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Effects of dietary lipid level on growth, fatty acid composition, digestive enzymes and expression of some lipid metabolism related genes of orange-spotted grouper larvae (*Epinephelus coioides* H.)

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Abstract

A 4-week study was conducted to investigate the effects of dietary lipid level on growth, fatty acid composition, activities of digestive enzymes and some lipid metabolism related genes of orange-spotted grouper larvae (*Epinephelous coioides*). Six isonitrogenous (57% crude protein) diets were formulated to contain graded contents of lipid (5.94%, 9.92%, 13.30%, 17.71% and 21.87% dry weight, respectively) using fish oil as main lipid source. Each diet was fed six times daily to triplicate groups of fish (71 ± 2 mg, 29 DAH). Results showed that the highest SGR and survival of larvae occurred in 17.71% and 13.30% lipid group respectively. The optimal dietary lipid requirement of larval grouper was estimated to be 15.99% based on SGR. Activities of pepsin and lipase were significantly higher in 17.71% lipid group compared to the rest ones. However, the highest activities of trypsin and amylase occurred in 9.92% and 5.94% lipid treatment respectively. The expression of fatty acid synthesis (FAS) decreased significantly with increasing dietary lipid, while the expression of lipoprotein lipase (LPL) and hormone sensitive lipase (HSL) significantly increased first and then decreased significantly. Compared to other treatment, larvae fed diets with 13.30% and 17.71% lipid have a significant higher relative expression of LPL and HSL respectively. Results of this study suggested that larvae fed the diet with appropriate lipid content showed higher growth performance. Moreover, dietary lipid content could also influence both *de novo* lipogenesis and lipolysis in transcriptional level.

Keywords: dietary lipid, growth, digestive enzyme, fatty acids, gene expression, *Epinephelus coioides*

Introduction

Orange-spotted grouper, *Epinephelus coioides*, is a popular fish cultured in Southeast Asia and a good candidate for intensive aquaculture due to its fast growth rats, efficient feed conversion and high market value (Millamena 2002). However, low survival of grouper larvae, mainly caused by egg quality, its cannibal behaviour and life history, seriously restricts the sustainable development of grouper cultivation. During the development of grouper larvae, the growth and configuration of spine of dorsal and ventral fin was the most obvious changes besides pigmentation, which make it be more sensitive to deficiency of nutrients (Xie, Weng, Su & Wang 2007; Liu, Zhang, Fang, Wang & Guan 2008). Rimmer (2004) also found that nutritional (particularly unsaturated fatty acid) requirements of larval grouper were likely to be a limiting factor in successful grouper larviculture. Although some studies on the nutrition of grouper have primarily focused on the juvenile stage (Luo, Liu, Mai, Tian, Liu, Tan & Lin 2005; Luo, Liu, Mai, Tian, Yang, Tan & Liu 2005; Ye, Liu, Tian,

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Mai, Du, Yang & Niu 2006), no information is available on the physiological effects of lipid on grouper larvae.

Dietary lipids are the main source of energy which also provide essential fatty acids (EFA), phospholipids, sterols and fat-soluble vitamins necessary for larval fish species (Sargent, McEvoy, Estevez, Bell, Bell, Henderson & Tocher 1999). A significant number of research has been performed evaluating the effects of lipid level on growth and survival of fish larvae. Results of these studies showed that appropriate lipid level could improve larval survival and growth, while excessive lipid content had adverse effects (Ai, Zhao, Mai, Xu, Tan, Ma & Liufu 2008). Optimal lipid level of marine fish larvae have been extensively studied using live prey with different oils (Zheng, Zhu, Han, Yang, Wu & Xie 2010). In recent years, formulated micro-diet, compared with live prey, has drawn much attention in aquaculture research to reduce cost and to balance the nutrition of fish larvae. However, only few studies assessing the optimal dietary lipid level in fish larvae have been conducted using formulated micro-diets (Ai *et al.* 2008; Zheng *et al.* 2010).

