ANIMAL SCIENCE

Fatty Acid Incorporation in the Muscle, Oxidative Markers, Lipid Peroxidation and PPAR-α and SREBP-2 Expression of Zebrafish Fed Linseed Oil and Clove Leaf Essential Oil

THIBÉRIO C. DA SILVA, KARINA S. UTSUNOMIYA, PEDRO LUIZ CASTRO, JOANA D'ARC M. ROCHA, JESUI V. VISENTAINER, ELIANE GASPARINO & RICARDO P. RIBEIRO

Abstract: The objective of this study is to assess, in zebrafish, the effects of combining linseed oil (LO) and clove leaf essential oil (CLEO) on the incorporation of fatty acids in the muscle, oxidative markers, lipid peroxidation and expression of the PPAR-α (Peroxisome Proliferator-Activated Receptor-α) and the SREBP-2 (Sterol Regulatory Element Binding Protein-2) genes. Six diets were prepared, containing combinations of LO (3, 6 and 9%) and CLEO (0.5 and 1%): 3% LO + 0.5% CLEO; 3% LO + 1% CLEO; 6% LO + 0.5% CLEO; 6% LO + 1% CLEO; 9% LO + 0.5% CLEO; 9% LO + 1% CLEO. Results showed increase in the incorporation of n-3 fatty acids in the muscle concomitantly with the addition of LO and CLEO. The activities of superoxide dismutase and catalase were reduced and the glutathione content had increased. Lipid peroxidation was lower in the treatment with 1% CLEO, regardless of LO content. The expression of the PPAR-α and the SREBP-2 genes was higher in animals fed 9% LO + 0.5% CLEO. Therefore, for a greater incorporation and protection against the oxidative damages of n-3 fatty acids, a combined use of 9% LO with 0.5% CLEO is recommended for zebrafish.

Key words: Antioxidant, α-linolenic acid, *Danio rerio*, eugenol, vegetable oil.

INTRODUCTION

Linseed oil stands out from other vegetable oils for being one of the richest sources of α-linolenic acid, a precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are physiologically important, with known immune functions (Nayak et al. 2017, Popa et al. 2012).

However, for a greater incorporation of these fatty acids in the muscle tissue, an antioxidant protection is required, as excessive amounts in the diet can increase the rate of lipid unsaturation in the tissues of fish and make them prone to attacks by free radicals (ROS), generated as by-products of normal cellular oxygen metabolism or by external tensions (Kiron et al. 1995). These ROS, such as hydrogen peroxide, superoxide and hydroxyl, can attack the phospholipid membrane of cells, react with cellular proteins and nucleic acids and damage these cells, causing immunosuppression (Sotoudeh et al. 2015).

The organism, to protect cells from damage, has developed protective mechanisms against the action of antioxidant enzymes, such as catalase (CAT), glutathione-peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR) and superoxide dismutase (SOD) (Halliwell & Gutteridge 2007). When the ROS production rate is greater than the elimination

capacity of the defense system, an exogenous antioxidant becomes necessary, which may come from diets. In this regard, clove oil presents the greatest antioxidant capacity among commonly marketed essential oils (Teixeira et al. 2013).

The main component of clove leaf oil is eugenol, to which many antioxidant properties are attributed. Gülçin et al. (2012) showed that clove oil inhibited 97.3% of lipid peroxidation in linoleic acid emulsion at a concentration of 15 μg/mL. The protective effect of 0.5% clove oil in the diet was reported in rohu carp fingerlings (*Labeo rohita*) through a reduction in SOD activity and in lipid peroxidation levels (Asimi & Sahu 2016).

In the present study, zebrafish was used to assess the effects of combining linseed oil as a source of α-linolenic acid, and clover leaf essential oil as a natural antioxidant, on the incorporation of fatty acids in the muscle, oxidative markers, lipid peroxidation, and expression of the PPAR-α and SREBP-2 genes.

MATERIALS AND METHODS

The experiment was run in the Ornamental Fish Laboratory of the PeixeGEN Research Center – Management, Enhancement and Molecular Genetics in Freshwater Fish Farming, at the State University of Maringá [*Universidade Estadual de Maringá*] – UEM. This project was approved by the Ethics Committee on Animal Use (CEUA) of said university, under protocol No. 8851180216.

