Effects of oxidised dietary fish oil and high-dose vitamin E supplementation on growth performance, feed utilisation and antioxidant defence enzyme activities of juvenile large yellow croaker (*Larmichthys crocea*)

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Abstract

This study was conducted to elucidate the effects of oxidised dietary lipids and high-dose vitamin E (VE) on growth performance and immune responses of large yellow croaker. Juvenile fish (initial average body weight of 7·82 (SEM 0·68) g) were fed diets containing either fresh fish oil (fresh diet, peroxide value (POV) = 1·72 mEq/kg) or fish oil oxidised to varying degrees (oxidised diets, POV = 28·29–104·21 mEq/kg), with or without supplementary 600 mg VE/kg diet, for 10 weeks in floating cages. Growth was significantly lower and feed intake (g/100 g body weight per d) was higher in fish fed the oxidised diet. Supplementation with VE increased the growth of fish fed the oxidised diets, but significantly decreased the growth of fish fed the fresh diet. Hepatosomatic index increased with increasing dietary POV and decreased with VE supplementation. Hepatic catalase activity, superoxide dismutase (SOD) activity and malondialdehyde content were significantly higher in fish fed the oxidised diets, and these values decreased significantly following VE supplementation. However, hepatic SOD activity was enhanced by VE supplementation in fish fed the fresh diet. Air-exposure mortality was significantly increased by dietary POV, and this effect was inhibited by VE supplementation. These results suggest that dietary oxidised fish oil could stimulate the activities of antioxidant defence enzymes in stressed large yellow croaker. High-dose VE supplementation can alleviate oxidative stress of large yellow croaker fed oxidised fish oil, but can exert deleterious effects on fish in the absence of oxidative stress.

Key words: Large yellow croaker: *Larmichthys crocea*: Oxidised fish oil: Vitamin E: Oxidative stress

Fish oil contains high amounts of *n*-3 highly unsaturated fatty acids (HUFA) such as EPA (20 : 5*n*-3) and DHA (22 : 6*n*-3), which are essential to many marine fish species because of deficiencies in the desaturation and elongation pathways necessary for their biosynthesis[1,2]. However, fatty acids are particularly susceptible to oxidation during feed processing and storage, especially in the absence of adequate amounts of antioxidants[3]. Oxidised fish oil is harmful to fish[4–6].

Vitamin E (VE) is one of the most extensively used antioxidants in foods and has been shown to reduce oxidative stress in fish[6–11]. A higher dose of VE is required in fish diets, particularly those with high levels of HUFA, because dietary lipid oxidation can reduce the VE content[7,12,13]. Moreover, several times the optimum dietary VE level is needed to enhance the non-specific immune responses of fish[14]. However, high doses of VE may exert pro-oxidative effects on human LDL and lipids in fish tissues[15–17].

In commercial situations, diets are often stored at room temperature for long periods of time, resulting in the oxidation of dietary lipids to varying degrees. Many previous studies have supplemented diets containing identical lipid oxidation values with graded levels of VE to evaluate optimal dietary VE concentrations under conditions of oxidative stress[4,6,8,11,18,19]. However, there is a lack of information with respect to the effects of VE under conditions of varying degrees of oxidative stress. The effects of the severity of oxidative stress on the role of dietary VE in marine fish are unclear.

Large yellow croaker (*Larmichthys crocea*) is a major commercially important marine fish in China and has been widely cultured in recent years. As a carnivorous species, its dietary lipid content is relatively high[20]. In subtropical and tropical regions such as southeast China, the climate is humid and the temperature is relatively high throughout the year, especially during the summer. Under such climatic conditions, it is probable that dietary

lipids would be easily oxidised to different degrees depending upon the conditions under which the diets are stored.

Therefore, in the present study, graded oxidative stress in juvenile large yellow croaker was induced by feeding diets in which the fish oil was oxidised to various degrees. High-dose (600 mg/kg diet) VE was supplemented in the diet to determine the effects on growth performance, activities of hepatic antioxidant defence enzymes and head kidney macrophages respiratory burst activity of fish suffering various stressful conditions. The purpose of this study was to investigate the combined effects of variable degrees of lipid oxidation and high-dose VE supplementation on oxidative-stressed marine fish.

