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Oxidized fish oil in the diet negatively affect rearing performance, health, and tissue fatty acid composition of juvenile spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869)

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Abstract
Lipids are essential in feeds for rearing marine fish. Inadequate handling and storage of feeds can cause lipid oxidation, increasing toxic compounds while decreasing the nutrition quality of feeds and negatively affecting both the physiology and rearing performance of fish. Here, the effect of increasing oxidized lipids intake was assessed on rearing performance and health of juvenile *L. guttatus*. Experimental feeds (EF) were manufactured with different levels of total peroxides (PV, 26–107 $meqO_2 \times kg^{-1}$) and anisidine value (AV, 74–154). A total of 270 fish (18 fish by tank, three replicates by treatment) with an initial average weight of 6.5 ± 1 g were reared in 3 m^3 tanks for 45 days. Survival and growth rate decreased with increasing PV and AV, particularly in fish fed with higher values of oxidized lipids. Viscerosomatic index was positively correlated with increase of oxidized lipids in EF, probably caused by increasing food retention into the gut. Glucose in plasma was inversely correlated with PV and AV in EF and together with the damage observed (i.e., inflammatory nodules, necrosis, and higher hepatosomatic index) suggests a decrease of liver metabolism. The increases of PV and AV on feeds were positively correlated with levels of triacylglycerides and total cholesterol in plasma, indicating an alteration in lipid metabolism. The total protein content in plasma and hematocrit was not different among treatments; in contrast, hemoglobin content decreased compared with control, suggesting an increase in the fragility of blood cells and oxygen transport capacity. Highly unsaturated fatty acid (HUFA) composition in the brain and liver was not different between treatments, but PV and AV were inversely correlated with docosahexaenoic acid (DHA) levels in neutral lipids and eicosapentaenoic acid (EPA) and arachidonic acid (ARA) levels in polar lipids in liver tissue, suggesting a specific demand to counteract cell damage caused by the accumulation of oxidized lipids. The content of HUFA in total lipids from muscle suggests this is an important storage tissue, whereas EPA level decreased with oxidized lipids in the diet. The results showed that increasing oxidized lipids on the diet compromise the health condition and rearing performance of juvenile rose snapper *L. guttatus*.

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Keywords Anisidine value · Eicosapentaenoic acid · Glucose · Hematocrit · Total peroxides

Introduction

Fish oil is an important ingredient in the manufacture of pelletized feeds for different aquatic species. This ingredient is rich in highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), but also of arachidonic acid (ARA, 20:4n-6) (Moffat and McGill 1993). These HUFA are essential biomolecules in fish nutrition, since several fish species lack the ability of desaturation and elongation pathways necessary for their biosynthesis (Tocher 2003). The considerable amount of HUFA contained in fish oil makes it more susceptible to lipid oxidation, particularly during industrial processing extraction (i.e., high temperature and pressure), but also during the use of fish oil for feed manufacture and handling (Vieira et al. 2017; Bonilla-Méndez and Hoyos-Concha 2018). Adverse environmental conditions during feed storage, such as high temperature (> 40 °C), exposure to sunlight, high humidity conditions, but also exposure to pro-oxidant elements as Cu and Fe and rusty metals during manufacture, are some of the common conditions that increase lipid oxidation in feeds for animal nutrition (Badui 2006; Sutton et al. 2006; Vieira et al. 2017). During the first stage of lipid oxidation, several hydroxyl- and peroxides are produced, which are then chemically transformed to aldehydes and ketones (i.e., epi-hydranal, malonaldehyde, and hexanaldehyde) (Vieira et al. 2017). These compounds are easily assessed by different chemical techniques, such as those that measure the reactive substances to thiobarbituric acid (TBARS), total peroxide value (PV), and anisidine value (AV) (Gray 1978; Badui 2006). Although the quality of commercial fish oils is not usually evaluated, the maximum lipid oxidation limits have been adopted for oil supplement manufacturers, establishing ≤ 5 meq $O_2 \times kg^{-1}$, ≤ 20, and ≤ 26 for PV, AV, and total oxidation value (FAO/WHO Codex Alimentarius Commission 2015). These parameters are also commonly used for assessing the quality of fish oil and pellet feeds to establish the effect of oxidized fish oil intake on physiological conditions and rearing performance in marine and freshwater fish species, specifically survival, growth rate, weight gained, and feed conversion rate (Koshio et al. 1994; Landines-Parra and Zambrano-Navarret, 2009; Zambrano-Navarrete 2011; Gao et al. 2012b). More recently, the detrimental effects of feeding oxidized fish oil have been evaluated on feeds for fish supplemented with vitamins E and C and minerals such as selenium; these compounds with antioxidant capacity, reduce oxidative stress, tissue damage, skeleton malformations, and tissue-specific oxidation of fatty acids; and affect hematological and biochemical blood parameters (Tocher et al. 2003; Lewis-McCrea and Lall 2007; Zhong et al. 2007, 2008; Gao et al. 2012a; Gao et al. 2013; Chen et al. 2013; Dong et al. 2014; Li et al. 2014; Gao and Koshio 2014;Wang et al. 2016; Rahimnejad et al. 2021). Furthermore, tissue damage and modified fatty acid composition, particularly in muscle, decrease the quality and shelf-life of fillets obtained from fish fed oxidized fish oil (Ruff et al. 2003; Gao and Koshio 2014). Additionally, fish fed oxidized fish oil has increased susceptibility to bacterial infection and decreased immune response and integrity of blood cells (Han et al. 2012; Rahimnejad et al. 2021), specific tissue damage and increased fish mortality (Daskalov et al. 2000; Gao et al. 2012b).

Nowadays, the spotted rose snapper (*L. guttatus*) is an important rearing resource in Mexico and Latin America, mainly by the development of floating marine cages where this

species performs well, but also by its domestication, since this species adapts well to captive conditions, accepts pelletized feeds, and grown well when rearing conditions are intensified; all these make this species an interesting economical alternative (Hernández et al. 2016; Martínez-Cordero et al. 2017). There is, however, still a lack of information on the impact of oxidized fish oil intake in this carnivorous species, which has very high dietary lipid requirement, and in addition, this species is tropical/subtropical and is reared at high environmental humidity (> 80%) and temperature (> 30 °C), increasing the probability of lipid oxidation in stores feed. Our goal is to evaluate the effect of different levels of oxidized fish oil in the feed on different qualitative and quantitative parameters such as growth and survival, histopathology, hematological and blood biochemistry, and tissue fatty acid composition of spotted rose snapper juvenile.

Material and methods

Oxidation of fish oil

The fish oils used to manufacture pelletized feed were oxidized by bubbling air using a mechanical pump and heated at 80 °C using an electric thermic plate. The same fish oil was used for all treatments, but in each treatment, fish oil was processed longer until the required oxidation was achieved, with a maximum time of 8 h for the highest oxidized fish oil value. Peroxide value (PV) and anisidine value (AV) were assessed in the fish oil according to the methods described below.

Experimental diets

Four practical pelletized diets with isonitrogenous and isolipidic content were formulated based on the nutritional requirements of *L. guttatus* (Abdo de la Parra et al. 2010; Silva-Carrillo et al. 2012). The ingredient composition used for all diets is shown in Table 1. All macro ingredients were previously grounded in a hammer mill, strained through a 250-μm mesh sieve, and homogenized in a Hobart mixer (AT-200, Troy, OH, USA). The micro-ingredients, such as soy lecithin, oxidized fish oil or non-oxidized fish oil as control, and water, were added and mixed until a homogenous paste was obtained. This mixture was pelleted using a meat grinder (22, TorRey, Monterrey, Mexico) with a 3-mm die. The pellets were dried in a forced-air oven at 36 ± 2 °C for 16 h. Finally, the pellets were manually cut at 1 cm, fine particles were removed using a sieve, and the food was stored in labeled plastic bags at − 20 °C until use.

