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ORIGINAL ARTICLE

Dietary ALA, But not LNA, Increase Growth, Reduce Inflammatory Processes, and Increase Anti-Oxidant Capacity in the Marine Finfish *Larimichthys crocea*

Dietary ALA, but not LNA, Increase Growth, Reduce Inflammatory Processes, and Increase Anti-oxidant Capacity in the Large Yellow Croaker

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Abstract Whilst aquaculture feed is increasingly formulated with the inclusion of plant oils replacing fish oil, and increasing research effort has been invested in understanding the metabolic effects of reduced dietary n-3 long chain poly unsaturated fatty acids (n-3 LC-PUFA), relatively little information is available on the potential direct metabolic roles of dietary alpha-linolenic acid (ALA, 18:3n-3) and alpha-linolenic acid/linoleic acid (LNA, 18:2n-6) ratio in cultured marine finfish species. In this study, four plant oil based diets, with varying ALA/LNA ratio (0.0, 0.5, 1.0 and 1.5) were fed to juvenile large yellow croakers (*Larimichthys crocea*) and compared to a fish oil-based control diet (CD) to evaluate the resulting effects on growth, nonspecific immunity, anti-oxidant capacity and related gene expression. High dietary LNA negatively impacted fish growth performance, nonspecific immunity and antioxidant capacity, but growth and immunity were maintained to levels comparable to CD by increasing the ratio of dietary ALA/LNA. The over-expression of genes associated with inflammation (cyclooxygenase-2 and interleukin-1β) and fatty acid oxidation (carnitine palmitoyl transferase I and acyl CoA oxidase) in croakers fed high concentrations of LNA were reduced to levels comparable to those fed CD by increasing dietary ALA/LNA. This study showed that dietary ALA, by increasing the overall n-3/n-6 PUFA ratio, exerts direct anti-inflammatory and antioxidant effects, similar to those exerted by dietary n-3 LC-PUFA.

Keywords Alpha-linolenic acid · Linoleic acid · Acyl CoA oxidase · Carnitine palmitoyl transferase · Cyclooxygenase · Interleukin · Large yellow croaker

Abbreviations

ALA	Alpha-linolenic acid
ACO	Acyl CoA oxidase
CAT	Catalase
COX-2	Cyclooxygenase-2
CPTI	Carnitine palmitoyl transferase I
FAS	Fatty acid synthase
G6PD	6-Phosphogluconate dehydrogenase
HSI	Hepatosomatic index
LNA	Linoleic acid
MDA	Malondialdehyde
ME	Malic enzyme
PI	Phagocytic index
SOD	Superoxide dismutase
T-AOC	Total antioxidant capacity
VSI	Viscerosomatic index

Introduction

Because of its availability and ability to meet essential fatty acid nutritional requirements, fish oil has been traditionally used by the aquaculture feed industry as the main dietary

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lipid source [1]. However, the limited global production of fish oil, coupled with its ever-increasing demand, is currently responsible for price hikes and concerns over the long term economic viability of commercial aquaculture [2, 3]. Consequently, in the last two decades, an intense global research effort has focused on identifying and implementing possible alternatives, with the vast majority of this work being implemented on salmonids [4–12], and Mediterranean, temperate marine finfish [13–17]. Plant oils have been viewed as the most likely candidates for the replacement of fish oil in aquafeed formulation, because of their relatively low price, global availability and stable supply. However, compared to fish oil which is rich in n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), the PUFA content of plant oils is fundamentally composed of linoleic acid (LNA, 18:2n-6), and in a few plant oils as alpha-linolenic acid (ALA, 18:3n-3) [18].

High inclusion of dietary LNA, which is abundant in several plant oils, such as soybean, corn, sunflower and safflower, has been shown to negatively affect (i) growth performance in salmonids [19, 20], black sea bream (*Acanthopagrus schlegeli*) [21] and gilthead sea bream (*Sparus aurata*) [22, 23]; (ii) nonspecific immunity of Atlantic salmon (*Salmo salar*) [24], gilthead sea bream [23], grouper (*Epinephelus malabaricus*) [25], and large yellow croaker (*Larimichthys crocea*) [26]; (iii) spawning performance and larval quality of tongue sole (*Cynoglossus semilaevis*) [27]; and (iv) fatty acid and lipid metabolism of prawn [28, 29], gilthead sea bream [30], and Atlantic salmon [31].

These negative effects of high LNA could be eliminated by increasing n-3/n-6 PUFA. Dietary n-3 LC-PUFA (primarily eicosapentaenoic acid, EPA, 20:5n-3 and docosahexaenoic acid, DHA, 22:6n-3) or ALA have been proven to be capable of efficiently eliminating negative effects from high LNA content by increasing the overall n-3/n-6 PUFA in salmon [31–33] and some freshwater fish species [34–37]. In comparison, remarkably less is known about the potential roles and effects of different dietary ALA/LNA ratios in marine fish species unable to bioconvert C_{18} PUFA into LC-PUFA. Wu and Chen [25] have reported ALA and LNA at a dietary level of 2 % and a ratio of 3:1 were beneficial to weight gain and non-specific cellular immune responses of juvenile grouper (*Epinephelus malabaricus*). Thus, it is interesting to elucidate mechanisms involved since ALA and LA are traditionally acknowledged as unessential fatty acids for marine fish species. Recently, Montero et al. [23] have first verified that high inclusion of LNA or low n-3 LC-PUFA could increase transcription of inflammation associated genes, e.g., TNF-α and IL-1β, in the intestine and head kidney of gilthead sea bream. Studies in mammals have shown that regulation of pro-inflammatory cytokine expression by n-3 LC-PUFA has been described to be mediated by an eicosanoid-independent mechanism [38]. However, as far as we know, the effects of dietary ALA/LNA on transcription of cyclooxygenase-2 (COX-2) and inflammation associated genes are still unknown in any marine fish species. Furthermore, marine finfish species are unable to bioconvert ALA into n-3 LC-PUFA [39], it is envisaged they could be valuable and interesting case study (model) to actually understand whether ALA has any effects of its own, or the potential effects of ALA are indirectly due to its bioconversion into n-3 LC-PUFA [40], without the confounding effect of the possible presence of EPA and DHA derived by its in vivo conversion.

The large yellow croaker, *L. crocea*, is an important marine fish species widely cultured in southeast China. Recently, its nutritional physiology has been studied [41–45], including its inability to convert ALA into n-3 LC-PUFA and the effects of high dietary LNA content on growth and health performance [26]. However, as with many other marine finfish species, little is yet known of the potential effects and mechanisms of dietary ALA/LNA ratio on the immune function, antioxidant capacity and lipid deposition in the large yellow croaker. Thus, the objectives of this study were to elucidate if the dietary ALA/LNA ratio affects growth performance, nonspecific immunity, antioxidant capacity and lipid deposition of large yellow croaker, and whether dietary ALA has similar efficacy as n-3 LC-PUFA in the reduction of inflammation and oxidative stress.

Materials and Methods

Ethics Statement

This study was conducted in Xiangshan Bay (Ningbo, China) with the permission of the Ningbo Marine Fisheries Bureau. All procedures involving animals implemented during this experiment were approved by the Institutional Animal Care and Use Committee of the Ocean University of China (protocol number 20001001). All possible efforts to minimize animal suffering were taken. At the beginning and termination of the experiment, all fish were fasted for 24 h, anesthetized by immersion in an eugenol bath (1:10,000), counted, individually weighed and sampled.

Experimental Design, Diets, Animals and Sampling

Five iso-nitrogenous (42 %) and iso-lipidic (13 %) experimental diets were formulated, with all diets containing 300 g/kg of fish meal and 20 g/kg of fish oil (basal diet), and varying only in an additional 70 g/kg of the added lipid source. A 100 % fish-oil-based diet was formulated with the addition of 70 g/kg fish oil to the basal diet, for

the control diet (CD). The other four experimental diets were formulated by adding 70 g/kg of four different blends of three plant oils (sunflower oil, palm oil and linseed oil). These blends were specifically formulated to achieve a graded dietary ALA/LNA ratio, varying from 0 to 1.5 (R-0.0, R-0.5, R-1.0 and R-1.5). Ingredient and fatty acid composition of the experimental diets are given in detail in Tables 1 and 2, respectively.

