*. 2011). In the presente experiment no significant differences

222   were observed for HSI. Pedron *et al.* (2008) obtained similar results when fed silver catfish

223   with different fiber levels in the diet for 120 days. The VSI and HSI indices also did not

224   change in channel catfish fed diets containing EO of oregano (Zheng *et al*. 2009). The VSI is

225   indicative of adaptation of the gastrointestinal tract to the type of food ingested. As the diet,

226 the digestive tract of the fish may increase in size and weight (volume) in an attempt to

227 increase the area of contact with the food and improve digestibility (Leenhouwers *et al.*

228 2006). In the present study the absorption area showed no significant difference between the

229 groups, however the number of villosities was higher in the treatment with increasing addition

230 of EO in the food. A greater number of villosities shows a greater absorption capacity of

231 nutrients and electrolytes (Naaburs 1995; Hampson 1996; Branco *et al.* 2010), as this increase

232 may be greater due to higher turnover rate caused by stimuli resulting from the action the

233 active principles of plants and their EOS that promote the rapid growth of villous (Branco *et*

234 *al.* 2010).

235 The CCK influences digestive processes and plays a role in the control of food intake

236 through peripheral satiety signals and a larger number of CCK cells demonstrates greater

237 absorptive capacity (Thavanathan & Volkoff 2006; Murashita *et al.* 2007; Hernández *et al.*

238 2012). Immunohistochemical studies in silver catfish showed that CCK cells are mainly

239 distributed in the anterior intestine (Hernández *et al.* 2012).

240 The main biological effect of NPY in fish is an increase in food consumption in a

241 dose-dependent manner (López-Patiño *et al.* 1999; Silverstein & Plisetskaya 2000; Kiris *et al.*

242 2007). Neuropeptide Y is expressed in the central nervous system of all vertebrates

243 investigated by Cerdá-Reverter *et al.* (2000) and in the hypothalamus (López-Patiño *et al.*

244 1999). In fish, NPY is found in the gut, with some interspecific variations in its regional

245 distribution. In silver catfish NPY-like immunoreactive neuroendocrine cells (NECs) were

246 identified in the three portions of the intestine, although the number and intensity of

247 immunoreaction were higher in the proximal segment (Hernández *et al.* 2012). The main

248 general effect of NPY is orexigenic, the higher number of NPY-like immunoreactive NECs in

249 the ascendant intestine of silver catfish could suggest that it has functions as a source of

250 peripheral signals to stimulate food intake in the absence of food at this location. There were

251 no significant differences in the amounts of CCK and NPY between the study groups,

252 demonstrating that the OE *A. triphylla* did not effect on these cells.

253   The addition of 2.0 mL EO per kg of diet increases length, weight, relative weight

254 gain, specific growth rate and consequently its use is recommended as a feed additive for

255 juvenile silver catfish.

256

257

258 **5. Acknowledgments**

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267

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383

384   Table 1- Formulation of the experimental diet.

Ingredients	(%)
Soybean meal	30
Meat and bone meal	35
Rice bran	12
Corn	15
Canola oil	3
Salt	1
Vitamin and mineral premix*	3
Phosphate dicalcium	1
Analyzed proximate composition	
Dry matter contente	92.36
Protein	46.17
Ether extract	10.54
Crude fiber	2.94
Mineral matter	14.29
Acid detergent fiber	2.91
Neutral detergent fiber	16.41

385   * Vitamin and mineral mixture (security levels per kilogram of product) - Folic acid: 250 mg, pantothenic acid:
386   5000 mg, antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg, copper: 2000 mg, iron: 820 mg, iodo: 100 mg,
387   manganese: 3750 mg, niacin: 5000 mg, selenium: 75 mg, vitamin A: 1000 000 UI, vitamin B1: 1250 mg,
388   vitamin B12: 3750 mcg, vitamin B2: 2500 mg, vitamin B6: 2485 mg, vitamin C: 28000 mg, vitamin D3: 500000
389   UI, vitamin E: 20000 UI, vitamin K: 500 mg, zinc: 17500 mg.
390

