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ADIÇÃO DO ÓLEO ESSENCIAL DE *Aloysia triphylla* EM DIETA PARA ZEBRAFISH

DISSERTAÇÃO DE MESTRADO

Daniane Cioccari Zago

Santa Maria, RS, Brasil

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ADIÇÃO DO ÓLEO ESSENCIAL DE *Aloysia triphylla* EM DIETA PARA ZEBRAFISH

Daniane Cioccari Zago

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Biodiversidade Animal, da Universidade Federal de Santa Maria (UFSM - RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas – Área de Biodiversidade Animal**.

Orientador: Prof. Dr. Mauro Alves da Cunha

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E-mail: danianezago@yahoo.com.br

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elaborada por
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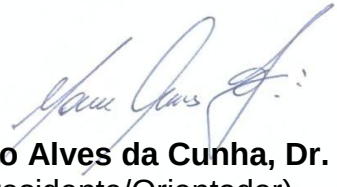

Mauro Alves da Cunha, Dr.
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“Poeta mesmo é a lagarta do
bicho-da-seda que quando cansa da
vida se transforma em borboleta.”
(Autor desconhecido)

RESUMO

Dissertação de Mestrado
Programa de Pós Graduação em Biodiversidade Animal
Universidade Federal de Santa Maria

ADIÇÃO DO ÓLEO ESSENCIAL DE *Aloysia triphylla* EM DIETA PARA ZEBRAFISH

AUTORA: DANIANE CIOCCARI ZAGO
ORIENTADOR: MAURO ALVES DA CUNHA
Data e local da defesa: Santa Maria, 18 de agosto de 2014.

O uso de óleos essenciais (OE) ou extratos vegetais em dietas para peixes tem demonstrado efeitos positivos no desempenho produtivo, na eficiência de utilização dos nutrientes, no aumento da taxa de crescimento, na resistência ao estresse, imunidade e proteção contra patógenos. A *Aloysia triphylla* é utilizada como condimento e para fins medicinais e seu OE destaca-se por possuir propriedades anestésicas e sedativas, bloqueadoras de cortisol, além de promover proteção oxidativa e crescimento de jundiás. O zebrafish possui muitas características semelhantes a dos mamíferos, sendo considerado organismo modelo para pesquisas, possui disponibilidade genômica funcional e rápido desenvolvimento, facilitando estudos comportamentais, nutricionais e de crescimento. Com o presente estudo objetivou-se avaliar os efeitos do OE de *A. triphylla* em zebrafish, analisando o crescimento, consumo de oxigênio, atividade exploratória, distância percorrida e cortisol corporal total. O experimento teve duração de 210 dias e o crescimento foi analisado no período de 90 dias. Foram avaliadas três dietas contendo (0.0; 1.0 e 2.0 mL/kg) de OE de *A. triphylla* adicionada à ração. A adição do OE à ração de zebrafish se mostrou eficiente reduzindo o consumo de oxigênio e atividade exploratória dos peixes tratados com dietas adicionadas OE à ração. Nenhuma diferença foi observada para o crescimento, distância percorrida e níveis de cortisol corporal total entre os tratamentos. Desta forma, conclui-se que a adição de 1.0 e 2.0 mL/kg de OE de *A. triphylla* adicionado à dieta é eficiente em promover redução do consumo de oxigênio e atividade exploratória em zebrafish.

Palavras-chave: Aquicultura. Produto natural. Metabolismo. Alimentação. Estresse.

ABSTRACT

Master Dissertation
Post-Graduation in Animal Biodiverity
Universidade Federal de Santa Maria

ADDITION *Aloysia triphylla* ESSENTIAL OIL IN ZEBRAFISH DIET

AUTHOR: DANIANE CIOCCARI ZAGO
ADVISOR: MAURO ALVES DA CUNHA
Date and place of defense: Santa Maria, August 18, 2014.

The use of essential oils (EO) or plant extracts in diets fish has demonstrated positive effects on growth performance, efficiency of nutrient utilization, increased growth rate and resistance to stress, immunity and protection against pathogens. The *Aloysia triphylla* is used as a condiment and for medicinal purposes and its EO stands out for having anesthetic and sedative properties, mitigate stress and promote oxidative protection and growth of silver catfish. The zebrafish has many characteristics similar to mammals and is considered a model organism for research, has functional genomics availability and rapid development, facilitating behavioral, nutritional and growth studies. The present study aimed to evaluate the effects of *A. triphylla* EO in zebrafish, analyzing the growth, oxygen consumption, exploratory activity, distance travelled and whole-body cortisol. The experiment lasted 210 days, and growth was evaluated at 90 days. Three diets (0.0, 1.0 and 2.0 mL/kg) with *A. triphylla* EO added to the diet were evaluated. The addition of EO to zebrafish diet proved efficient by reducing oxygen consumption and exploratory activity of fish treated with diets OE. No difference was observed for the growth, distance travelled and whole-body cortisol between treatments. Thus, it is concluded that addition of 1.0 and 2.0 mL/kg *A. triphylla* EO added to the diet is effective in promote reduction of oxygen consumption and exploratory activity in zebrafish.

Keywords: Aquaculture. Natural product. Metabolism. Food. Stress.

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1 INTRODUÇÃO

1.1 Características do zebrafish

O zebrafish (*Danio rerio*), comumente chamado de zebrafish ou paulistinha (Figura 1) é um peixe ornamental, que pertence à família Cyprinidae; de água doce; onívoro e que regularmente consome insetos terrestres e aquáticos, assim como fitoplâncton. Essa espécie é nativa do sul da Ásia, originária das partes baixas dos principais rios da Índia, Bangladesh e Nepal (DAMMSKI et al.; 2011).