All dry ingredients were finely ground and sieved at 320 μm before being blended with their respective oil mixtures, and then mixed. Water (200 g/kg) was then added to the mixture, and the resulting dough was loaded into an automatic pellet-making machine (Weihai, Shandong province, China). The resulting pellets (4 mm × 5 mm) were then dried for 12 h in a ventilated oven at 40 °C. After drying, the experimental diets were

Table 1 Formulation (g/kg) and proximate composition (mg/g) of the experimental diets

Ingredients (g/kg dry diet)	Dietary treatments[1]				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
White fish meal[2]	300	300	300	300	300
Soybean meal[2]	269.5	269.5	269.5	269.5	269.5
Wheat meal[2]	225	225	225	225	225
Casein[2]	50	50	50	50	50
Mineral premix[3]	20	20	20	20	20
Vitamin premix[4]	20	20	20	20	20
Attractant[5]	4	4	4	4	4
Mold inhibitor[6]	1	1	1	1	1
Yttrium oxide	0.5	0.5	0.5	0.5	0.5
Lecithin	20	20	20	20	20
Fish oil[7]	90	20	20	20	20
Sunflower oil[8]	–	70	20	–	–
Palm oil[9]	–	–	30	30	–
Linseed oil[10]	–	–	20	40	70
Proximate composition (mg/g wet diet)					
Moisture	46.1	45.3	44.6	47.8	46.9
Crude protein	401.4	405.7	402.2	403.9	404.5
Crude lipid	128.6	128.1	124.8	132.4	129.4
ALA/LNA	0.26	0.03	0.45	0.90	1.51

[1] Experimental diets abbreviations: *CD* fish oil based diet (control diet), *R-0.0* plant oil based diet with ALA/LNA ratio of 0, *R-0.5* plant oil based diet with ALA/LNA ratio of 0.5, *R-1.0* plant oil based diet with ALA/LNA ratio of 1, *R-1.5* plant oil based diet with ALA/LNA ratio of 1.5

[2] White fish meal: crude protein 74.3 % dry matter, crude lipid 6.6 % dry matter; soybean meal: crude protein 49.4 % dry matter, crude lipid 0.9 % dry matter; wheat meal: crude protein 16.4 % dry matter, crude lipid 1.0 % dry matter; casein: 93 % crude protein and 1 % crude lipid, Alfa Aesar, Avocado Research Chemicals Ltd, UK

[3] Mineral premix (mg or g kg^{-1} diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3 (1 %), 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1 %), 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; Ca $(IO_3)_2$, 180 mg; $MgSO_4 \cdot 7H_2O$, 1,200 mg; zeolite, 18.35 g

[4] Vitamin premix (mg or g kg^{-1} diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B_{12}, 10 mg; vitamin B_6, 20 mg; folic acid, 20 mg; vitamin B_1, 25 mg; vitamin A, 32 mg; vitamin B_2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2,000 mg; microcrystalline cellulose, 16.47 g

[5] Attractant: glycine and betaine

[6] Mold inhibitor: contained 50 % calcium propionic acid and 50 % fumaric acid

[7] Fish oil: palmitic acid (16:0) content, 15.77 % total fatty acids (TFA); oleic acid (18:1n-9) content, 3.29 % TFA; linoleic acid (18:2n-6) content: 2.12 % TFA; alpha-linolenic acid (18:3n-3) content: 0.42 % TFA; ARA content, 0.23 % TFA; EPA content, 13.34 % TFA; DHA content, 10.53 % TFA, bought from the Great Seven Biotechnology Co, Ltd, Qingdao, China

[8] Sunflower oil: palmitic acid (16:0) content, 7.56 % TFA; oleic acid (18:1n-9) content, 12.89 % TFA; linoleic acid (18:2n-6) content: 58.85 % TFA; alpha-linolenic acid (18:3n-3) content: 0.08 % TFA, bought from the Liqun supermarket, Qingdao, China

[9] Palm oil: palmitic acid (16:0) content, 34.44 % TFA; oleic acid (18:1n-9) content, 47.31 % TFA; linoleic acid (18:2n-6) content: 12.56 % TFA; alpha-linolenic acid (18:3n-3) content: 0.24 % TFA, bought from the Liqun supermarket, Qingdao, China

[10] Linseed oil: palmitic acid (16:0) content, 5.25 % TFA; oleic acid (18:1n-9) content, 19.40 % TFA; linoleic acid (18:2n-6) content: 13.52 % TFA; alpha-linolenic acid (18:3n-3) content: 47.53 % TFA, bought from the Liqun supermarket, Qingdao, China

Table 2 Fatty acid composition of the experimental diets (mole%)

Fatty acid	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
14:0	7.2	2.7	2.8	2.8	2.6
16:0	22.1	16.2	23.5	23.1	15.1
18:0	4.1	4.0	4.0	4.1	4.2
20:0	1.2	0.4	0.4	0.4	0.4
∑SFA[1]	34.6	23.3	30.7	30.3	22.3
16:1	7.5	3.3	3.3	3.2	3.1
18:1	18.9	24.0	23.2	26.8	21.4
∑MUFA[2]	26.5	27.3	26.5	30.0	24.5
18:2n-6 (LNA)	14.5	43.2	23.2	18.2	18.8
20:4n-6	2.1	1.6	1.5	1.5	1.5
∑n-6PUFA	16.5	44.8	24.7	19.7	20.4
18:3n-3 (ALA)	3.8	1.5	10.4	16.3	28.5
20:5n-3	9.1	3.3	3.3	3.4	3.7
22:6n-3	7.7	2.9	2.8	3.0	3.1
∑n-3PUFA	20.6	7.6	16.5	22.7	35.3
ALA/LNA	0.3	0.0	0.5	0.9	1.5
n-3/n-6PUFA	1.3	0.2	0.7	1.2	1.7
n-3LC-PUFA[3]	16.8	6.2	6.1	6.4	6.8
ARA/EPA	0.2	0.5	0.5	0.4	0.4
DHA/EPA	0.8	0.9	0.9	0.9	0.9
DBI[4]	166.8	158.0	143.5	156.5	191.0

Some fatty acids, of which the contents were minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C20:3n-6, C22:5n-3, are not listed in the Table

[1] *SFA* saturated fatty acids

[2] *MUFA* mono-unsaturated fatty acids

[3] *n-3 LC-PUFA* n-3 highly-unsaturated fatty acids, EPA + DHA

[4] *DBI* ∑ % of unsaturated fatty acids×number of double bonds of each unsaturated fatty acid

packed in double air-tight plastic bags and stored at −20 °C until used.

The feeding experiment was conducted at an experimental floating sea cage facility at Xiangshan Bay (Ningbo, China). Approximately 1,000 juvenile large yellow croaker (*L. crocea*) were sourced from a local commercial farm. Juveniles were maintained in the experimental facility (a large floating sea cage; 3 m × 3 m × 3 m) and fed the control diet (CD) for 2 weeks to acclimatize the fish to the experimental conditions.

Fish (initial body weight 7.56 ± 0.6 g) were randomly distributed into 15 floating sea cages at 60 fish per cage (1 m × 1 m × 1.5 m), and then the five experimental diets were randomly allocated to triplicate groups ($n = 3$, $N = 15$). Fish were fed twice daily (05:00 and 17:00) to apparent satiation for 70 days. Water temperature, salinity and dissolved oxygen were measured daily, with temperature ranging from 24.0 to 30.0 °C, salinity from 32 to 36 ‰, and dissolved oxygen at about 7 mg l^{-1} for the duration of the experimentation.

At the termination of the experiment, all fish were fasted for 24 h, anesthetized by immersion in eugenol bath (1:10,000), counted and individually weighed. Blood samples were obtained from the caudal vasculature of five fish from each cage with 27-gauge needles and 1-ml syringes and allowed to clot at room temperature for 4 h and then at 4 °C for further 6 h. The clot was removed and residual blood cells were separated from the serum by centrifugation (836 g, 10 min, 4 °C). The serum was then frozen in liquid nitrogen and stored at −80 °C until subsequent analysis. The liver and kidney of five fish from each cage were sampled, pooled into 1.5-ml tubes (RNAase-Free, Axygen, USA), frozen in liquid nitrogen and then stored at −80 °C until subsequent analysis of gene expression associated with inflammation and fatty acid oxidation. Liver and equal pieces of muscle from another ten fish in each cage were pooled into 10-ml tubes, frozen in liquid nitrogen and then stored at −80 °C for analysis of fatty acid composition, moisture and crude lipid.