The experimental and control diets were analyzed for dry matter, crude protein (N × 6.25), ether extract (lipids), ash, nitrogen-free extract, and gross energy contents according to standard methods (AOAC 2011). Briefly, the dry matter (method 4.1.06) was assessed by gravimetry drying the sample in an oven at 105 °C for 12 h. The protein content (method 954.01) was determined using a LECO FP-528 nitrogen analyzer (LECO Instrument Corporation, St. Joseph, MI, USA). The lipid content (method 4.5.05) was evaluated by extraction with petroleum ether using a Foss Soxtec Avanti 2050 Automatic System (Foss Soxtec, Hoganäs, Sweden). The ash content (method 31.1.05) was measured by calcination of the samples at 550 °C for 6 h in a muffle furnace (Fisher, Scientific International, Inc. Pittsburg, PA, USA). Finally, the gross energy of samples of each diet was analyzed using an adiabatic calorimeter. The peroxide value (PV) was assessed according to the method

Table 1 Formulation and proximate composition of the experimental diets

Ingredients	%
Fish meal[a]	52.60
Squid meal[b]	6.00
Krill meal[c]	7.59
Fish oil[d]	8.78
Dextrin[d]	17.47
Vitamin premix[f]	0.60
Mineral premix[f]	0.23
Carotenoids[g]	0.08
Corn gluten[g]	2.00
Soy lecithin[g]	1.50
Vitamin C[g]	0.10
Alginate[d]	3.00
BHT[e]	0.05
Proximal composition	
Crude protein	46.34 ± 0.02
Ether extract	14.92 ± 0.17
Ash	15.08 ± 0.01
NFE[h]	23.65 ± 0.17
Gross energy (kJ g^{-1})[n]	20.84 ± 0.50

[a]"Premium" grade fish meal, Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, Mexico

[b]Squid meal was made from fresh squid mantle

[c]PROAQUA, S.A. de C.V. Mazatlán, Sinaloa, Mexico

[d]Droguería Cosmopolita, S.A. de C.V. México, D.F., Mexico

[e]Sigma-Aldrich Chemical, S.A. de C.V. Toluca, Mexico State, Mexico

[f]Trouw Nutrition México S.A. de C.V. (by cortesy)

*Vitamin premix composition: vitamin A, 10,000,000 IU or mg/g; vitamin D3, 2,000,000 IU; vitamin E, 100,000 g; vitamin K3, 4.00 g; thiamine B1, 8.00 g; riboflavin B2, 8.70 g; pyridoxine B6, 7.30; vitamin B12, 20.00 mg; niacin, 50.00 g; pantothenic acid, 22.20 g; inositol, 153.80 g; nicotinic acid, 160.00 g; folic acid, 4.00 g;, 80 mg; biotin, 500 mg; vitamin C, 100.00 g; choline 300.00 g, excipient q.s. 2000.00 g

**Mineral premix composition: manganese, 100 g; magnesium, 45.00 g; zinc, 160 g; iron, 200 g; copper, 20 g; iodine, 5 g; selenium,400.00 mg; cobalt 600.00 mg. Excipient q.s. 1500.00 g

[g]DSM Nutritional Products Mexico S.A. de C.V., El Salto, Jalisco, Mexico

[h]Nitrogen-free extract (including fiber) = 1000 − (g kg crude protein^{-1} + g kg crude fat^{-1} + g kg ash^{-1})

[n]The gross energy was calculated according to the physiological fuel values of protein 20.93 kJ g^{-1}; lipids 37.68 kJ g^{-1}; and nitrogen-free extract, 16.75 kJ g^{-1} (Shiau and Chou 1991)

965.33 of A.O.A.C. (1990), while anisidine value (AV) was analyzed according to the Cd-1890 method of A.O.A.C. described in IAFMM (1981), and the sum of both values is hereby referred to as total oxide value (TOV) in pelletized feds and shown in Table 2.

Table 2 Peroxide value (PV, $meqO_2 \times kg^{-1}$), anisidine value (AV), and total oxide value assessed in the experimental diets

Diet	PV	AV	TOV
Control	10.22 ± 0.1	21.21 ± 1.42	30
OX-100	26.20 ± 0.14	74.73 ± 4.11	100
OX-200	58.69 ± 0.33	140.93 ± 0.88	200
OX-230	84.16 ± 0.23	145.13 ± 1.78	230
OX-260	106.95 ± 0.29	154.17 ± 1.33	260

Lipids from pellets were extracted with a mixture of chloroform and methanol (2:1) according to Bligh and Dyer (1959) and trans-esterified with boron trifluoride-methanol (or BF3 in 14% methanol; Supelco, Bellefonte, PA, USA), and fatty acid methyl esters (FAME) were analyzed in a gas chromatograph (GC Agilent 6890 M) equipped with DB-23 silica column (30 m × 0.25 mm ID × 0.25 µm film thickness) and a flame ionization detector, using helium as carrier gas (0.7 mL × min^{-1}), and a temperature ramp from 110 to 220 °C (Palacios et al. 2005). Fatty acids were individually identified by comparing the retention time of each compound with those of standards (Sigma; Bellefonte, PA, USA). The fatty acid composition of pelletized feds is shown in Table 3.

Fish origin and rearing conditions

Spotted rose snapper juveniles used in this experimental trail were obtained from a single spawning batch in the facilities at the Marine Fish Reproduction and Rearing Laboratory in the Center for Research in Food and Development (CIAD), following the protocols for spawning and larvae rearing established by Ibarra-Castro and Alvarez-Lajonchere (2011) and Ibarra-Castro et al. 2020, Ibarra-Castro et al. 2020a). A total of healthy (i.e., no apparent skin or fin lesions, scales lost, normal vigorous swimming behavior) 270 fish (6.5 g of initial weight) were randomly sampled and distributed (18 per treatment in triplicate) into each 1 m^3 fiberglass tank. Each tank was covered with a 0.5-cm mesh net; also, each tank received aeration, continuous seawater flow (1.5 L × min^{-1}), and natural light conditions (12L:12D). Temperature and dissolved oxygen of rearing seawater were registered daily with an YSI 85 multiparameter oximeter (YSI Inc., Yellow Springs, OH, USA) and were maintained at 29.9 ± 0.09 °C and 7.07 ± 0.23 mg × L^{-1}. Fish were manually fed three times per day (9:00, 12:00 and 16:00 h) ad libitum with the experimental diets for 45 days. Unconsumed food was siphoned daily 1 h after feeding and dried in an oven at 60 °C to determine the feed intake (TFI).

Survival, growth, and biometric indexes

Survival of fish was daily verified. At the beginning and every 2 weeks until the end of experimental trial, three fish from each treatment were individually anesthetized using a clove oil solution (0.2 mL × L^{-1}) to obtain the length and weight from each fish. At the end of the experiment, three fish from each tank were euthanized without exsanguination (Fernandes et al. 2017) using a clove oil overdose (0.5 mL × L^{-1}). The liver, viscera, and mesenteric fat were dissected to assess the biometric indexes. Weight gain (WG), growth rate (GR), specific growth rate (SGR), survival (S), food conversion rate (FCR), total feed

