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Nutritional programming of large yellow croaker (*Larimichthys crocea*) larvae by dietary vegetable oil: effects on growth performance, lipid metabolism and antioxidant capacity†

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Abstract

The nutritional status experienced in the early development of life plays a vital role in the long-term metabolic state of the individual, which is known as nutritional programming. The present study investigated the long-term effects of vegetable oil (VO) nutritional programming during the early life of large yellow croaker. First, larvae were fed either a fish oil (FO) diet or a VO diet for 30 d. Subsequently, under the same conditions, all fish were fed a commercial diet for 90 d and thereafter challenged with an FO or VO diet for 30 d. The results showed that growth performance was significantly lower in larvae fed the VO diet than in those in fed the FO diet in the stimulus phase. Notably, VO nutritional history fish showed lower levels of liver lipids liver total triglycerides and serum nonesterified free fatty acids than the FO nutritional history fish when juveniles were challenged with the VO diet, which was consistent with the expression of lipogenesis-related genes and proteins. Moreover, the VO nutritional history fish showed lower liver damage and higher antioxidant capacity than FO nutritional history fish when challenged with the VO diet. In summary, this study showed that a short VO stimulus during the early life stage of large yellow croaker, had a long-term effect on lipid metabolism and the antioxidant system. Specifically, VO nutritional programming had a positive effect on alleviating abnormal lipid deposition on the liver, liver damage, and the reduction of hepatic antioxidant capacity caused by a VO diet.

Keywords: **Nutritional programming: Vegetable oil: Fish oil: Lipid metabolism: Antioxidant capacity**

With the rapid development of world aquaculture and the decline in fish oil (FO) production, searching for suitable FO substitutes has become a research hotspot[1,2]. Due to the high availability, steady growth in production and stable prices, vegetable oil (VO) is often used as a substitute for FO. However, compared with FO, VO contains no long-chain PUFA, such as EPA and DHA, while the content of α-linolenic acid and linoleic acid (LA) are higher[3,4]. Previous studies have established that excessive dietary FO replaced by VO often leads to abnormal lipid deposition, reduction of antioxidant capacity and nonspecific immunity in large yellow croaker (*Larimichthys crocea*)[5,6], black carp (*Mylopharyngodon piceus*)[7], Atlantic salmon (*Salmo salar* L.)[8] and blunt snout bream (*Megalobrama amblycephala*)[9]. Consequently, it is imperative to find nutritional measures to alleviate the adverse effects of the substitution of VO for FO.

Nutritional programming proposed by Lucas[10] means that an early stimulus or insult during a 'critical window' period may reset physiological pathways with long-term consequences[11]. These 'programming effects' can be reflected in gene expression changes in key metabolic pathways and critical organ development later in life[12–14]. In mammalian research, nutritional programming is usually associated with various metabolic diseases such as obesity, insulin resistance, glucose intolerance, hypertension and hypertriglyceridaemia[15,16]. In recent years, nutritional programming has also been studied in fish, which is a promising strategy to increase aquaculture production at a lower cost and with fewer environmental impacts[17]. VO

Abbreviations: DGAT1, diacylglycerol acyltransferase 1; FO, fish oil; LA, linoleic acid; MDA, malondialdehyde; SOD, superoxide dismutase; SREBP1c, sterol-regulatory element binding protein 1 c; VO, vegetable oil.

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nutritional programming may be one of the ways through which the utilisation of VO in juvenile fish can be improved and reduce the adverse effects caused by FO substitution. Clarkson *et al.*[18] confirmed that short exposure to a vegetable-based diet during the first exogenous feeding of Atlantic salmon could promote the use of a similar diet when challenged in later life. Turkmen *et al.*[19] reported that nutritional programming of gilthead sea bream (*Sparus aurata*) through broodstock feeding could improve utilisation of low-FM/FO diets and growth of juvenile fish. In general, nutritional programming studies carried out on fish include high carbohydrates[20–22], plant protein[23–25], methionine[26] and micronutrients[27,28] or nonnutritional stimuli such as hypoxia[13,29]. However, to our knowledge, few reports have focused on the alleviation of the negative effects of VO replacement through VO nutritional programming.

Large yellow croaker is a crucial marine fish in China with high yield (254 062 tons in 2020) and great popularity among consumers[30,31]. Due to the enormous scale of farming, VO has been widely used in diets, which has caused growth retardation, abnormal lipid deposition on the liver and decreased antioxidant capacity[5,32,33]. The purpose of the present study was to investigate the long-term effects of early VO nutritional programming on the growth performance, lipid metabolism and antioxidant capacity of large yellow croaker, particularly when juveniles were fed the VO diet.

Materials and methods

Ethics statement

The present study was conducted in strict accordance with the Management Rule of Laboratory Animals (Chinese Order No. 676 of the State Council, revised 1 March 2017) and approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Experimental design

Larvae were obtained from Xiangshan Harbour Aquatic Seeds Company, Ningbo, China. The experimental design is shown in Fig. 1. The feeding experiment was conducted in the Marine and Fishery Science and Technology Innovation Base, Ningbo, China.

Stimulus phase. This phase was aimed at performing nutritional conditioning on larvae. Fish larvae were fed rotifers (*Brachionus plicatilis*) from 3 to 8 d after hatching (DAH), *Artemia nauplii* from 6 to 11 DAH and live copepods (*Calanus sinicus*) and the FO diet from 10 to 14 DAH. After 14 DAH, large yellow croaker larvae were fed either a 30-d FO diet (Table 1) or a VO diet (Table 1) (Fig. 1, Stimulus phase). The two treatment groups were named F and V, respectively. Each group was conducted in triplicate tanks. Larvae were distributed in six blue plastic tanks (water volume 700 l, 5000 larvae/tank). Throughout the experiment, the environmental conditions (water temperature: 24·6 ± 1·12°C; dissolved oxygen: 6·57 ± 0·39 mg/l; pH: 8·15 ± 0·18; salinity: 22·52 ± 1·47 %) were maintained within the specified range, and 150–200 % of the water was renewed daily. Larvae were fed seven times daily (06:30, 08:30, 10:30, 13:30, 15:30, 17:30 and 23:00) to apparent satiation.

Growth phase. All fish were fed a commercial diet (New Love Larva Yu Bao, Hayashikane Sangyo Co., Ltd.) for 90 d (Fig. 1, Growth phase). According to the difference in nutritional history, the two treatment groups were named FC and VC. Each group was conducted in triplicate tanks. Fish were distributed in six blue plastic tanks (water volume 700 l, 100 fish/tank). Throughout the experiment, the environmental conditions (water temperature: 27·19 ± 0·72°C; dissolved oxygen: 6·57 ± 0·39 mg/l; pH: 8·15 ± 0·18; salinity: 22·52 ± 1·47 %) were maintained within the specified range, and 150–200 % of the water was renewed daily. Fish were fed two times daily (06:30 and 17:30) to apparent satiation.

Challenge phase. At the end of the growth trial, juvenile fish were subjected to a 30-d challenge test with an FO or VO diet (Fig. 1, Challenge phase). According to the differences in nutritional history and challenge diets, the three treatment groups were named FCF, FCV and VCV. Each group was conducted in triplicate tanks. Fish were distributed in nine blue plastic tanks (water volume 700 l, 30 fish/tank). Throughout the experiment, the environmental conditions (water temperature: 28·4 ± 0·97°C; dissolved oxygen: 6·57 ± 0·39 mg/l; pH: 8·15 ± 0·18; salinity: 22·52 ± 1·47 %) were maintained within the specified range, and 150–200 % of the water was renewed daily. Fish were fed two times daily (06:30 and 17:30) to apparent satiation.

Sampling and growth monitoring

At the termination of each phase, larvae or juvenile fish were fasted for 24 h before sampling and then anaesthetised (eugenol, 1:10 000).

Stimulus phase. The visceral mass was collected from forty larvae of each tank, which was immediately frozen in liquid nitrogen and stored at −80°C for gene expression analysis. Eighty other whole larvae were sampled from each tank, immediately frozen in liquid nitrogen and stored at −80°C for fatty acid analysis. The total number was determined by counting the individuals remaining in each tank. Except for the larvae used in the next phase of the experiment, three hundred fish were collected to measure proximate composition. Fifty larvae from each tank were collected randomly to monitor the final body weight and the final body length.