Preparation of experimental diets and food management

Six experimental diets were prepared in accordance with the nutritional recommendations proposed by Siccardi et al. (2009), containing the following levels of inclusion for linseed oil (LO) and clove leaf essential oil (CLEO): Diet 1: 3% LO + 0.5% CLEO; Diet 2: 3% LO + 1% CLEO; Diet 3: 6%

LO + 0.5% CLEO; Diet 4: 6% LO + 1% CLEO; Diet 5: 9% LO + 0.5% CLEO; Diet 6: 9% LO + 1% CLEO.

The experimental design was completely randomized with three repetitions and six treatments. Diet composition and fatty acid profile are displayed in Tables I and II, and determined in accordance with the AOAC (2005) and Figueiredo et al. (2016).

Corn oil was used to keep the diets isoenergetic. The ingredients were ground in a hammer mill with a sieve measuring 0.3 mm in diameter. The feed was processed in an extruded manner (Ex-Micro® extruder with 1.0 mm in diameter). The fish were fed four times a day (8:00, 11:00, 14:00 and 17:00) until apparent satiety.

Fish and experimental conditions

This experiment used 360 male zebrafish (*D. rerio*) aged 50 days after hatching, with an average weight of 0.29 ± 0.04 g and average total length of 30.67 ± 0.71 mm. The animals were distributed in 18 glass tanks with a useful volume of 50 liters (20 animals per tank), individually equipped with an internal filter, a 50 w thermostat and constant aeration by means of a central air blower.

The values for average water temperature, pH and dissolved oxygen were 26.23 ± 0.54 °C, 7.3 ± 0.01, and 6.6 ± 0.32 mg/L, which are within the comfortable range for the species (Westerfield 2007). At the end of the experiment (55 days), the fish were euthanized (methanesulfonate (MS-222, tricaine), at 250 mg/L for 10 minutes) to have their weight (g) and length (mm) taken. The animals were then dissected to have their livers removed, which were used to analyze gene expression (five livers from each experimental unit) and antioxidant markers and lipid peroxidation (five livers from each experimental unit). The samples were immediately stored in liquid nitrogen, then transferred to a freezer at -80 °C.

Table I. Percentage composition of the experimental diets.

Ingredients		Experimental diets						
		Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
Soy protein isolate		50.90	50.90	50.90	50.90	50.90	50.90	50.90
Corn		20.00	20.00	20.00	20.00	20.00	20.00	20.00
Corn gluten		7.06	7.06	7.06	7.06	7.06	7.06	7.06
Corn oil		10.00	6.50	6.00	3.50	3.00	0.50	0.00
LO		0.00	3.00	3.00	6.00	6.00	9.00	9.00
CLEO		0.00	0.50	1.00	0.50	1.00	0.50	1.00
Broken rice		5.00	5.00	5.00	5.00	5.00	5.00	5.00
Dicalcium phosphate		3.63	3.63	3.63	3.63	3.63	3.63	3.63
Lysine		1.14	1.14	1.14	1.14	1.14	1.14	1.14
[1]Mineral and vitamin supplement		1.00	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine		0.43	0.43	0.43	0.43	0.43	0.43	0.43
Calcitic limestone		0.41	0.41	0.41	0.41	0.41	0.41	0.41
Common salt		0.30	0.30	0.30	0.30	0.30	0.30	0.30
L-Tryptophan		0.09	0.09	0.09	0.09	0.09	0.09	0.09
Centesimal composition								
Crude protein		51.82	51.26	51.15	50.97	51.06	50.85	51.47
Energy (kj/g)		17.16	17.28	17.57	17.23	17.41	17.68	17.13
Ethereal extract		11.26	11.09	11.52	11.63	11.06	11.81	11.32
Calcium		1.10	1.26	1.03	1.16	1.15	1.32	1.01
Total phosphorus		1.33	1.14	1.29	1.22	1.17	1.55	1.10

[1]Guaranteed levels per kilo of product: vit. A - 500,000 IU; vit. D3 - 200,000 IU; vit. E - 5,000 mg; vit. K3 - 1,000 mg; vit. B1 - 1,500 mg; vit. B2 - 1,500 mg; vit. B6 - 1,500 mg; vit. B12 - 4,000 mg; Folic acid - 500 mg; calcium pantothenate - 4,000 mg; biotin - 50 mg; inositol - 10,000; nicotinamide - 7,000; choline - 40,000 mg; cobalt - 10 mg; copper - 500 mg; iron - 5,000 mg; iodine - 50 mg; manganese - 1,500 mg; selenium - 10 mg; zinc - 5,000 mg.