Figura 1 – Zebrafish produzido e cultivado pelo Laboratório de Biologia Molecular da Universidade Federal do Rio Grande (FURG).

Esta espécie possui muitas características semelhantes a dos mamíferos, podendo ser considerada um modelo alternativo e complementar de vertebrados (BRAGA et al.; 2013). Como resultado destas características, muitos laboratórios começaram a explorar as vantagens de utilizar o zebrafish para estudos de doenças humanas como porfiria, doenças sanguíneas (WANG et al.; 1998; AMATRUDA & ZON, 1999) e comportamentais como ansiedade (LEVIN et al.; 2007; BENCAN et al.; 2009; ROSEMBERG et al.; 2011, 2012; CACHAT et al.; 2010). Também são muito utilizados em estudos de toxicologia, desenvolvimento, neurobiologia e genética molecular; sendo proposto como um possível organismo modelo para estudos de nutrição e crescimento em peixes (DE-SANTIS & JERRY, 2007). O zebrafish é uma importante ferramenta para a realização de pesquisas de biologia do desenvolvimento dos vertebrados porque possui um desenvolvimento rápido e os

embriões são transparentes, permitindo a visualização do desenvolvimento de órgãos e tecidos (FLINN, 2008; RUBINSTEIN, 2003). Seu uso para pesquisas possui várias vantagens, tais como: baixo custo, pouco espaço requerido para manutenção, rápido desenvolvimento, atingindo maturidade sexual em cerca de três meses após a fecundação, grande número de descendentes e estágios de desenvolvimento bem caracterizados (BRAGA et al.; 2013). Devido o seu pequeno tamanho, o comportamento pode ser facilmente observado e quantificado em um ambiente controlado (BEIS & STAINIER, 2006). No entanto, sua utilização para pesquisas faz com que estejam sempre sujeitos a fatores estressantes como manipulação e transporte, levando ao estresse, podendo desencadear doenças, redução na taxa de crescimento e consequentemente afetar sua sobrevivência.

1. 2 Óleos essenciais (OE) e extratos vegetais

Os óleos essenciais e extratos vegetais são compostos de origem vegetal, formados principalmente por metabólitos secundários (monoterpenos e sesquiterpenos) nas plantas aromáticas (BAKKALI et al.; 2008). Destacam-se por serem substâncias naturais e seguras para o meio ambiente, para a saúde dos animais e para os seres humanos (JINTASATAPORN & BONALDO, 2012). Estes compostos vêm sendo amplamente utilizados em dietas para peixes e seu uso tem demonstrado efeitos positivos, como exemplo, o chá verde (*Camellia sinensis*) adicionado à dieta de linguados (*Paralichthys olivaceus*) se mostrou eficiente, melhorando seu ganho de peso (CHO et al.; 2007). Esse mesmo resultado foi obtido em tilápias do nilo (*Oreochromis niloticus*) alimentadas com a adição de alho (*Allium sativum*) à dieta (SHALABY et al.; 2006). Robalos (*Lates calcarifer)* alimentados com dieta contendo alho (*Allium sativum*) melhoraram a imunidade e resistência à infecção por *Vibrio harveyi* (TALPUR & IKHWANUDDIN, 2012).

Além disso, os OE possuem propriedades anestésicas, sendo uma alternativa natural ao uso de anestésicos sintéticos (HELDWEIN et al.; 2012). Estudos feitos com jundiás (*Rhamdia quelen*), verificaram que o OE de *Lippia alba*, pertencente a família Verbenaceae e conhecida popularmente como erva cidreira, se mostrou eficiente para sedação e anestesia, além de inibir a elevação do cortisol plasmático

após o estresse de manejo (CUNHA et al.; 2010). Em outro estudo o mesmo OE adicionado à dieta influenciou beneficamente os biomarcadores de estresse oxidativo (SACCOL et al.; 2013). O óleo de cravo (*Syzygium aromaticum*) se mostrou eficaz na redução da resposta ao estresse de curto prazo, sendo recomendado como um anestésico alternativo em truta arco-íris (*Oncorhynchus mykiss*) (WAGNER et al.; 2003).

1.3 A planta *Aloysia triphylla*

A *Aloysia triphylla* (L'Hérit) Britton (Figura 2), pertencente a família Verbenaceae, é conhecida, popularmente, como limonete ou cidrão (PAULUS et al.; 2013). Nativa da América do Sul (Chile, Argentina, Uruguai a Peru), atinge até três metros de altura. Suas folhas e flores são aromáticas, com odor semelhante ao do limão. Usada como condimento e para fins medicinais, agindo como sedativo brando, auxiliando na digestão e contra resfriados (PAULUS et al.; 2013).

Figura 2 – *Aloysia triphylla*.
Fonte: http://www.burncoose.co.uk

Possui 41 compostos ativos, sendo que seus componentes majoritários são α – citral (29,41%) e β – citral (20,78%) (Tabela 1).

Tabela 1 – Composição química dos constituintes do OE 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-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	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 identif compounds			**97.42**

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

Estudos feitos recentemente com a adição de 2.0 mL/kg de OE de *Aloysia triphylla* na ração de jundiás (*Rhamdia quelen*), alimentados por 60 dias, mostraram melhora em ganho de peso final e relativo, taxa de crescimento específico e

comprimento (ZEPPENFELD, 2014). O OE de *A. triphylla* se mostrou eficaz como anestésico para jundiás e camarões (PARODI et al.; 2014, 2012). Além disso, a adição do OE na água de transporte reduziu o estresse em jundiás, demostrado por níveis mais baixos de cortisol plasmático (ZEPPENFELD, 2014; GRESSLER et al.; 2012) e promoveu proteção oxidativa (GRESSLER et al.; 2012).