Growth phase and challenge phase. For gene expression analysis, the liver of fish was collected and stored at −80°C. For biochemical analysis, whole fish, liver and muscle tissue were collected and stored at −20°C. Fish blood was collected from the caudal vein, placed at 4°C for coagulation and stratification and then centrifuged (2300 ***g***, 10 min) to obtain the serum. The serum was stored at −80°C for further analysis. Faeces were collected by siphoning after 5 h of feeding and frozen at −20°C. In the growth phase, the total fish in each tank were collected to

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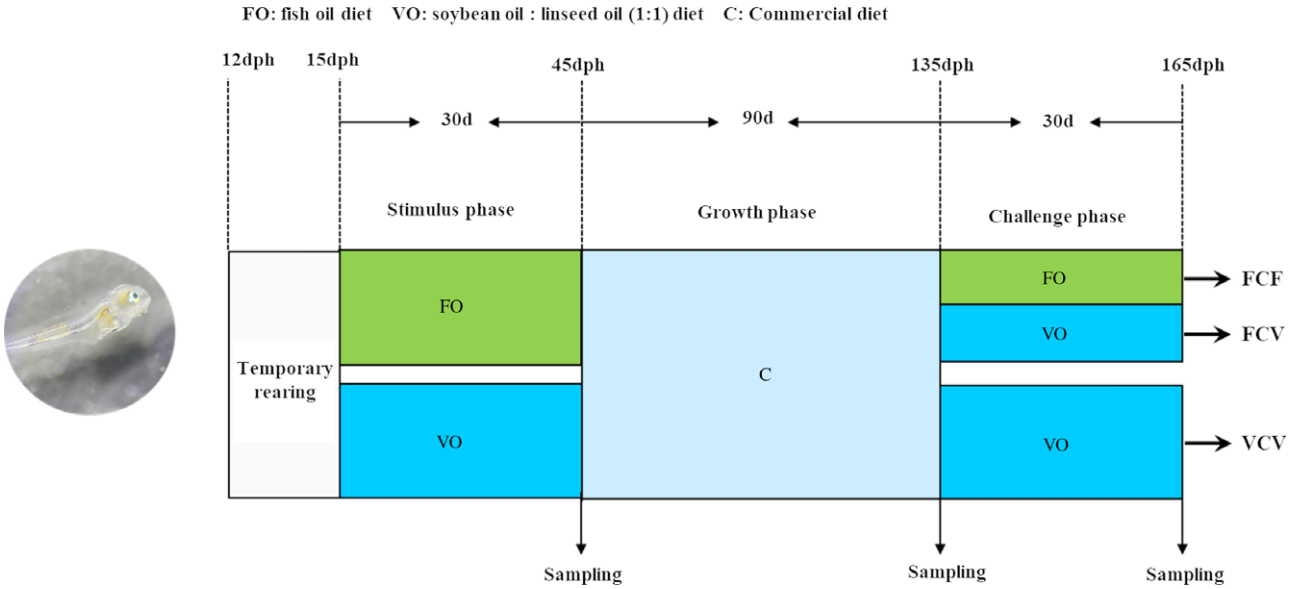

Fig. 1. Experimental design. FO or VO dietary stimulus was applied to large yellow croaker larvae for 30 d, and then juveniles were fed the commercial diet for 90 d. Subsequently, juveniles were fed to a 30 d challenge test with a FO or VO diet.

Table 1. Formulation and proximate composition of the experimental diets

Ingredient (% dry diet)	FO	VO
White fish meal*	27·00	27·00
Krill meal*	22·00	22·00
Squid meal†	5·00	5·00
Wheat gluten*	9·00	9·00
Corn gluten meal*	5·00	5·00
Yeast extract‡	3·00	3·00
α-Starch*	10·00	10·00
Sodium alginate	2·00	2·00
Vitamin premix§	1·50	1·50
Mineral premix‖	1·00	1·00
Ascorbyl polyphosphate	0·10	0·10
Attractant mixture	2·00	2·00
Antioxidant	0·05	0·05
Mould inhibitor	0·05	0·05
Yttria	0·10	0·10
Choline choride	0·20	0·20
Linseed oil*	0·00	3·50
Soybean oil*	0·00	3·50
Fish oil*	7·00	0·00
Soybean lecithin*	5·00	5·00
Total	100·00	100·00
Proximate composition (%)		
Crude protein	52·00	52·43
Crude lipid	14·88	14·12

* Commercially available from Great Seven Biotechnology Co., Ltd in Shandong, China; elementary composition (dry matter): White fish meal, crude protein, 71.73 %, crude lipid, 4.76 %; Krill meal, crude protein, 64.86 %, crude lipid, 8.0 %.
† Commercially available from market squid, then drying and crushing; elementary composition (dry matter): Squid meal, crude protein, 81.81 %, crude lipid, 5.16 %.
‡ Commercially available from Zhejiang Dongcheng Biotechnology Co., Ltd.
§ Composition of vitamin premix (mg or g^{kg-1}): vitamin A palmitate, 3 000 000 mg; vitamin D_3 1 200 000 mg; DL-α-vitamin E 40.0 g/kg; menadione, 8.0 g/kg; thiamine-HCl, 5.0 g/kg; riboflavin, 5.0 g/kg; D-calcium pantothenate, 16.0 mg/kg; pyridoxine-HCl, 4.0 mg/kg; inositol, 200.0 mg/kg; biotin, 8.0 mg/kg; folic acid, 1.5 mg/kg; 4-aminobenzoic acid, 5.0 mg/kg; niacin, 20.0 mg/kg; vitamin B_{12}, 0.01 mg/kg; L-ascorgyl-2-monophosphate-Na (35 %) 2000.0 mg/kg.
‖ Composition of mineral premix (g kg^{-1} premix): $Ca(H_2PO_4)\cdot H_2O$, 675.0; $C_OSO_4\cdot H_2O$, 0.15; $CuSO_4\cdot H_2O$, 5.0; $FeSO_4\cdot 7H_2O$, 50.0; KCl, 0.1; $MgSO_4\cdot 2H_2O$, 101.7; $MnSO_4\cdot 2H_2O$, 18.0; NaCl, 80.0; $NaSeO_3\cdot H_2O$, 0.05; $ZnSO_4\cdot 7H_2O$, 20.0.

monitor the final body weight; thirty fish were collected to measure the final body length. In the challenge phase, the total fish in each tank were collected to monitor the final body weight and body length.

Biochemical analysis

Proximate composition and fatty acid profile analysis. The diet, whole fish, liver and muscle proximate composition (crude protein, crude lipid and moisture) were measured according to the instructions of the Association of Official Analytical Chemists[34]. The moisture content of the samples was determined by drying to a constant weight at 105°C. Crude protein was determined by measuring the n content ($n \times 6{\cdot}25$) through the Kjeldahl method. Crude lipids were quantified with ether extraction by the Soxhlet method. The fish tissue (liver and muscle) was freeze-dried in a lyophilised chamber (α 1–4 LDplus; Christ), which was used for fatty acid profile analysis.

The fatty acid profile was determined according to the protocol of Li *et al.*[5]. Fatty acids were esterified into fatty acid methyl esters by KOH–ethanol and methanolic hydrogen chloride. Fatty acid methyl ester was separated and measured by an HP6890 GC (Agilent Technologies Inc.). The results were expressed as the percentage of each fatty acid in the total fatty acid content.

Plasma biochemical indices. The contents of total cholesterol, TAG, total protein, HDL-cholesterol, LDL-cholesterol, aspartate aminotransferase and alanine transaminase were measured by an automatic biochemical analyser (Cobas c311, Roche). The NEFA and alkaline phosphatase level was measured by commercial assay kits (Nanjing Jiancheng Bioengineering Institute), following the manufacturer's instructions.