Determination of fatty acids and muscle in the zebrafish

The incorporation of fatty acids in the muscle was determined in accordance with Figueiredo et al. (2016).

Assessment of antioxidant activity and lipid peroxidation

Zebrafish were euthanized and the livers were removed immediately and freeze-clamped in liquid nitrogen. Tissue samples were homogenized in a van Potter homogenizer with 10 volumes of icecold 0.1M potassium phosphate buffer (pH 7.4). The protein content in the total homogenate was determined as described by Lowry et al. (1951).

Lipid peroxidation was evaluated in liver homogenates by thiobarbituric acid reactive substances (TBARS), predominantly malondialdehyde (MDA) (Ohkawa et al. 1979). An aliquot of liver homogenate (50 mg protein) was added to 4 ml of a solution containing 0.4% SDS, 7.5% acetic acid and 0.25% TBA. After 1 h incubation at 95 °C, the MDA-TBA complex was extracted with 1 mL n-butanol/pyridine 15:1 (v/v) and the absorbance was determined

Table II. Fatty acid profile of the experimental diets (% of total fatty acids).

Fatty acids	Experimental diets						
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7
16:00	11.56	10.81	11.01	9.29	9.80	7.95	8.57
18:00	4.87	5.51	5.14	5.08	5.46	5.40	5.45
20:00	0.54	0.54	0.46	0.42	0.43	0.44	0.38
22:00	0.63	0.59	0.51	0.44	0.45	0.43	0.31
24:00	0.26	0.26	0.28	0.22	0.22	0.23	0.24
18:1n-9	28.31	31.11	26.40	29.78	31.06	31.36	27.95
18:1n-7	1.49	1.42	1.05	1.15	1.13	1.12	0.69
20:1n-9	0.33	0.34	0.24	0.27	0.29	0.28	0.20
18:2n-6	46.58	37.69	36.67	30.17	29.44	27.80	19.44
18:3n-3	5.43	11.71	12.89	23.17	21.69	24.99	27.65
[1]ΣSat	17.85	17.72	17.40	15.46	16.37	14.45	14.95
[2]ΣMon	30.13	32.87	27.69	31.21	32.48	32.76	28.83
[3]ΣPol	52.01	49.41	49.55	53.34	51.13	52.79	47.09
n6/n3	8.57	3.22	2.85	1.30	1.36	1.11	0.70

[1]**Sum of saturated fatty acids; [2]Sum of monosaturated fatty acids, and [3]Sum of polyunsaturated acids.**

at 532 nm. The amount of lipoperoxides was calculated from the standard curve prepared with 1,1',3,3'-tetraethoxypropane, and the values were expressed as nmol MDA/mg protein ($\varepsilon = 1.56 \times 10^5$ M^{-1} × cm^{-1}).

The GSH content was measured in liver homogenates using o-phthalaldehyde (OPT) (Hissin & Hilf 1976). The samples were added to a medium containing 0.1 M phosphate buffer and 5.0 mM EDTA (pH 8.0). The reaction was started by adding 100 μL of OPT solution (1 mg/mL, in methanol). The fluorescent product GSH-OPT was measured fluorometrically (350 nm excitation and 420 nm emission) after an incubation period of 15 min at room temperature. The results were expressed as μg GSH/mg protein.

The antioxidant enzymatic activities were assessed in the homogenate supernatant. Superoxide dismutase (SOD) activity was estimated by its capacity to inhibit pyrogallol autoxidation in alkaline medium at 420 nm

(Marklund & Marklund 1974). The amount sufficient to inhibit the enzyme reaction by 50% (IC$_{50}$) was defined as 1 unit of SOD, and the results were expressed as U SOD/mg protein. Catalase (CAT) activity was estimated by measuring the change in absorbance at 240 nm using H$_2$O$_2$ as substrate and expressed as H$_2$O$_2$ consumed/min × mg protein ($\varepsilon = 33.33$ M^{-1} × cm^{-1}) (Aebi 1984). Glutathione peroxidase (GPx) activity was estimated by measuring the change in absorbance at 340 nm due to NADPH consumption in the presence of H$_2$O$_2$, GSH and glutathione reductase and expressed as nmol of NADPH oxidized/min × mg protein ($\varepsilon = 6220$ M^{-1} × cm^{-1}) (Paglia & Valentine 1967).