1.4 Objetivo

Avaliar os efeitos do OE de *A. triphylla* adicionado à dieta de zebrafish em parâmetros metabólicos e comportamentais.

1.5 Metas específicas

Verificar a adição de diferentes concentrações do OE de *A. triphylla* em dieta para zebrafish:

- Na promoção do crescimento
- Na redução do consumo de oxigênio
- Na redução da atividade exploratória
- Na redução da distância percorrida
- Na redução do cortisol corporal total

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MANUSCRITO

Adição do óleo essencial de *Aloysia triphylla* em dieta para zebrafish

Daniane Cioccari Zago[1], Carlos Frederico Ceccon Lanes[2], Daniela Volcan Almeida[2], Gessi Koakoski[3], Berta Heinzmann[4], Bernardo Baldisserotto[1], Leonardo José Gil Barcellos[5], Luis Fernando Marins[2,6] & Mauro Alves da Cunha*[1]

[1]Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, UFSM, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil.

[2]Instituto de Oceanografia, Universidade Federal do Rio Grande, FURG, Rio Grande, Av. Itália, Km 8, Rio Grande, RS, 96201-900, Brazil.

[3]Departamento de Farmacologia Aplicada a Produção Animal, Universidade Federal de Santa Maria, UFSM, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil.

[4]Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, UFSM, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil.

[5]Curso de Medicina Veterinária, Universidade de Passo Fundo, UPF, Campus I, Bairro São José, Caixa Postal 611, Passo Fundo, RS, 99001-970, Brazil.

[6]Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, FURG, Rio Grande, Av. Itália, Km 8, Rio Grande, RS, 96201-900, Brazil.

* Corresponding author

Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, UFSM, Av. Roraima, 1000, Santa Maria, RS, 97105-900, Brazil.

Fone: + 55 55 3320-9382 Fax: + 55 55 3220-8241

E-mail adress: cunha.mauroalves@gmail.com

Resumo

O objetivo deste estudo foi analisar os efeitos do óleo essencial (OE) de *Aloysia triphylla* adicionado à dieta de zebrafish (*Danio rerio*), no crescimento, consumo de oxigênio, atividade exploratória, distância percorrida e cortisol corporal total. Juvenis de zebrafish (11.02 ± 0.5 mg e 0.92 ± 0.01 cm) foram divididos em três tratamentos: controle (sem adição de OE), T1 (1mL/kg de OE adicionado à dieta) e T2 (2 mL/kg de OE adicionado à dieta) e alimentados três vezes ao dia “ad libitum” durante 210 dias. Cada tratamento foi realizado em quadruplicata. O crescimento foi avaliado a cada 15 dias pelo período de 90 dias e ao final do experimento, o consumo de oxigênio, atividade exploratória, distância percorrida e cortisol corporal total também foram avaliados. Não houve diferença significativa entre os tratamentos nos parâmetros de crescimento, distância percorrida e cortisol corporal total. No entanto, os tratamentos T1 e T2 apresentaram uma redução no consumo de oxigênio e atividade exploratória quando comparados com o controle. Desta forma, conclui-se que a adição de 1.0 e 2.0 mL/kg de OE de *Aloysia triphylla* adicionado à dieta é eficiente em promover a redução do consumo de oxigênio e da atividade exploratória em zebrafish.

Palavras-chave: Aquicultura. Produto natural. Metabolismo. Alimentação. Stress.

Abstract

The aim of this study was to analyze the effects of *Aloysia triphylla* essential oil (EO) added in zebrafish diet, on growth, oxygen consumption, exploratory activity, distance traveled and whole-body cortisol. Juveniles zebrafish (11.02 ± 0.5 mg e 0.92 ± 0.01 cm) were divided into three treatments: control (without EO), T1 (1 mL/kg of EO added to the diet) and T2 (2 mL/kg of EO added to the diet) and fed three times a daily "ad libitum" for 210 days. Each treatment was performed in quadruplicate. The growth was evaluated every 15 days for a period of 90 days and at the end of the experiment, oxygen consumption, exploratory activity, distance travelled and whole-body cortisol were also evaluated. There was no significant difference between treatments in growth parameters, distance travelled and whole-body cortisol. However, treatments T1 and T2 showed a reduction in oxygen consumption and exploratory activity when compared with control. Thus, it is concluded that the addition of 1.0 and 2.0 mL/kg of *Aloysia triphylla* EO added to the diet is effective in promoting the reduction of oxygen consumption and exploratory activity in zebrafish.

Keywords: Aquaculture. Natural product. Metabolism. Food. Stress.

Introduction

The zebrafish (*Danio rerio*) is a fish that has many characteristics and can be considered alternative and complementary model vertebrates, thus, widely used in research (BRAGA et al.; 2013). Have similar organs and cells of the mammalian and as a result of these characteristics many laboratories have begun to explore the advantages of using the zebrafish for studies of human diseases (RUBINSTEIN, 2003), behavioral (BENCAN et al.; 2009; ROSEMBERG et al.; 2011, 2012; CACHAT et al.; 2010) of nutrition and growth (DE-SANTIS & JERRY, 2007). Currently, the need for a well characterized model fish to produce results applicable to fish in aquaculture has been satisfied by zebrafish, due to the availability of functional genomics and molecular biology data to facilitate the analysis of growth, reproduction, meat quality and Biology the disease, with the corresponding development of vaccines and therapies (ALLESTRÖM et al.; 2006; DAHM & GEISLER, 2006). Its use for research has several advantages such as: low cost, rapid development, reaching sexual maturity in about three months after fertilization, transparent embryos, large numbers of offspring and developmental stages well characterized (BRAGA et al.; 2013). In addition, its small size facilitates behavioral studies, and can be easily observed and quantified in an environment controlled (BEIS & STAINIER, 2006). However, because this species widely used for and research, is always subject to stressors such as transport, handling and biometrics, leading to stress, may affect their survival.