Methods

Diet preparation

White fishmeal and menhaden fish oil without antioxidant were used as the main protein and lipid sources in the diet (Table 1). The peroxide value (POV) of fresh fish oil (FFO) was 1·72 mEq/kg. FFO was oxidised to three different degrees (low, moderate and high) by heating at 50°C with vigorous aeration while monitoring the POV at 4-h intervals. The final POV values for the lowly (LO), moderately (MO) and highly oxidised (HO) fish oil were 28·29, 62·79 and 104·21 mEq/kg, respectively.

Table 1. Formulation of the experimental basal diet

Ingredients	%
Fishmeal*	43·0
Soyabean meal*	15·0
Fish oil*	7·0
Beer yeast*	3·0
Wheat meal	25·3
Lecithin	2·5
Attractant†	0·3
Mould inhibitor‡	0·1
Vitamin premix§	2·0
Mineral premix‖	2·0

* Fishmeal obtained from Russia AKROS Fishing Co. Ltd; soyabean meal obtained from Liulu Oli Lit; fish oil obtained from Shengda Fish Meal & Oil Co. Ltd; beer yeast obtained from Cishan Fisheries.
† Attractant: glycine and betaine.
‡ Mould inhibitor contained 50 % calcium propionic acid and 50 % fumaric acid.
§ Vitamin premix (mg or g/kg diet): thiamin, 25 mg; riboflavin, 45 mg; pyridoxine HCl, 20 mg; vitamin B_{12}, 0·1 mg; vitamin K_3, 10 mg; inositol, 800 mg; pantothenic acid, 60 mg; niacin acid, 200 mg; folic acid, 20 mg; biotin, 1·20 mg; retinyl acetate, 32 mg; cholecalciferol, 5 mg; DL-α-tocopherol, 120 mg; ascorbic acid, 2000 mg; choline chloride, 2500 mg; microcrystalline cellulose, 14·67 g.
‖ Mineral premix (mg or g/kg diet): NaF, 2 mg; KI, 0·8 mg; $CoCl_2.6H_2O$ (1 %), 50 mg; $CuSO_4.5H_2O$, 10 mg; $FeSO_4.H_2O$, 80 mg; $ZnSO_4.H_2O$, 50 mg; $MnSO_4.H_2O$, 60 mg; $MgSO_4.7H_2O$, 1200 mg; $Ca(H_2PO_3)_2.H_2O$, 3000 mg; NaCl, 100 mg; zoelite, 15·45 g.

After oxidisation, diets containing either fresh or oxidised fish oil were stabilised with 150 mg ethoxyquin/kg diet. The proximate composition of the experimental diets is shown in Table 2.

A previous study in our laboratory demonstrated that approximately 60 mg DL-α-tocopheryl acetate (α-Toc)/kg diet was adequate to maintain normal physiological function in juvenile large yellow croaker[21]. In the present study, eight experimental diets were designed to investigate the effects of 600 mg/kg VE (α-Toc) and oxidised fish oil (LO, MO and HO) on large yellow croaker: two FFO diets (FFO and FFO/VE), two LO fish oil diets (LO and LO/VE), two MO fish oil diets (MO and MO/VE) and two HO fish oil diets (HO and HO/VE). Experimental diets were sealed in plastic bags and stored indoors in wooden boxes at room temperature until use. Samples were taken from these diets and stored at −20°C for future chemical determinations.

Experimental procedures

Fish rearing and sampling were carried out according to the experimental procedures of the key laboratory of mariculture (Ministry of Education of China), Ocean University of China. Juvenile large yellow croaker *L. crocea* was obtained from a local commercial hatchery farm in Xiangshan Bay, Ningbo, China. Upon arrival, they were reared in open floating sea cages (3·0 × 3·0 × 3·0 m) and were fed the control diet for 2 weeks to acclimate to the experimental diet and culture conditions. At the end of the acclimatisation period, the fish were starved for 24 h, anaesthetised with eugenol (1:10 000; Shanghai Reagent) and weighed. Fish of similar sizes (7·82 (SEM 0·68) g) were distributed into twenty-four sea cages (1·5 × 1·5 × 2·0 m), and each cage was stocked with sixty fish. Each diet was randomly assigned to triplicate cages. The fish were hand-fed to apparent satiation twice daily (04.30 and 16.30 hours). Fish were considered satiated when they did not exhibit feeding behaviour towards the pellets. The weight of diet for each cage before feeding was recorded and the remaining diet was weighed. Daily consumption of feed in each cage was recorded. Smaller pellets (1·5 mm) were fed to fish during weeks 1–4 and the larger pellets (2·5 mm) during weeks 5–10. The feeding trial lasted for 10 weeks. Water column characteristics were monitored weekly in the morning. During the trial, water temperature ranged from 26·5 to 29·5°C, the salinity was about 28 g/l and dissolved O_2 content was approximately 7 mg/l.