Chemical Analysis

Samples of muscle and liver were freeze dried to constant weight at −50 °C (Christ ALPHA 1–4 freeze dryer, Christ, Germany) to determine moisture content. Crude protein was determined by digestion using the Kjeldahl method (Kjeltec FOSS 2,300, Tecator, Sweden) and estimated by multiplying nitrogen by 6.25. Crude lipid was measured gravimetrically after ether extraction by the Soxhlet method (Soxhlet Extraction System B-811, BUCHI, Switzerland). The fatty acid profiles were analyzed using the procedures previously described by Metcalfe et al. [46] with minor modifications [43]. After extraction and *trans*-methylation, fatty acid methyl esters were separated, identified and quantified by gas chromatography, using an HP6890 (Agilent Technologies Inc., Santa Clara, California, USA) with a fused silica capillary column (007-CW, Hewlett Packard, Palo Alto, CA, USA) and a flame ionization detector. The oven temperature was programmed to rise from 150 up to 200 °C at a rate of 15 °C/min, from 200 to 250 °C at a rate of 2 °C/min, whilst the injector and the detector were both at 250 °C. The resulting peaks were corrected by the theoretical relative FID response factors [47], and then divided by the fatty acid methyl ester molecular weight. Fatty acids were eventually reported as mol% of total identified fatty acids.

Functional Immune Assay

Phagocytic index The phagocytic index (PI) was measured according to the method of Pulsford et al. [48] with some

Table 3 Real-time quantitative PCR primers used in the present study

Gene	Nucleotide sequence (5′–3′)	Size (bp)	Reference
COX-2	F ACCATCTGGCTGCGGGAAC R GAATGAGTCGTGTGGTCTGGAAG	100	JX520677
IL-β	F AGCCAATCTGGCAAGGATCA R GCTGATGAACCAGTTGTTGT	121	DQ306711
TNF-α	F CGTCCTGGTGTTTGCTTGGT R TGTTTTCTCGGCAGTCGTCTT	100	Zuo et al. [26]
CPTI	F GCTGAGCCTGGTGAAGATGTTC R TCCATTTGGTTGAATTGTTTACTGTCC	159	JX434612
ACO	F TTCTCAACGACCCAGACTTTAAGG R GTTGCCTCTGACCATATTCTTATAGC	167	JX456348
PPARα	F GTCAAGCAGATCCACGAAGCC R TGGTCTTTCCAGTGAGTATGAGCC	82	Zhao et al. [56]
FAS	F CAGCCACAGTGAGGTCATCC R TGAGGACATTGAGCCAGACAC	126	JX456351
ME	F TGCCCATCATTTACACTCCAAC R ATCCGTCACGCACACAGC	164	JX456349
G6PD	F ATGGCGGCAACTCTGAATAC R GCGATGCTCTGGAAGATGTC	184	JX456352
β-Actin	F TTATGAAGGCTATGCCCTGCC R TGAAGGAGTAGCCACGCTCTGT	107	Yao et al. [55]

modifications. Briefly, head kidneys of 6 fish per cage were collected, homogenized in a modified formula L-15 culture medium (10 μg/ml heparin; 200 U/ml penicillin/streptomycin; hepas 15 mmol/l; 1 % foetal bovine serum) and then filtered through 100 μm nylon mesh. The resulting cell suspensions were enriched by centrifugation (836 g, 25 min, 4 °C) on 34/51 % Percoll (Pharmacia, USA) density gradient. The cells were collected at the 34–51 % interface and washed twice using the L-15 culture medium, described above. 100 μl cell suspensions were stained with 100 μl trypan blue (0.4 %, Sigma, USA) at 23.5 °C for 1 min, and then examined under microscope (40×) to determine the cell concentration and viability. The cell viability was more than 95 % and final cell concentration was adjusted to approximately 1×10^7 leucocytes/ml. One-hundred μl cell suspensions of head kidney leucocytes and 100 μl yeast suspension (Bakers yeast, Type II, Sigma, USA, 1 × 108 cells/ml) were mixed in a 2 ml sized plastic tube and cultured at 23.5 °C for 40 min. To calculate the PI, an aliquot of the mixture was loaded into a hemacytometer (Shanghai Qiujing Biochemical Reagent and Apparatus Co., Ltd., Shanghai, China) and 200 cells were counted for each sample, and the number of cells containing ingested yeast was recorded.

Respiratory burst activity The production of intracellular superoxide anion (O^{2-}) was assessed for five fish per cage by nitroblue tetrazolium (NBT) (Sigma, USA) reduction following the method of Secombes [49], with minor modifications. Specifically, a 100 μl cell suspension of head kidney was stained with 100 μl NBT (1 mg/ml) and 100 μl phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) (1 μg/ml) for 45 min, which was used to induce O^{2-} production. Absolute (100 %) methanol was added to terminate the staining. Each tube was washed three times with 70 % methanol and air-dried. Then, 120 μl 2 M KOH and 140 μl dimethyl sulfoxide (DMSO, Sigma, USA) were added, and absorbance was measured at 630 nm with a spectrophotometer using KOH/DMSO as a blank.

Lysozyme activity The lysozyme activity in serum was measured according to the method described by Ellis [50]. Briefly, a sample of 0.05 ml serum was added to 1.4 ml of a suspension of Micrococcus lysodeikticus (Sigma) (0.2 mg/ml) in 0.1 M sodium phosphate buffer (pH 6.8). The reaction was carried at 25 °C and absorbance was measured at 530 nm after 0.5 and 4.5 min incubation. Each unit was defined as the amount of sample causing a decrease in absorbance of 0.001/min.

Hepatic antioxidant capacity assays

Liver samples were first ground into powder in liquid nitrogen, homogenized in 9 volumes of ice cold phosphate solution (pH 7.4), and then centrifuged at 4,000 g for 10 min at 4 °C. Total soluble protein was determined by the method described by Bradford [51] using bovine serum albumin (BSA) as standard. Samples were stored at −80 °C until subsequent use for the assay of antioxidant capacity.

Superoxide dismutase (SOD) activity Superoxide dismutase activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer

(pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome C and 0.024 IU/ml xanthine oxidase. The reaction was triggered by the addition of the xanthine oxidase. Results were expressed in units of SOD per milligram soluble protein and each unit was defined as the amount of enzyme necessary to produce 50 % inhibition of the ferricytochrome C reduction rate measured at 550 nm [52].

Total antioxidant capacity (T-AOC) A commercial kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) was used to assay T-AOC. Briefly, the method is based on the reduction of Fe^{3+}–Fe^{2+} and subsequent Fe^{2+}-phenanthroline complex formation. Absorbance was read at 520 nm with distilled H_2O as blanks. Results were expressed in units of T-AOC per milligram soluble protein and each unit was defined as the amount of enzyme which increased the absorbance by 0.01/min at 37 °C.

Catalase (CAT) activity Hepatic CAT was assayed using the commercial kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China). Briefly, after 1 min CAT reaction with H_2O_2, sulphuric acid (2 mol/l) was quickly added to stop the reaction [53]. The remaining H_2O_2 was quantified by reaction with ammonium molybdate, which generate a light-yellow complex. The CAT activity was calculated by assaying the absorbance at 405 nm, with distilled H_2O as blanks. Results were expressed in units of CAT per mg soluble protein, and each unit was defined as the amount of enzyme necessary to resolve one μmol H_2O_2/sec at 37 °C.

Malondialdehyde (MDA) content MDA was measured using the thiobarbituric acid (TBA) assay kit (Nanjing Jiancheng Bio-engineering Institute, China). Briefly, MDA reacts with thiobarbituric acid to produce a pink-colored material that can be readily measured spectrophotometrically to give an overall indication of the level of lipid peroxidation [54]. For each replicate sample, 100 μl supernatant of liver homogenate was incubated with 20 % trichloroacetic acid and 0.67 % thiobarbituric acid at 95 °C for 40 min. Absorbance was then read at 532 nm, with distilled H_2O as blanks. Results were converted to nmol/mg of MDA, after calibration using a standard sample of 10 nmol/ml malonaldehyde diethyl acetate.