Essential oils (EO) and plant extracts have been used in fish as natural alternative to use of traditional chemical anesthetics, minimizing the stress of physical damage. Recent studies have demonstrated a reduction in the release of cortisol and improvements in oxidative stress parameters (GRESSLER et al.; 2012). Furthermore, the use of EO have demonstrated positive effects as additives in the diets, improving immunity (TALPUR & IKHWANUDDIN, 2012; SIVARAM et al.; 2004), growth (SHALABY et al.; 2006) and blood biochemical levels, such as glutamic pyruvic transaminase, and LDL cholesterol (CHO et al.; 2007).

The plant *Aloysia triphylla* belongs to Verbenaceae, is popularly used as a condiment and for medicinal purposes, acting as a sedative, aiding digestion and against colds (PAULUS et al.; 2013). Has anesthetic and sedative effects

(ZEPPENFELD et al.; 2014b; PARODI et al.; 2012, 2014), cortisol blockers; as growth promoters and oxidative protection (ZEPPENFELD 2014a; GRESSLER et al.; 2012) have been described by the research group of the Laboratory of Physiology of Fish (LAFIPE) of the Universidade Federal de Santa Maria (UFSM). The EO of this plant was patented as anesthetic for aquatic animals (Patent N^0 PI 01609000590-5) and recently to promote growth in aquatic animals (Patent N^0 BR 102014013628-2, em 05.06.2014). Therefore, this study aimed to evaluate the effects of *A. triphylla* EO added in zebrafish diet, in the growth, oxygen consumption, exploratory activity, distance travelled and cortisol.

Material and methods

Fish and experimental conditions

Juveniles zebrafish with 40 days of age (11.02 ± 0.53 mg e 0.92 ± 0.01 cm) were obtained from own production of the Instituto de Ciências Biológicas da Universidade Federal do Rio Grande (ICB/FURG). Fish were randomly distributed in twelve plastic boxes (15 L), 15 fish per plastic box, and were kept in a recirculation system comprising a biological filter and ultraviolet light for sterilization of water. During the experiment, photoperiod was controlled (12h with 12h light and no light). The temperature (28°C), pH (± 7) and dissolved oxygen (± 8 mg/L) were maintained within physiological limits considered great for the successful development of the specie (Dammski et al., 2011). The experimental protocol was approved by the Ethical and Animal Welfare Committee of the FURG under registration number P002/2014.

Plant species

Aerial parts of the plants *A. triphylla* were obtained from culture in Centro de Educação Superior Norte (CESNORS) - Campus Frederico Westphalen. The plant was collected in June 2010 and the identification was performed by the botanist Dr. Gilberto Dolejal Zanetti from the Department of Industrial Pharmacy, UFSM, and a

voucher specimen (SMDB 11169) was deposited in the herbarium of the Department of Biology.

Essential oil extraction

The *A. triphylla* EO was obtained from fresh plant leaves by hydrodistillation for 3 h using a Clevenger type apparatus European Pharmacopoeia (2007) and was stored at - 20º C in amber glass bottles. The density was approximately 0.9 g mL^{-1}.

The composition of the EO was analyzed using gas chromatography-mass spectrometry (GC-MS). The GC-MS TIC analysis was performed using an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass selective detector under the following conditions: HP-5 MS column (5% phenyl-95% methylsiloxane, 30 m x 0.25 mm x 0.25 µm); EI-MS: 70 e V; operating conditions: split inlet, 1:100; temperature program, 40-260^0 C; 40^0 C for 4 min; ramp rate, 4^{0}C/min; carrier gas, He; flow rate, 1 mL/min; injector and detector temperature, 220^{0}C; interface temperature, 250^{0}C. The constituents of the EO were identified by comparing the mass spectra with a mass spectral library (NIST, 2002) and by comparing the Kovats retention index with data from the literature (Adams, 2001).

Diets and experimental design

The diets were formulated based on the study of LAZZARI et al. (2008), and analysis of its composition showed: protein 41.35% and 7.46% lipid. For the production of control diet was used, soybean meal, meat and bone meal, rice meal, corn meal, canola oil, salt, vitamins and minerals (premix) and dicalcium phosphate. In diets with EO, the same ingredients were used, but with the added concentration of 1 mL/kg of EO (T1) and 2 mL/kg of EO (T2) (Table 1). The fish were divided into three treatments: Control (no added EO of *A. triphylla*), T1 (1 mL/kg of EO) and T2 (2 mL/kg of EO) and were fed three times daily, at 9 am, 1 pm and 4 pm "ad libitum".

The cleaning of the boxes was done 30 minutes after the feeding of juveniles, through the siphoning process for the removal of waste (scraps of food and feces). The volume of water was replenished in the same physical and chemical conditions found in aquariums.

Analysis of growth performance

Biometrics in fish were taken on day zero and every fifteen days for a period of 90 days. In each biometric, fish were anaesthetized using 100 mg/L of trichinae methane sulfonate (MS-222), weighed, measured and photographed.

The ImageJ software was used to measure the length of the fish from the photos. After biometrics each copy returned to its box in the respective treatment.