Activities of antioxidant enzymes and malondialdehyde content assay. Total antioxidant capacity, reduced GSH content, superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in the liver was determined by commercial assay kits (Nanjing Jiancheng Bioengineering Institute), following the manufacturer's instructions.

cDNA synthesis and RT–qPCR. Total RNA was extracted from the visceral mass or liver using RNAiso Plus (Takara). RNA was reverse-transcribed to cDNA with a Prime Script-RT reagent Kit (Takara). The mRNA level was measured by RT–qPCR using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) using the supplied primers (online Supplementary Table 2). According to Zuo *et al.*[35], the fluorescence data obtained were normalised to *β-actin* by the $2^{-\Delta\Delta CT}$ method.

Western blot analysis. Liver protein was extracted by RIPA (Solarbio) with protease and phosphatase inhibitor cocktails (Roche). Protein was separated on a 10 % SDS–PAGE gel for 1 h at 150 V and then transferred to PVDF membranes (Millipore) for 1 h at 100 V. Then, the PVDF membranes were blocked for 1 h at room temperature and incubated with a primary antibody in Tris-buffered saline with Tween overnight in the 4°C freezers. After washing with Tris-buffered saline with Tween, the membranes were incubated with the secondary antibody in Tris-buffered saline with Tween for 1 h at room temperature. Next, the signal was measured by using ELC reagents (Beyotime Institute of Biotechnology). The intensity of the protein bands was quantified by using ImageJ 1·44p software (U.S. National Institutes of Health).

The antibody used were sterol-regulatory element binding protein 1 c (SREBP1c) (WL02093, Wanleibio), diacylglycerol acyltransferase 1 (DGAT1) (Gensript), acetyl-CoA carboxylase (3662, CST), acyl-CoA oxidase (ab184032, Abcam, UK), PPAR*α* (117 362, Absin), carnitine palmitoyl transferase 1 *α* (15184-1-AP, Proteintech) and glyceraldehyde-3-phosphate dehydrogenase (R001, Goodhere).

Calculations and statistical analysis

$$\text{Survival rate } (\%) = N_t \times 100/N_i$$

$$\text{Specific growth rate } (\%d^{-1}) = (\text{Ln}W_t - \text{Ln}W_i) \times 100/d$$

$$\text{Feed intake } (\%) = W_f/((W_i + W_t)/2)/d \times 100$$

$$\text{Feed efficiency ratio } (\%) = (W_t - W_i)/W_f$$

$$\text{Apparent digestibility coefficients of dry matter } (\%) = (1 - Y_2O_3 \text{ in the diet}/Y_2O_3 \text{in faeces}) \times 100$$

$$\text{Apparent digestibility coefficients of lipid } (\%) = (1 - Y_2O_3 \text{ in the diet} \times \text{lipid in feces}/Y_2O_3 \text{in faeces} \times \text{lipid in diet}) \times 100$$

$$\text{Hepatosomatic index (HSI, \%)} = \text{liver weight/fish weight} \times 100$$

$$\text{Visceral somatic index } (\%) = \text{viscera weight/fish weight} \times 100$$

where N_t and N_i represent the final and initial numbers of larvae or juvenile fish in each tank, respectively; W_t is the final body weight and W_i is the initial body weight; W_f is the weight of feed; and d is the experimental duration in days.

Statistical analysis was performed in SPSS 23.0 (SPSS Inc.), and the results are presented as the mean ± SE of the mean (SEM) (n 3). Data from each treatment were subjected to ANOVA followed by Tukey's test or independent-samples t-test. For statistically significant differences, $P < 0{\cdot}05$ was needed.

Results

Survival and growth performance

The survival rate was slightly lower in larvae fed the VO diet than in those fed the FO diet in the stimulus phases ($P > 0{\cdot}05$) (Table 2). During the growth phase and challenge phase, there were no effects of nutritional history on the survival rate of juveniles. Final length, final weight and specific growth rate were significantly lower in larvae fed the VO diet than in those fed the FO diet in the stimulus phase ($P < 0{\cdot}05$) (Table 2). However, fish with a VO nutritional history showed higher specific growth rate than fish with a FO nutritional history in the growth phase ($P < 0{\cdot}05$) (Table 2). Like the survival rate, although the nutritional history of juvenile fish was different, there was no significant difference in the growth performance among treatments during the challenge phase.

Feed utilisation

No significant difference in feed intake, feed efficiency ratio or apparent digestibility coefficients of dry matter was observed in juvenile fish in the challenge phase. Juveniles fed the FO diet showed significantly higher apparent digestibility coefficients of lipid than other groups in the challenge phase ($P < 0{\cdot}05$) (Fig. 2).

Body composition, tissue lipids and TAG content

No significant difference in crude protein and crude lipid was observed in whole larvae or fish among groups during the three phases. FCV-fish showed significantly higher liver lipid and TAG content than FCF-fish in the challenge phase ($P < 0{\cdot}05$) (Table 3). Although no significant difference between FCV-fish and VCV-fish in liver lipid and TAG content was observed, there was a trend that VO nutritional history reduced the liver lipid content of fish when juveniles were challenged with the VO diet.

Fatty acid composition

In the stimulus phase, the whole larvae SFA (14:0 and 16:0) and 16:1*n*-7 were significantly lower in larvae fed the VO diet than in larvae fed the FO diet ($P < 0{\cdot}05$) (Table 4). However, the contents of 18:1*n*-9, 18:2*n*-6, 18:3*n*-3, ΣMUFA, Σ*n*-6 PUFA and Σ*n*-3 PUFA was significantly higher in larvae fed the VO diet than in larvae fed the FO diet ($P < 0{\cdot}05$) (Table 4).

During the challenge stage, the liver fatty acid content (14:0, 16:0, 16:1*n*-7, 20:4*n*-6, 20:5*n*-3, 22:6*n*-3 and Σ*n*-3 PUFA) was

Table 2. Survival and growth rate of fish during each of the three nutritional phases
(Mean values with their standard errors of the mean, *n* 3)

		F				V	
Phase	Parameter	Mean			SEM	Mean	SEM
Stimulus phase	Initial length (mm)	5·39			0·31	5·39	0·31
	Final length (mm)	17·50			0·23*	16·54	0·21
	Initial weight (mg)	4·71			0·17	4·71	0·17
	Final weight (mg)	114·31			5·25*	93·72	3·00
	Specific growth rate (%/d)	10·62			0·15*	9·97	0·11
	Survival (%)	24·15			2·07	21·46	1·50
Growth phase		FC				VC	
	Initial length (mm)	17·50			0·23*	16·54	0·21
	Final length (mm)	90·38			2·25	86·78	1·21
	Initial weight (mg)	114·31			5·25*	93·72	3·00
	Final weight (g)	11·83			0·29	11·05	0·02
	Specific growth rate (%/d)	5·15			0·03	5·31	0·00*
	Survival (%)	95·59			0·01	94·56	0·01
Challenge phase		FCF		FCV		VCV	
		Mean	SEM	Mean	SEM		
	Initial length (cm)	9·04	0·22	9·04	0·22	8·68	0·12
	Final length (cm)	12·12	0·26	11·75	0·40	11·59	0·01
	Initial weight (g)	11·83	0·29	11·83	0·29	11·05	0·02
	Final weight (g)	28·57	2·16	26·36	3·03	25·21	0·26
	Specific growth rate (%/d)	2·92	0·25	2·62	0·41	2·75	0·03
	Survival (%)	85·36	2·32	81·35	4·81	82·50	3·44

* $P < 0.05$, significant differences between groups.
In this phase, data in the same row sharing the same superscript letter are not significantly different, determined by Tukey's test ($P > 0.05$).

significantly higher in FCF-fish, while 18:2*n*-6, 18:3*n*-3 and Σ*n*-6 PUFA content was significantly lower than in other groups ($P < 0·05$) (Table 5).

Like the liver, the muscle fatty acid content (14:0, 16:0, 16:1*n*-7, 20:4*n*-6, 20:5*n*-3, 22:6*n*-3 and Σ*n*-3 PUFA) was significantly higher in the FCF-fish than in the other groups ($P < 0·05$) (Table 6). In addition, lower content of 18:1*n*-9, 18:2*n*-6, 18:3*n*-3, Σ*n*-6 PUFA and Σ*n*-3 PUFA was observed in FCF-fish than in other groups ($P < 0·05$) (Table 6).