Table 2- Growth performance of the silver catfish *Rhamdia quelen* fed with diets containing different concentrations of *Aloysia triphylla* essential oil (EO)

	Diet (mL EO per kg of diet)				
	0	0.25	0.5	1.0	2.0
IW (g)	3.9 ± 0.1^a	4.1 ± 0.1^a	3.9 ± 0.1^a	4.09 ± 0.1^a	3.9 ± 0.1^a
LS (cm)	11.92 ± 0.5^a	11.54 ± 0.5^a	11.17 ± 0.5^a	11.90 ± 0.5^a	13.19 ± 0.5^b
FW (g)	14.4 ± 0.4^a	16.5 ± 0.7^a	14.48 ± 0.4^a	15.1 ± 0.6^a	23.6 ± 0.6^b
RWG (%)	272.9 ± 17.8^a	304.3 ± 21.8^a	243.2 ± 24.3^a	291.9 ± 20.5^a	522.0 ± 26.8^b
SGR(% day^{-1})	2.1 ± 0.1^a	2.2 ± 0.1^a	1.9 ± 0.1^a	2.1 ± 0.1^a	2.9 ± 0.1^b
HSI (%)	1.7 ± 0.1^a	1.8 ± 0.1^a	1.6 ± 0.1^a	1.7 ± 0.2^a	1.6 ± 0.1^a
VSI (%)	57.2 ± 4.8^a	83.3 ± 13.8^a	75.7 ± 8.0^a	59.5 ± 7.5^a	52.1 ± 5.1^a
CF (g cm^{-3})	0.9 ± 0.02^a	0.9 ± 0.01^a	0.9 ± 0.03^a	1.0 ± 0.03^a	1.0 ± 0.03^a

IW- initial weight, LS- length standard, FW- final weight, RWG- relative weight gain, SGR- specific growth rate, HSI- hepatosomatic index,VSI- viscerosomatic index, CF- condition factor. Values are expressed as the mean ± SEM (n=4). Different letters in the rows indicate significant difference by one-way ANOVA and Tukey test (P<0.05).

Table 3- Histological and immunohistochemical parameters of the intestine of silver catfish *Rhamdia quelen* fed with diets containing different concentrations of *Aloysia triphylla* essential oil (EO). Data are means of independent measurements cross-sectional of the proximal intestine for 12 fish per diet, except for EH for which data are means of 30 measurements per fish.

	Diet (mL EO per kg of diet)				
	0	0.25	0.5	1.0	2.0
NF (500μm)	3.2 ± 0.2^a	3.7 ± 0.2^{ab}	3.8 ± 0.2^{ab}	4.21 ± 0.2^{bc}	4.75 ± 0.3^c
FH (μm)	678.2 ± 30.5^a	691.7 ± 72.4^a	530.1 ± 30.1^b	560.5 ± 16.9^{ab}	567.1 ± 21.5^{ab}
TEA (mm^2/100μm linear)	16.1 ± 0.9^a	20.9 ± 3.1^a	16.2 ± 1.6^a	15.5 ± 1.0^a	18.7 ± 1.7^a
EH (μm)	36.9 ± 1.7^{ab}	40.3 ± 1.7^a	38.7 ± 1.5^{ab}	33.8 ± 0.5^b	34.8 ± 1.0^{ab}
CCK (N°/1000μm linear)	3.7 ± 0.2^a	4.4 ± 0.2^a	3.7 ± 0.2^a	4.4 ± 0.1^a	4.1 ± 0.3^a
NPY (N°/1000μm linear)	3.1 ± 0.3^a	3.8 ± 0.1^a	3.5 ± 0.1^a	4.2 ± 0.2^a	3.8 ± 0.1^a

NF- number of folds, FH- folds height, TEA- total epithelium area, EH- mean enterocyte height, CCK- mean number of cells, NPY- mean number of cells. Different letters in the rows indicate significant difference by one-way ANOVA and Tukey test (P<0.05).