RNA extraction and real-time quantitative PCR Total RNA was extracted from liver and kidney using Trizol Reagent (Invitrogen, USA) and electrophoresed on a 1.2 % denaturing agarose gel to determine RNA quality. The RNA was treated with RNA-Free DNase (Takara, Japan) to remove contaminating DNA and reverse transcribed to cDNA by PrimeScriptTM RT reagent Kit (Takara, Japan). First strand cDNA was diluted four times using sterilized, double-distilled water. Real-time quantitative PCR was carried out in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany). The amplification was performed in a total volume of 25 μl, containing 1 μl of each primer (10 μM), 1 μl of the diluted first strand cDNA product, 12.5 μl of 2 × SYBR® Premix Ex TaqTM^{II} (Takara, Japan) and 9.5 μl of sterilized double-distilled water. The real-time quantitative PCR program was as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s. The primer sequence for β-actin, COX-2, IL-β, TNF-α, CPTI, ACO and PPARα were designed following the published sequences from large yellow croaker on gene bank or published papers [55, 56] and listed in Table 3. PCR fragments amplified by each pair of primers were sequenced, blasted and analyzed to assure the specificity of each primer pair. At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Standard curves were made with five different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analyzed according to the following equation $E = 10^{(-1/\text{Slope})}-1$. The primer amplification efficiency was 1.020 for β-actin, 1.004 for COX-2, 0.9954 for IL-β, 1.0188 for TNF-α, 1.1190 for CPTI, 1.0813 for ACO and 0.9924 for PPARα. The absolute ΔC_T values between the target gene and inner control gene were all close to zero and indicate that $\Delta\Delta C_T$ calculation for the relative quantification of target genes could be used. To calculate gene expression, the comparative CT method ($2^{-\Delta\Delta t}$ method) was used as described by Yao et al. [55].

Calculations and Statistical Analysis

Growth parameters were calculated using standard formulae, including weight gain rate (WGR, %), feed intake (FI, %/d), feed conversion ratio (FCR), survival rate (SR, %), hepatosomatic index (HSI, %), and viscerosomatic index (VSI, %).

All data were subjected to a one-way analysis of variance (ANOVA) and differences between means were tested by Tukey's multiple range test. A significant level of $P < 0.05$ was used for all statistical tests, and the results were presented as mean ± SEM (standard error of the mean) ($n = 3$). All statistical analyses were performed by SPSS 16.0 for Microsoft Windows® (Redmond, WA, USA).

Results

Survival Rate, Growth Performance and Somatic Indices

The experimental diets were iso-nitrogenous and iso-lipidic (Table 1), and varied only in their fatty acid composition. The CD was the highest in n-3 LC-PUFA (19.21 %), whilst the four plant oil- based diets contained a similar amount of n-3 LC-PUFA (~7 %) with ALA/LNA ratios of 0.03, 0.45, 0.90 and 1.51, for R-0.0, R-0.5, R-1.0

and R-1.5, respectively (Table 2). All diets were readily accepted by the fish (Table 4). No statistically significant differences in feed intake or feed efficiency ratio (FER) were detected among dietary treatments. Survival rate of experimental fish increased significantly from 92.8 to 98.3 % as dietary ALA/LNA increased from 0.0 to 0.5, and then decreased to 93.4 % with further increase of this ratio. Significantly lower final body weight (FBW) and weight gain rate (WGR) were observed in R-0.0 compared to other experimental diets. VSI of fish in R-0.0 was the highest (6.73 %), and then significantly decreased to a value comparable with that in CD (6.34 %) as dietary ALA/LNA increased to 1.5. As dietary ALA/LNA increased from 0.0 to 0.5, HSI increased from 1.65 to 1.90 %, and then decreased to 1.38 %, comparable with that in CD, with further increase of this ratio to 1.5 (Table 4).

Immunological and Antioxidant Parameters

As dietary ALA/LNA increased from 0.0 to 1.0, the phagocytic index (PI) increased significantly from 30.1 to 41.5 % comparable to that in fish fed CD (44.0 %), and then decreased to 19.2 % in fish fed R-1.5. The respiratory burst activity (RBA) and serum lysozyme activity (LMA) showed a similar trend to that recorded for PI, relative to dietary ALA/LNA increase. Respiratory burst activity for CD (RBA = 0.71) and R-1.0 (RBA = 0.68) was significantly higher than that in R-0.0 (RBA = 0.40) and R-1.5 (RBA = 0.45). LMA in FO (172.00 unit ml^{-1}) and R-1.0 (168.00 unit ml^{-1}) was significantly higher than that in R-0.0 (100.47 unit ml^{-1}) and R-1.5 (104.50 unit ml^{-1}). No statistically significant differences were observed for serum SOD among the five dietary treatments (Table 5).

Catalase (CAT) in the liver of CD was significantly lower than that in R-0.0, R-0.5 and R-1.0. Hepatic CAT activity increased significantly from 133.09 to 163.37 U/mg protein as dietary ALA/LNA increased from 0.0 to 0.5 and decreased with further increase of ALA/LNA to 1.5. As dietary ALA/LNA increased, T-AOC activity in liver of fish fed the plant oil diets increased from 2.11 to 4.15 U/mg protein with increasing ALA/LNA in diet, all significantly higher than that in CD. Hepatic SOD and MDA content in CD was comparable with that in R-0.0. Hepatic SOD activity increased significantly from 176.42 to 276.49 U/mg protein as dietary ALA/LNA increased from 0.0 to 0.5, and decreased with further increase of this ratio. Hepatic MDA content decreased significantly from 6.08 to 3.72 nmol/mg protein when ALA/LNA increased from 0.0 to 1.0, and then increased to 5.37 nmol/mg protein when the ratio was 1.5 in diet (Table 6).

Proximate Composition of Liver and Muscle

Crude lipid of liver and muscle in CD (24.55 and 12.12 %) was significantly higher than that in fish fed the four plant oil based treatments (20.43–21.96 and 8.09–10.63 %, respectively). No statistically significant differences were detected in moisture or lipid content of liver and muscle relative to dietary ALA/LNA (Table 7).

Table 4 Growth, survival and biometrical parameters of large yellow croaker fed the experimental diets with different dietary ALA/LNA ratio (mean ± SEM, $n = 3$)

	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
FBW[1]	43.96 ± 0.79^{a}	35.99 ± 0.60^{b}	44.84 ± 0.73^{a}	47.30 ± 0.53^{a}	43.45 ± 1.45^{a}
WGR[2]	481.50 ± 10.46^{a}	376.07 ± 7.90^{b}	493.15 ± 9.59^{a}	525.63 ± 7.04^{a}	474.76 ± 19.14^{a}
SR[3]	94.10 ± 1.21^{ab}	92.8 ± 0.56^{a}	98.3 ± 0.96^{b}	93.3 ± 0.98^{ab}	93.4 ± 1.92^{ab}
FI[4]	20.11 ± 1.02	19.88 ± 0.98	21.45 ± 0.83	22.07 ± 1.15	20.29 ± 0.94
FER[5]	0.91 ± 0.05	1.14 ± 0.08	1.06 ± 0.11	1.01 ± 0.09	1.11 ± 0.15
VSI[6]	6.34 ± 0.09^{ab}	6.73 ± 0.09^{c}	6.61 ± 0.10^{bc}	6.70 ± 0.07^{bc}	6.27 ± 0.02^{a}
HSI[7]	1.37 ± 0.02^{a}	1.65 ± 0.01^{b}	1.90 ± 0.05^{c}	1.77 ± 0.05^{bc}	1.38 ± 0.04^{a}

Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$

[1] *FBW* final body weight (g)

[2] *WGR* weight gain rate (%) = 100 × [(FBW−IBW)/IBW]

[3] *SR* survival rate (%) = 100 × final fish number/initial fish number

[4] *FI* feed intake (g/kg average body weight/day) = feed consumed (g)/[(IBW+FBW)/2/1,000 (kg)]/days

[5] *FER* feed efficiency ratio = wet weight gain (g)/dry feed consumed (g)

[6] *VSI* viscerosomatic index, (%) = 100 × (visceral weight/bodyweight)

[7] *HSI* hepatosomatic index, (%) = 100 × (liver weigh/body weight)

Table 5 Immunological parameters of large yellow croaker fed the experimental diets with different dietary ALA/LNA ratio (mean ± SEM, $n = 3$)

	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
PI[1]	44.0 ± 1.7^{a}	30.1 ± 2.9^{bc}	37.5 ± 3.8^{ab}	41.5 ± 2.7^{ab}	19.2 ± 3.4^{c}
RBA[2]	0.71 ± 0.02^{a}	0.40 ± 0.02^{b}	0.55 ± 0.01^{cd}	0.68 ± 0.04^{ad}	0.45 ± 0.02^{bc}
LMA[3]	172.00 ± 3.46^{a}	100.47 ± 9.40^{b}	133.50 ± 9.50^{ab}	168.00 ± 8.00^{a}	104.50 ± 5.00^{b}
SOD[4]	102.98 ± 3.42	101.88 ± 9.28	92.50 ± 11.14	96.97 ± 4.58	91.42 ± 7.84

Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$

[1] *PI* phagocytic index (%) = leucocytes with ingested yeast/200 observed leucocytes under microscope

[2] Respiratory burst of activity

[3] *LMA* lysozyme activity (unit ml^{-1})