Gene expression analysis

Total RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad CA, USA), in accordance with the manufacturer's standards. To assess total RNA concentration, the samples

were measured with the aid of a PICODROP® spectrophotometer (PicodropLimited, Hinxton, United Kingdom). RNA integrity was evaluated in 1% agarose gel stained with SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad CA, USA) and visualized in a transilluminator with ultraviolet light.

The RNA samples were treated with DNase I (Invitrogen, Carlsbad, CA, USA) for removal of possible residues of genomic DNA, in accordance with the manufacturer's recommendations. Complementary DNA was made using the SuperScrippt TM III First-StrandSyntesisSuper Mix kit (Invitrogen, Carlsbad CA, USA), in accordance with the manufacturer's standards. In a sterile and RNA-free tube, 6 µl of total RNA, 1 µL of oligo (dT) (50 µ Moligo (dT) 20, and 1 µL of annealing buffer were added. The reaction was incubated for 5 minutes at 65 ºC, then put on ice for 1 minute. Afterwards, 10 µL of 2x First-StrandReaction Mix solution and 2 µL of a solution containing the SuperScript III reverse transcriptase enzyme and RNAse inhibitor were added. The solution was incubated for 50 minutes at 50 ºC and, then, for 5 minutes at 85 ºC, being immediately put on ice. The samples were stored at -20 ºC until the moment of use.

For real-time PCR (qRT-PCR) to be assessed, the primers of the SREBP-2 (Sterol regulatory element-binding protein 2) and the PPAR-α (Peroxisome proliferator-activated receptor-α) genes were designed in accordance with the sequences and uploaded to www.ncbi.nlm.nih. gov for zebrafish (*D. rerio*) via www.idtdna.com (Table III). For endogenous control, the ß-actin gene was used.

For the qRT-PCR reactions, a SYBR® GREEN PCR Master Mix fluorescent dye (AppliedBiosystems USA) was used, in accordance with the manufacturer's recommendations. All analyses were carried out in a volume of 25 µL and in duplicates. The reactions were conducted in strips in a StepOne Plus device. Primer and cDNA concentrations were determined through efficiency tests, using three primer concentrations (100, 200 and 400 nM) and four cDNA concentrations (10, 100, 200 and 400 ng). The 2-ΔCT method was used for relative quantification analyses, with data being expressed in arbitrary unit (AU).

Statistical analysis

The results were expressed as mean ± sd. The data were analyzed on Statistical Analysis Software 9.3 (SAS 2011) and subjected to factorial analysis of variance to allow determining the interaction between the LO and CLEO factors (LOxCLEO) or the LO and CLEO isolated factors. The data were also checked for homogeneity of variances and normality by Levene's test and the Kolmogorov-Smirnov test, respectively. The

Table III. Primers used in this study.

Primer	Sequence	Amplicon (pb)	Access number
SREBP-2	F:GATGCTGGTATTGGTGGTTTATG R:AGTGTCTGTGTGTGAGAGATTG	149	NM_001089466.1
PPAR-α	F:GAACCGAAACAAGTGCCAATAC R:GGATCTCTGCCTTCAACCTTAG	122	NM_001102567.1
β-actin	F: CAAACGAACGACCAACCTAAAC R:TACCTCCCTTTGCCAGTTTC	108	NM_131031.1

results were compared through Tukey's test, and P values <0.05 were considered significant.

RESULTS

Incorporation of Fatty Acids in Zebrafish Muscle

Table IV shows the isolated effects of fatty acids analyzed in the muscle of zebrafish. Saturated fatty acids did not differ between treatments (P>0.05). The monounsaturated, arachidonic (ARA, 20:4n-6) and n-6 acids showed isolated effect for the LO factor (P<0.05) (Table IV), with the content of monosaturated fatty acid increasing with ascending LO levels, whereas ARA and n-6 content reduced. For the n-6/n-3 ratio, there was isolated effect for the LO and CLEO factors (P <0.05), indicating a decrease in their results.

Table V shows the interaction effects (P<0.05), with linoleic acid (18:2n-6) eicosadienoic acid (20:2n-6) and dihomo-gamma-linolenic acid

(20:3n-6) presenting a reduction in their content with increasing LO and CLEO levels in the diet, whereas the 9% LO% + 1% CLEO diet resulted in lower means.