Biochemical analysis

Fish were starved for 24 h at the end of the feeding trial. The total number and mean body weight of the fish in each cage were measured. Ten fish were randomly sampled from each cage for

Table 2. Proximate composition of the experimental diets (%, dry weight basis)

Proximate composition (%)	Diets							
	FFO	FFO/VE	LO	LO/VE	MO	MO/VE	HO	HO/VE
Crude protein	43·4	43·4	43·6	43·9	43·8	43·5	43·7	43·9
Crude lipid	12·9	13·1	12·9	13·3	13·1	13·0	12·7	13·2
Ash	12·4	12·4	13·8	14·1	13·6	14·1	14·0	14·3
Analysed α-Toc (mg/kg)	108·2	681·9	112·0	689·3	102·9	701·3	110·8	695·8

FFO, fresh fish oil; VE, vitamin E; LO, lowly oxidised fish oil; MO, moderately oxidised fish oil; HO, highly oxidised fish oil; α-Toc, DL-α-tocopheryl acetate.

individual proximate composition analysis. Proximate composition analyses of feed ingredients, experimental diets and fish carcass were performed according to the standard methods[22]. Samples of the diets and fish were dried to a constant weight at 105°C to determine moisture content. Protein content was determined by measuring N content (N × 6·25) using the Kjeldahl method. Body lipid levels were quantified by diethyl ether extraction using Soxhlet. Lipids in the liver were extracted as described by Folch et al.[23]. Ash was measured by combustion at 550°C. All measurements and determinations were performed in triplicate. Fatty acid profiles were analysed using a HP6890 GC (Agilent Technologies Inc.) as described by Zuo et al.[24]. The α-Toc concentrations of diets were determined by HPLC with a fluorescence detector according to Salo-Väänänena et al.[25].

Antioxidant enzyme activity and respiratory burst activity

Blood samples were collected from the caudal vein from three fish per cage with a 27-G needle and a 1-ml syringe, allowed to clot at room temperature for 4 h and then at 4°C for further 6 h. Following centrifugation (836 **g**, 10 min, 4°C), serum samples were immediately frozen in liquid N_2 and stored at −80°C for later analysis of antioxidant defence enzyme activities. Activities of superoxide dismutase (SOD) and catalase (CAT) were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's protocol. Respiratory burst activity of head kidney macrophages was measured according to a method previously described in our laboratory by Zuo et al.[24].

Hepatic malondialdehyde

Livers were dissected from three fish per cage, immediately frozen in liquid N_2 and stored at −80°C before analysis. Malondialdehyde (MDA), one of the metabolites derived from lipid peroxidation, was measured using a thiobarbituric acid reactive substances assay kit (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's protocol.

Air-exposure mortality

After blood and tissue sampling, twenty fish were randomly collected from each cage, exposed to air at an ambient temperature of approximately 28°C for 12 min and then put back into the water. Accumulated mortality during the subsequent 20 min was monitored and was defined as air-exposure mortality (AEM).

Calculations and statistical analysis

The following variables were calculated:

Survival (%) = $100 \times N_t/N_0$,

Specific growth rate (SGR, %/d) = $100 \times (\ln W_t - \ln W_0)/t$,

Feed intake (FI, g/100 g BW/d) = $D_f \times 100/((W_t + W_0)/2 \times t)$,

Feed efficiency (FE) = $(W_t - W_0)/D_f$,

Hepatosomatic index (HSI, %) = $100 \times$ liver wet weight/W_t,

where N_0 and N_t represent the initial and final numbers of fish in each cage, respectively. BW is the wet body weight, W_t is the final mean body weight (g), W_0 is the initial mean body weight (g), t is the experimental duration in days and D_f is the dry diet intake (g).

All data were subjected to a one-way ANOVA test, and differences between the means were tested by Tukey's multiple-range test. The level of significance was set at $P < 0·05$, and the results are presented as mean values with their standard errors. All the statistical analyses were performed using Statistica 6.0 (StatSoft).