Previous research have also shown that dietary lipid level have an impact on lipid metabolic enzymes in fish (Alvarez, Diez, Lopez-Bote, Gallego & Bautista 2000; Han, Wen, Zheng & Li 2011). Higher dietary lipid content may increase the activity of lipoprotein lipase (LPL) and hormone sensitive lipase (HSL), but depress the activity of fatty acid synthase (FAS). FAS plays a crucial role in *de novo* lipogenesis by catalysing all the reaction steps in the conversion of acetyl-CoA and malony-CoA to saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0) (Sargent 1989). LPL is an important regulator of lipid and lipoprotein metabolism, which is involved in the hydrolysis of triglyceride (TG) from TG-rich lipoproteins (Eckel 1989; Wang & Eckel 2009). HSL is expressed predominantly in adipose tissue, where it is believed to be the key enzyme in lipolysis of stored TG (Haemmerle, Zimmermann, Hayn, Theussl, Waeg, Wagner, Sattler, Magin, Wagner & Zechner 2002). Based on these observations, it was postulated that the expression of lipid metabolism related genes (FAS, LPL and HSL) in orange-spotted grouper is affected by dietary lipid level, thus offering a potential mechanism for the modulation of growth performance and body composition content.

The growth performance of larvae was also affected by its ability to absorb nutrients (Xie, Ai, Mai, Xu & Ma 2011). Meanwhile, the ability of larvae to assimilate required nutrients depends on dietary composition and on their capacity of modulating their digestive enzymes and metabolic processes (Cahu & Zambonino Infante 2001). The digestive tract of marine fish larvae begins to develop during the early stages (Krogdahl & Sundby 1999). Many studies have reported that dietary composition could affect the development of fish larvae (Infante & Cahu 2007). Therefore, activities of digestive enzymes were an important index to reflect the effects of nutrient on larval development. Considering the role of dietary lipid in larval development, research on lipid nutrition may play a crucial role in weaning for grouper larvae.

The aim of this study was to determine the effects of dietary lipid levels on growth, survival, activities of digestive enzymes, fatty acid composition and expression of some lipid metabolism related genes of larval grouper and seek appropriate lipid content in micro-diet for grouper larvae.

Materials and methods

Experimental diets

Five isonitrogenous experimental diets of 57% crude protein (Yu, Ai, Mai, Ma, Cahu & Zambonino Infante 2012) were formulated to contain five lipid levels (5.94%, 9.92%, 13.30%, 17.71% and 21.78% dry matter) by adding grade levels of fish oil. Defatted fish meal, white fish meal, low temperature (LT)-krill meal, squid meal and hydrolysed fish meal, together with casein were used as protein sources. Fish oil and soy lecithin were used as the main lipid sources. Ingredients and nutrients composition of the five experimental diets are given in details (Tables 1 and 2).

All solid ingredients were ground into fine powder through 75 μm mesh. Ingredients of all diets were blended manually and then by machine. After that, the oil mixture (fish oil and lecithin) were added to one diet to mix the other ingredient thoroughly and then water were incorporate to make stiff dough. Pellets were pelleted by an automatic pellet-making machine (Weihai, Shandong province, China) and dried for about 8 h in a ventilated oven at 45°C. After drying, the diets were

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Table 1 Formulation and proximate analysis of the experimental diets (g kg^{-1} dry weight)

Ingredients (g kg^{-1} dry weight)	Dietary lipid content (%)				
	5.94	9.92	13.3	17.71	21.87
Casein*	190	190	190	190	190
White fish meal†	130	130	130	130	130
Defatted fish meal‡	200	200	200	200	200
LT-Krill meal§	100	100	100	100	100
Squid meal¶	40	40	40	40	40
Hydrolysed fish meal**	60	60	60	60	60
LT-Yeast	20	20	20	20	20
Alginate sodium	10	10	10	10	10
Wheat starch	161	126	91	56	21
Vitamin premix††	15	15	15	15	15
Mineral premix‡‡	15	15	15	15	15
Attractant§§	15	15	15	15	15
Antioxidant	1	1	1	1	1
Choline chloride	2	2	2	2	2
Anchovy oil	0	35	70	105	140
Soy lecithin	40	40	40	40	40
Mould inhibitor¶¶	1	1	1	1	1
Proximate composition (n = 3)					
Crude protein	57.14	56.79	56.68	56.56	55.77
Crude lipid	5.94	9.92	13.3	17.71	21.78
Ash	12.87	13.65	13.57	13.31	13.34

*Crude protein 87.91% dry matter, crude lipid 1.69% dry matter; Qingdao Great Seven Bio-Tech., China.

†Crude protein 71.18% dry matter, crude lipid 5.32% dry matter; Qingdao Great Seven Bio-Tech., China.

‡Crude protein 73.36% dry matter, crude lipid 1.52% dry matter; White fish meal were defatted with ethanol [fish meal: ethanol = 1:2 (w:v)] at 37°C for three times. Qingdao Great Seven Bio-Tech., China.