3 DISCUSSÃO GERAL

O uso de substâncias no transporte para reduzir as respostas ao estresse pode ser benéfico para os peixes, especialmente quando os animais forem transportados por longas distâncias. Recomenda-se que os peixes sejam sedados (perda de reatividade a estímulos externos e diminuição da taxa metabólica) para o transporte, mas o equilíbrio deve ser mantido (SUMMERFELT & SMITH, 1990; PIRHONEN & SCHRECK, 2003). A manutenção do equilíbrio parcial e capacidade de natação são importantes para evitar danos físicos resultante de colisões (COOKE et al., 2004).

Investigamou-se as possíveis eficiências do uso de um anestésico e sedativo de origem natural no transporte de jundiá. A adição do óleo essencial (OE) de *Aloysia triphylla* na água de transporte dos jundiás, realizado neste estudo, demonstrou ser eficaz para a sedação e manutenção do equilíbrio dos peixes durante o período de 6 horas de transporte, não ocorrendo lesões nem mortalidade ao final dos experimentos. Neste estudo os níveis de oxigênio dissolvido foram significativamente mais elevados no final do transporte dos peixes em todos os tratamentos, provavelmente devido à adição de oxigênio puro aos sacos e também à movimentação da água no interior dos sacos produzida pelo deslocamento do veículo durante o transporte. Resultados semelhantes em jundiás transportados por 4h na mesma densidade de carga foram observados por Carneiro et al. (2009).

Outros parâmetros avaliados relacionados com a água de transporte apresentaram resultados interessantes. Os valores de amônia total (TAN) do grupo controle no final do transporte foram superiores aos valores de concentração letais (96h) para jundiás em pH 6,0 determinados por Miron et al. (2008), mas no presente estudo não ocorreu mortalidade. No entanto, os níveis de TAN e amônia não ionizada (NH_3) no final do transporte com OE *A. triphylla* adicionado à água foram menores que os valores letais, portanto, jundiás poderiam ser transportados por um longo período nas condições utilizadas neste experimento com o uso deste na água de transporte. A diminuição da excreção de amônia ocorrida neste estudo nos tratamentos com adição do OE na água está de acordo com resultados de Becker et al. (2012) para jundiás transportados com eugenol (1,5 ou 3,0 uL / L) e OE *L. alba* (10 ou 20 uL / L).

Tem sido descrito por alguns autores que o aumento dos níveis de cortisol plasmático é resposta típica ao estresse geradas pelos procedimentos de transporte (IWAMA et al., 2004; URBINATI & CARNEIRO, 2004). Os resultados obtidos neste trabalho demonstram que o transporte com o uso de OE *A. Triphylla* adicionado à água promove uma redução

significativa nos níveis de cortisol plasmático em comparação com o grupo controle antes e após o transporte. Provavelmente houve uma liberação de catecolaminas, resultando em menor fluxo sanguíneo branquial. Durante situações estressantes catecolaminas aumentam o fluxo sanguíneo e a permeabilidade branquial facilitando o transporte de oxigênio para atender a demanda dos tecidos. Em peixes de água doce a mudança na permeabilidade branquial leva a distúrbios osmorregulatórios, como a redução dos níveis plasmáticos de íons (McDONALD & MILLIGAN, 1997). A adição do OE de *A. triphylla* à água de transporte diminuiu a perda de Na^+, Cl^- e K^+ evitando alterações nos níveis desses íons no plasma.

Outra possibilidade é que jundiás transportados com o OE de *A. triphylla* na água de transporte podem reduzir a freqüência de ventilação (e consequentemente o fluxo de água através das brânquias), como observado quando transportado com o OE de *L. alba* (BECKER et al., 2012).

A adição do OE de *A. triphylla* na água de transporte reduziu os níveis de glicose tanto no fígado quanto no músculo, o que indica que o metabolismo de carboidratos foi usado para fornecer energia durante o transporte. Além disso, ocorreu um aumento da glicogenólise e redução dos níveis de lactato no fígado nestes grupos. Isto sugere que o metabolismo aeróbio foi a principal fonte de energia necessária durante o transporte. No músculo, os níveis de glicose mais baixos foram associados com os níveis de lactato mais elevados, o que sugere o uso concomitante das vias anaeróbias para produção de energia no músculo de peixes transportados com OE de *A. triphylla*. Barbosa et al. (2007) relataram aumento semelhante dos níveis de lactato plasmáticos de *Brycon amazonicus* submetidos a banho de anestésico com eugenol.