The amount of α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosaexanoic acid (DHA, 22:6n-3) increased concomitantly with the combined levels of inclusion of LO and CLEO (P <0.05). Consequently, the increase in n-3 in the zebrafish muscle occurred together with the addition of LO and CLEO, and higher means were found in the diet with greater inclusion levels of both oils used (9% LO + 1% CLEO) (Table V).

Assessment of antioxidant activity and peroxidation level

SOD activity showed isolated effect for both factors (P <0.05), while CAT showed isolated effect only for LO (P<0.05). Both SOD and CAT showed a decrease in their activity. TBARS showed isolated effect for CLEO (P<0.05), with

Table IV. Isolated effect on the fatty acid profile (% of the total) in the muscle of zebrafish (*Danio rerio*) fed diets containing linseed oil (LO) and clove leaf essential oil (CLEO).

Components	Fatty acid				
	[1]ΣSat	[2]ΣMono	[3]ARA	n-6	n-6/n-3
Effects					
LO	0.293	0.018	<0.001	<0.001	<0.001[*]
CLEO	0.104	0.338	0.550	0.729	<0.001[*]
LOxCLEO	0.534	0.06	0.229	0.167	0.289
Factors					
LO					
3%	27.74±1.39	36.39±1.28 b	2.35±0.20 a	27.71±1.03 a	3.37±0.13 a
6%	27.61±1.31	38.31±1.54 a	1.72±0.24 b	22.46±0.61 b	1.93±0.09 b
9%	26.94±1.18	39.26±1.37 a	1.43±0.13 c	18.64±0.65 c	1.21±0.05 c
CLEO					
0.5%	28.12±0.99	38.28±1.05	1.86±0.47	22.75±3.22	2.29±0.33 a
1%	26.74±0.81	37.72±1.11	1.80±0.42	22.93±4.58	2.05±0.30 b

[1]ΣSat: Sum of saturated fatty acids. [2]ΣMon: Sum of monounsaturated fatty acids. [3]Arachidonic acid (20:4n-6). Data are presented as mean ± standard deviation. *Values followed by different letters in the column are statistically different (P<0.05).

decreased concentration as a function of CLEO levels. GSH content showed interaction effect (P <0.05), and a reduction was found in their content with 0.5% CLEO associated with 6 and 9% LO in the diets (Table VI).

Gene expression analysis

The assessed genes showed interaction effect between factors (P<0.05) (Table VII), with PPAR-α and SREBP-2 presenting similar behaviors, and with the groups of fish fed diet containing 9 and 0.5% LO and CLEO showing means higher than those of the other treatments.

DISCUSSION

The profile of the fatty acids in the zebrafish muscle was clearly affected by the lipid composition of the experimental diets. This observation is consistent with the general concept that the composition of fatty acids in fish tissue is largely a reflection of diets (Benítez-Dorta et al. 2013, Sotoudeh et al. 2015).

The increased content of monounsaturated fatty acids can be explained by the increase in the diet, considering that LO is usually composed of about 18% of oleic acid (Ganorkar & Jain 2013), in addition to corn oil contributing to keeping the levels of the monounsaturated acids, though at low LO replacement rates. In a study conducted by Araújo et al. (2016), the inclusion of 9% LO and corn oil in diets for zebrafish did not cause significant differences (P >0.05) in the composition of monounsaturated acid in the carcasses of the animals.

The muscular content of linoleic acid (18:2n-6) decreased in response to the lipid composition of the diets and as a precursor to the n-6

Table V. Effect of the experimental diets on the profile of fatty acids (% of the total) in the zebrafish muscle (*Danio rerio*) fed a diet containing linseed oil (LO) and clove leaf essential oil (CLEO).