Table 3 Fatty acid composition (%) assessed in the experimental diets

	Treatments					*P*
	Control	OX-100	OX-200	OX-230	OX-260	
14:0	6.0 ± 0.0a	6.3 ± 0.2a	6.1 ± 0.1a	6.0 ± 0.0a	5.6 ± 0.0b	<0.01
16:0	18.7 ± 0.1ab	19.4 ± 0.3ab	19.1 ± 0.0a	18.7 ± 0.3ab	18.3 ± 0.0b	<0.05
18:0	4.2 ± 0.0ab	4.3 ± 0.1a	4.2 ± 0.0ab	4.1 ± 0.1b	4.2 ± 0.0ab	<0.05
24:0	3.7 ± 0.0a	3.5 ± 0.1ab	3.5 ± 0.1b	3.4 ± 0.0b	3.4 ± 0.0b	<0.01
16:1n-7	6.6 ± 0.1ab	6.5 ± 0.2a	6.7 ± 0.1a	6.6 ± 0.0ab	6.2 ± 0.0b	<0.05
18:1n-9	11.6 ± 0.2ab	11.3 ± 0.3ab	11.3 ± 0.1b	11.4 ± 0.3b	12.2 ± 0.1a	<0.05
18:1n-7	3.2 ± 0.0ab	3.2 ± 0.0a	3.1 ± 0.0ab	3.1 ± 0.0b	3.0 ± 0.0b	<0.01
20:1n-9	1.2 ± 0.0a	1.1 ± 0.0bc	1.0 ± 0.0b	1.0 ± 0.0b	1.1 ± 0.0ab	<0.01
18:2n-6	5.2 ± 0.2e	6.3 ± 0.1d	7.0 ± 0.3c	8.0 ± 0.1b	9.1 ± 0.0a	<0.01
18:3n-3	1.2 ± 0.1d	1.4 ± 0.0c	1.4 ± 0.0c	1.6 ± 0.0b	1.8 ± 0.0a	<0.01
18:4n-3	1.6 ± 0.0a	1.6 ± 0.0a	1.6 ± 0.0a	1.6 ± 0.0a	1.5 ± 0.0b	<0.01
20:4n-6	1.9 ± 0.0a	1.9 ± 0.0ab	1.7 ± 0.0b	1.7 ± 0.0b	1.7 ± 0.0b	<0.01
20:5n-3	18.8 ± 0.1a	18.7 ± 0.1ab	18.8 ± 0.3ab	17.8 ± 0.0bc	17.1 ± 0.0c	<0.01
22:6n-3	14.3 ± 0.2	14.3 ± 0.6	13.3 ± 0.2	13.3 ± 0.4	13.1 ± 0.1	NS
∑ SAT	32.6 ± 0.2a	32.1 ± 0.2ab	32.9 ± 0.2a	32.2 ± 0.4ab	31.5 ± 0.1b	<0.01
∑ MONO	23.3 ± 0.3	22.7 ± 0.5	22.9 ± 0.2	22.7 ± 0.4	23.3 ± 0.1	NS
∑ PUFA	44.0 ± 0.5	45.2 ± 0.3	44.2 ± 0.4	45.1 ± 0.9	45.2 ± 0.2	NS
∑ HUFA	37.2 ± 0.3a	37.1 ± 0.2a	35.2 ± 0.0ab	35.0 ± 0.9ab	33.9 ± 0.1b	<0.01
∑ (n-3)	35.9 ± 0.3a	36.0 ± 0.1a	34.4 ± 0.0ab	34.4 ± 0.9ab	33.4 ± 0.1b	<0.01
∑ (n-6)	6.3 ± 0.2e	7.4 ± 0.1d	8.1 ± 0.3c	9.0 ± 0.1b	10.0 ± 0.0a	<0.01
(n-3)/(n-6)	5.7 ± 0.1a	4.9 ± 0.0b	4.2 ± 0.2c	3.8 ± 0.1d	3.3 ± 0.0e	<0.01
22:6/20:5	0.8 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	NS
20:5/22:6	1.3 ± 0.0	1.3 ± 5.6	1.4 ± 0.0	1.3 ± 0.0	1.3 ± 0.0	NS
20:4/20:5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
20:5/20:4	10.0 ± 0.0	10.2 ± 0.2	10.5 ± 0.4	10.5 ± 0.1	10.0 ± 0.1	NS
Unsat. I.	235.24 ± 1.6	236. 8 ± 0.6	228.5 ± 0.9	229.8 ± 4.4	227.5 ± 0.8	NS

∑*SAT*, sum of total saturated fatty acids; ∑*MONO*, sum of monounsaturated fatty acids; ∑*PUFA*, sum of total polyunsaturated fatty acids; ∑*HUFA*, sum of highly unsaturated fatty acids; ∑*(n-3)*, sum of fatty acids of n-3 series; ∑*(n-6)*, sum of fatty acids of n-6 series; and *Unsat I.*, unsaturation index. *NS*, not significant.

intake (TFI), condition factor (CF), hepatosomatic index (HSI), viscerosomatic index (VSI), and mesenteric fat index (MFI) were assessed as follows:

$$\mathrm{WG(g)} = \mathrm{final\ weight\ (g)–initial\ weight\ (g)}$$

$$\mathrm{GR\ (g)} = \frac{\mathrm{final\ weight–initial\ weight}}{\mathrm{initial\ weight}} \times 100$$

$$\mathrm{SGR\ (\% \times day–1)} = \frac{\mathrm{ln\ final\ weight– ln\ \ initial\ weight}}{\mathrm{time\ in\ days}} \times 100$$

$$\mathrm{S}\ (\%) = \frac{\text{final count}}{\text{initial count}} \times 100$$

$$\mathrm{FCR} = \frac{\text{food consumed (g)}}{\text{weight gained (g)}} \times 100$$

$$\mathrm{TFI} = \frac{\text{total feed intake (g)}}{\text{number of fish}}$$

$$\mathrm{CF} = \frac{\text{body weight (g)}}{\text{body lenght}^3\ \text{(cm)}} \times 100$$

$$\mathrm{HSI}\ (\%) = \frac{\text{liver weight (g)}}{\text{body weight (g)}} \times 100$$

$$\mathrm{VSI}\ (\%) = \frac{\text{viscera weight (g)}}{\text{body weight (g)}} \times 100$$

$$\mathrm{MFI}\ (\%) = \frac{\text{mesenteric fat (g)}}{\text{body weight (g)}} \times 100$$

Hematological and biochemical blood parameters

At the end of the trail, the fish were deprived of food for 24 h, and 10 fish per tank were euthanized (Fernandes et al. 2017) without exsanguination using a clove oil overdose (0.5 $\mathrm{mL} \times \mathrm{L}^{-1}$). Blood samples were taken from each fish by puncturing the caudal vein using 1-mL tuberculin syringe with heparin as anticoagulant. Hematocrit was assessed according to Del Rio-Zaragoza et al. (2008). Hemoglobin concentration was assessed using the commercial kit (Randox) based on the cyanmethemoglobin method. Total protein and triacylglyceride content were determined in plasma, which was obtained by centrifugation ($8853 \times g$ for 5 min) of blood samples. Total protein content was assessed by biuret method using a colorimetric kit (BioSystems). Triacylglyceride and total cholesterol content were assessed following the commercial kit suggestions (MexLab Group). Absorption samples were read in a spectrophotometer at 545, 520, and 505 nm, respectively (Silva-Carrillo et al. 2012; Hernández et al. 2014).

Lipid extraction and fatty acid composition

Total lipids and fatty acid composition from brain, liver, and muscle samples from fish of each treatment were extracted and assessed as previously described for foods pellet manufactured. The reserve and membrane lipid fractions obtained from liver and brain tissues were separated using silica-packed microcolumns according to Palacios et al. (2005). The fatty acid composition in muscle tissues was obtained from a composite sample of both fractions (total lipids).

Histological analyses

A portion of brain, liver, and muscle was systematically dissected from six fish per treatment. Samples were fixed in neutralized formalin 10% during 24 h and processed by histological conventional methods described in Tonguthai et al. (1999). Sections of 5 μm of each tissue was obtained with a manual microtome (Leica 820). Each slide was stained 5 min with Harris's hematoxylin and counterstained for 12 min with eosin-phloxine. The histological slides were analyzed with an optic microscope (Nikon Eclipse E200). The pathological alterations were classified as scores from 1 to 3 based only in the extension of the injury (Median Alteration Value MAV) according to Schwaiger et al. (1997). Tissue alterations were classified by ranking the severity of tissue lesions as follow: ranking grade 1 = no pathological alterations, grade 2 = focal mild to moderate changes, and grade 3 = extended severe pathological alterations.

Statistical analysis

Data obtained from the trail were analyzed by one-way analysis of variance (ANOVA) using feed treatment as independent variable and rearing performance, blood and biochemical parameters, and fatty acid composition as the dependent variables. When a significant effect occurred, the means were compared with a post hoc Tukey's test to assess significant ($P < 0.05$) differences. Median Alteration Value (MAV) recorded from histology preparations was analyzed using a contingency table. Percentage results were transformed to arcsine before analyses (Zar 1984), but only untransformed means are provided. Data are reported as mean ± standard error and were analyzed with Statistica v. 6.1 (Statsoft, Inc., Tulsa, OK, USA).