O estresse afeta negativamente o metabolismo das proteínas, inibindo a síntese de e estimulante catabolismo proteico (VAN DER BOON et al., 1991; MOMMSEN et al., 1999). Jundiás do tratamento controle e 30 µL / L EO *A. triphylla* apresentaram baixos níveis de proteína no fígado comparados aos peixes transportados com 40 µL / L EO *A. triphylla*, indicando o papel protetor do OE contra o estresse do transporte sobre o metabolismo de proteínas. Este resultado favorece o uso de proteínas para outras funções vitais, uma vez que o metabolismo de carboidratos foi suficiente para atender a demanda de energia para as 6 horas de transporte.

Outros parâmetros bioquímicos, tais como atividades enzimáticas, também são muito importantes para compreendermos como os peixes respondem ao transporte e, se a adição de substância com atividade sedativa e/ou anestésica a água pode ser benéfica ou prejudicial aos peixes. O estresse oxidativo é caracterizado pelo desbalanço entre os pró-oxidantes e os

antioxidantes, resultando em potenciais danos celulares (SIES, 1991; HALLIWELL & 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; TRENZADO et al., 2006).

Em uma situação de estresse oxidativo, os peixes podem apresentar uma reação típica de espécies reativas ao oxigênio (ROS) envolvendo lipoperoxidação (LPO), que pode ser quantificada pelo aumento dos níveis de TBARS (AHMAD et al., 2000; SEVGILER et al., 2004). Por outro lado, o efeito deletério de ROS pode ser equilibrado pela produção das defesas antioxidantes (AHMAD et al., 2000), com a CAT (HALLIWELL & GUTTERIDGE, 1999). Neste estudo, os níveis mais baixos de TBARS no fígado e músculo de jundiás transportado com EO *A. triphylla* foram observados em comparação ao grupo controle. Também os níveis de TBARS mais baixos foram observados no fígado de jundiás transportados por 5h ou 6h com o EO *Lippia alba* (Azambuja et al., 2011).

Os principais constituintes do Óleo essencial *A. Triphylla* (OEA) são o β-citral (20,78%) e o α-citral (29,41%) (anexo E). De acordo com Gressller, et al (2012) citando Edris (2007) , o citral é um monoterpenóide que induz glutationa S-transferase (GST). A GST catalisa reações de conjugação entre GSH e moléculas oxidadas. Atua na remoção dos xenobióticos e produtos de LPO, transformando o composto tóxico em uma forma facilmente excretável (LUSHCHAK & BAGNYUKOVA, 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 a atividade da CAT renal foi significativamente menor nos tratamentos com OE quando compardos ao grupo controle. 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). Uma aumento significativo da atividade da CAT foi observado nas brânquias e no fígado de jundiás transportados com 40 μL /L do OE, esse aumento da defesa antioxidante no fígado também foi observado pós a anestesia por imersão com o mesmo óleo em estudo de Gressler et al. (2012). Este aumento da atividade antioxidante possivelmente contribuiu para impedir a oxidação lipídica no tecido hepático.

A modificação oxidativa é responsável pela formação de grupos carbonila em proteínas, devido ao radical hidroxila (OH ⁻) altamente reativo, que é uma das ROS gerada no processo que conduz ao estress oxidativo (OLIVER, 1987). Neste estudo, o teor de proteína carbonila foi menor no rim e fígado de peixes transportados com EO de *A. triphylla* quando comparados ao grupo controle, demonstrando o papel protetor da EO de *A. triphylla* em jundiás.

Diante dos resultados obtidos no estudo com a utilização do OE de *A. triphylla* na água de transporte de jundiás verificamos ser este OE bastante promissor e foi realizado um segundo estudo incorporando o OE de *A. triphylla* na ração de alimentação dos jundiás para verificar possíveis ações benéficas com um promotor de crescimento nos peixes.