[4] *SOD* superoxide dismutase (unit ml^{-1})

Table 6 Hepatic anti-oxidative parameters of large yellow croaker fed experimental diets with different dietary ALA/LNA ratio (mean ± SEM, $n = 3$)

	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
SOD[1]	164.79 ± 2.90^{a}	176.42 ± 7.09^{a}	276.49 ± 31.78^{b}	200.22 ± 11.27^{a}	222.55 ± 7.40^{ab}
MDA[2]	6.14 ± 0.33^{a}	6.08 ± 0.59^{a}	4.21 ± 1.25^{ab}	3.72 ± 0.69^{b}	5.37 ± 0.71^{ab}
T-AOC[3]	0.58 ± 0.07^{a}	2.11 ± 0.46^{b}	3.80 ± 0.24^{b}	3.31 ± 1.24^{b}	4.15 ± 1.75^{b}
CAT[4]	71.84 ± 4.94^{a}	133.09 ± 14.02^{bc}	163.37 ± 8.09^{b}	131.56 ± 8.48^{bc}	106.20 ± 11.63^{ac}

Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$

[1] *SOD* superoxide dismutase (unit mg^{-1} protein)

[2] *MDA* malondialdehyde (nmol mg^{-1} protein)

[3] *T-AOC* total anti-oxidative capacity (unit mg^{-1} protein)

[4] *CAT* catalase (unit mg^{-1} protein)

Table 7 Moisture and lipid content (percentage of live weight) in liver and muscle of large yellow croaker fed experimental diets with different ALA/LNA ratio (mean ± SEM, $n = 3$)

Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$

	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
Liver					
Moisture	60.14 ± 0.99	63.93 ± 3.19	60.46 ± 0.69	61.39 ± 3.59	63.65 ± 0.93
Lipid	24.55 ± 2.11^{a}	20.43 ± 3.16^{b}	21.96 ± 0.25^{b}	21.46 ± 2.73^{b}	21.64 ± 2.09^{b}
Muscle					
Moisture	70.68 ± 0.24	74.59 ± 1.47	71.86 ± 0.16	73.58 ± 0.16	73.69 ± 1.29
Lipid	12.12 ± 0.25^{a}	8.09 ± 0.13^{b}	10.63 ± 0.71^{b}	9.44 ± 0.50^{b}	9.99 ± 0.19^{b}

Fatty Acid Profile in Liver and Muscle

In liver and muscle, LNA and total n-6 PUFA increased significantly in R-0.0 compared to CD, but decreased significantly with the increase of dietary ALA/LNA. The concentration of ALA, n-3 PUFA, the ALA/LNA and n-3/n-6 PUFA ratios in liver and muscle increased significantly with the increase of dietary ALA/LNA ratio. On the contrary, the content of LNA and n-6 PUFA recorded the opposite trend as dietary ALA/LA increased (Tables 8, 9).

The LC-PUFA (primarily EPA, DHA and ARA) contents of liver and muscle were significantly higher in fish fed CD compared to the other diets. The n-3 LC-PUFA of liver and muscle in fish fed the four plant oil treatments were about half of that observed with CD (6.5 and 10.7 mol%). However, no statistically significant differences were detected in the n-3 LC-PUFA content of liver and muscle relative to dietary ALA/LNA ratio between these diets (Tables 8, 9). ARA/EPA in liver first increased from 1.3 to 1.5 % as dietary ALA/LNA ratio increased from 0.0 to 1.0, and then

Table 8 Fatty acid composition (mol%) in the liver of juvenile large yellow croaker fed the diets with different ALA/LNA ratio (mean ± SEM, $n = 3$)

	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
14:0	5.1 ± 0.6^{a}	1.9 ± 0.0^{b}	1.9 ± 0.0^{b}	1.8 ± 0.1^{b}	2.1 ± 0.3^{b}
16:0	26.1 ± 1.9^{a}	16.8 ± 0.5^{b}	21.5 ± 0.4^{c}	23.4 ± 0.6^{ac}	19.4 ± 1.6^{bc}
18:0	0.2 ± 0.0^{a}	3.1 ± 0.1^{b}	3.1 ± 0.1^{b}	3.0 ± 0.2^{b}	4.1 ± 0.3^{b}
20:0	2.1 ± 0.4^{a}	0.2 ± 0.0^{b}	0.2 ± 0.1^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}
∑SFA[1]	33.5 ± 2.5^{a}	21.9 ± 4.3^{b}	26.8 ± 0.3^{bc}	28.4 ± 0.5^{c}	25.8 ± 0.8^{d}
16:1	29.5 ± 3.9^{a}	5.7 ± 0.1^{b}	6.7 ± 0.2^{b}	8.6 ± 0.3^{c}	7.7 ± 0.6^{c}
18:1	14.4 ± 1.0^{a}	15.8 ± 3.7^{a}	35.6 ± 0.9^{c}	37.9 ± 1.3^{c}	31.2 ± 0.6^{b}
∑MUFA[2]	43.9 ± 3.9^{a}	21.6 ± 3.6^{b}	42.3 ± 1.1^{a}	46.5 ± 1.4^{a}	39.0 ± 0.9^{ab}
18:2n-6 (LNA)	13.3 ± 1.1^{a}	40.4 ± 0.8^{b}	22.8 ± 1.3^{c}	20.2 ± 0.8^{c}	19.2 ± 1.7^{c}
20:4n-6	0.4 ± 0.0^{a}	1.7 ± 0.1^{b}	1.8 ± 0.1^{b}	1.7 ± 0.0^{b}	1.7 ± 0.0^{b}
∑n-6PUFA	13.7 ± 1.1^{a}	42.1 ± 0.8^{b}	24.6 ± 1.3^{c}	22.0 ± 0.6^{c}	20.9 ± 0.8^{c}
18:3n-3 (ALA)	2.8 ± 0.1^{a}	1.1 ± 0.0^{a}	7.1 ± 0.9^{b}	9.9 ± 0.8^{c}	15.7 ± 2.0^{d}
20:5n-3	3.9 ± 0.3^{a}	1.3 ± 0.1^{b}	1.3 ± 0.0^{b}	1.2 ± 0.0^{b}	1.3 ± 0.2^{b}
22:6n-3	2.2 ± 0.1^{a}	1.5 ± 0.0^{b}	1.3 ± 0.5^{b}	1.2 ± 0.1^{c}	1.3 ± 0.1^{bc}
∑n-3PUFA	8.9 ± 0.4^{a}	3.8 ± 0.1^{b}	9.7 ± 0.6^{a}	12.3 ± 0.9^{c}	18.3 ± 0.9^{d}
ALA/LNA	0.2 ± 0.0^{a}	0.0 ± 0.0^{b}	0.3 ± 0.0^{a}	0.5 ± 0.0^{c}	0.8 ± 0.3^{d}
n-3/n-6PUFA	0.7 ± 0.0^{a}	0.1 ± 0.0^{b}	0.4 ± 0.1^{c}	0.6 ± 0.0^{a}	0.9 ± 0.3^{d}
n-3LC-PUFA[3]	6.1 ± 0.3^{a}	2.8 ± 0.1^{b}	2.6 ± 0.5^{b}	2.4 ± 0.2^{b}	2.6 ± 0.2^{b}
ARA/EPA	0.1 ± 0.0^{a}	1.3 ± 0.1^{b}	1.4 ± 0.3^{b}	1.5 ± 0.8^{b}	1.3 ± 0.2^{b}
DHA/EPA	0.6 ± 0.1^{a}	1.2 ± 0.1^{b}	1.0 ± 0.4^{b}	1.1 ± 0.1^{b}	1.0 ± 0.2^{b}
DBI[5]	113.3 ± 0.5^{a}	127.6 ± 4.2^{a}	128.8 ± 3.3^{a}	123.8 ± 3.5^{a}	153.1 ± 4.4^{b}

Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$; Some fatty acids, of which the contents were minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C20:3n-6, C22:5n-3, are not listed in the Table

[1] *SFA* saturated fatty acids

[2] *MUFA* mono-unsaturated fatty acids

[3] *n-3 LC-PUFA* n-3 highly-unsaturated fatty acids, EPA+DHA

[4] *DBI* ∑ % of unsaturated fatty acids×number of double bonds of each unsaturated fatty acid

Table 9 Fatty acid composition (mol%) in the muscle of juvenile large yellow croaker fed the diets with different ALA/LNA ratio (mean ± SEM, $n = 3$)