Results

Dietary fatty acid profiles

Lipid oxidation increased the proportions of SFA and MUFA fatty acids, and decreased the proportions of EPA, DHA and total HUFA in the experimental diets (Table 3).

Survival, growth, feed utilisation and hepatosomatic index

There were no significant differences ($P > 0·05$) in the rate of survival, which ranged from 93·7 to 97·5 %, among the treatments (Fig. 1(A)). For the VE non-supplemented groups, the specific growth rate was significantly ($P < 0·05$) lower in fish fed the oxidised diets than in fish fed the FFO diet. VE supplementation significantly ($P < 0·05$) decreased the growth rate of fish fed FFO. However, VE supplementation increased the growth rate of fish fed the oxidised diets (Fig. 1(B)).

FI of fish fed MO and HO were significantly ($P < 0·05$) higher than fish fed FFO and LO. VE supplementation increased the FI of fish fed FFO and LO, but decreased the FI of fish fed MO and HO (Fig. 1(C)). No statistically significant differences ($P > 0·05$) were detected in FE between treatments (Fig. 1(D)). The hepatosomatic index (HSI) of fish increased with increasing dietary POV and decreased with VE supplementation. The HSI of fish in the HO group was significantly higher ($P < 0·05$) than in the HO/VE group (Fig. 1(E)).

Table 3. Fatty acid composition (% total fatty acids) of the experimental diets containing different levels of oxidised fish oil

	Diets			
	Fresh fish oil	Lowly oxidised fish oil	Moderately oxidised fish oil	Highly oxidised fish oil
C16:0	20·07	20·02	20·68	20·97
C18:0	3·36	3·37	3·46	3·52
C20:0	5·14	5·25	5·32	5·37
ΣSFA	28·58	28·64	29·46	29·86
C16:1	6·81	6·82	7·06	7·16
C18:1n-7	10·05	10·18	10·36	10·53
C18:1n-9	2·38	2·42	2·44	2·47
ΣMUFA	19·23	19·42	19·87	20·15
C18:2n-6	1·98	1·97	1·96	1·98
C18:3n-3	1·35	1·33	1·30	1·31
C20:5n-3 (EPA)	0·61	0·60	0·57	0·57
C22:6n-3 (DHA)	9·70	9·54	8·84	8·91
ΣHUFA	10·31	10·14	9·41	9·48

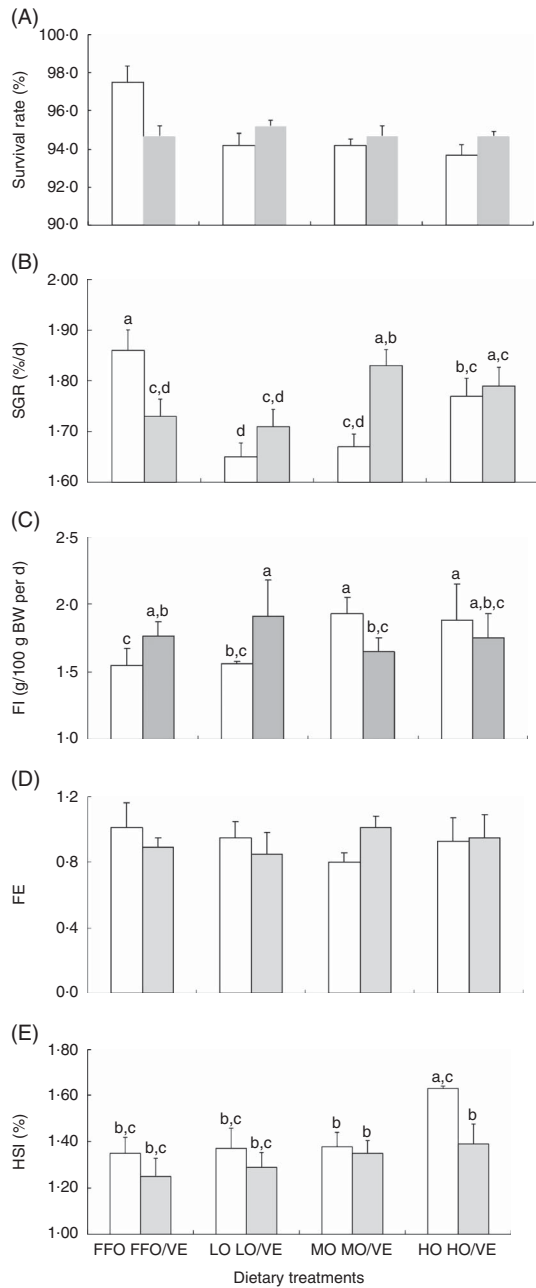HUFA, highly unsaturated fatty acids (EPA and DHA in the present study).