Results

Survival, growth, and feed utilization

Fish survival was significantly ($P < 0.05$) different among treatments, with highest value in fish fed the treatment with the lowest oxidized oil (OX-100) and the control (87% and 78%, respectively), while lowest survival (33%) was recorded in fish fed with the intermediate oxidized fish oil treatment (OX-230, Fig. 1A).

Growth rate significantly ($P < 0.05$) decreased with increase the level of oxidized fish oil in the diet, with higher value (267%) in the control diet and lowest (109%) in fish fed with the intermediate oxidized fish oil (OX-230) in the diet (Fig. 1B).

Specific growth rate significantly ($P < 0.05$) decreased with increasing oxidized fish oil in the diet, with higher values (3.1–3.4% × day^{-1}) in fish fed OX-100 and control, and lower values (2.5% × day^{-1}) in fish fed OX-230 and OX-260 (Fig. 1C).

Weight gain was similar after 2 weeks among the fish fed the different treatment. There were significant differences ($P < 0.05$) by the 4th week between fish fed OX-100 and control when compared with the other three treatments. By the 6th week, these differences were more evident (Fig. 1D).

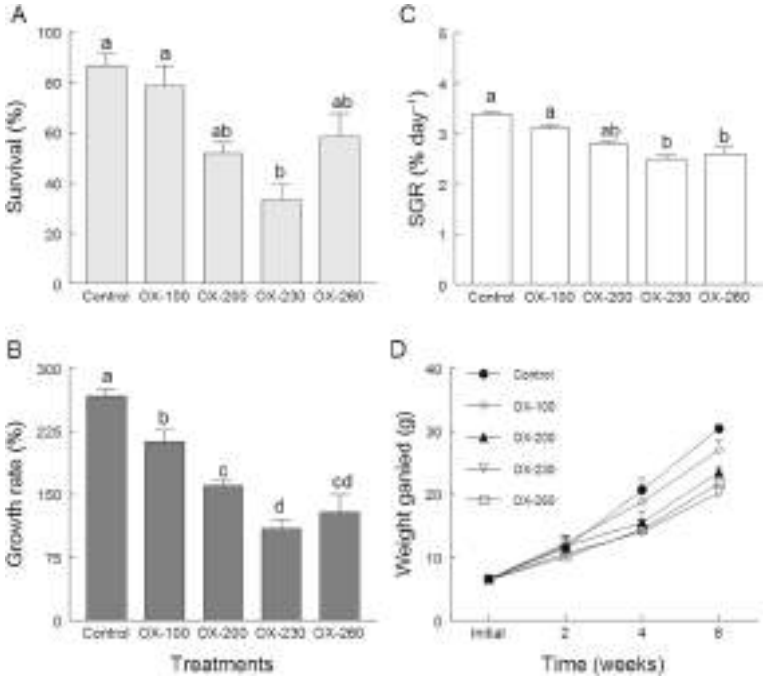

Fig. 1 **A** Survival (%), **B** growth rate (%), **C** specific growth rate (% × day^{-1}), and **D** weight gained (g) of juveniles of rose snapper *L. guttatus* fed with different levels of oxidized fish oil. Values are means ± standard error (SE) and were analyzed by one-way analysis of variance (ANOVA) and Tukey's test to assess significant differences. Means with different letters indicate statistically significant differences ($P < 0.05$)

The total feed individually consumed (Table 4) by fish tended to decrease when oxidized fish oil increased in the diet, although it did not reach significant difference among treatments (39.5 ± 6.5 to 29.1 ± 2.3 g) or in comparison to the control diet (39.3 ± 5.0 g). No significant differences were observed in the food conversion ratio (FCR), although the values for treatments were slightly higher (1.91 ± 0.34% to 2.45 ± 0.43%) compared with the control (1.65 ± 0.16%, Table 4).

Table 4 Total feed intake (TFI, %), food conversion rate (FCR, %), condition factor (CF, %), viscerosomatic index (VSI, %), hepatosomatic index (HSI, %), and mesenteric fat index (MFI, %), assessed in juvenile rose snapper *L. guttatus* fed with different levels of oxidized fish oil

	Treatments					*P*
	Control	OX-100	OX-200	OX-230	OX-260	
TFI (g)	39.32 ± 5.03	39.52 ± 6.50	32.28 ± 5.85	33.02 ± 3.69	29.12 ± 2.33	NS
FCR (%)	1.65 ± 0.16	1.97 ± 0.45	1.91 ± 0.34	2.45 ± 0.43	1.98 ± 0.25	NS
CF (%)	2.17 ± 0.04	2.15 ± 0.02	1.80 ± 0.05	1.69 ± 0.08	1.54 ± 0.03	NS
VSI (%)	9.31 ± 0.44b	9.92 ± 0.54b	9.93 ± 0.22b	12.86 ± 0.25a	10.56 ± 0.41ab	0.04
HSI (%)	1.03 ± 0.08	1.25 ± 0.04	1.18 ± 0.02	1.49 ± 0.13	1.46 ± 0.03	NS
MFI (%)	3.09 ± 0.14	3.31 ± 0.13	4.45 ± 0.33	3.84 ± 0.10	4.20 ± 0.27	NS

NS, not significant

Biological indexes

The factor condition (FC) of fish tended to decrease with increasing oil oxidation in the diet, but it did not reach significant differences among treatments (2.15 ± 0.10% to 1.54 ± 0.10%) and control (2.17 ± 0.10%; Table 4). The viscerosomatic index (VSI) was significantly higher (12.86 ± 0.81%; $P < 0.05$) in fish fed OX-230, compared to fish fed with the control diet (9.31 ± 1.44%) and the OX-100 and OX-250 (values, respectively), with intermediate values for the highest OX-260 (10.56 ± 0.41%). The hepatosomatic (HSI) and mesenteric fat (MFI) indexes tended to increase with the intake of oxidized fish oil, but without reaching significant differences (Table 4).

Hematological and biochemical blood parameters

The content of triacylglycerides and cholesterol in plasma significantly ($P < 0.05$) increased in fish fed oxidized fish oil (Fig. 2A and B). Triacylglycerides were lowest in the control and OX-100, significantly increased in the OX-200 and OX-260, and were highest in the fish from OX-230 treatment. Cholesterol was lowest in the control group, followed by fish in the OX-100, OX-200, and OX-230 and highest in the OX-260 fish. In contrast, glucose in plasma decreased with intake of oxidized fish oil, with higher values in fish from the control group, OX-100 and OX-200, and lower values in the OX-230 and OX-260 (Fig. 2C). Hemoglobin decreased in fish fed OX-100 compared with control fish, with intermediate values for the rest of the treatments (Fig. 2D). The hematocrit in fish

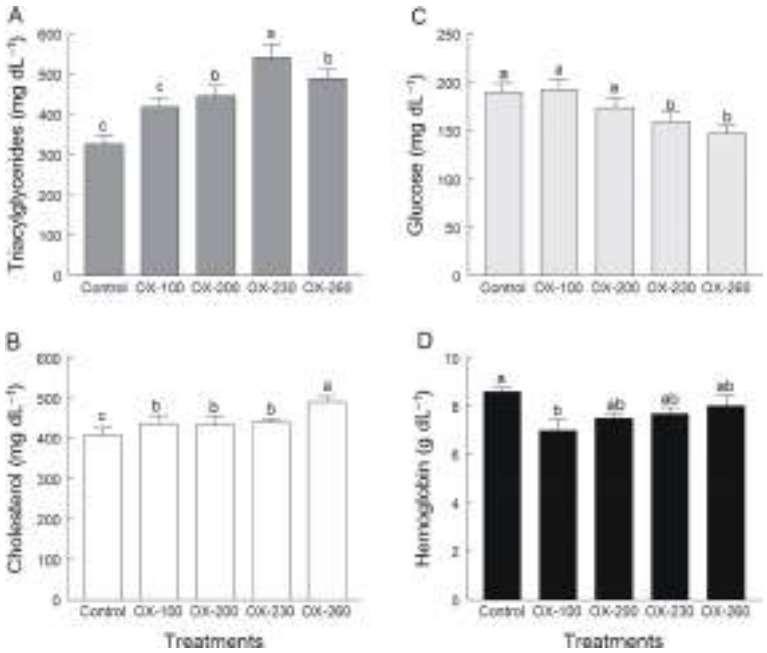

Fig. 2 **A** Triacylglycerides, **B** cholesterol, **C** glucose, and **D** hemoglobin concentration (mg × dL^{-1}) in plasma of juveniles of rose snapper *L. guttatus* fed with different levels of oxidized fish oil. Values are means ± standard error (SE); see Fig. 1 for statistical analysis

fed with oxidized fish oil was in the range of 43 to 52%, while fish in control condition of 56%, but no significant differences were observed between treatments and control condition (Table 5). The total protein content in the plasma of fish fed with oxidized fish oil was in the range of 4.7 to 4.8 g × dL^{-1}, while in fish in the control condition of 4.3 g × dL^{-1}, no significant differences were observed between treatments and control condition (Table 5).