Components	Fatty acid composition						
	18:2n-6	20:2n-6	20:3n-6	18:3n-3	20:5n-3	22:6n-3	n-3
Effects							
LO	<0.001	<0.001	<0.001	<0.001	<0.001	0.005	<0.001
CLEO	0.09	<0.001	<0.001	<0.001	<0.001	0.014	<0.001
LOxCLEO	<0.001	<0.001	<0.001	<0.001	<0.008	0.013	<0.014
Outcome							
3%LO+0.5%CLEO	22.30±0.51 a	0.29±<0.01 a	1.52±0.07 a	4.10±0.06 e	0.31±0.05 e	3.04±0.56 b	7.45±0.67 e
3%LO+1%CLEO	21.97±2.06 a	0.20±0.02 bc	1.38±0.11 c	5.20±0.11 d	0.42±0.02 d	3.31±0.09 b	8.94±0.21 d
6%LO+0.5%CLEO	19.13±0.76 b	0.22±<0.01 c	1.23±0.03 af	11.42±0.31e	0.58±0.03 c	3.66±0.29 b	11.42±0.31 c
6%LO+1%CLEO	19.35±0.14 b	0.21±<0.01 c	1.14±0.07df	7.17±0.58 c	0.60±0.02 bc	3.53±0.09 b	11.83±0.08 c
9%LO+0.5%CLEO	15.32±0.20 c	0.21±0.01 bc	1.30±0.06 b	9.96±0.43 b	0.69±0.06 b	3.33±0.18 ab	13.97±0.67 b
9%LO+1%CLEO	15.56±0.55 c	0.18±<0.01 c	0.87±0.02 e	11.17±0.61 a	0.87±0.02 a	4.31±0.01 a	16.35±0.59 a

Data are presented as mean ± standard deviation. Values followed by different letters in the column are significantly different (P<0.05).

series; consequently, there was a decrease in the eicosadienoic (20:2n-6), linolenic dihomo-gamma (20:3n-6) and arachidonic (ARA, 20:4n-6) acids. The inclusion of increasing levels of LO and, therefore, gradual amounts of α-linolenic acid (18:3n-3) in the diet resulted in an increase in 18:3n-3 in the muscle and its long-chain counterparts, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexanoic (DHA, 20:4n-3).

In a study carried out by Tocher et al. (2001), a diet formulated to supply 1% of α-linolenic and linoleic acid for zebrafish and tilapia, the same pattern was observed for desaturation and elongation of fatty acids. In general, freshwater fish have the ability to elongate and desaturate

n-3 and n-6 fatty acids (C18) for their respective long-chain counterparts (C20 and C22); however, accumulation in the tissue depends on the quantity supplied in the food (Liu et al. 2013, Qiu et al. 2017).

Unlike DHA, there was low accumulation of EPA in the zebrafish muscle. Fatty acids, regardless of chain size or number of unsaturations, are important sources of energy, but DHA tends to be preserved in the phospholipid bilayer of membranes, playing structural and functional roles (Tocher 2010). The present study, based on the results found, supports the hypothesis that EPA is probably directed to meet different metabolic needs, such as conversion to DHA,

Table VI. Antioxidant and lipid peroxidation markers (TBARS) assessed in zebrafish (*Danio rerio*) fed experimental diets containing linseed oil (LO) and clove leaf essential oil (CLEO).

Components	Antioxidant markers			
	[1]SOD	[2]CAT	[3]TBARS	[4]GSH
Effect				
LO	0.004	0.021	0.446	0.370
CLEO	<.001	0.228	0.043	<.001
LOxCLEO	0.089	0.936	0.803	0.034
Outcome				
LO				
3%	1.13±0.32 a	3.47±0.99 a	2.16±0.61	-
6%	0.92±0.47 ab	3.59±1.11 a	1.79±0.55	-
9%	0.64±0.25 b	1.90±1.42 b	1.90±0.27	-
CLEO (%)				
0.5%	1.51±0.33 a	3.21±1.34	2.26±0.51 a	-
1.0%	0.63±0.28 b	2.88±1.02	1.89±0.63 b	-
LO+CLEO				
3%LO+0.5%CLEO	-	-	-	2.88+011 c
3%LO+1%CLEO	-	-	-	3.22+0.31 abc
6%LO+0.5%CLEO	-	-	-	2.53+0.14 c
6%LO+ 1%CLEO	-	-	-	3.94+0.34 ab
9%LO+0.5%CLEO	-	-	-	2.47+0.35 c
9%LO+1%CLEO	-	-	-	4.46+0.31 a

[1]Superoxide dismutase (U of SOD mg^{-1}). [2]Catalase (ε, 33.33 M^{-1} × cm^{-1}). [3]Tiobarbituric acid reactive substances (MDA mg^{-1} of protein). [4]Glutathione (*µ*g GSH mg-1). Data are presented as mean ± *standard* deviation. Values followed by different letters in the column are statistically different (P<0.05).

synthesis of eicosanoids and energy production through β-oxidation, causing its reduction in tissues (Glencross et al. 2014, Rosenlund et al. 2016, Thomassen et al. 2012).