O crescimento dos peixes observado neste segundo estudo foi promissor, a adição de 2,0 mL de EO de *A. triphylla* por kg de ração na dieta aumentou comprimento dos jundiás, peso final, ganho de peso relativo, taxa de crescimento específico e integridade intestinal quando comparado ao grupo controle. O uso de OE de orégano (*Origanum heracleoticum L.*) em dietas para bagre do canal (*Ictalurus punctatus*) aumentou significativamente o ganho de peso e conversão alimentar (ZHENG et al., 2009). Em *Oncorhynchus mykiss* a adição de timol-carvacrol originado de *Origanum vulgare* na dieta resultou em aumento do crescimento final, o peso corporal e a conversão alimentar (AHMADIFAR et al., 2011). No presente estudo, a área de absorção mostrou nenhuma diferença significativa entre os grupos, no entanto, o número de vilosidades foi aumentando significativamente com a adição crescente do OE na ração. Um maior número de vilosidades mostra uma maior capacidade de absorção de nutrientes e eletrólitos (NAABURS, 1995; HAMPSON, 1996; BRANCO et al., 2010).

A colecistocinina (CCK) é secretada pelas células I presentes especialmente no duodeno e no jejuno, atuando, principalmente, por mecânismo telécrino (POLAK et al.,1993). Ela estimula a contração da vesícula biliar, a secreção pancreática de amilase, atividade motora intestinal, a secreção de pepsina gástrica e a secreção das glândulas de Brunner(em mamíferos), além de inibir a secreção ácida das células parietais, a atividade motora e o esvaziamento gástrico, a contração do esfíncter inferior esofágico, a contração do esfíncter de Oddi e a absorção de fluidos e eletrólitos no jejuno e no íleo (GRANNER, 1988; POLAk et al., 1993). A colecistocinina também inibe a ingestão de alimentos, atuando por mecanismo neurócrino no centro da fome e da saciedade no SNC. A liberação de CCK pelas células I é estimulada pela presença de nutrientes no duodeno, como peptídios, gorduras e carboidratos. A CCK liberada atua sobre o pâncreas exócrino, resultando na liberação das enzimas pancreáticas na luz duodenal (tripsina, amilase e lipase). Essas, por sua vez, inibem a

liberação da CCK pelas células I, exercendo um "feedback" negativo e, completando, assim, o ciclo de ação desse hormônio (SWENSON & REECE 1996).

O neuropeptídeo tirosina (NPY), localiza-se nos sistemas nervoso central e periférico, desempenhando a função de neurotransmissor ou neuromodulador, relacionado à noradrenalina (POLAK et al., 1993). O NPY presente no sistema nervoso simpático está freqüentemente, localizado nos neurônios noradrenérgicos e funciona, aparentemente, tanto como vasoconstritor quanto como co-transmissor, juntamente com a noradrenalina. O NPY produz vários efeitos sobre o sistema nervoso central, incluindo aumento na ingestão de alimento (REID, 1998). O NPY encontrado em alguns neurônios secretomotores do sistema nervoso entérico pode inibir a secreção de água e eletrólitos no intestino (KATZUNG, 1998). Também foi encontrado em células L, coexistindo com PYY e glicentina.

Em peixes, como em outros vertebrados, os processos digestivos, tais como motilidade, secreção, absorção e imunidade são modulados pelo sistema neuroendócrino. O hipotálamo desempenha um papel importante na regulação de várias funções gastrointestinais, produzindo fatores que estimulam (orexígenos) ou inibem (anorexígenos) a ingestão integra diversos sinais periféricos (LIN et al., 2000; VOLKOFF et al., 2005). Estes sinais periféricos podem vir de: (1) sistema nervoso autônomo (simpático e parassimpático), (2) sistema nervoso entérico, e (3) sistema neuroendócrino difuso (SNED) (LE BAIL & BOEUF, 1997; JENSEN, 2001; BUDDINGTON & KROGDAHL, 2004). O SNED de peixes mostra semelhanças com o seu homólogo de mamíferos no que diz respeito aos processos regulatórios. No entanto, o SNED apresenta características funcionais únicas relacionadas ao habitat (de água doce ou salgada), estação, período reprodutivo ou desenvolvimento fase (BUDDINGTON & KROGDAHL, 2004).