Fig. 1. Survival rate (A), specific growth rate (SGR) (B), feed intake (FI) (C), feed efficiency (FE) (D) and hepatosomatic index (HSI) (E) of juvenile large yellow croaker fed diets containing oxidised fish oil supplemented with vitamin E (VE) for 10 weeks. Values are means, with their standard errors represented by vertical bars. [a,b,c,d] Mean values with unlike letters were not significantly different ($P > 0.05$) among treatments. BW, body weight; FFO, fresh fish oil; LO, lowly oxidised; MO, moderately oxidised; HO, highly oxidised.

Body composition

No statistically significant differences ($P > 0.05$) in whole-body composition were detected among treatments, with the exception of lipid content ($P < 0.05$). Fish fed the oxidised diets had a lower lipid content than fish fed FFO, and their lipid content increased with VE supplementation. Dietary inclusion of VE increased lipid content in the liver of fish in all the groups, with significant differences detected in fish fed FFO and LO diets ($P < 0.05$) (Table 4).

Table 4. Body composition (% wet weight) of juvenile large yellow croaker fed diets containing oxidised fish oil supplemented with vitamin E (VE) for 10 weeks*

					Diets					
	FFO	FFO/VE	LO	LO/VE	MO	MO/VE	HO	HO/VE	Pooled SEM	P
Moisture (%)	76·8	77·6	77·9	77·4	78·2	77·4	77·3	77·3	0·41	0·232
Protein (%)	14·7	14·4	14·5	14·9	14·6	14·9	15·0	14·8	0·23	0·784
Lipid (%)	5·3[a]	5·1[a,b]	4·5[b,c]	5·1[a,b]	4·2[c]	4·7[a,b,c]	4·8[a,b]	4·9[a,b]	0·82	0·045
Lipid content in liver (%)	36·9[a]	44·3[b]	33·8[a]	45·2[b]	34·0[a]	40·2[a,b]	37·6[a]	40·8[a,b]	0·38	0·039
Ash (%)	3·7	3·8	3·9	3·7	3·8	3·7	3·6	3·9	0·09	0·473

FFO, fresh fish oil; LO, lowly oxidised fish oil; MO, moderately oxidised fish oil; HO, highly oxidised fish oil.
[a,b,c] Mean values within the same row with unlike superscript letters were not significantly different ($P > 0.05$, Tukey's test).
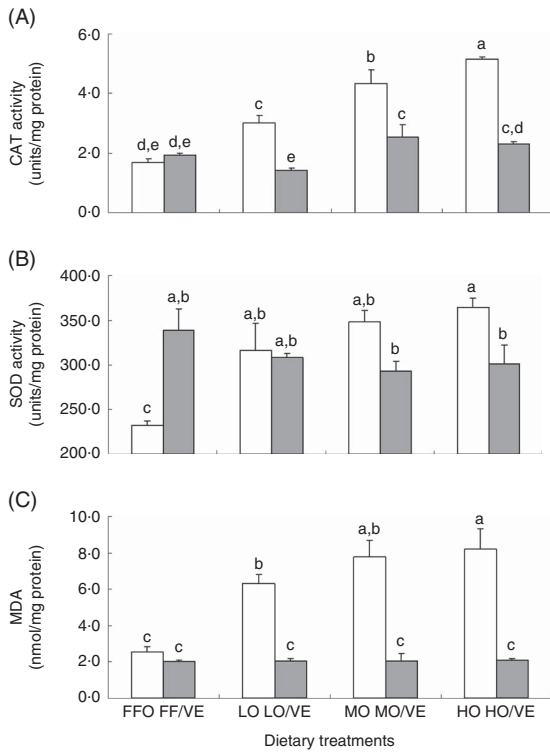* Data represent the mean of triplicate groups.