Fatty acid composition in fish tissues

The analysis of fatty acid composition in the fish tissues was focused on the content of arachidonic (ARA), eicosapentaenoic (EPA), and docosahexaenoic acid (DHA). As mentioned before, these fatty acids are essential for most marine fishes and are expected that pro-oxidant compounds contained in the oxidized fish oil would have a stronger effect on fatty acids with more double bonds that are more prone to oxidation. Fatty acids in phospholipids in membranes (polar lipids) are expected to be more oxidized as they are exposed to an aqueous ambient, while fatty acids in triacylglycerides (neutral lipids) are expected to be less affected since they are contained in anhydrous lipid droplets.

Fatty acid composition in fish liver

A higher content of EPA was assessed in fish liver in neutral lipids, while ARA and DHA content were higher in polar lipids (Fig. 3). The content of ARA, EPA, and DHA, in both lipid fractions (neutral and polar), was not affected by fish intake of oxidized fish oil (Fig. 3A to F).

Fatty acid composition in fish brain

Contrary to observed in the fish liver, the content of ARA, EPA, and DHA was higher in fish brain in neutral lipid fraction, compared with those observed in polar lipid fraction. In both lipid fractions, the content of DHA was higher, followed of EPA and ARA. The content of ARA and EPA was very similar in the fish fed with oxidized fish oil and control treatment. The content of ARA, EPA, and DHA, in both lipid fractions (neutral and polar), was not affected by fish intake of oxidized fish oil (Fig. 4A to F).

Fatty acid composition in fish muscle

A higher content of DHA, followed of EPA and ARA, was observed in fish muscle. Only the content of EPA significantly ($P < 0.05$) decreased with fish intake of oxidized fish oil (Fig. 5).

Table 5 The hematocrit (%) in blood and total protein content (g × dL^{-1}) in plasma assessed in juvenile rose snapper *L. guttatus* fed with different levels of oxidized fish oil

	Treatments					*P*
	Control	OX-100	OX-200	OX-230	OX-260	
Hematocrit (%)	55.78 ± 1.32	52.56 ± 1.10	49.12 ± 1.52	47.60 ± 2.12	43.29 ± 2.47	NS
Tot. protein (g × dL^{-1})	4.32 ± 0.08	4.75 ± 0.12	4.80 ± 0.16	4.75 ± 0.17	4.77 ± 0.14	NS

NS, not significant

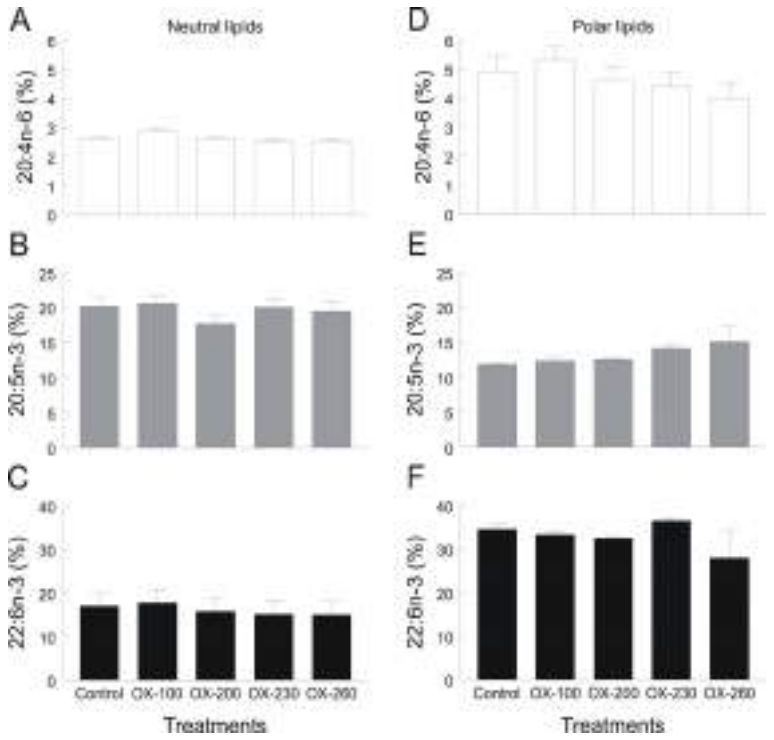

Fig. 3 **A–D** Arachidonic, **B–E** eicosapentaenoic, and **C–F** docosahexaenoic fatty acid composition (%) in neutral and polar lipids in the liver of juveniles of rose snapper *L. guttatus* fed with different levels of oxidized fish oil. Values are means ± standard error (SE); see Fig. 1 for statistical analysis

Histological analyses

Representative images of a longitudinal cross section of the liver from the fish fed with the experimental feeds are showed in Fig. 6. A higher proportion of tissue pathologies, as inflammatory nodules and necrosis, were observed and quantified in the fish fed with the higher content of oxidized fish oil (OX-230 and OX-260), while these pathologies were not observed in the fish fed with lowest content of oxidized fish oil and control condition (Fig. 6). No significant ($\chi 2 = 5.4$, $P = 0.249$) differences were observed in the proportion of pathological alterations (median alteration value, MAV) in the liver of fish fed with oxidized fish oil; however, focused pathologies of grade 2 increased particularly in fish fed with highest content of oxidized fish oil (OX-230 and OX-260) (Fig. 7). No pathologies were observed in brain or muscle of fish fed with oxidized fish oil (Figs. 8 and 9). Visible lesions on the skin and fins of the fish were recorded, and the caudal fin of fish was mainly damaged, particularly in fish fed with the higher content of oxidized fish oil (Fig. 10).

Discussion

The increase of oxidized fish oil in the diet caused a detrimental effect in survival, growth, and hematological and blood biochemistry. Furthermore, specific tissue pathologies and peroxidation of specific fatty acids in the tissues of spotted rose snapper *L. guttatus*