Diversos estudos com técnicas de imunohistoquímica revelaram a presença de células de CCK imunoreativas do cérebro e do trato gastrointestinal de várias espécies de peixes (JENSEN et al., 2001; KAMISAKA et al., 2001; KU et al., 2004;. LEE et al., 2004; VIGLIANO et al., 2011).

Em *R. quelen*, as células imunoreativas CCK foram identificados apenas no intestino ascendente e descendente. No entanto, o maior número foi encontrado na porção ascendente, o que difere do descrito por Ku et al. (2004) para *Zacco platypus* uma vez que nesta espécie as células de CCK imunoreativas estão distribuídas ao longo de todo o intestino. Em contraste, a distribuição de células de CCK imunoreativas semelhantes a que ocorre em *R. quelen* foram relatadas em *O. mykiss* (JENSEN et al., de 2001), *S. trutta* (BOSI et al., 2004) e *O. bonariensis* (VIGLIANO et al., 2011).

Considerando as funções biológicas da CCK, este padrão de distribuição em juvenis de *R. quelen* pode indicar um papel principal do intestino ascendente na contração da vesícula biliar, secreção enzimas pancreáticas, estimulação da motilidade gastrointestinal e inibição do esvaziamento gástrico (JENSEN, 2001).

A regulação da ingestão de alimentos em vertebrados é um complexo processo em que várias vias neurais desempenham importantes papéis. Entre as várias moléculas de sinalização envolvidas nestas vias esta o neuropeptídeo Y (NPY), que desempenha um papel no controle do consumo de alimentos e do peso corporal. O NPY é um dos mais potentes estimulantes de ingestão de alimentos em mamíferos, mas muito pouco se sabe sobre suas ações em peixes. O principal efeito biológico do NPY em peixes é um aumento na consumo de alimentos de forma dose dependente, tal como foi demonstrado pela administração central ou periférica do péptido (LÓPEZ-PATI et al., 1999; SILVERSTEIN & PLISETSKAYA, 2000; KIRIS et al., 2007). O neuropeptídeo Y é amplamente expresso no sistema nervoso central de todos os vertebrados investigados (CERDÁ-REVERTER et al., 2000) e, principalmente, no hipotálamo (López-Pati et al., 1999). Por outro lado, o NPY é também encontrado no intestino de peixes, com algumas variações na sua distribuição. Em *Scleropages Jardini, Chitala chitala, Gnathonemus petersii e Lepisosteus Osseus*, NPY imunoreativo só foi encontrado no células neuroendócrinas (CNE) do intestino anterior (GROFF & YOUSON, 1997; AL-MAHROUKI E YOUSON, 1998), enquanto em *O. bonariensis*, foi encontrado em CNE do intestino anterior e posterior, com um número significativamente maior no anterior (VIGLIANO et al., 2011).

Cinar et al. (2006) observaram que a CNE de NPY-imunoreativas foram distribuídas no estômago e intestino médio de *Pseudophoxinus antalyae.* Domeneghini et al. (1999) mostraram NPY imunopositividade nas fibras nervosas associadas com as células do músculo estriado do esôfago em *Acipenser transmontanus*. Em contraste, em *R. quelen* imunoreatividade para NPY não estava presente tanto nas CNE localizados no esôfago ou no estômago, ou nas fibras nervosas de qualquer segmento do trato digestivo investigado. No entanto, o NPY imunorreativo no CNE foram identificados nas três partes do intestino, embora o número e intensidade de imunoreação foram maiores no segmento proximal. Tendo em conta que o principal efeito geral de NPY é orexígeno e o maior número de NPY imunoreativos foi encontrado no intestino ascendente de *R. quelen,* sugere-se que este local possui funções como uma fonte de sinais periféricos para estimular ingestão de alimentos na ausência de comida no local (HERNANDEZ et al., 2012).