The specific growth rate was calculated to compare fish growth, defined as the increase in growth during the period analyzed. The condition factor was calculated, relates the weight and length of the fish. The indices were calculated using the following equations:

SGR = 100 x (In final weight – In initial weight)/days of experiment, where: ln= logarithm neperian.

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

Behavioral analysis

Exploratory activity

The horizontal exploratory activity was measured at the end of the experiment.

The fish were placed in an arena measuring 30 x 20 cm with 1L of water, the same utilized in the treatments. The arena was divided into 6 horizontal quadrants and 4 vertical quadrants, each quadrant measuring 5 cm.

Were used 16 fish of each treatment and individually placed in the arena. The fish were habituated for 30 seconds and subsequently filmed over a period of 150 seconds to determine the number of crossings in the squares.

Distance travelled

Filming of the previous experiment were used to analyze the total distance travelled (m). The data were automatically measured at a rate of 30 frames/s, using appropriate video-tracking software (ANY-maze, Stoelting CO, USA).

Analysis of oxygen consumption

The oxygen consumption was measured at the end of the experiment. The fish were fasted for 24 hours before the start of the measurements. Eight males fish of each treatment (control, T1 and T2) were used to avoid the undesirable oxygen consumption by females due to egg production.

The fish were placed individually in glass recipients with 0,07 L the same water of the system, oxygenated until saturation, with temperature of 27° C and habituated for 10 minutes. A recipient without fish was used for the control, and recipients sealed with plastic and cover for 2 minutes, to be opened afterwards to assess the initial oxygenation. All recipients were well sealed for 1 hour in order to prevent the passage of atmospheric oxygen to the aquarium water and then open for the final measure of oxygen consumption.

The oxygen concentration was measured in the water, inside the recipients, with a portable oximeter (DMO - 2, Digimed). This has been calculated during 1 hour by the following equation:

OC = (Io - Fo) x V/W, where: Io is the initial concentration of oxygen (mgO_2/L), Fo is the final concentration of oxygen (mgO_2/L), V is the volume of the tank (L), W is the weight of fish (kg).

Cortisol extraction and analysis

The evaluation of total cortisol was performed at the end of the experiment, no stimulation of stressor, fish were captured and immediately frozen for 60 s, and stored at - 80° C until analysis. Whole-body cortisol was measured in duplicate samples of tissue extract with a commercially available ELISA kit (EIAgen ™ CORTISOL test, BioChemImmunoSystems), according to the methodology described by (OLIVEIRA et al., 2014). From 12 samples (4 samples for each treatment) constituted from a pool of 3 fish.

Statistical Analysis

The results are expressed as the mean ± standard error (S.E) and p-values were considered significant for ($P<0.05$). The Shapiro-Wilk test was performed to

evaluate the homogeneity of the variances. In the analysis of the growth performance comparisons between different treatments were made using repeated measures analysis of variance (ANOVA). The data of oxygen consumption and exploratory activity were analyzed using one-way ANOVA followed by Holm-Sidak test. The data of distance and the total cortisol levels were analyzed using one-way ANOVA, followed by Kruskall-Walls test.

Results

The effects of adding the *A. triphylla* EO in zebrafish diet were observed by reducing oxygen consumption (Figure 2) (P=0.001) and reduced exploratory activity (Figure 3) (P=0.002). However, zebrafish fed diet containing *A. triphylla* EO showed no significant differences in any growth parameters: weight, specific growth rate, condition factor and length (Figure 1). The same result was obtained for the distance travelled (Figure 4) (P= 0.59) and the whole-body cortisol (Figure 5) (P=0.74), when compared with the control group.

Discussion

Oxygen consumption

The metabolism can be defined as the energy necessary to perform the vital functions of an organism, can be measured by oxygen consumption. Several studies found decreased metabolism in fish, after the anesthetic bath, indicated by decreased oxygen consumption. PARK et al. (2009) revealed that the anesthetic lidocaine hydrochloride decreased metabolic activity in winter flounder (*Pleuronectes americanus*), leading to a lower oxygen consumption. Blue cod (*Parapercis colias*) exposed to anesthetics hydrogen sulphide and isoeugenol decreased oxygen consumption (FORGAN & FORSTER, 2010). The same result was obtained in silver catfish (*Rhamdia quelen*) anesthetized with *A. triphylla* EO, that decreased metabolism, demonstrated by the reduction of CO_2 in the water used to transport

(PARODI et al. 2014). In our study, the fish treated with EO decreased oxygen consumption compared to their controls. The reduction of oxygen consumption observed due to anesthetic exposure is likely associated with reduced aerobic ATP production (FORGAN & FORSTER, 2010). The results of the current study indicate that the reduction of oxygen consumption presented by the groups fed diets containing EO, can possibly be explained by the reduction of metabolism, that can be directly related to the anesthetic and sedative effects presents in the *A. triphylla* EO (ZEPPENFELD et al.; 2014b; PARODI et al.; 2012, 2014).

Exploratory activity and Distance travelled

The zebrafish as a model organism for behavioral studies has been widely used in the last decade. Tests as exploratory activity and distance travelled have been performed to analyze the behavior of this species, and often the tests used are adaptations of established protocols in rodents (MAXIMINO et al.; 2010; LEVIN et al.; 2007). Exploratory activity in zebrafish can be defined as a complex set of behaviors directed toward the exploration of new environments, can be measured by quantifying the ratio of its activity in different sections of the area of a tank (KALUEFF et al.; 2013). Exposure to new environments (novelty) has an anxiogenic character in zebrafish and this exhibition increase behavioral responses as motivation to explore an environment (CACHAT, et al.; 2010). The exploratory activity may be related to locomotion and other parameters, such as anxiety behavior type (KALUEFF et al.; 2013). In our study, the fish of the groups treated with EO reduced horizontal exploratory activity. However, the distance of the groups treated with EO was not changed compared to their controls. In another studies, the reduction of exploratory activity suggested to be indicative of reduced anxiety levels.