§Crude protein 63.76% dry matter, crude lipid 12.95% dry matter; Shandong Keruier Biological Products, Jinan, China.

¶Crude protein 61.72% dry matter, crude lipid 3.16% dry matter; Zhejiang Jinhaiyun Biology, Wenzhou, China.

**Crude protein 77.10% dry matter, crude lipid 4.60% dry matter. Zhejiang Jinhaiyun Biology, Wenzhou, China.

††Vitamin premix (IU or g kg^{-1} vitamin premix): retinal palmitate, 3 000 000 IU; cholecalciferol, 1 200 000 IU; DL-α-tocopherol acetate, 40.0 g; menadione, 8.0 g; thiamin-HCl, 5.0 g; riboflavin, 5.0 g; D-calcium pantothenate, 16.0 g; pyridoxine-HCl, 4.0 g; meso-inositol, 200.0 g; D-biotin, 8.0 g; folic acid, 1.5 g; para-aminobenzoic acid, 5.0 g; niacin, 20.0 g; cyanocobalamin, 0.01 g; ascorbyl polyphosphate (contained 25% ascorbic acid), 100.0 g.

‡‡Mineral premix (g kg^{-1}): $Ca(H_2PO_4)_2{\cdot}H_2O$, 675.0; $CoSO_4{\cdot}4H_2O$, 0.15; $CuSO_4{\cdot}5H_2O$, 5.0;$FeSO_4{\cdot}7H_2O$, 50.0; KCl, 50.0; KI, 0.1; $MgSO_4.2H_2O$, 101.7;$MnSO_4{\cdot}4H_2O$, 18.0; NaCl, 80.0; $Na_2SeO_3{\cdot}H_2O$, 0.05; $ZnSO_4{\cdot}7H_2O$, 20.0.

§§Attractant (g 100 g^{-1}): betaine, 50; glycine, 15; alanine, 10; argine, 10; taurine, 10; inosine-5′-monophosphoric acid, 5.

¶¶Contained 50% calcium propionic acid and 50% fumaric acid.

Table 2 Fatty acid composition of the experimental diets (% identified fatty acids)

Fatty acid	Dietary lipid content (%)				
	5.94	9.92	13.30	17.71	21.87
14:0	4.29	4.54	4.95	5.17	5.28
16:0	21.93	22.87	19.38	19.81	18.41
18:0	3.43	3.01	2.59	2.61	2.13
20:0	0.00	0.31	0.33	0.41	0.39
∑SFA	29.65	30.72	27.25	28.00	26.20
16:1	5.21	5.69	6.99	6.97	7.48
18:1	18.08	18.51	19.46	20.19	20.98
∑MUFA	23.29	24.19	26.45	27.16	28.46
18:2n-6	21.99	15.75	13.98	12.00	11.50
20:4n-6	0.43	0.43	0.47	0.65	0.60
∑n-6PUFA	22.42	16.19	14.45	12.64	12.11
18:3n-3	2.64	2.36	2.37	2.14	2.24
20:5n-3	6.73	6.92	7.25	7.31	7.36
22:6n-3	6.29	7.60	8.15	8.55	8.85
∑n-3PUFA	15.66	16.87	17.77	17.74	18.44
n-3/n-6PUFA	0.70	1.04	1.23	1.40	1.52
n-3LC-PUFA	13.01	14.52	15.40	15.86	16.20
DHA/EPA	0.93	1.10	1.12	1.17	1.20

SFAs, saturated fatty acids; MUFAs, mono-unsaturated fatty acids; n-6 PUFAs, n-6 poly-unsaturated fatty acids; n-3 PUFAs, n-3 poly-unsaturated fatty acids; n-3 LC-PUFAs, n-3 long chain-polyunsaturated fatty acids; DHA/EPA, 22:6n-3/20:5n-3.

broken to sizes ranged from 250 to 380 μm for the larvae between 29 and 45 days after hatching and 380 to 550 μm for the larvae thereafter, and then were stored at −20°C until used.

Experiment procedure

The feeding trial was carried out at Hainan Virtue Wealth Aquatic Technology Development, and larval grouper were obtained from a local fish rearing farm in Yandun, Hainan, China. A total of 2100 larvae of the 29 day after hatch (DAH) age, a more formal staging reference, were distributed into 15 white plastic tanks (water volume 100 L) at a stocking density of 140 individuals per tank which were placed in an indoor concrete pond.