The CLEO supplied in the diet contributed to a greater retention of fatty acids, playing its role as an antioxidant for having mainly eugenol (81 to 86%) in its chemical composition, in addition to β-karyophylene (17.4%) and α-humulene (2.1%), protecting them against the action of free radicals and improving the assimilation of these lipids (Jirovetz et al. 2006, Sohilait 2015). It was proven in the study by Sotoudeh et al. (2015) that antioxidants from diets can lead to this result; in common trout (*Salmo trutta caspius*), for instance, α-tocopherol promoted the protection of EPA and DHA against oxidation in the cell membrane, enabling greater incorporation.

In general, wild freshwater fish are characterized by a 5 to 6:1 ratio of n-6/n-3 (Dabrowski & Portela 2006). As in the present study, Tonial et al. (2009) also found a reduction in the n-6/n-3 ratio (1:1) for Nile tilapia fed a diet containing 7% linseed oil compared to the group fed 7% soybean oil, in addition to a significant difference (P <0.05) in growth improvement. Research aimed at lowering this ratio to increase the nutritional value of food, without harming health and, consequently, to improve the productive performance of animals, is important for aquaculture.

TBARS level is used to measure the extent of lipid peroxidation (Bartoskova et al. 2014). In the present study, CLEO was responsible for reducing lipid peroxidation. This result can be explained by the presence of eugenol, the active ingredient of CLEO and a phenolic compound that acts as an antioxidant, sequestering hydroxyl (OH⁻) reactive species, and responsible for causing the oxidation of polyunsaturated fatty acids and, consequently, inhibiting the

Table VII. Expression of the PPAR-α and SREBP-2 genes assessed in zebrafish (*Danio rerio*) fed experimental diets containing linseed oil (LO) and clove leaf essential oil (CLEO).

Components	Genes	
	[1]PPAR-α	[2]SREBP-2
Effect		
LO	0.089	<0.001
CLEO	0.151	<0.001
LOxCLEO	0.010	<0.001
Outcome		
3%LO+0.5%CLEO	0.13±0.01 b	0.11±0.01 b
3%LO+1%CLEO	0.61±0.27 ab	0.20±0.13 b
6%LO+0.5%CLEO	0.32±0.24 ab	0.19±0.18 b
6%LO+ 1%CLEO	0.21±0.01 b	0.50±0.01 b
9%LO+0.5%CLEO	1.12±0.27 a	2.89±0.17 a
9%LO+1%CLEO	0.18 ±0.10 b	0.24±0.14 b

[1]Peroxisome proliferator-activated receptor alpha (PPAR-α). [2]Sterol regulatory element-binding proteins 2 (SREBP-2). Data are presented as mean ± standard deviation. Values followed by different uppercase letters in the column are statistically different (P<0.05).

chain reaction (Pereira & Maia, 2007, Lima & Bezerra 2012).

Moreover, a decrease in the enzymatic activities of SOD, CAT and GSH confirms the antioxidant property of CLEO, reducing the substrate of action of these enzymes, which, consequently led to a decrease in their activities (Elia et al. 2006, Hou et al. 2015). This result is confirmed in a study carried out with *Rhamdia quelen* fish (Azambuja et al. 2011), mice (Sheweita et al. 2016) and *Macrobrachium rosenbergii* shrimp (Cagol et al. 2020), in which the use of essential oils with antioxidant properties reduced the action of these enzymes.

Polyunsaturated fatty acids, especially those of the n-3 series, are natural PPAR-α activators (Michalik et al. 2006, Kamalam et al. 2013). As expected, this study found an increase in PPAR-α expression in the zebrafish liver; this happened as a consequence of diets containing

increasing amounts of α-linolenic acid, and of a better protection against lipid peroxidation at the level of 0.5% CLEO.