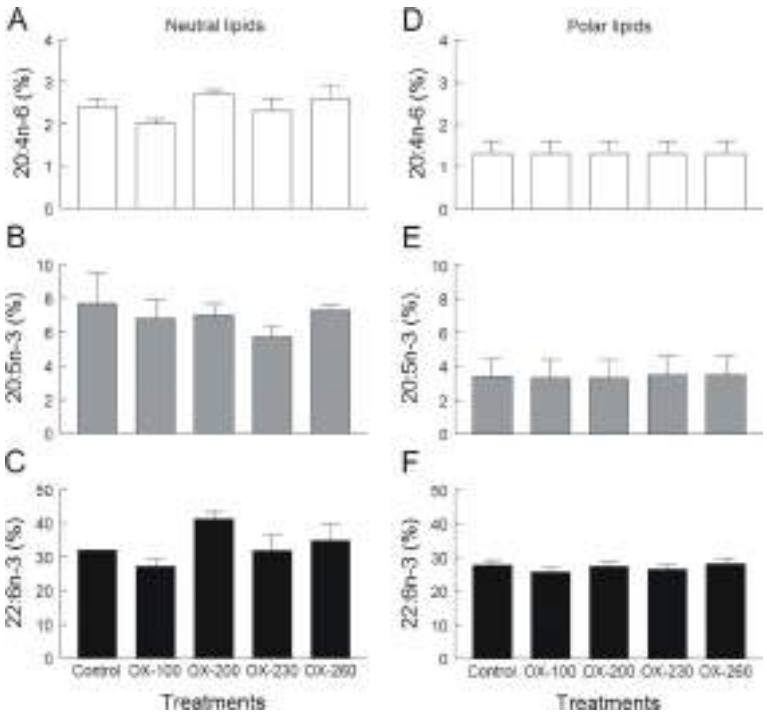

Fig. 4 **A–D** Arachidonic, **B–E** eicosapentaenoic, and **C–F** docosahexaenoic fatty acid composition (%) in neutral and polar lipids in the brain of juveniles of rose snapper *L. guttatus* fed with different levels of oxidized fish oil. Values are means ± standard error (SE); see Fig. 1 for statistical analysis

juveniles were observed. The results demonstrate that this fish species is quite sensitive to oxidized fish oil intake, since rearing performance significantly decreased, reaching a lower survival, growth rate, specific growth rate, and weight gained at the higher content of oxidized fish oil in the diet (Fig. 1). The content of PV, AV, and total oxidation value determined in both the fish oil used as ingredient and feeds used in this trail was above of those adopted by fish oil supplement manufacturers (FAO/WHO Codex Alimentarius Commission 2015); therefore, this study represents the first evaluation of the impact of feed lipid oxidation on the nutrition and physiology on rose snapper, as well as the need to control the quality of commercial fish oil used in the nutrition of aquatic organisms. It has been previously described that aldehydes produced by the oxidation of lipids could have interacted with available proteins in the experimental feeds, changing their chemical configuration and decreasing their availability and absorption, with the concomitant detrimental effect on the growth of the fish (Chopin et al. 2007). However, the effect of oxidized fish oil intake seems to be species-specific, since some report a detrimental effect in the rearing performance (i.e., lower survival or weight decrease) in some species of fish (Koshio et al. 1994; Daskalov et al. 2000; Tocher et al. 2003; Gao et al. 2012a, 2012b; Chen et al. 2013; Dong et al. 2014; Wang et al. 2016; Fontagné-Dicharry et al. 2018), while other fish species seems to be more resilient to feed with oxidized fish oil (Lewis-McCrea and Lall 2007; Zhong et al. 2008; Chen et al. 2012; Han et al. 2012; Gao et al. 2013; Boglino et al. 2014; Dong et al. 2014).

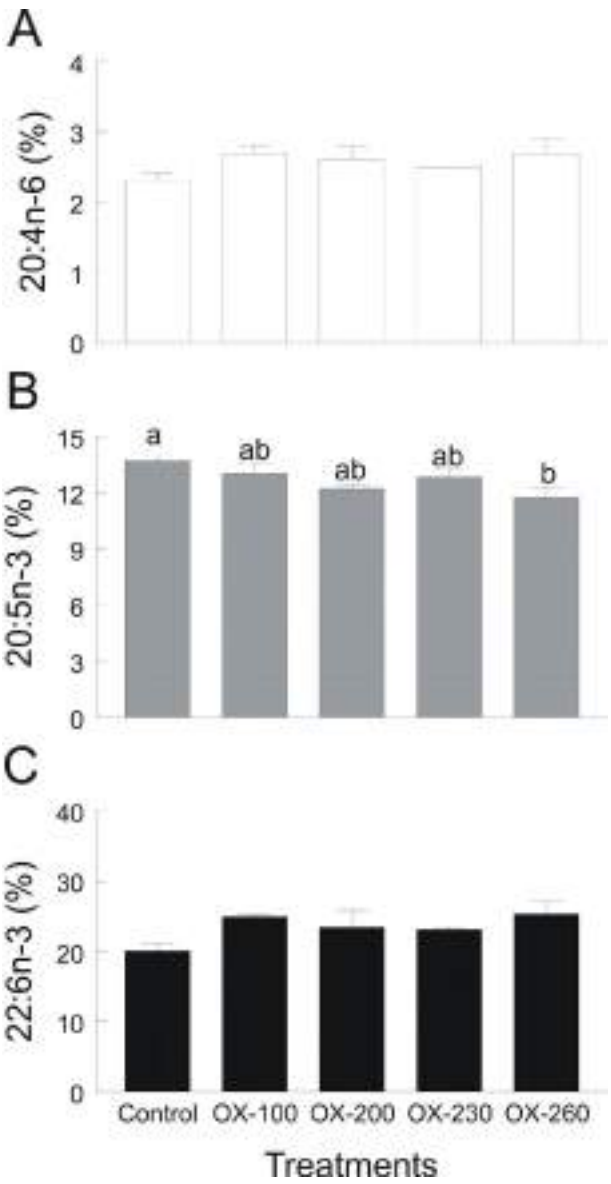

Fig. 5 **A** Arachidonic, **B** eicosapentaenoic, and **C** docosahexaenoic fatty acid composition (%) in total lipids in the muscle of juveniles of rose snapper *L. guttatus* fed with different levels of oxidized fish oil. Values are means ± standard error (SE); see Fig. 1 for statistical analysis

The increase of triacylglycerides and cholesterol in plasma with oxidized lipids (Fig. 2A and B), higher than those reported for spotted rose snappers in good health conditions (Hernández et al. 2019), suggests a possible alteration in lipid metabolism in liver, which have reported before in some other fish species (Dong et al. 2014) fed different levels of oxidized lipids. In agreement, we observed that the liver was larger, probably caused by inflammation because of the presence of nodules and necrosis in the liver tissue. Another possibility is increased lipoprotein production or decreased intake of lipoproteins. The decrease in plasma glucose (Fig. 2C), together with high levels of lipids, could indicate that glucose has been liberated to blood from

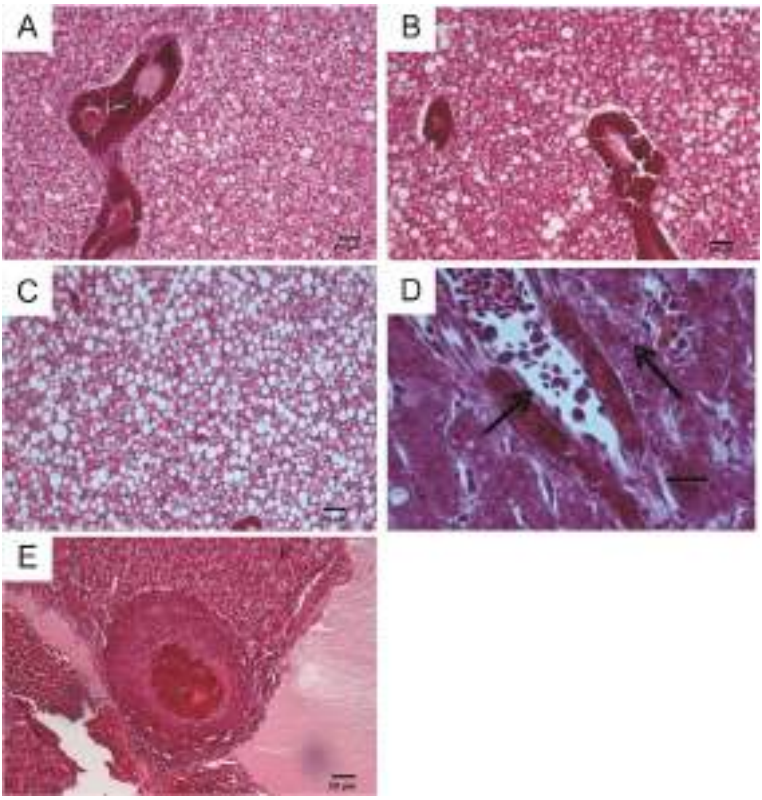

Fig. 6 Microphotographs of histological sections in the liver of juveniles of rose *snapper L. guttatus* fed with **A** control, liver with normal content of lipids; **B** OX-100, increase in the content of lipids; **C** OX-200, fatty liver; **D** OX-230, necrosis of the liver (arrows indicate picnosis of the nuclei of liver cells and necrosis); and **E** inflammatory nodule, inflammation, and necrosis of the liver of fish fed with OX-260