Zebrafish exposed to nicotine showed reduced exploratory activity (time spent in top and bottom) (LEVIN et al.; 2007). According to Egan et al. (2009) Acute exposure to caffeine produced anxiogenic behavioral responses in zebrafish, shown by the reduction of entries in the upper area. Although the results show that exploratory activity was reduced by exposure to EO, possibly because the existing properties in the EO, future studies are necessary to evaluate the effect of EO with anxiety parameters related in zebrafish.

Growth performance

The zebrafish have recently been proposed as a possible model organism for nutritional genomic studies, aiming to influence somatic growth in finfish (DE-SANTIS & JARRY, 2007; DAHM & GEISLER, 2006). However, it is known that growth is linked to productivity and profitability in aquaculture. According to Moriyama et al. (2000) the growth is under genetic control, but also depends upon nutritional and environmental factors, may also be affected by stress. In recent years, EO have long been used in fish diet to improve the growth, showing did not match results.

In our study, the EO in the diet showed no significant difference in zebrafish growth. The same results were found in studies with rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) fed with diet additives propolis (KASHKOOLI et al.; 2011; UCZAY et al.; 2011). In addition, the *Lippia alba* (*L. alba*) EO, plant belonging to the Verbenaceae (same family as the EO used in this study), added in concentrations (0.25, 0.5, 1.0 and 2.0 mL per kg of EO) in the diet of silver catfish (*Rhamdia quelen*), did not induce significant differences between fish fed the EO (SACCOL et al.; 2013). The present study had longer duration when compared with the period used for (ZEPPENFELD, 2014a) that showing in recent studies silver catfish (*R. quelen*) fed for 60 days with diet containing 2 mL/kg of *A. triphylla* EO presented improvement in weight gain. The same results were obtained by (ZHENG et al*.;* 2009) in catfish (*Ictalurus punctatus*) fed for 56 days with diet containing oregano (*Origanum heracleoticum L*.) EO.

Despite the proven benefits EO and plant extracts in fish, the levels used in this study did not provide evidence of the positive effect of *A. triphylla* EO in zebrafish growth. Thus, it is possible that this result is related to the time of administration of the EO as the species used.

Whole-body cortisol

Practices performed in studies using animal models in laboratories, such as transportation, handling and biometrics often expose the fish to stress factors can affect their performance. A high level of plasma cortisol is a primary indicator of stress in fish (BARTON, 2002) and the stress response is considered an adaptive reaction of extreme importance for the regulation of homeostasis (ASHLEY, 2007).

According to Rhamsay et al. (2006), unstressed zebrafish has cortisol levels ranging from 2.1(fasted control) a 4.7 ng g^{-1} (fed control). For Barcellos et al. (2007) cortisol levels in zebrafish completely isolated from the predator range from 6.78 ng g^{-1}, similar to the value found in the present study (7.5 ng g^{-1}). However, in the until present, its known that is not established baseline cortisol for zebrafish (RHAMSAY et al.; 2006). The difference between cortisol levels on non-stressed fish among these studies may be attributed to different life-stage, rearing temperature, feeding level and in the type, severity, and timing of stressors (Barcellos et al.; 2007). In our study, there was no difference in whole-body cortisol in fish treated with EO added to the diet for 210 days, maybe explained by the fact that the treatments have not previously been challenged to stressors. Despite the long period of use of *A. triphylla* EO in zebrafish diet, this caused no change in basal cortisol levels of fish, and this interesting result, showing that EO was not a stressor to animals.

Conclusion

The *Aloysia triphylla* essential oil is effective in reducing the consumption of oxygen and exploratory activity, its use is indicated in concentrations between 1.0 and 2.0 mL/kg in handling conditions that may affect water quality. However, more studies are needed to confirm the mechanism of action of the *Aloysia triphylla* essential oil in the zebrafish diet.

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Table 1 – Basic formulation (%) of the diet.

Ingredients	(%)
Soybean meal	30
Meat and bone bran	35
Rice bran	12
Corn	15
Canola oil	3
Salt	1
Vitamin and mineral (premix)*	3
Phosphate dicalcium	1

*Vitamin and mineral mixture (security levels per kilogram of product) - folic acid: 250 mg, pantothenic acid: 5.000 mg; antioxidant: 0.60 g, biotin: 125 mg, cobalt: 25 mg copper: 2.000 mg, iron: 820 mg iodine: 100 mg, manganese: 3.750 mg, niacin: 5.000 mg, selenium: 75 mg, vitamin A: 1.000.000 UI vitamin B1: 1.250 mg, vitamin B12: 3.750 mcg, vitamin B2: 2.500 mg, Vitamin B6: 2.485 mg vitamin C: 2.8000 mg, vitamin D3: 500.000 UI, vitamin E: 20.000 UI, vitamin K : 500 mg, zinc: 17.500 mg.