Serum biochemical indices

During the challenge stage, FCV-fish had significantly higher serum nonesterified free fatty acid than other groups ($P < 0·05$) (Table 7). However, neither nutritional history nor different oil source stimulation had significant effects on serum TAG, total cholesterol, LDL-cholesterol, HDL-cholesterol and total protein.

Expression of liver lipid metabolism-related genes and proteins

During the stimulus phase, the expression of a lipogenesis-related gene (*acc1*) was significantly higher in larvae fed the VO diet than in larvae fed the FO diet ($P < 0·05$) (Fig. 3(a)). However, no significant difference was observed in other lipogenesis or lipolysis-related genes ($P > 0·05$) (Fig. 3(a)). Meanwhile, higher expression of long chain-PUFA (LC-PUFA) synthesis genes (△*6fad* and *elovl4*) was observed in larvae fed the VO diet than in larvae fed the FO diet ($P < 0·05$) (Fig. 3(b)). For lipid transport-related genes, compared to larvae fed the FO diet, dietary VO downregulated the expression of *apob100* and *apobAI* ($P < 0·05$) (Fig. 3(c)).

During the growth phase, no significant difference was observed in lipogenesis or lipolysis-related genes ($P > 0·05$) (Fig. 4(a)). Effects of VO nutritional history on the expression of LC-PUFA synthesis and lipid transport genes still existed after the 90-d growth trial. Higher expression of △*6fad* and *elovl5* was observed in VC fish than in FC fish ($P < 0·05$) (Fig. 4(b)). Meanwhile, lower expression of *mtp* was also observed in VC fish than FC fish ($P < 0·05$) (Fig. 4(c)).

During the challenge phase, the expression of lipogenesis-related genes (*srebp1c*, *dgat1*, *acc1*, and *fas*) and proteins (SREBP1c, DGAT1 and acetyl-CoA carboxylase) in FCV-fish was higher than that in FCF- or VCV-fish (Fig. 5(a) and Fig. 6). Neither nutritional history nor VO stimulation had a significant effect on the genes involved in lipolysis among treatments (Fig. 5(a)). Higher expression of the LC-PUFA synthesis gene (△*6fad*) was observed in VCV-fish than FCF-fish (Fig. 5(b)). Furthermore, the lipid transport-related gene expression (*apob100* and *mtp*) of FCF-fish was significantly higher than that of the other groups ($P < 0·05$) (Fig. 5(c)).

Live damage and antioxidant capacity

During the challenge stage, the activities of alanine transaminase and aspartate aminotransferase were significantly higher in FCV-fish than in other groups ($P < 0·05$) (Fig. 7(a)). Similarly, FCV-fish showed significantly higher alkaline phosphatase activity than FCF-fish ($P < 0·05$) (Fig. 7(a)). The lowest activities of SOD, total antioxidant capacity and GSH were observed in FCV-fish (Fig. 7(b)). Meanwhile, FCV-fish had significantly higher MDA

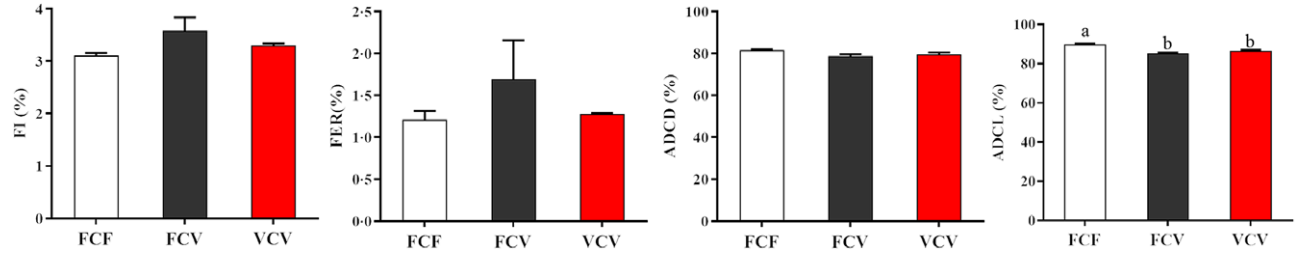

Fig. 2. Feed utilisation of juvenile large yellow croaker in the challenge phase. FI, feed intake; FER, feed efficiency ratio; ADCD, apparent digestibility coefficients of dry matter; ADCL, apparent digestibility coefficients of lipid. Values are means (n 3), with their standard errors represented by vertical bars. Bars bearing the same letters were not significantly different ($P > 0{\cdot}05$, Tukey's test).

Table 3. Body composition, liver and muscle lipid content in fish during each of the three nutritional phases (Mean values with their standard errors of the mean, n 3)

		FO				VO	
Phase	Parameter	Mean			SEM	Mean	SEM
Stimulus phase	Whole larvae crude protein (%, d.w.)	56·07			1·85	55·25	1·95
	Whole larvae crude lipid (%, d.w.)	26·52			0·92	25·23	1·22
Growth phase		FC				VC	
	Whole fish crude protein (%, d.w.)	60·75			0·94	61·70	1·02
	Whole fish crude lipid (%, d.w.)	20·29			0·03	18·77	1·03
Challenge phase		FCF		FCV		VCV	
		Mean	SEM	Mean	SEM		
	Whole fish crude protein (%, d.w.)	54·87	1·50	53·46	1·06	53·22	0·52
	Whole fish crude lipid (%, d.w.)	31·92	1·60	31·68	1·09	31·22	0·55
	Liver crude lipid content (%, d.w.)	56·47	2·33^{b}	64·23	1·69^{a}	60·69	0·68^{ab}
	Muscle crude lipid content (%, d.w.)	38·28	4·01	40·67	4·47	41·38	0·98
	Liver TAG (mmol/g)	63·05	10·58^{b}	106·82	6·06^{a}	74·71	4·91^{ab}

d.w.: dry weight.
In this phase, $^{*}P < 0.05$, significant differences between groups.
In this phase, data in the same row sharing the same superscript letter are not significantly different, determined by Tukey's test ($P > 0.05$).

Table 4. Fatty acid composition of whole larvae in the stimulus phase† (Mean values with their standard errors of the mean, n 3)

	F		V	
Fatty acid (% total fatty acids)	Mean	SEM	Mean	SEM
14:0	7·13	0·26*	4·44	0·56
16:0	45·49	0·85*	38·16	0·57
18:0	16·40	0·42	17·50	1·17
20:0	0·62	0·05	0·45	0·06
ΣSFA	69·64	1·06*	60·53	1·37
16:1*n*-7	3·43	0·09*	1·94	0·13
18:1*n*-9	9·99	0·16	13·47	0·33*
ΣMUFA	13·42	0·07	15·42	0·27*
18:2*n*-6	6·40	0·48	12·34	0·97*
20:4*n*-6	0·20	0·02	0·14	0·01
Σ*n*-6 PUFA	6·61	0·51	12·47	0·98*
18:3*n*-3	0·56	0·08	3·76	0·38*
20:5*n*-3	2·06	0·33	1·90	0·17
22:6*n*-3	2·76	0·37	2·43	0·22
Σ*n*-3 PUFA	5·38	0·31	8·09	0·42*
n-3/*n*-6PUFA	0·82	0·04*	0·65	0·01
Σ*n*-3LC-PUFA	4·82	0·28	4·33	0·10

* $P < 0.05$, significant differences between groups.
† Some fatty acids, of which the contents are minor, trace amount or not detected, such as 4:0, 6:0, 8:0, 10:0, 11:0, 12:0, 13:0, 24:0, 14:1, 20:1*n*-9, 22:1*n*-11, 20:2*n*-6, 23:0, 20:3*n*-6, are not listed in the table.