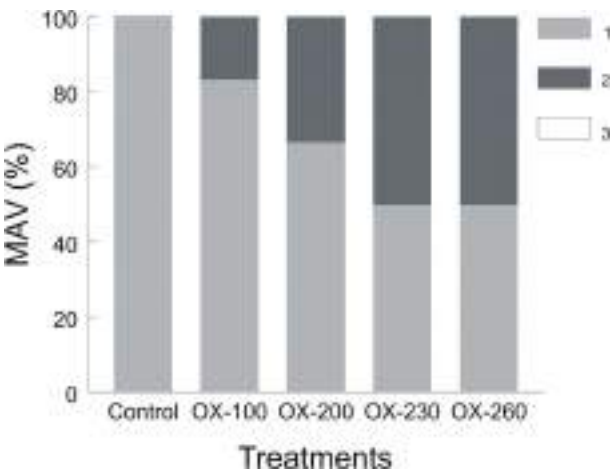

Fig. 7 Media alteration value (MAV, %) of the lesions observed in the liver of juveniles rose snapper *L. guttatus* fed with different levels of oxidized fish oil. Tissue alterations were recorded according to described by Schwaiger et al. (1997) and the following classification: grade 1 = non-pathological alteration, grade 2 = focused alteration, and grade 3 = profuse alteration

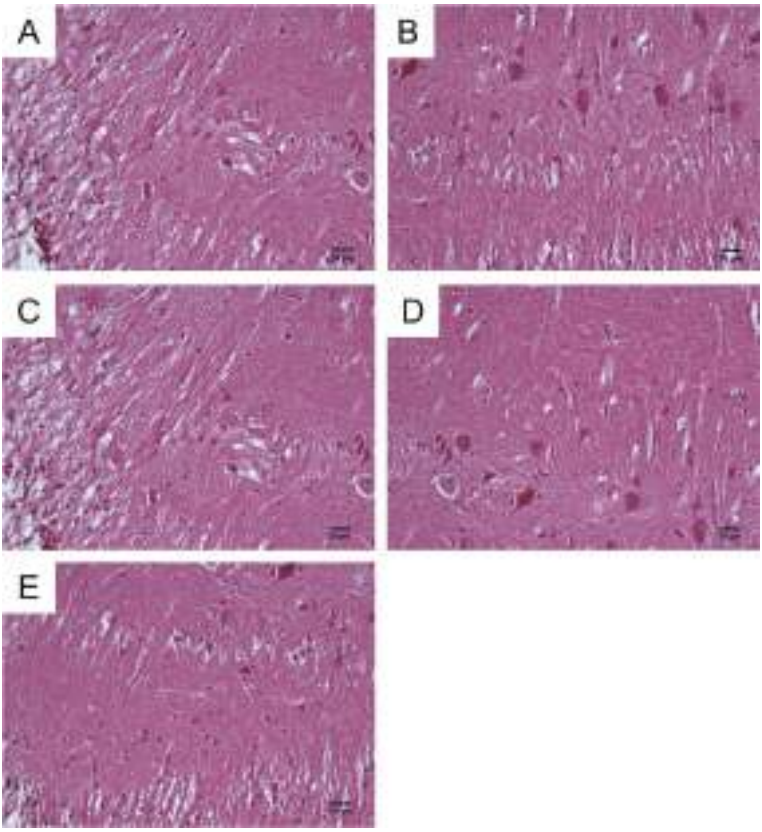

Fig. 8 Microphotographs of histological sections (without visible pathologies) in the brain of juveniles of rose *snapper L. guttatus* fed with **A** control, **B** OX-100, **C** OX-200, **D** OX-230, and **E** OX-260 diets with different levels of oxidized fish oil

liver to be used for synthesis of saturated fat (i.e., 27.6% and 27.5%, and 23.9% of total saturated fatty acids for treatments OX-200 and OX-230, and control conditions, respectively; data not showed), which was stored in the liver increasing its size. Moreover, the increase in VSI (Table 4) as lipid oxidation in feed increases is a form of accumulating oxidized fats in a less metabolically active tissue, stored as to not induce more damage in metabolically active tissues: this is further confirmed by a positive correlation between viscerosomatic index and oxidized fish oil intake (PV, $r = 0.87$ and AV, $r = 0.87$). Other tissues that are metabolically more active were not affected by lipid oxidation in feed (Table 4). In accordance, no apparent damage was observed in the histological sections in the brain or in the muscle tissues (Figs. 8 and 9). The differences observed in tissue damage may be due to the differential accumulation of fish oil oxidation products, although it is also closely linked to the antioxidant capacity among tissues (Tocher et al. 2003; Chen et al. 2012; Dong et al. 2014). Antioxidant capacity involves specific antioxidant enzymes (i.e., superoxide dismutase, catalase), some biochemical compounds (i.e., pigments, vitamins, fatty acids), and secondary metabolites (i.e., polyphenols, flavonoids) which effectively counteract the production of several pro-oxidant chemical compounds by oxidative stress (Landines-Parra and Zambrano-Navarrete 2009). In this trial, the antioxidant capacity in the tissues of the fish fed with the oxidized oil was not assessed; however, it is inferred that the

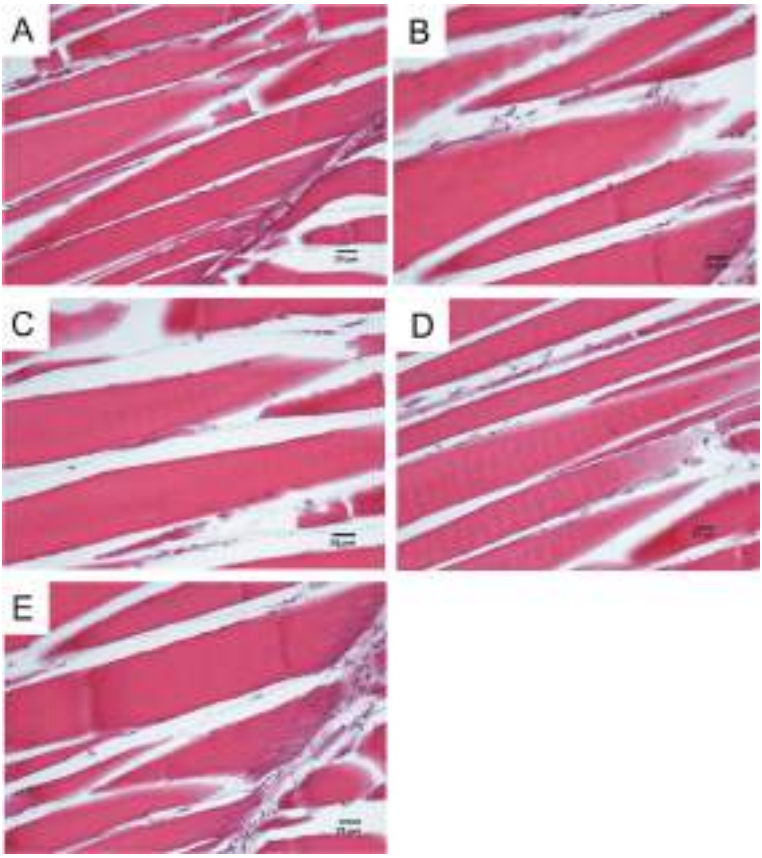

Fig. 9 Microphotographs of histological sections (without visible pathologies) in the muscle of juveniles of rose *snapper L. guttatus* fed with **A** control, **B** OX-100, **C** OX-200, **D** OX-230, and **E** OX-260 diets with different levels of oxidized fish oil

antioxidant capacity was probably higher in the liver and muscle and lesser extent in the brain. This is despite the damage observed in the liver and lipid oxidation that occurred in the muscle tissue. In general, the antioxidant capacity is considerable higher in the liver compared with that occurred in the brain tissues in mammals (Limón-Pacheco and Gonsebatt 2009). In contrast that occurred in other vertebrates, the information is scarce in marine fishes, particularly in regard to the relationship between the accumulation of lipid oxidation products and the antioxidant activity in fish tissues (Chen et al. 2012).