Before the experiment, rotifers (*Artemia nauplii*) and micro-diet (the experiment diet with 13.30% lipid) were used alternately to wean the larvae for 3 days, and then 200 larvae were randomly sampled to determine the wet body weight and body length. At the beginning, the fish were fasted for 24 h and each diet was randomly assigned to triplicate groups of fish. Fish were fed to apparent

satiation six times daily manually (07:00, 09:00, 11:00, 13:00, 15:00 and 17:00 hours) for 4 weeks. During the rearing period, the water temperature ranged from 28 to 30°C, and salinity from 28.5 to 31.0‰. Approximately 300% of water volume was renewed daily and there was a light permanent agitation by means of air bubbling. The photoperiod was set to a 12L:12D cycle. The intensity of light provided by incandescents was about 7.9 WM^{-2} during daytime at the water surface. Organic matter floating on the water surface was skimmed with a polyvinylchloride pipe in time and accumulations of feed and faeces at the tank bottoms were syphoned twice daily. At the termination of the experiment, all grouper have already finished morphogenesis from larvae to juvenile, and were also fasted for 24 h before harvest and 30 fish were randomly sampled in each tank to determine wet body weight of the juvenile. Survival was determined by counting individuals remaining in each tank. The remaining fish of each tank were collected and immediately frozen into liquid nitrogen and then stored at −80°C for enzymatic assays.

Biochemical analysis

The chemical composition of diets and fish were determined according to the procedures of the Association of Official Analytical Chemists (AOAC 2003). To determine the dry mater content of diets and fish, they were dried to a constant weight at 105°C. Crude protein was determined by the Kjeldahl method ($N \times 6.25$) and crude lipid was determined by ether extraction using a Soxhlet extractor. Ash content was determined by a muffle furnace (550°C) for 24 h and weighing residue. The procedure for analysis of the fatty acid profiles was based on the method described by Metcalfe, Schmitz and Pelka (1966) with some modification (Ai *et al.* 2008). Fatty acid methyl esters from fatty acids (FA) of diets and fish tissue samples were prepared and quantified using HP6890 gas chromatograph (Agilents Technologies, Santa Clara, CA, USA) with a fused silica capillary column (007-CW; Hewlett Packard, Palo Alto, CA, USA) and a flame ionization detector. The column temperature was programmed to rise from 150°C up to 200°C at a rate of 15°C min^{-1}, from 200°C to 250°C at a rate of 2°C min^{-1}. Injector and detector temperature was 250°C respectively. Upelco 37 Compoent FAME Mix (Nu-Chek, Elysian, MN, USA) and HPCore ChemStation workstation (Palo Alto, CA, USA) was used to identify and quantify each fatty acid after separation using gas chromatography.

Digestive enzymes

Nine fish of each replicate tank, which were stored at −80°C, were dissected to remove the entire digestive tract on glass plate maintained at 0°C, and then separate stomach and intestine. The dissected samples, 0.2–0.3 g, were homogenized in 2 mL cold (0°C) ultrapure water, and then were centrifuged at 3300 *g* for 3 min. The supernatant was collected for further assay. The activity of pepsin was determined according to Anson (1938). Bovine haemoglobin (Sigma H-2625; St. Louis, MO, USA) was used as substrate. Soluble fractions were determined by Folin-phenol reagent (AppliChem A-5084). One unit was defined as 1 μg tyrosine by hydrolysing bovine haemoglobin in 1 min at 37°C. The activity of trypsin was measured according to Holm, Krogdahl and Hanssen (1988), and BAPNA (benzoyl-D-L-arginine-p-nitroanilide) (Sigma B-4875) was used as substrate. One unit was defined as 1 μmol PNA (p-nitroanilide) released by catalysing BNPNA in 1 min at 37°C. The activity of amylase was assayed as described by Métais and Bieth (1967). Starch soluble (Sigma S-9765) was used as substrate. One unit was defined as 1 mg soluble starch hydrolysed totally in 30 min. The activity of lipase was determined according to the methods of Brockman (1981). PVA (polyviny alcohol)-olive oil emulsion was used as substrates. One unit was defined as 1 μmol fatty acid released by hydrolysing lipid in 1 min at 37°C. Protein was determined by the Bradford (1976) procedure using bovine serum albumin (Sigma A-2153) as a standard and enzyme activities are expressed as specific activity (U/mg protein).