Table 5. Fatty acid composition in the liver of juvenile large yellow croaker in the challenge phase* (Mean values with their standard errors of the mean, n 3)

	FCF		FCV		VCV	
Fatty acid (% total fatty acids)	Mean	SEM	Mean	SEM	Mean	SEM
14:0	4·30	0·30^{a}	3·03	0·29^{b}	3·56	0·11^{ab}
16:0	45·36	0·35^{a}	40·84	0·21^{b}	43·68	0·89^{b}
18:0	15·45	0·68	18·30	1·59	16·56	0·41
20:0	0·25	0·00^{b}	0·27	0·00^{a}	0·23	0·01^{b}
ΣSFA	65·36	0·73	62·45	1·52	64·04	1·14
16:1*n*-7	7·42	0·16^{a}	4·94	0·24^{b}	5·33	0·07^{b}
18:1*n*-9	16·35	0·56	17·43	0·87	15·83	0·14
ΣMUFA	23·77	0·58	22·38	1·11	21·16	0·21
18:2*n*-6	4·55	0·33^{b}	8·58	1·60^{a}	8·53	0·62^{a}
20:4*n*-6	0·21	0·03^{a}	0·08	0·01^{b}	0·07	0·01^{b}
Σ*n*-6 PUFA	4·76	0·35^{b}	8·66	1·61^{a}	8·60	0·63^{a}
18:3*n*-3	0·63	0·08^{b}	3·23	0·66^{a}	3·19	0·30^{a}
20:5*n*-3	1·95	0·26^{a}	0·85	0·08^{b}	0·73	0·03^{b}
22:6*n*-3	1·51	0·31^{a}	0·71	0·10^{b}	0·62	0·06^{b}
Σ*n*-3 PUFA	4·08	0·44	4·79	0·46	4·54	0·40
n-3/*n*-6PUFA	0·87	0·12	0·62	0·18	0·53	0·01
Σ*n*-3LC-PUFA	3·46	0·23^{a}	1·56	0·20^{b}	1·35	0·11^{b}

* Some fatty acids, of which the contents are minor, trace amount or not detected, such as 4:0, 6:0, 8:0, 10:0, 11:0, 12:0, 13:0, 24:0, 14:1, 20:1*n*-9, 22:1*n*-11, 20:2*n*-6, 23:0, 20:3*n*-6, are not listed in the table.
Data in the same row sharing the same superscript letter are not significantly different, determined by Tukey's test ($P > 0.05$).

Table 6. Fatty acid composition in the muscle of juvenile large yellow croaker in the challenge phase*
(Mean values with their standard errors of the mean, *n* 3)

Fatty acid (% total fatty acids)	FCF		FCV		VCV	
	Mean	SEM	Mean	SEM	Mean	SEM
14:0	8·58	0·49[a]	6·10	0·18[b]	6·03	0·18[b]
16:0	42·51	0·62[a]	38·02	0·10[b]	37·32	0·28[b]
18:0	8·91	0·53	8·67	0·24	8·60	0·25
20:0	0·35	0·01[a]	0·26	0·01[b]	0·27	0·00[b]
ΣSFA	60·34	0·50[a]	53·04	0·07[b]	52·21	0·51[b]
16:1*n*-7	5·14	0·05[a]	3·57	0·04[b]	3·51	0·02[b]
18:1*n*-9	12·29	0·34[b]	14·25	0·21[a]	14·11	0·13[a]
ΣMUFA	17·44	0·38	17·82	0·22	17·62	0·14
18:2*n*-6	7·80	0·13[b]	13·43	0·24[a]	13·88	0·33[a]
20:4*n*-6	0·27	0·01[a]	0·15	0·02[b]	0·15	0·01[b]
Σ*n*-6 PUFA	8·07	0·14[b]	13·58	0·23[a]	14·03	0·33[a]
18:3*n*-3	1·41	0·04[b]	7·13	0·16[a]	7·60	0·24[a]
20:5*n*-3	4·24	0·15[a]	2·76	0·14[b]	2·81	0·08[b]
22:6*n*-3	4·67	0·16[a]	2·88	0·25[b]	2·94	0·08[b]
Σ*n*-3 PUFA	10·31	0·27[b]	12·77	0·26[a]	13·35	0·60[a]
n-3/*n*-6PUFA	1·28	0·01[a]	0·94	0·03[b]	0·95	0·01[b]
Σ*n*-3LC-PUFA	8·90	0·31[a]	5·64	0·38[b]	5·75	0·15[b]

* Some fatty acids, of which the contents are minor, trace amount or not detected, such as 4:0, 6:0, 8:0, 10:0, 11:0, 12:0, 13:0, 24:0, 14:1, 20:1*n*-9, 22:1*n*-11, 20:2*n*-6, 23:0, 20:3*n*-6, are not listed in the table.
Data in the same row sharing the same superscript letter are not significantly different, determined by Tukey's test ($P > 0.05$).

Table 7. Serum metabolite profiles of fish in the challenge phase
(Mean values with their standard errors of the mean, *n* 3)

Parameter	FCF		FCV		VCV	
	Mean	SEM	Mean	SEM	Mean	SEM
TAG (mmol/l)	1·35	0·31	2·26	0·19	1·43	0·29
TC (mmol/l)	1·75	0·28	2·05	0·18	1·49	0·13
TP (g/l)	19·22	0·89	20·27	0·14	18·97	0·43
HDL-cholesterol (mmol/l)	0·51	0·07	0·68	0·07	0·57	0·10
LDL-cholesterol (mmol/l)	0·11	0·02	0·09	0·02	0·09	0·02
NEFA (mmol/l)	0·49	0·04[b]	0·67	0·05[a]	0·50	0·02[b]

TC, total cholesterol; TP, total protein; NEFA, nonesterified free fatty acids.
Data in the same row sharing the same superscript letter are not significantly different, determined by Tukey's test ($P > 0.05$).

content than the other groups ($P < 0·05$) (Fig. 7(b)). Furthermore, the lowest antioxidant-related gene (*sod2* and *nrf2*) expression was observed in FCV-fish (Fig. 7(c)).

Discussion

Previous studies have extensively demonstrated that early life history may profoundly affect metabolic adaptability and disease risk in adult mammals, so nutritional programming (metabolic programming) has become a hot research area[13]. In recent years, nutritional programming has also been gradually applied to aquatic animals, which has been considered a promising strategy to enhance VO or vegetable protein utilisation in aquafeed[18,36]. The 'critical window' is an extremely crucial aspect of nutritional programming. For mammals, it is mostly during gestation or lactation, while for fish, it is usually during the egg or larval stage, or during the spawning season (broodstock)[11]. For fish, the embryonic and larval stages are characterised by organogenesis, establishment of metabolic pathways and strong metabolic plasticity[17]. Therefore, most studies have chosen to conduct nutritional/non-nutritive stimulation at the first feeding or embryonic stage. In the present study, we started the VO stimulus at 15 dph (completely exogenous feeding starts at 7 dph). The start of this stimulation was slightly later than the embryonic stage or first feeding. There are many reasons for the choice of this stimulation time point: (1) large yellow croaker larvae are still in the process of organ formation and rapid development during 15–45 dph[37]; (2) large yellow croaker larvae are not like rainbow trout[13], Atlantic salmon[18] and other fish in consuming formulated microdiets during first feeding and (3) there are also studies that chose to stimulate at a later time[36,38] or at multiple stages[39]. The results of this study also partially prove that slightly later stimulation in the larval phase was effective in inducing significant physiological and metabolic changes. This provides the possibility to conduct nutritional programming studies for fish species with extremely high mortality in the early stage, that is, to conduct nutritional stimulation later. For the current study, the aim was to investigate the potential long-term metabolic effects of early short-term nutritional programming of large yellow croaker.