	Dietary treatments				
	CD	R-0.0	R-0.5	R-1.0	R-1.5
14:0	5.4 ± 0.2^{a}	2.3 ± 0.1^{b}	2.3 ± 0.1^{b}	2.5 ± 0.0^{b}	2.3 ± 0.1^{b}
16:0	23.3 ± 0.1^{a}	17.7 ± 0.2^{b}	22.0 ± 0.5^{a}	23.0 ± 0.5^{a}	17.5 ± 0.5^{b}
18:0	3.1 ± 0.2^{a}	4.4 ± 0.1^{ab}	5.7 ± 0.9^{b}	4.8 ± 0.4^{b}	4.8 ± 0.4^{b}
20:0	1.7 ± 0.1^{a}	0.1 ± 0.0^{b}	0.2 ± 0.1^{b}	0.3 ± 0.0^{b}	0.2 ± 0.0^{b}
∑SFA[1]	33.5 ± 0.2^{a}	24.6 ± 0.3^{b}	30.2 ± 0.5^{c}	30.6 ± 0.9^{ac}	24.7 ± 0.7^{b}
16:1	27.1 ± 1.2^{a}	4.2 ± 0.1^{b}	5.1 ± 0.1^{b}	5.3 ± 0.4^{b}	4.7 ± 0.0^{b}
18:1	14.1 ± 1.7^{a}	23.4 ± 0.3^{b}	26.3 ± 1.8^{b}	27.2 ± 0.8^{b}	22.54 ± 0.4^{b}
∑MUFA[2]	41.1 ± 1.3^{a}	27.7 ± 0.3^{b}	31.4 ± 1.9^{b}	32.6 ± 1.2^{b}	27.3 ± 0.4^{b}
18:2n-6 (LNA)	12.0 ± 0.6^{a}	39.1 ± 0.6^{b}	22.7 ± 2.6^{c}	15.1 ± 0.8^{d}	16.04 ± 0.4^{d}
20:4n-6	0.5 ± 0.0^{a}	1.6 ± 0.1^{b}	1.7 ± 0.1^{b}	1.6 ± 0.1^{b}	1.6 ± 0.1^{b}
∑n-6PUFA	12.5 ± 0.6^{a}	40.7 ± 0.6^{b}	24.4 ± 2.5^{c}	16.74 ± 0.8^{d}	17.7 ± 0.3^{d}
18:3n-3 (ALA)	2.7 ± 0.1^{a}	1.3 ± 0.0^{a}	8.4 ± 1.0^{b}	14.2 ± 1.0^{c}	23.9 ± 0.7^{d}
20:5n-3	5.9 ± 0.3^{a}	2.7 ± 0.0^{b}	2.7 ± 0.0^{b}	2.9 ± 0.2^{b}	3.0 ± 0.1^{b}
22:6n-3	4.4 ± 0.3^{a}	3.0 ± 0.1^{b}	3.0 ± 0.1^{b}	3.1 ± 0.1^{b}	3.4 ± 0.1^{b}
∑n-3PUFA	12.9 ± 0.6^{a}	7.1 ± 0.1^{b}	14.0 ± 1.1^{a}	20.1 ± 1.3^{c}	30.3 ± 0.7^{d}
ALA/LNA	0.2 ± 0.0^{a}	0.0 ± 0.0^{b}	0.4 ± 0.1^{a}	0.9 ± 0.0^{c}	1.5 ± 0.0^{d}
n-3/n-6PUFA	1.0 ± 0.0^{a}	0.2 ± 0.0^{b}	0.6 ± 0.1^{c}	1.2 ± 0.0^{d}	1.7 ± 0.0^{e}
n-3LC-PUFA[3]	10.2 ± 0.5^{a}	5.8 ± 0.1^{b}	5.7 ± 1.1^{b}	6.0 ± 0.6^{b}	6.4 ± 0.2^{b}
ARA/EPA	0.1 ± 0.0^{a}	0.6 ± 0.0^{b}	0.6 ± 0.0^{b}	0.6 ± 0.1^{b}	0.6 ± 0.0^{b}
DHA/EPA	0.8 ± 0.0^{a}	1.1 ± 0.0^{b}	1.1 ± 0.0^{b}	1.1 ± 0.0^{b}	1.1 ± 0.1^{b}
DBI[5]	130.4 ± 3.0^{a}	148.1 ± 1.1^{b}	139.9 ± 1.3^{ab}	144.6 ± 3.7^{b}	173.2 ± 2.4^{c}

Mean values with the different superscript letters within the same row are significantly different at $P < 0.05$; Some fatty acids, of which the contents were minor, trace amount or not detected, such as C22:0, C24:0, C14:1, C20:1n-9, C22:1n-11, C20:2n-6, C20:3n-6, C22:5n-3, are not listed in the Table

[1] *SFA* saturated fatty acids

[2] *MUFA* mono-unsaturated fatty acids

[3] *n-3 LC-PUFA* n-3 highly-unsaturated fatty acids, EPA+DHA

[4] *DBI* ∑ % of unsaturated fatty acids×number of double bonds of each unsaturated fatty acid

decreased significantly to 1.3 % with further increase of the ratio to 1.1 ($P > 0.05$) (Table 8). There were no statistically significant differences in ARA and ARA/EPA of muscle as dietary ALA/LNA ratio increased (Table 9). Hepatic double bond index (DBI) was highest in R-1.5 (153.1 mol%), significantly higher than that in other four treatments (about 120.0 mol%) (Table 8). DBI of muscle in CD was significantly lower than that in other treatments except for R-1.0. Also, the highest DBI was observed in R-1.5 (173.2 mol%), significantly higher than all other treatments except for R-1.0 (Table 9).

Expression of Inflammation, Fatty Acid Oxidation and Synthesis Associated Genes

Transcription of COX-2, IL-1β and TNF-α in liver, kidney and muscle was increased by more than 1.0-fold in fish fed R-0.0 compared to CD, and then transcription of COX-2 and IL-1β decreased as dietary ALA/LNA increased. Hepatic transcription of COX-2 and TNF-α was significantly lower by more than 0.40-fold in R-0.5, R-1.0 and R-1.5 than for R-0.0. Hepatic mRNA levels of IL-1β significantly decreased by about 1.0-fold in R-0.5, R-1.0 and R-1.5 than that in R-0.0 (Fig. 1a). Transcription of COX-2 and IL-1β in kidney was decreased by more than 0.50-fold in R-1.0 and R-1.5 compared to R-0.0 fed fish, respectively (Fig. 1b). Transcription of COX-2 and IL-1β in muscle was decreased by more than 10-fold and 2-fold in treatments with ALA/LNA equal to or higher than 0.5 (Fig. 1c).

The transcription rates of CPTI and ACO in liver and kidney were more than 2.0-fold higher in R-0.0 than in fish fed CD and the plant oil diets in liver but not kidney, and then rates decreased to levels comparable to CD as dietary ALA/LNA was 0.5 or higher ($P < 0.05$) (Fig. 2a, b). PPARα mRNA levels in muscle significantly decreased as dietary ALA/LNA increased from 0.0 to 1.5. There were no statistically significant differences in transcription of CPT I and ACO in muscle among the five dietary treatments (Fig. 2c).