Fig. 2. Hepatic catalase (CAT) activity (A), superoxide dismutase (SOD) activity (B) and malondialdehyde (MDA) content (C) of juvenile large yellow croaker fed diets containing oxidised fish oil supplemented with vitamin E (VE) for 10 weeks. Values are means, with their standard errors represented by vertical bars. [a,b,c,d,e] Mean values with unlike letters were not significantly different ($P > 0.05$) among treatments. FFO, fresh fish oil; LO, lowly oxidised; MO, moderately oxidised; HO, highly oxidised.

Fig. 3. Respiratory burst activity of head kidney macrophages from juvenile large yellow croaker fed diets containing oxidised fish oil supplemented with vitamin E (VE) for 10 weeks. Values are means, with their standard errors represented by vertical bars. OD, optical density; FFO, fresh fish oil; LO, lowly oxidised; MO, moderately oxidised; HO, highly oxidised.

Fig. 4. Air-exposure mortality (AEM) of juvenile large yellow croaker exposed to air for 20 min after being fed diets containing oxidised fish oil supplemented with vitamin E (VE) for 10 weeks. Values are means, with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were not significantly different ($P > 0.05$) among treatments. FFO, fresh fish oil; LO, lowly oxidised; MO, moderately oxidised; HO, highly oxidised.

Antioxidant enzyme activities and respiratory burst activities

As dietary POV increased in the groups not supplemented with VE, the activities of CAT and SOD increased significantly ($P < 0.05$). VE supplementation significantly ($P < 0.05$) reduced the activities of CAT and SOD in fish fed the oxidised diets. In contrast, the activities of CAT and SOD were increased by VE supplementation in fish fed the FFO diet (Fig. 2(A) and (B)). Hepatic MDA levels increased significantly ($P < 0.05$) with each increase in dietary POV (Fig. 2(C)). VE supplementation decreased the concentration of MDA in all groups, irrespective of dietary POV. No significant ($P > 0.05$) differences in MDA concentration were observed among the VE supplementation groups (Fig. 2(C)).

No significant ($P > 0.05$) difference in respiratory burst activities (optical density 630 values) was detected among treatments, although fish fed oxidised diets had lower respiratory burst activities than those fed FFO (Fig. 3).

Air-exposure mortality

AEM increased significantly ($P < 0.05$) with increasing dietary POV (Fig. 4). VE supplementation decreased AEM in fish fed oxidised diets and increased AE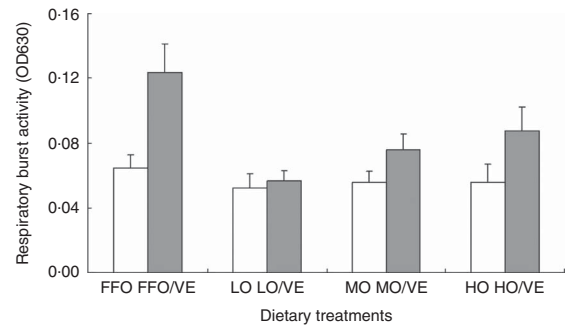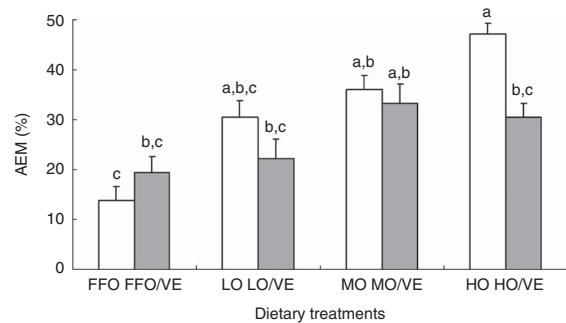M in fish fed FFO. The positive effect of VE supplementation was most evident among fish receiving the HO diet, where a significant reduction in AEM ($P < 0.05$) was observed.