In this study, early short VO intervention significantly reduced the growth performance of larvae. Similarly, Turkmen *et al.*[40] found that feeding the low *n*-3 LC-PUFA (main lipid source: linseed oil and soybean lecithin) diet led to a lower larval weight, total length and survival rate of gilthead seabream larvae. This may be due to the low content of ARA, EPA or DHA in the VO diet, which cannot satisfy the nutritional requirements of large yellow croaker larvae. Another reason might be the low acceptance of VO diets by large yellow croaker larvae causing a decrease in the feeding rate and, ultimately, growth retardation, although this could not be accurately measured in the stimulus phase of the present study. Similarly, studies have demonstrated that Atlantic salmon[18] and turbot[41] are reluctant to eat a vegetable-based diet, especially when it is first introduced. After the 90-d growth phase, regardless of the nutritional history, no significant difference in final body weight was observed among the groups. Since the VC groups had smaller initial weights (93·72 ± 3·00 mg) in the growth phase than the FC group (114·31 ± 5·25 g), the final body weight was similar between the two groups (FC:11·83 ± 0·29; VC: 11·05 ± 0·02), and VO history fish showed higher specific growth rate than FO history fish in the growth phase. The compensatory growth of fish during the growth phase may be one of the reasons for this phenomenon[42]. Comparable results were also found in yellow perch (*Perca flavescens*)[43] and European sea bass[12]. Indeed, this may be limited by fish species, stimulation type and stimulation duration, and the clarification of which will require additional studies. The analysis of growth-related gene changes through nutrigenomics, and microarray technology can provide a more accurate explanation. During the challenge phase, VO nutritional history did not affect the utilisation of the VO diet (Feed intake, feed efficiency ratio, apparent digestibility coefficients of lipid and apparent digestibility coefficients of dry matter) or the growth performance of juvenile large yellow croaker. This

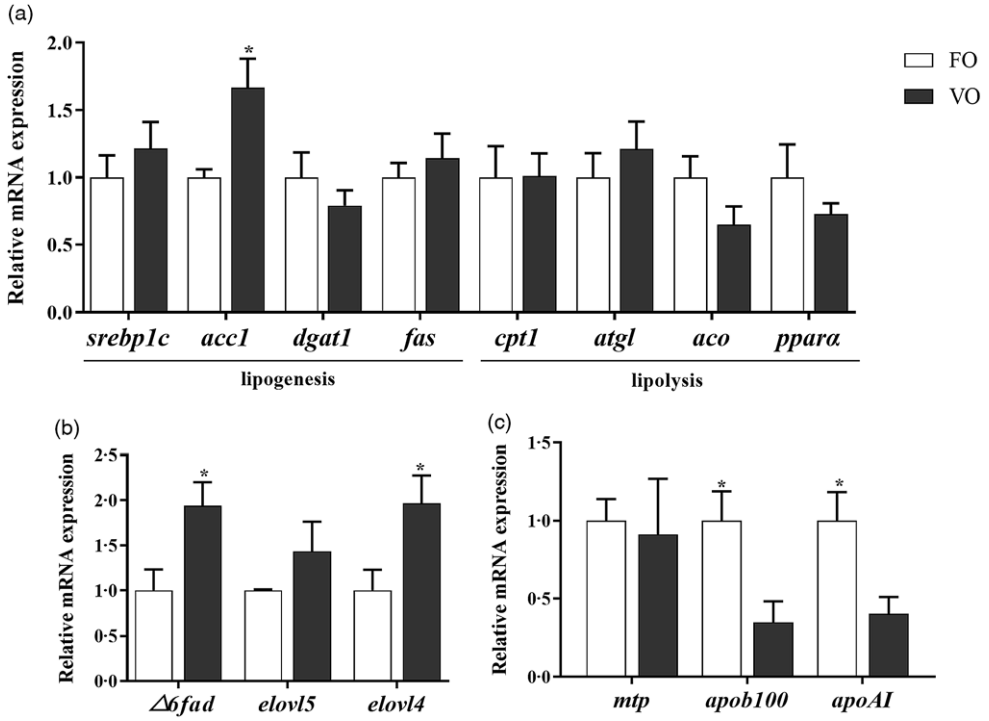

Fig. 3. Expression of lipid metabolism-related genes in the visceral mass of large yellow croaker larvae during the stimulus phase. (a) Lipid lipogenesis and lipolysis; (b) LC-FUFA synthesis and (c) lipid transport. Values are means (n 3), with their standard errors represented by vertical bars. *$P < 0{\cdot}05$, significant differences between groups.

indicated that the early VO stimuli did not cause long-term effects on the overall growth performance at the late juvenile stage, even when faced with a VO challenge. Similarly, the growth of European sea bass juveniles was not affected by larval nutritional conditioning (low/high LC-PUFA) when fed an LC-PUFA-deprived experimental diet[38]. However, those results differed from studies in Atlantic salmon[18] and rainbow trout (*Oncorhynchus mykiss*)[36], in which early nutritional exposure through a vegetable or plant-based diet improved the utilisation of a vegetable or plant-based diet when challenged later in life. Balasubramanian *et al.*[44] found that early feeding exposure to a plant-based diet could alter the expression of genes related to visual transduction processes, olfactomedin, a member of the taste receptor family (Tas1r2) and appetite regulating factors in rainbow trout juveniles, thereby increasing their utilisation and acceptance of plant-based diet to achieve growth-promoting effects. How nutritional programming affects the expression of the above genes may be related to epigenetic modifications represented by DNA methylation[45]. In addition, nutritional programming can also affect growth by directly affecting the expression of protein synthesis genes. Zhu *et al.*[46] demonstrated that early leucine programming could affect the methylation and expression of genes involved in protein synthesis in zebrafish. Whether early nutritional programming has long-term effects on the growth performance of different fish remains uncertain, and experimental results may be influenced by a variety of factors, such as fish species, causes, critical window and duration (persistence).

It is widely known that the fatty acid profile of farmed fish can reflect the fatty acid composition of the diet[47]. In the present study, the effect of the current diet on fish body fatty acid composition was significantly stronger than that of nutritional history. Furthermore, the early VO nutritional stimulus had no major effect on the final fatty acid compositions of either whole body or tissues. Previous studies have shown that nutritional programming could also improve the LC-PUFA synthesis ability of fish. European seabass larvae were given either a high (2·2 %) or a low (0·8 %) LC-PUFA diet from the first feeding, followed by a commercial diet for 3 months. When juveniles were challenged with an LC-PUFA depleted diet (0·5 %) for 2 months, low LC-PUFA diet nutritional history fish had higher DHA content in polar lipids[38,48]. In the present study, nutritional history did not significantly affect the LC-PUFA content of muscle or liver when the juveniles were challenged with the VO diet. VO nutritional history had a long-term effect only on the expression of the critical gene (△*6fad*) in the synthesis of LC-PUFA. Although early VO stimulation could continuously upregulate the gene expression of fatty acid desaturase, it was not enough to completely compensate for the lack of EPA and DHA in the VO diet during the challenge phase. Comparable results have been reported in Atlantic salmon[18]. The mechanism of nutritional programming regulating the fatty acid synthesis pathways and key molecular marker gene expression (such as △*6fad*) may be related to the methylation level of the promoter region of △*6fad*. Xu *et al.*[49] indicated that the △*6fad* gene expression regulated by dietary fatty acids was significantly negatively correlated with the methylation rate of the △*6fad* gene promoter.

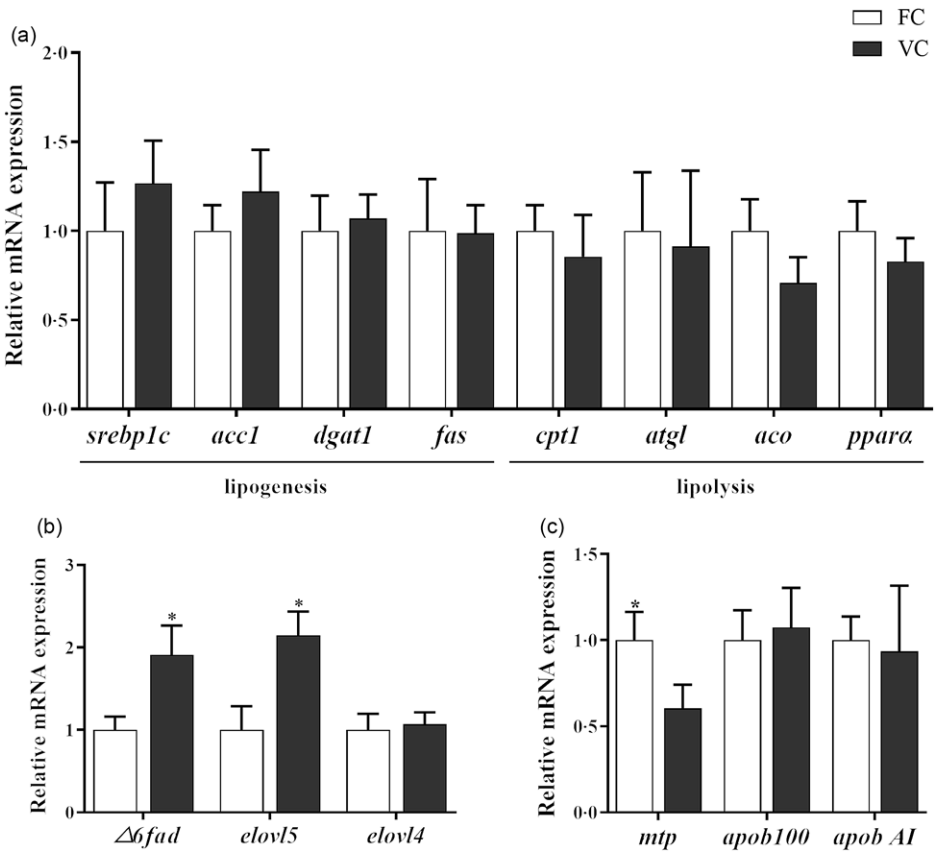

Fig. 4. Expression of lipid metabolism-related genes in the liver of large yellow croaker during the growth phase. (a) Lipid lipogenesis and lipolysis; (b) LC-FUFA synthesis and (c) lipid transport. Values are means (n 3), with their standard errors represented by vertical bars. *$P < 0{\cdot}05$, significant differences between groups.