Em nosso estudo não houve diferenças significativas nos valores de CCK e NPY entre os grupos de estudo, demonstrando que o OE *A. triphylla* não efeito sobre essas células. Apesar disso ainda há muitos aspectos a serem explorados em relação a adição de óleos esseciais na ração para peixes e ao mecanismo de ação destes óleos no trato digestório e sua atuação no controle do apetite dos peixes.

4 CONCLUSÕES

A adição de OE de *A. triphylla* na água de transporte reduziu o estresse de transporte em jundiás, como demonstrado por níveis mais baixos de cortisol plasmático, a excreção de amônia e mudanças ionoregulatórias. Além disso, alterações nos parâmetros metabólicos e de estresse oxidativo sugerem a presença de mecanismos compensatórios para melhorar o estado oxidativo dos peixes. Assim, a utilização de óleo essencial de *A. triphylla* na água é aconselhável para o transporte de jundiás, na melhor concentração de 40 μL / L.

A adição de 2,0 mL de OE de *A. triphylla* por kg de ração na alimentação melhora o comprimento, peso final, ganho de peso relativo, taxa de crescimento específico e, consequentemente, o seu uso é recomendado como aditivo na alimentação de juvenil de jundiá.

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ANEXOS

ANEXO A – Espécime de jundiá (*Rhamdia quelen*) utilizado nos experimentos (FONTE: Carla Cristina Zeppenfeld).

ANEXO B – Partes aéreas da espécie *A. tryphilla* (FONTE: Arquivo pessoal).

ANEXO C – Sistema de recirculação utilizado no experimento (FONTE: Carla Cristina Zeppenfeld).

ANEXO D – Filtros biológicos do sistema de recirculação utilizado no experimento (FONTE: Carla Cristina Zeppenfeld).

ANEXO E – Tabela1. Composição química dos constituintes do óleo essencial de *A. triphylla*

IK$_t$	IK$_c$	Compounds	%
964	969	β-pinene	1.07
971	971	3-octanol	0.29
1017	1010	limonene	11.90
1027	1030	Cis-ocimene	0.83
1090	1081	α-Pinene oxide	0.10
1090	1084	Linalool	0.69
1126	1123	Myrtanal	0.30
1146	1132	citronellal	0.76
1146	1140	isopulegol	0.19
1155	1154	2-pinen-4-ol	1.46
1168	1164	Pulegone (p-Menth-4(8)-en-3-one)	0.44
1179	1173	α-terpineol (p-menth-1-en-8-ol)	2.24
1217	1220	Cis geraniol	0.51
1228	1225	Citronellol ou linalool acetate	1.34
1240	1240	β-citral	20.78
1259	1249	Trans- geraniol	0.55
1271	1274	α-citral	29.41
1331	1333	δ-elemene	0.13
1364	1363	Neryl acetate	0.35
1367	1377	α-cubebene	0.16
1384	1385	Geranyl acetate	2.98
1418	1422	caryophyllene	5.64
1459	1456	α-caryophyllene	0.29
1463	1463	Aromadendrene	0.17
1471	1478	Acoradiene	0.18
1475	1485	Geranil propionate	1.13
1481	1487	Germacrene D	1.55
1495	1502	bicyclogermacrene	1.16
1507	1517	β-bisabolene	0.57
1509	1519	cis-α-bisabolene	0.24
1521	1522	τ-cadinene	0.15
1527	1529	δ- cadinene	0.11
1564	1575	nerolidol	0.78
1586	1589	spathulenol	0.58
1594	1593	Caryophyllene oxide	2.33
1604	1598	cubenol	3.29
1613	1604	cedrol	0.16
1619	1624	Humulane-1,6-dien-3-ol	0.28
1648	1658	τ-cadinol	1.72
1682	1672	α-Bisabolol	0.38
1688	1689	Cedr-8-en-13 ol	0.23
Total percentage of identified compounds			**97.42**

IK^t= retention índex reference; **IK^c** = retention índex calculated; **%** = relative percentage.

ANEXO F – Normas revista Aquaculture Nutrition

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