Discussion

In the present study, large yellow croaker fed diets containing oxidised fish oil without supplemental VE exhibited poor growth. This is in accordance with previous studies[6,26–28]. It was also reported that feed oxidisation decreased FI, and consequently the growth rate, of Atlantic salmon (*Salmo salar*)[29]. Decreases in food intake due to the presence of oxidised lipids in the diet may be the result of altered odour or palatability of the diet[30]. However, in the present study, the FI of fish was not depressed by oxidised feed, indicating that large yellow croaker tolerate quite high levels of oxidation products before they avoid the diet because of unpleasant taste or odour. This finding is in agreement with that of Hamre *et al.*[29], who reported that FI in Atlantic salmon fed the oxidised diet was not significantly lower compared with fish fed the control diet. The FE was not significantly different among treatments, indicating that the oxidised fish oil was effectively absorbed. These results are in

agreement with studies in Siberian sturgeon (*Acipenser baeri*)[31]. The decreased growth of fish fed oxidised diets is likely a result of increased oxidative stress caused by both the reduced nutritional value of the diet due to the loss of HUFA[32–34] and the production of toxic peroxidation products[28,35]. These mechanisms are in line with the observations from the present study, in which the activities of the two primary free-radical scavenging enzymes, CAT and SOD, were significantly increased with increasing dietary oxidised oil POV. The HSI and hepatic MDA contents were also increased with increasing dietary POV. These results clearly demonstrate that oxidised fish oil could stimulate the activities of antioxidant defence enzymes in stressed large yellow croaker and imply that fish were suffering from graded oxidative stress. Similar findings were reported in previous studies where the growth of fish fed diets containing oxidised oil was inversely proportional to the responses observed in the activities of hepatic antioxidant defence enzymes[8,28,36].

High-dose VE inclusion significantly enhanced the growth of large yellow croaker fed the oxidised oil diets in this study. This growth-promoting effect of VE is consistent with previous findings in tilapia[37], grass carp[38], Japanese flounder[6] and gilthead sea bream[8]. The positive effects of VE on large yellow croaker were likely due to an alleviation of the oxidative stress induced by the oxidised oil in the diet. VE can act as a scavenger of free radicals and a singlet O_2 quencher[39,40], thereby lowering the burden placed upon antioxidant enzymes. This is consistent with the observed marked decline in the activities of hepatic antioxidant defence enzymes, which suggests significant reductions in levels of oxidative stress among large yellow croaker. In accordance, hepatic MDA concentrations, HSI values and AEM of fish were lowered by dietary VE supplementation. A similar observation was reported in grass carp where hepatic MDA content was significantly decreased following supplementation with VE[38]. In the present study, VE may have also exerted beneficial effects through the enhancement of immunity in large yellow croaker. This is in accordance with the observations of significantly lower AEM of fish fed the oxidised diets supplemented with VE. Similarly, Ortuño *et al.*[41] found that dietary administration of high doses of vitamins C and E reduced stress in sea bream (*Sparus aurata*) when exposed to a combination of stressors that included a 2-min air exposure.

It is noteworthy that the inclusion of a high concentration of dietary VE resulted in a significant decrease in the growth of fish fed the fresh oil diet in this study. This deleterious effect could potentially be caused by an excess dose of VE accumulated in the liver and other tissues[4,12,14,26,42,43]. VE stored in animal tissues is partially utilised for halting the free radical cascade initiated by lipid peroxyl radicals and other assorted products of lipid peroxidation[44]. Excess supplementation of VE to the biological system may lead to acceleration of lipid peroxidation, increasing accumulation of hydroperoxide in blood and reduced erythrocyte osmotic fragility of fish[6,16,17,45,46]. Moreover, high dietary concentrations of VE (5000 mg of α-Toc/kg of diet) have been shown to cause reduced concentrations of erythrocytes in trout blood[47]. In addition, it was reported that an excess amount of dietary α-tocopherol could enhance lipid

deposition in fish liver[16]. Consistently, in the present study, the lipid content in the liver was increased by VE supplementation, indicating that excess VE promotes lipid accumulation, resulting in poor liver health condition in large yellow croaker. Inclusion of oxidised oil in fish diets decreased tissue α-tocopherol concentrations[7]. Therefore, it could be that these adverse effects of high-dose VE would be most evident in the FFO/VE group, due to a lack of reaction substrates in the form of oxidised oil supplied in the diet. Our results indicate that the degree of oil oxidation (i.e. the severity of oxidative stress) could be a key factor influencing the physiological effects of VE.

In conclusion, the ingestion of oxidised oil in the diet significantly decreased growth, but activated antioxidant defence enzymes, in large yellow croaker. Inclusion of high-dose VE supplementation can alleviate oxidative stress and enhance the immune response of stressed fish fed oxidised fish oil. However, high doses of VE can also exert deleterious effects on non- or less-stressed large yellow croaker.

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