High-level VO in diets often leads to abnormal lipid deposition on the liver and damages the health of the fish, such as sharpsnout seabream (*Diplodus puntazzo*)[50], gilthead sea bream (*Sparus aurata* L.)[51] and large yellow croaker[6]. To further investigate the effects of nutritional programming on lipid metabolism in large yellow croaker, we studied the body composition, serum biochemical indices, lipid metabolism-related genes and proteins of fish. In the present study, fish with a VO nutritional history showed lower liver lipid content, liver TAG, and serum NEFA than fish with an FO history when challenged with the VO diet. The expression of lipogenesis-related genes (*srebp1c*, *acc1*, *dgat1* and *fas*) and proteins (SREBP1c, acetyl-CoA carboxylase and DGAT1) further confirmed this result, although nutritional history did not significantly affect the expression of lipolysis-related genes. For the lipid transport genes, regardless of the nutritional history in the larval stage, the VO diet significantly reduced the expression of *mtp* and *apob100* in the challenge stage. The above results indicate that VO nutritional programming in the larval stage may alleviate the abnormal lipid deposition in the liver of juvenile fish caused by the VO diet to a certain extent by reducing lipogenesis. Less research has focused on the effect of VO nutritional programming on abnormal liver lipid deposition, while most studies have focused on the utilisation of VO and vegetable protein[18,36,38], although studies on regulating liver lipid deposition through nutritional programming in mammals have been conducted. Cai *et al.*[52] reported that dietary betaine supplementation of the maternal pig during gestation attenuated hepatic lipogenesis in neonatal piglets via epigenetic (DNA methylation and histone methylation) and glucocorticoid receptor-mediated mechanisms. Heng *et al.*[53] found that heat stress of the maternal pig regulated early fat deposition partly through modification of m6A RNA methylation in neonatal piglets. The mechanism of regulating fish liver lipid metabolism through VO nutritional programming may also be related to epigenetic modification (DNA methylation, histone methylation and RNA methylation, etc.), which needs more research to be confirmed.

Replacement of FO by VO will cause lipid accumulation in the liver and further leads to liver damage and decreased antioxidant capacity. Tan *et al.*[54] found that dietary VO could reduce the liver antioxidant capacity of Japanese sea bass (*Lateolabrax japonicus*). Thus, the present study further investigated the effect of VO nutritional programming on relieving liver damage and reducing hepatic antioxidant capacity. Aspartate aminotransferase, alanine transaminase and alkaline phosphatase are biomarkers of liver damage that are released from hepatocytes into the blood during liver injury[55,56]. During the challenge stage, the activities of alanine transaminase, aspartate aminotransferase and alkaline phosphatase were lower in the VO nutritional history fish than in the FO nutritional history fish when juveniles were challenged with the VO diet, indicating that early VO nutritional programming may relieve liver

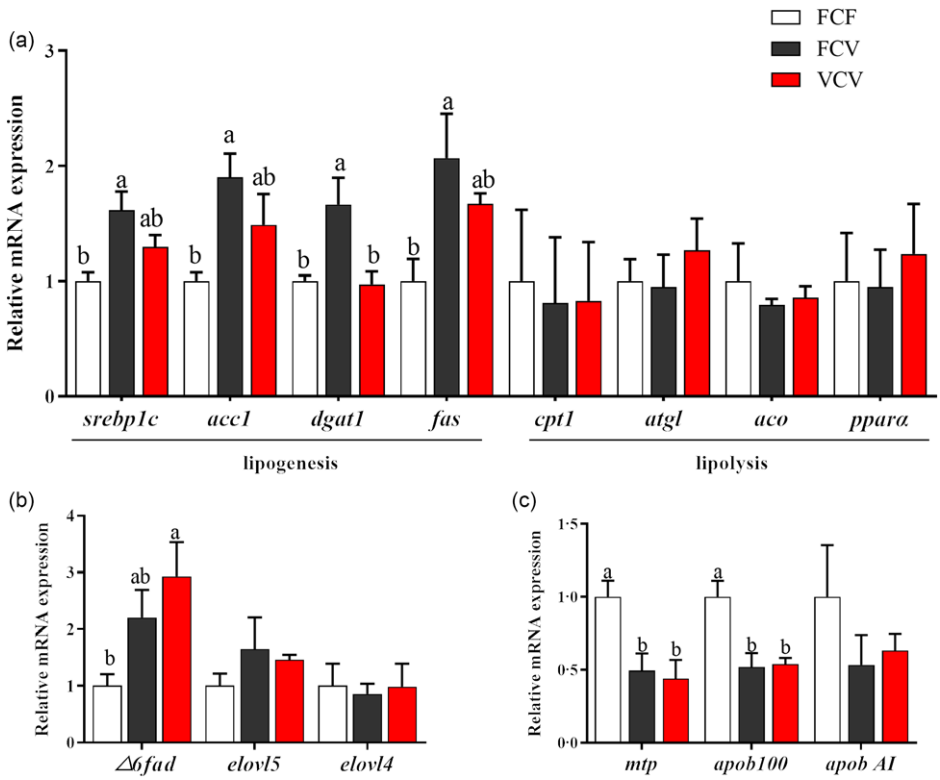

Fig. 5. Expression of lipid metabolism-related genes in the liver of large yellow croaker during the challenge phase. (a) Lipid lipogenesis and lipolysis; (b) LC-FUFA synthesis and (c) lipid transport. Values are means (n 3), with their standard errors represented by vertical bars. Bars bearing the same letters were not significantly different ($P > 0{\cdot}05$, Tukey's test).