The result of this study corroborates the assay carried out by Li et al. (2015) to assess the effects of n-3 series fatty acids on lipid metabolism in grass carp (*Ctenopharyn godonidellus*), which reports increased PPAR-α expression in the liver and muscle of fish fed a diet containing a greater quantity of alpha-linolenic acid, EPA and DHA. Jin et al. (2017) also confirmed an increase in PPAR-α when the dietary content of n-3 rose from 0.23% to 1.29% in diets for juvenile snapper (*Acanthopagrus schlegelii*). In rainbow trout (*Oncorhynchus mykiss*), there was greater activation in fish fed LO in their diet compared to soybean oil and fish oil, which have a lower amount of alpha-linolenic acid (Dong et al. 2017).

PPAR-α is known to play a critical role in regulating lipid homeostasis (Yessoufou et al. 2009), activating lipid catabolism by regulating the expression of target genes that encode enzymes involved in peroxisomal and mitochondrial β-oxidation (Walczak & Tontonoz 2002, Lu et al. 2014). A higher PPAR-α expression is also associated with decreased lipid deposition in the hepatocyte (Yoon 2009). In the research by Lu et al. (2014), carried out with Wuchang bream (*Megalobrama amblycephala*), a reduction in PPAR-α led to an accumulation of fat in the hepatocyte, followed by nuclear atrophy, characterizing steatosis, with the fish showing reduced growth as well. The opposite result has been reported in fish with greater PPAR-α expression and greater β-oxidation.

As for SREBP-2, its main function is to control cholesterol biosynthesis (Fonseca-Alaniz et al. 2006). In particular, SREBP-2 is a transcription factor that resides in the endoplasmic reticulum, which has an activity dependent on cholesterol and, therefore, is deeply involved in the regulation of the expression of genes related to its metabolism (Sato 2010). The increase in SREBP-2 expression in the present study can be explained by the fact that vegetable oils and diets have a cholesterol deficiency (Phillips et al. 2002). In addition, other sterols such as sitosterol, stigmasterol, campesterol and brassicasterol are present in said oils, which can inhibit the absorption of cholesterol and are unlikely to be absorbed by fish (Ostlund 2004, Tocher et al. 2008).

In a study conducted by Castro et al. (2016), they observed that cholesterol concentration was lower in fish fed vegetable oil compared to the group treated with fish oil. Leaver et al. (2008) assessed the replacement of fish oil with vegetable oils (canola, soy and linseed) in diets for salmon (*Salmo salar*). Their findings indicate an increase in SREBP-2 expression in all diets with vegetable oil, being therefore an important regulator of cholesterol levels.

CONCLUSION

There was a greater incorporation of n-3 fatty acids, as well as reduced lipid peroxidation and antioxidant activity. The expression analysis pointed to an increase in β-oxidation, which is directly related to a reduction in liver fat accumulation and a decrease in cholesterol synthesis. Therefore, a combination of 9% linseed oil with 0.5% clove leaf essential oil is recommended.

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THIBÉRIO C. DA SILVA[1]
https://orcid.org/0000-0002-9725-0887

KARINA S. UTSUNOMIYA[2]
https://orcid.org/0000-0001-5373-6466

PEDRO LUIZ CASTRO[2]
https://orcid.org/0000-0001-7351-6938

JOANA D'ARC M. ROCHA[1]
https://orcid.org/0000-0002-7131-2349

JESUI V. VISENTAINER[2]
https://orcid.org/0000-0003-3412-897X

ELIANE GASPARINO[2]
https://orcid.org/0000-0002-2611-9259

RICARDO P. RIBEIRO[2]
https://orcid.org/0000-0002-4781-375X

[1]Universidade do Estado do Amapá, Av. Presidente Vargas, 650, 68900-070 Macapá, AP, Brazil

[2]Universidade Estadual de Maringá, Departamento de Zootécnia, Av. Colombo, 5790, Zone 7, 87020-900 Maringá, PR, Brazil

Correspondence to: **Thibério Carvalho da Silva**
E-mail: thiberiocs@hotmail.com

Author contributions

Thiberio Carvalho da Silva execute an experiment, analyzed the data and wrote the manuscript. Karina Sayuri Utsunomiya and Eliane Gasparino were responsible for carrying out the analysis of the antioxidant activity and lipid peroxidation. Jesui Vergilio Visentainer performed the fatty acid analysis. Joana D'Arc Mauricio Rocha to analyze the data and review the manuscript. Pedro Luiz Castro and Ricardo Pereira Ribeiro contributed to the analysis of gene expression. All authors discussed the results and approved the final version of the manuscript.