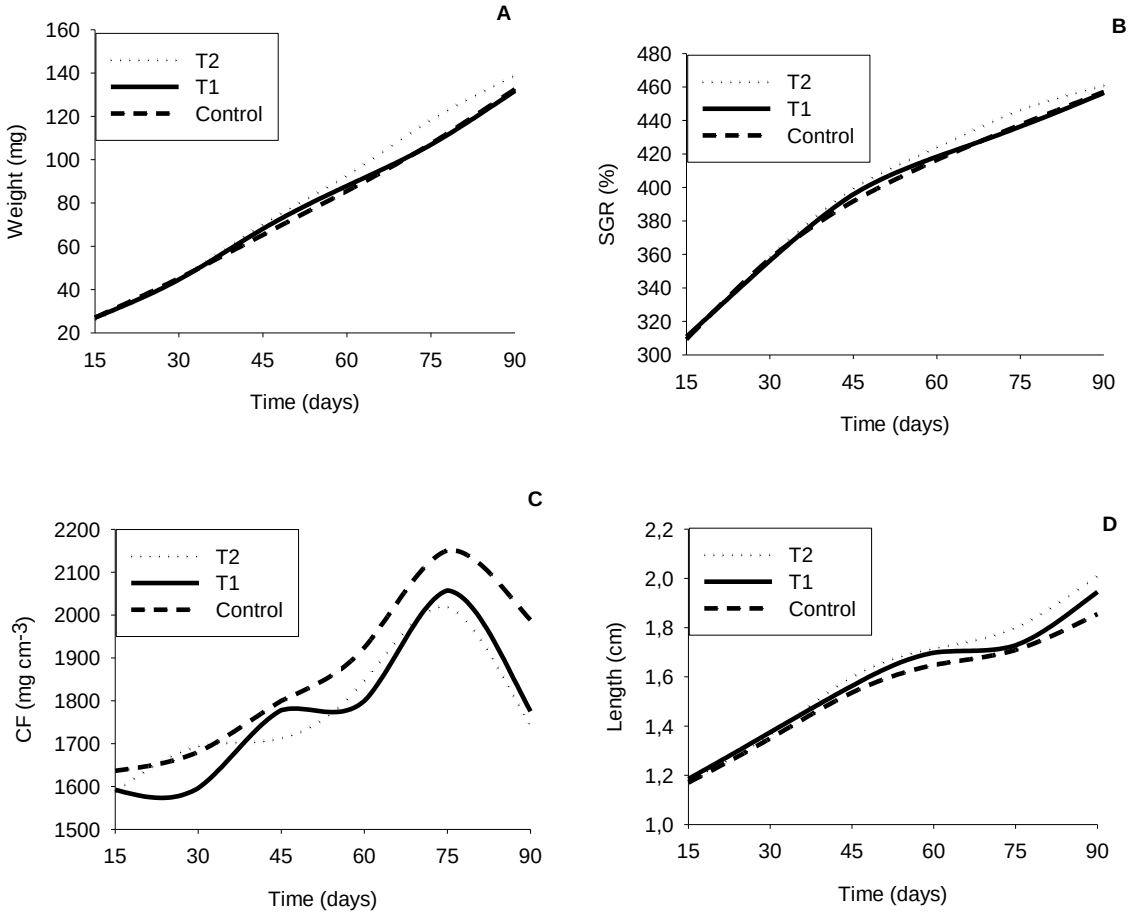

Figure 1 – Growth performance in zebrafish (*Danio rerio*) fed diets containing different concentrations of *A. triphylla* essential oil (EO). A - Weight. B - Specific growth rate (SGR). C - Condition factor (CF). D - Length. Where, control (no addition of OE), T1 (1 mL/kg of OE) and T2 (2 mL/kg of OE). Data were analyzed by repeated measures analysis of variance (ANOVA) ($P<0.05$).

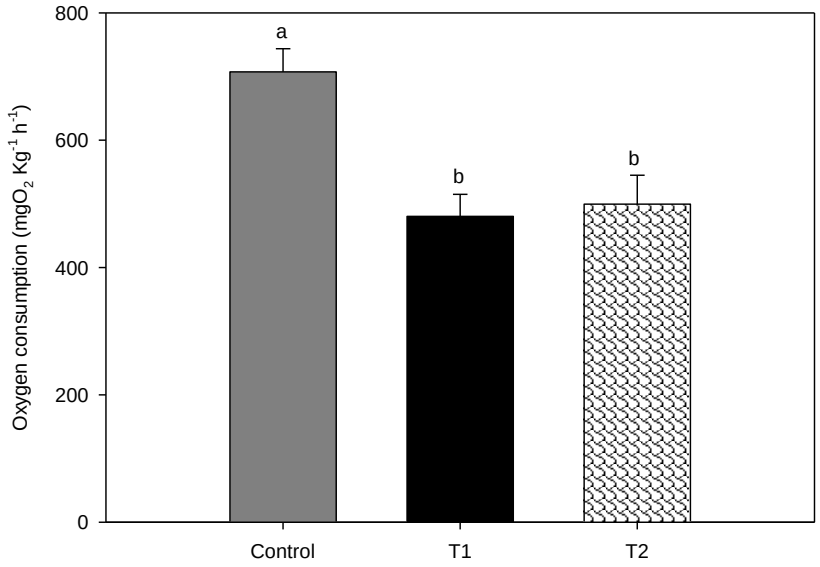

Figure 2 – Oxygen consumption in zebrafish (*Danio rerio*) fed diets containing different concentrations of *A. triphylla* EO. Where, control (no addition of OE), T1 (1 mL/kg of OE) and T2 (2 mL/kg of OE). Values are expressed as mean ± SE (n = 8). Different letters in columns indicate a significant difference between treatments based on one-way ANOVA followed by Holm-Sidak test as post hoc ($P<0.05$).