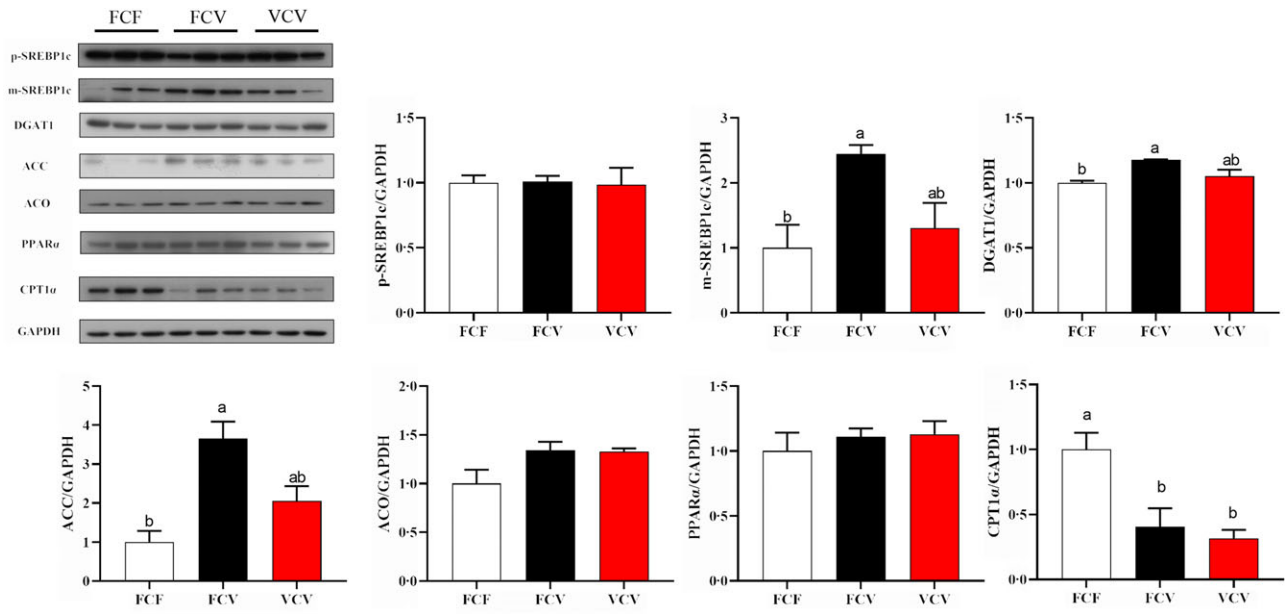

Fig. 6. Expression of lipid lipogenesis and lipolysis-related proteins in the liver of large yellow croaker during the challenge phase. p-SREBP1c, precursor SREBP1c; m-SREBP1c, mature SREBP1c. Values are means (n 3), with their SE represented by vertical bars. Bars bearing the same letters were not significantly different ($P > 0{\cdot}05$, Tukey's test).

damage caused by the VO diet in later life. Similarly, studies of nutritional programming affecting liver function have also been reported in mammals, where protein restriction during pregnancy and lactation or maternal high fat intake can cause liver damage in offspring[57,58]. Oxidative stress causes overproduction of reactive oxygen species, leading to lipid

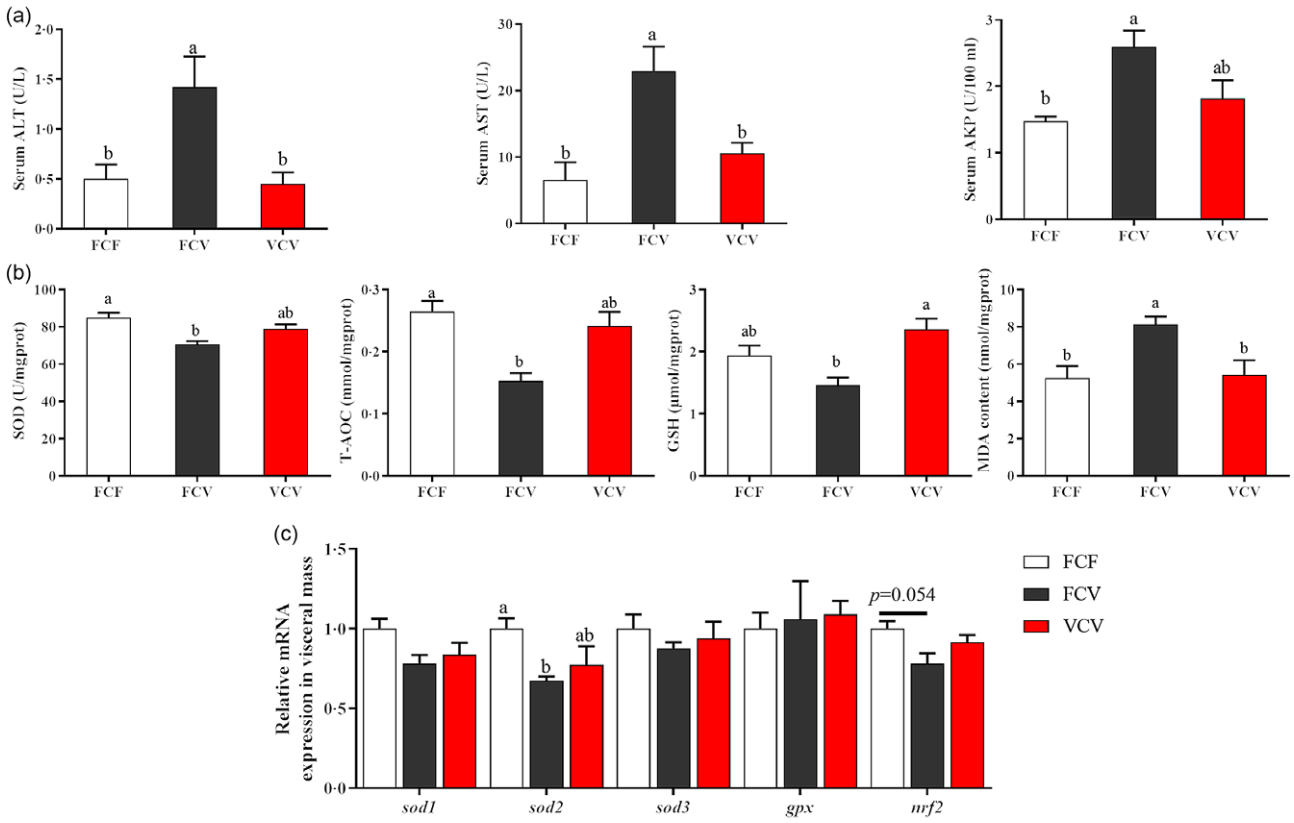

Fig. 7. Liver damage and antioxidant capacity-related parameters of large yellow croaker during the challenge phase. (a) Liver damage: serum ALT, AST and AKP; (b) antioxidant parameters: liver SOD, T-AOC, GSH and MDA and (c) antioxidant genes. Values are means (n 3), with their standard errors represented by vertical bars. Bars bearing the same letters were not significantly different ($P > 0{\cdot}05$, Tukey's test).

peroxidation and DNA damage[59]. As one of the metabolites derived from lipid peroxidation, MDA is a widely known biomarker of oxidative stress damage[60]. In this study, fish with a VO nutritional history showed significantly lower MDA content in the liver than fish with a FO nutritional history when challenged with the VO diet. Furthermore, antioxidant enzymes and substances (SOD, total antioxidant capacity and GSH) were higher in fish with a VO nutritional history than fish with a FO nutritional history when challenged with the VO diet, despite statistically significant differences only occurring in GSH. In addition, antioxidant genes (*sod2* and *nrf2*) showed similar trends. Related results have been confirmed in mammals. Specifically, when maternal mice were fed a high-fat diet, adult offspring developed hypertension combined with increased MDA and decreased activity of SOD, CAT and GPx[61]. Nutritional programming can also be used to alleviate oxidative stress. Prenatal exposure to quercetin resulted in upregulation of Nrf2 and Sod2 in the liver of foetus at Day 14·5 of gestation and in both the liver and lung tissue of adult mice[62]. Barden *et al.*[63] demonstrated that high-dose *n*-3 PUFA supplementation in pregnancy can regulate foetal oxidative stress. There are few related studies in fish. Parental se supplementation of rainbow trout has long-lasting effects on antioxidant metabolism even if it reduce the hypoxic stress resistance of the progeny[64]. However, early LC *n*-3 PUFA programming has no remarkable difference in the antioxidant activities of SOD, total antioxidant capacity, CAT and MDA of Siberian sturgeon at all three stages (early programming period, intermediate period and adaptability period)[65]. In general, it could be speculated that early VO nutritional programming of large yellow croaker larvae may relieve liver damage and reduce hepatic antioxidant capacity caused by the VO diet in later life. However, the mechanism by which VO nutritional programming regulates hepatic antioxidant capacity and liver damage is still unclear. Although the stimulating factors are different, studies on mammals have shown that metabolic diseases such as oxidative stress and liver damage are mostly related to epigenetic modifications such as DNA methylation[66–68]. Compared with mammals, there are fewer studies on nutritional programming in fish, and the underlying mechanism needs further study.

Conclusion

While no significant effects of nutritional programming on juvenile growth and survival were observed in this study, a short VO stimulus during the early life of large yellow croaker had a long-term effect on lipid metabolism and the antioxidant system. Specifically, VO nutritional programming had a positive effect on alleviating abnormal lipid deposition on the liver, liver damage and the reduction of hepatic antioxidant capacity caused by a VO diet. Further research is needed to better understand the

potential epigenetic, metabolic and molecular mechanisms involved in the nutritional programming of large yellow croaker.

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The authors declare that they have no conflict of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114522001726

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