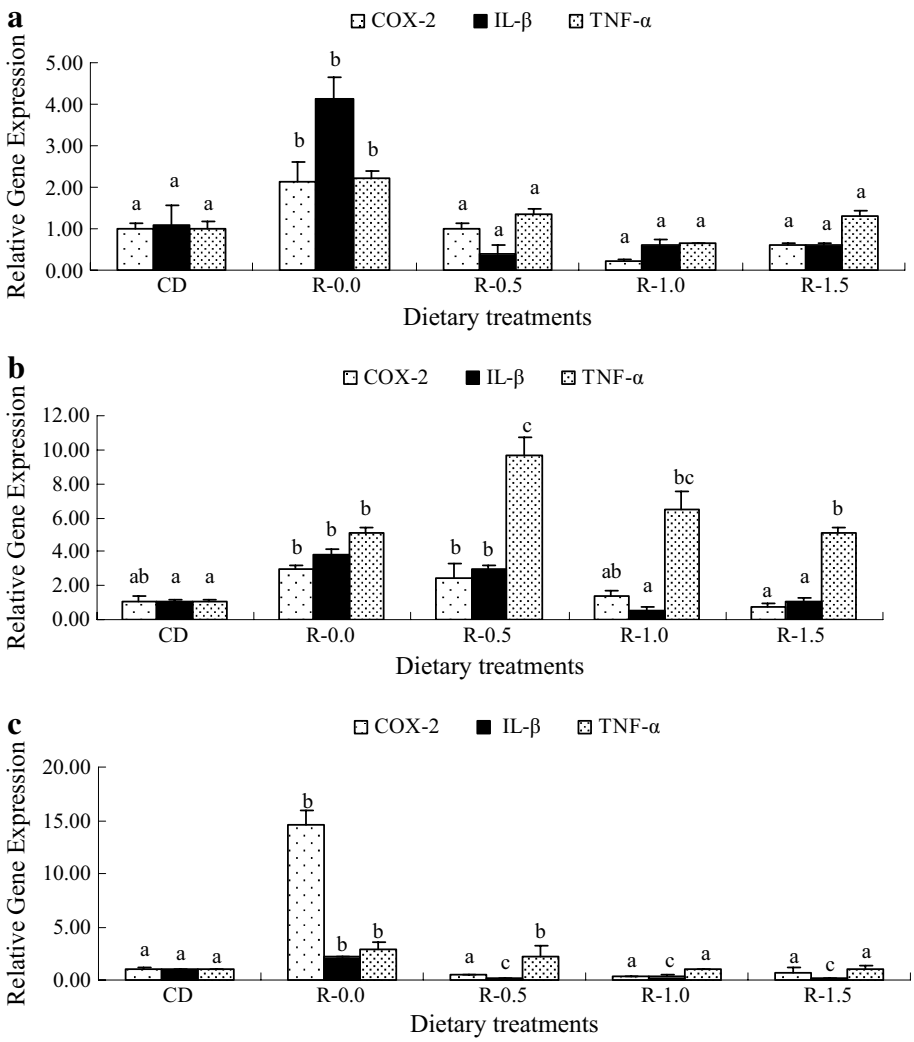

Fig. 1 Effects of dietary ALA/LNA ratio (18:3n-3/18:2n-6) on relative mRNA expression of inflammation associated genes in the liver (**a**), kidney (**b**) and muscle (**c**) of juvenile large yellow croaker. Values are means ± SEM ($n = 3$). *Bars* bearing with different letters are significantly different by Tukey's test ($P < 0.05$). *COX-2* cyclooxygenase-2, *IL-β* interleukin beta, *TNF-α* tumor necrosis factor alpha

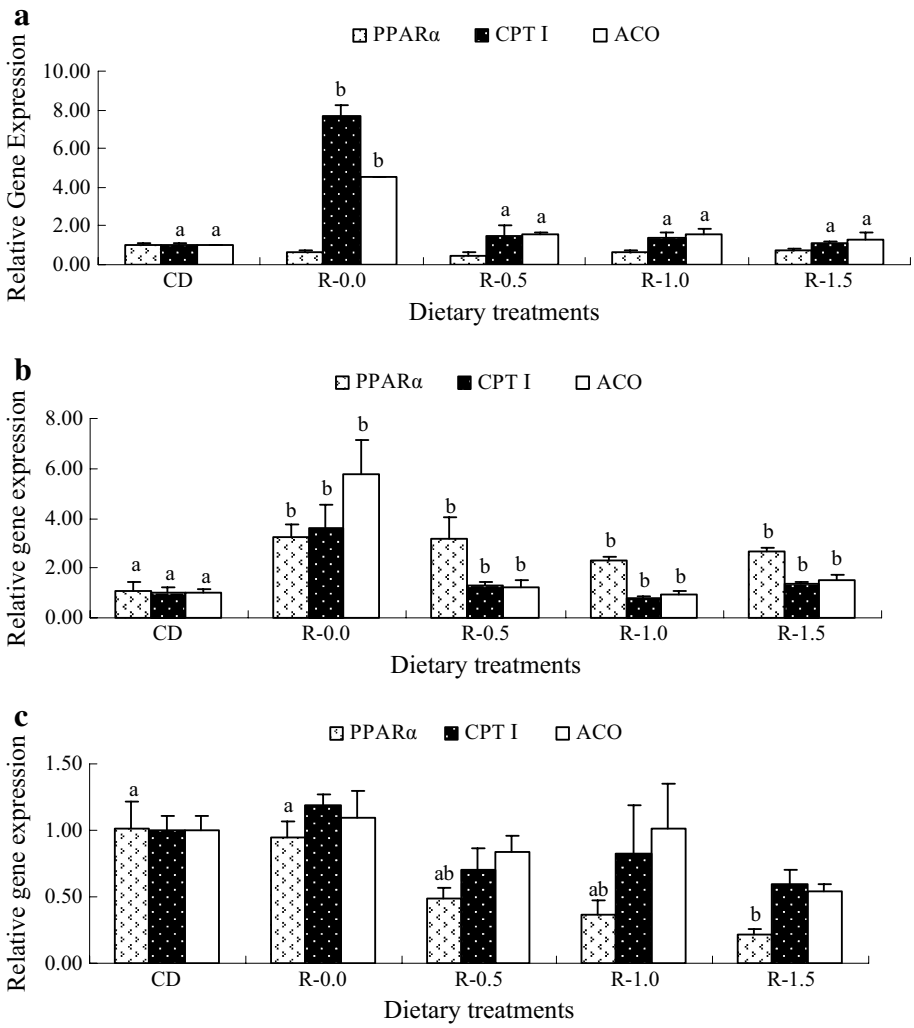

Fig. 2 Effects of dietary ALA/LNA ratio (18:3n-3/18:2n-6) on relative mRNA expression of fatty acid oxidation associated genes in the liver (**a**), kidney (**b**) and muscle (**c**) of juvenile large yellow croaker. Values are means ± SEM ($n = 3$). *Bars* bearing with different letters are significantly different by Tukey's test ($P < 0.05$). *PPARα* peroxisome proliferative activated receptor alpha, *CPTI* carnitine palmitoyl transferase I, *ACO* acyl coenzyme A oxidase

The transcription rates of FAS and ME in liver and kidney of yellow croaker were significantly higher by more than 1.0-fold compared to those fed CD, and then decreased to levels similar to CD as dietary ALA/LNA reached 1.0 and 1.5 (Fig. 3a, b). The transcription of FAS, ME and G6PD in muscle was decreased by about 0.50-fold in R-0.5, R-1.0 and R-1.5, compared to those fed R-0.0 (Fig. 3c).

Discussion

Performance of juvenile large yellow croaker fed the diet with the highest LNA content (R-0.0) was negatively impacted. This is consistent with previous findings with different marine finfish species which reported that high dietary LA coupled with low dietary LC-PUFA resulted in growth retardation in salmonids [19, 20], black sea bream [21], and gilthead sea bream [22, 23]. The vast majority of marine finfish have long been known to lack the capacity to bioconvert C_{18} PUFA (i.e., ALA and LNA) into LC-PUFA [57, 58]. In this study, no differences in either n-3 LC-PUFA or n-6 LC-PUFA were observed in tissues of yellow croaker fed the four experimental plant oil-based diets, despite large differences in dietary ALA and LNA supply. This confirms that the large yellow croaker is similarly incapable of converting C_{18} PUFA into LC-PUFA. Thus, the negative effects of high dietary LNA could be eliminated by increasing the ratio of dietary ALA to LNA rather than n-3 LC-PUFA to LNA.

When dietary ALA/LNA ratio increased to 0.5 or higher, the resulting n-3/n-6 PUFA ratio in liver and muscle increased to levels comparable to fish fed CD. On the other hand, the tissue n-3 LC-PUFA content was significantly higher in fish fed CD compared to fish fed the four plant oil based diets which is consistent with findings in other marine fish species [5–11]. Nevertheless, this study showed that yellow croaker can efficiently accumulate ALA in their tissues, which can help provide a more physiologically favorable n-3/n-6 PUFA ratio.