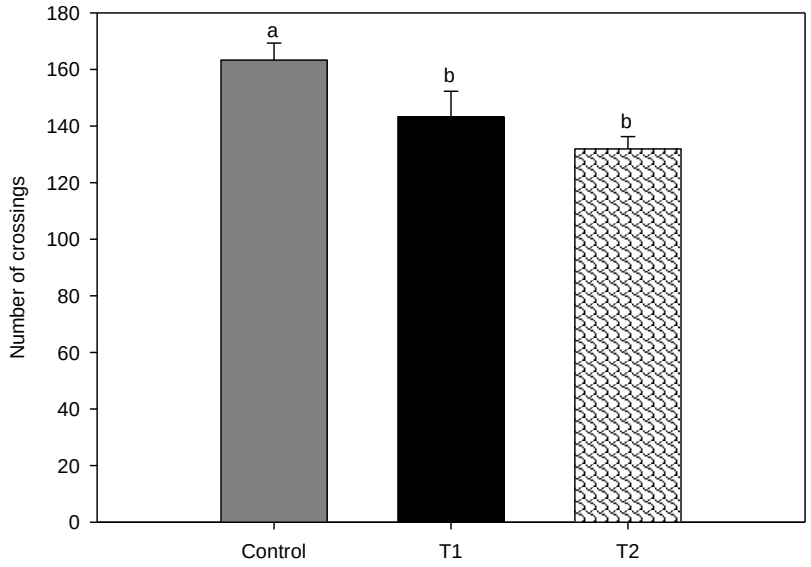

Figure 3 – Exploratory activity in zebrafish (*Danio rerio*) fed diets containing different concentrations of *A. triphylla* EO. Where, control (no addition of OE), T1 (1 mL/kg of OE) and T2 (2 mL/kg of OE). Values are expressed as mean ± SE (n = 16). Different letters in columns indicate a significant difference between treatments based on one-way ANOVA followed by Holm-Sidak test as post hoc ($P<0.05$).

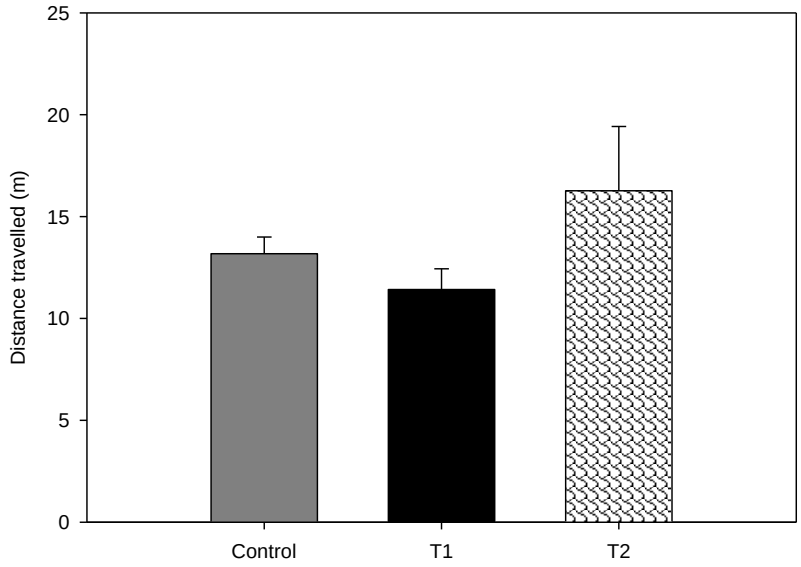

Figure 4 – Total distance travelled in zebrafish (*Danio rerio*) fed diets containing different concentrations of *A. triphylla* EO. Where, control (no addition of OE), T1 (1 mL/kg of OE) and T2 (2 mL/kg of OE). Data were analyzed by one-way ANOVA followed by Kruskall-Walls test ($P<0.05$).

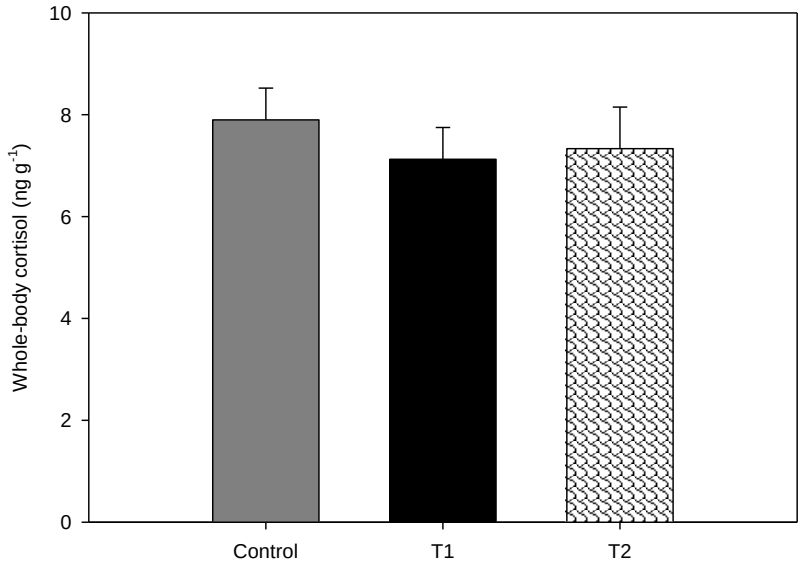

Figure 5 – Levels of whole-body cortisol in zebrafish (*Danio rerio*) fed diets containing different concentrations of *A. triphylla* EO. Where, control (no addition of OE), T1 (1 mL/kg of OE) and T2 (2 mL/kg of OE). Data were analyzed by one-way ANOVA followed by Kruskall-Walls test ($P<0.05$).

CONCLUSÃO GERAL

O óleo essencial de *Aloysia Triphylla* é eficaz na redução do consumo de oxigênio e atividade exploratória, sendo indicado seu uso nas concentrações entre 1.0 e 2.0 mL/kg em condições de manejo que possam afetar a qualidade da água.