Hemoglobin content decreased significantly (Fig. 2D, $P < 0.05$) in fish fed with oxidized fish oil, suggesting an increase in fragility of blood cells and certainly in the oxygen transport capacity. This result, along with decreased in the hematocrit, may compromise the rearing of spotted rose snapper *L. guttatus* when some environmental stress eventually occurs, particularly during decrease of oxygen levels or hypoxia condition.

The content of highly unsaturated fatty acids (HUFA) decreased in tissues of fish fed with different levels of oxidized fish oil (Zhong et al. 2007; Gao et al. 2012a; Dong et al. 2014). In contrast, in the present work, the levels of HUFA in liver and brain tissues were not different between treatments (Figs. 3 and 4), in accordance to the results of histology. However, the

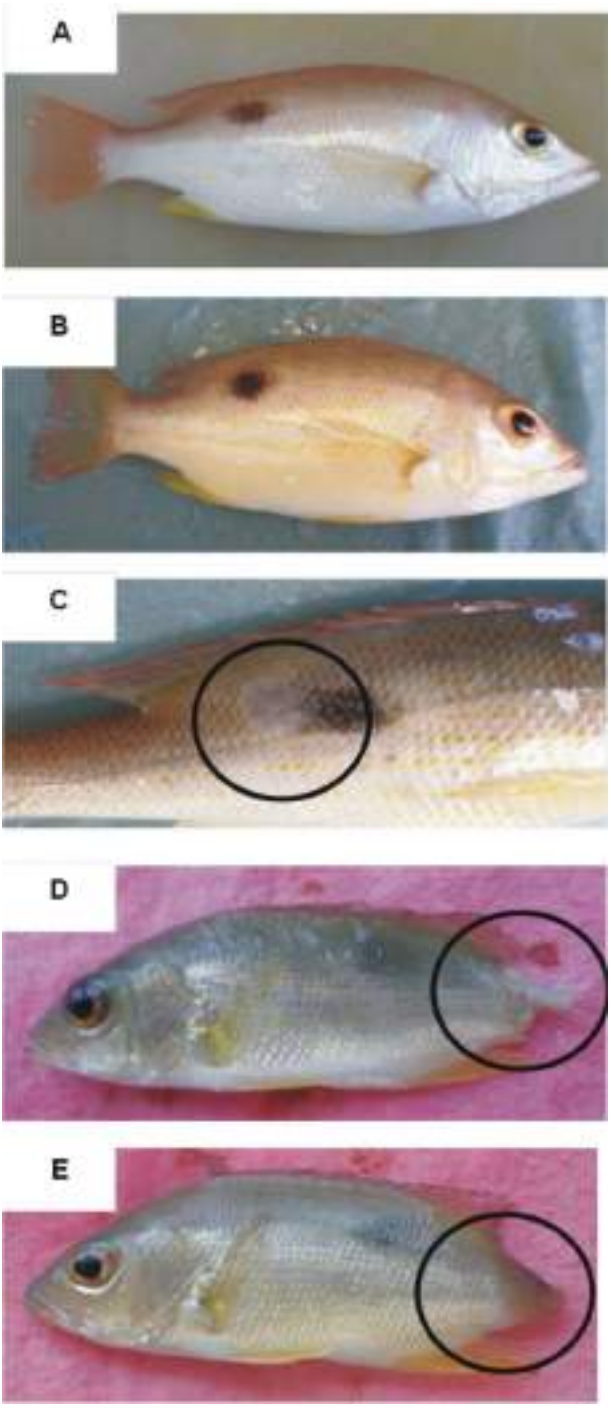

Fig. 10 Photographs of juveniles of rose *snapper L. guttatus* fed with **A** control, **B** OX-100, **C** OX-200, **D** OX-230, and **E** OX-260 diets with different levels of oxidized fish oil. The lesions observed in the skin and caudal fins are shown inside of black circles

values of PV and AV contained in experimental feeds (Table 2) were inversely correlated ($r = -0.90$ and -0.90, $P < 0.05$) with docosahexaenoic acid (DHA) levels in neutral lipids, and eicosapentaenoic acid (EPA) ($r = -0.90$ and -0.90, $P < 0.05$) and arachidonic acid (ARA) ($r = -0.87$ and -0.87, $P < 0.05$) levels in polar lipids assessed in the liver tissue, indicating a specific demand to counteract cell damage caused by oxidized lipids. This also suggests that it is probably other compounds involved as antioxidants (i.e., vitamins, pigments, polyphenols) that counteract the accumulation of fish oil oxidation products. Without discard the differences that could exist in the activity of antioxidant enzymes between tissues as discussed above. In contrast, the content of EPA in total lipids in muscle decreased slightly but significantly (13.7 to 11.7%) in fish fed with highest content of oxidized fish oil, while no differences were observed in the content of ARA (2–3%) and DHA (20 to 25%) (Fig. 5). This result suggests a particular susceptibility to oxidation of the HUFA, specifically EPA, contained in the fish muscle. Therefore, the oxidation of lipids contained in the diet could affect the content of HUFA, with higher nutritional value in the fillet of rose snapper and consequently decrese its commercial value, as has been observed in other fish species (Gao and Koshio 2014). Also, the physical appearance of fish was significantly affected with oxidized fish oil intake. Skin lesions appeared in fish fed OX-200 treatment, while bacterial proliferation and destruction of pectoral and caudal fins occurred in treatments with higher levels of lipid oxidation (Fig. 10). Although pathological or parasitological examinations were not carried out on the fish, it is not ruled out that the consumption of oxidized oil makes the fish more susceptible to diseases that frequently appear during their rearing. The increased susceptibility of the fish to disease, together with the lesions observed on the skin and fins, could explain the poor survival observed in fish fed high levels of oxidized fish oil. In agreement, similar lesions (i.e., inflammation and hemorrhage in dorsal, pectoral, and caudal fins) and poor survival have described in *Micropterus salmoides* fed with different levels of oxidized fish oil in the diet (Chen et al. 2012).

In conclusion, this study demonstrates that the increase of oxidized lipids (> 100 meq $O_2 \times kg^{-1}$) in the diet compromise the health condition and rearing performance of juvenile rose snapper *L. guttatus*. Particularly, oxidized lipids affected the metabolism of liver, reflected in the decrease of glucose, and increased triacylglycerides and cholesterol in plasma. Decreased hemoglobin in plasma along with hematocrit in fish fed with oxidized lipids may compromise the rearing of this fish species, particularly during stressful conditions of hypoxia. The increased hepatosomatic index suggests damage to this tissue, further evidenced by the presence of nodules and necrosis, respectively. Visceral index was increased in fish fed more oxidized lipids in feed, suggesting accumulation of toxic lipids to avoid damage to other tissues. The EPA content in the muscle tissue was significantly affected by oxidation of lipids in the diet. The decrease in the content of lipids of high nutritional value, together with the visible lesions on the skin and fins of the fish fed with oxidized lipids, causes a decrease in the quality of the fillet and the high commercial value of this species of fish.

Author contribution Gabriel Á. Quintero-Martínez: Conceptualization, experimental trail performance, samples and data analysis, writing-review and editing. Crisantema Hernández: Conceptualization, funding acquisition, project administration, investigation, data analysis, writing- review and editing. Elena Palacios: Investigation, samples and data analysis, writing- review and editing. María Cristina Chávez Sánchez: Histopathology–review and editing. Leonardo Ibarra-Castro: Broodstock, larval rearing, juvenile's production, review and editing. Miguel Ángel Hurtado-Oliva: Investigation, funding acquisition, data analysis, writing-original draft.

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Data availability Supporting data of this study are available upon reasonable request.

Declarations

Ethics approval The animal study was reviewed and approved by Comité de Etica en Investigación del Centro de Investigación en Alimentación y Desarrollo, A.C. (endorsed by the Conbioetica-National Ethics Commission).

Competing interests The authors declare no competing interests.

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