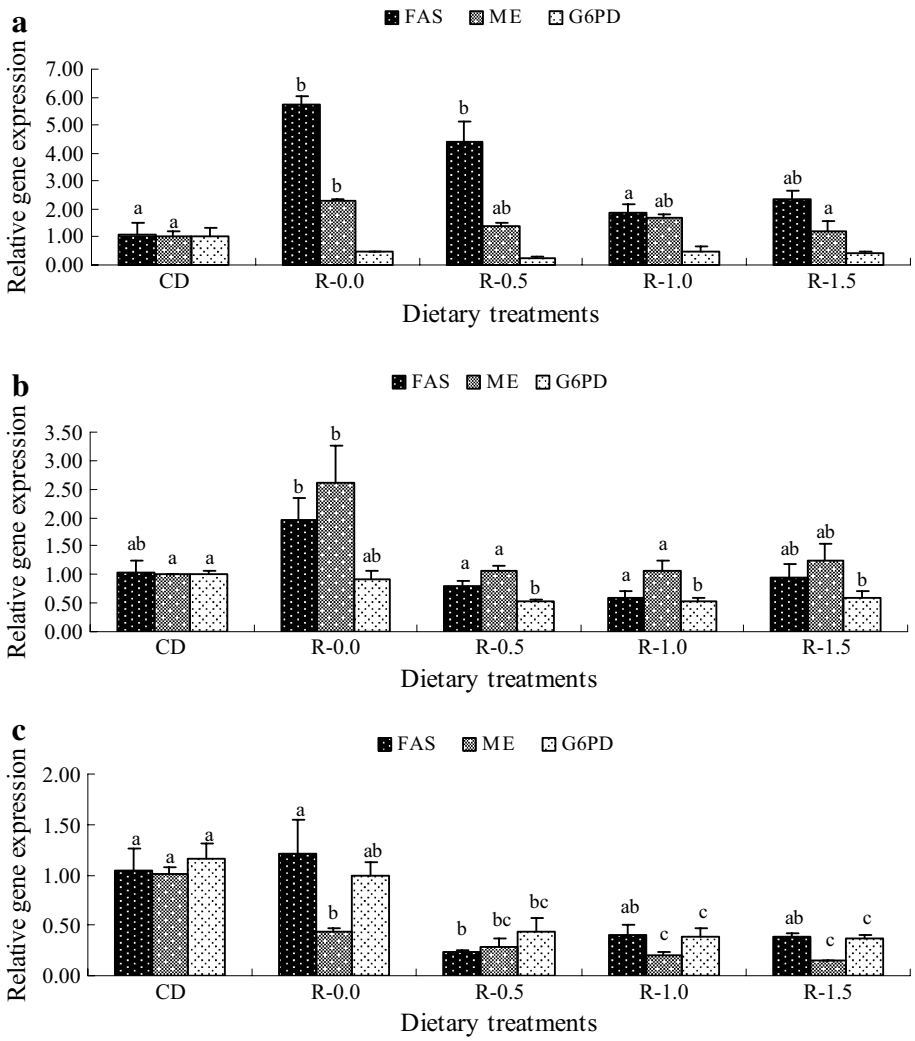

Fig. 3 Effects of dietary ALA/LNA ratio (18:3n-3/18:2n-6) on relative mRNA expression of fatty acid synthesis associated genes in the liver (**a**), kidney (**b**) and muscle (**c**) of juvenile large yellow croaker. Values are means ± SEM ($n = 3$). *Bars* bearing with different letters are significantly different by Tukey's test ($P < 0.05$). *FAS* fatty acid synthase, *ME* malic enzyme, G6PD 6-phosphogluconate dehydrogenase

The transcription rates of fatty acid synthesis associated genes (FAS, ME and 6PGD) in liver and muscle were all significantly lower as the dietary ALA/LNA ratio increased. Previous studies have shown that n-3 LC-PUFA and ALA rich diets inhibited the activities of FAS, G6PD and ME in carp, rainbow trout, Atlantic salmon and turbot [59, 60]. This could be accomplished by decreasing inflammation level reflected by transcription of COX-2, IL-1β and TNF-α, which has been found to be crossed linked with fatty acid synthesis [61]. In the present study, transcription of fatty acid oxidation associated genes was significantly higher in three tissues of fish fed R-0.0 compared to that fed CD and other three plant oil based diet. This was inconsistent with the findings of some previous studies which have found that dietary n-3 LC-PUFA increased mitochondrial fatty acid oxidation by stimulating the activity of CPT I [60, 62]. Compared to n-3 LC-PUFA and ALA, LNA is poorly oxidized and thus difficult to remove [1]. Thus, fatty acid oxidation transcription could be increased to remove the negative effects of high LNA deposition, which was about 40 mol% in both liver and muscle. Furthermore, the high rate of fatty acid oxidation in R-0.0 could be related to high inflammation, which has been found to contribute to oxidative stress [26].

Thus, the overall combined effect of the recorded modification of fatty acid synthesis (anabolism) and fatty acid oxidation (catabolism) was that lipid deposition of liver and muscle did not increase as dietary ALA/LNA ratio increased. This is inconsistent with the findings of a previous study which reported elevated lipid deposition in yellow catfish in response to increased dietary ALA/LNA ratio [34]. However, the yellow catfish is a carnivorous freshwater species, and this may account for the different response to dietary ALA/LNA ratio compared to the present study. On the other hand, the lipid deposition in yellow croaker fed the CD (fish oil based, rich in n-3 LC-PUFA) was significantly higher than those fed the four plant oil based diets. This is consistent with the findings of a previous study on gilthead sea bream which showed that dietary n-3 LC-PUFA increase body lipid retention [30]. Thus, it

seems plausible to conclude that dietary n-3 LC-PUFA play a greater role, compared to dietary ALA, in modulating lipid deposition.

In mammals, dietary ALA has been reported to suppress inflammation, although to a much lower extent when compared to n-3 LC-PUFA [63]. However, it is unclear whether ALA could play a direct role in modulating inflammation, or if the effects recorded in mammals were due to its partial conversion to n-3 LC-PUFA [37, 64]. In the present study, phagocytic index, respiratory burst and lysozyme activity were all significantly lower in fish fed diets with the highest dietary LNA (R-0.0) content, compared to CD, R-0.5 and R-1.0. It should be noted that transcription rates of inflammation associated genes (COX-2, TNF-α and IL-1β) in kidney and liver were significantly higher in fish fed R-0.0 compared to the other diets. Accordingly, a series of published studies clearly showed that high dietary LNA could exert deleterious effects on health of gilthead sea bream, by directly affecting the fatty acid composition of immune cells, altering eicosanoids production, and even chronically increasing the basal expression of inflammation associated genes [13, 22, 23, 65, 66]. Thus, it is possible that higher levels of inflammation could account for the low nonspecific immunological parameters of fish fed R-0.0. The results of the present study suggest that, independently to total dietary n-3 LC-PUFA availability, the increase of the dietary ALA/LNA ratio (0.5 or 1.0) may benefit fish immunity by modulating inflammation in lowering the transcriptional levels of COX-2 and IL-1β to a level similar to that recorded in fish fed high dietary n-3 LC-PUFA (CD). However, it should be admitted that the present study cannot differentiate between the possibly beneficial effects of a higher dietary ALA, or lower dietary LNA.

Large yellow croaker fed the diet with the highest dietary ALA/LNA ratio (R-1.50) showed significantly lower activities for the immunological parameters measured in this study, although the transcription rates of inflammation genes were comparable with that observed in CD. Previous studies on rodents [67] and non-human primates [68] showed an immune suppressive effect of dietary ALA, which was suggested to be accomplished by decreased α-tocopherol in the blood. In the present study, α-tocopherol content was not measured and therefore no speculation could be done relative to the possible effects of dietary ALA/LA ratio and α-tocopherol in the blood. Nevertheless, the significantly lower antioxidant capacity (lower activity of SOD and CAT, as well as higher MDA content in serum) and higher hepatic double bond index (DBI) of fish fed diet with the highest ALA/LA ratio (R-1.5), warrant future studies on the effects of dietary ALA and the possible modified α-tocopherol content in the blood, which could affect the antioxidant activity and the overall immune status in fish species.

The recorded antioxidant activity of yellow croaker fed CD was significantly lower than what would be expected, considering that in mammals dietary n-3 LC-PUFA can improve antioxidant defense [69]. This could be attributed to that the higher levels of n-3 LC-PUFA in tissues of fish fed CD [45]. Since the same amount of vitamin E was included in all diets, it is possible that the imbalance in the ratio of n-3 LC-PUFA to vitamin E could have accelerated the oxidation of excess n-3 LC-PUFA into ROS [70, 71] and caused increased activities of antioxidant enzymes (SOD, CAT and T-AOC) and MDA production.

To conclude, the present study showed that when dietary fish oil is replaced with plant oil blends to produce a dietary ALA/LNA ratios of 0.5–1.0, there is a beneficial effect on fish growth performance, nonspecific immunity and hepatic antioxidant capacity. Dietary ALA/LNA ratio modulated the mRNA expression of inflammation associated genes (COX-2 and IL-1β), fatty acid oxidation associated genes (PPARα, CPT I and ACO) and fatty acid synthesis associated genes (FAS, G6PD and ME). Although ALA is not considered an essential fatty acid for marine fish species, it has similar efficacy as n-3 LC-PUFA in the modulation of inflammation and oxidative stress in large yellow croaker. Further study is needed to investigate the effects of this important fatty acid on other metabolic processes such as lipid transportation and distribution in marine finfish.

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