Total RNA extraction, reverse transcription and sub cloning

Total RNA was extracted from grouper liver using TRIzol Reagent (Takara, Tokyo, Japan) followed by quality measurement on a 1.2% denaturing agarose gel and yield determination on NanoDrop® ND-1000 (Wilmington, DE, USA). First strand cDNA was synthesized using PrimeScript™ RT

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reagent Kit (Takara) following the manufacturer recommendations. To obtain the first fragment of HSL cDNA of grouper, degenerate polymerase chain reaction (PCR) primers were designed based on highly conserved regions from HSL sequences of other fish in Genbank and were synthesized by Biosune Biotech (Shanghai, China): HSL F: 5′-GCC G(C/T)T GCC T(G/C)G GCT TTC-3′; R: 5′-G(G/T)G A(C/T)T T(G/C)G AGG TCT G(G/T)G-3′. PCR program was carried out in Eppendorf Mastercycler Gradient (Eppendorf, Hamburg) and the PCR conditions were: 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 40 s at 72°C; another 10 min at 72°C. The amplification products were separated by electrophoresis on a 1.5% agarose gel for length difference, and then the target band was ligated into the pEASY-T1 vector (TransGen Biotech, Beijing, China). Two microlitres of each ligation reaction were transformed into the competent cells of *Escherichia coli* TOP10, and then plated on LB agar plates overnight at 37°C. White colonies were selected and transferred to LB both containing ampicillin, cultured at 37°C for 8 h under shaking at 200 rpm. Specific primers for HSL and M13 were used to select the plasmid clones, and only plasmid clones containing a target insert were sequenced in Biosune Biotech (Shanghai, China). Figure 1 showed the partial nucleotide sequence and deduced amino acid sequence of *E. coioides* HSL cDNA. The sequence (Genbank accession number: KF049203) revealed a fragment of 591 bp highly homologous to gilthead seabream (92%), black seabream (91%) and tilapia (88%) HSL.

Real-time quantitative PCR analysis of FAS, LPL and HSL expression

The total RNA extracted from visceral mass were treated with Recombinant DNase I (RNase-free) (Takara) to remove possible DNA contaminant and reverse transcribed to cDNA by PrimeScript™ RT reagent Kit (Takara) following the instructions. The real-time RT-PCR primer pairs (Table 3) were designed by Primer Primier 5.0 based on the nucleotide sequences of FAS (GenBank: FJ196231), LPL (GenBank: EU683732), HSL (GenBank: KF049203) and β-actin (GenBank: AY510710) of grouper. Real-time RT-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 2 × SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara), 0.5 μL each of primers (10 μmol L^{-1}), 1 μL of cDNA mix. The real-time RT-PCR program was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s and 72°C for 20 s. At the end of each PCR reaction, melting curve analysis of amplification products was carried out to confirm that a single 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 analysed

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1     GCCGTTGCCTCGGCTTTCAGTTCACTCCAGCCATCCGGCCCTGTCTCCAGACCATCGCTA
1        R  C  L  G  F  Q  F  T  P  A  I  R  P  C  L  Q  T  I  A
61    TTGGCCTTGTGGCCTTTGGAGAAAACTACAGACGCCATCAGTCAGGAATAGGTGTAGCAG
20     I  G  L  V  A  F  G  E  N  Y  R  R  H  Q  S  G  I  G  V  A
121   CCAGCTCTTTCTTTACCTCAGGGAAGTACGCCATTGACCCAGAGTTGAGAGGGGCAGAAT
40     A  S  S  F  F  T  S  G  K  Y  A  I  D  P  E  L  R  G  A  E
181   TTGAACGCATCACCCAGAACCTGGACGTCCACTTCTGGAAGACCTTCTGGAACATCACAG
60     F  E  R  I  T  Q  N  L  D  V  H  F  W  K  T  F  W  N  I  T
241   AGACAGAAGTCCTGTCTAGTCTTGCCAGTATGACATCCACGCCAGTTAAGGTGAACCGGG
80     E  T  E  V  L  S  S  L  A  S  M  T  S  T  P  V  K  V  N  R
301   CTCTTTCTGTGCCCCCCGTGCCCTTTGACCTTCCCCTGGCAGCCAACCACAGAGCATCCG
100    A  L  S  V  P  P  V  P  F  D  L  P  L  A  A  N  H  R  A  S
361   TGACCATAGCCCCACCATCAGCACACATTGGCACTGCTCCAGTTCAGATGAGGCTCATCT
120    V  T  I  A  P  P  S  A  H  I  G  T  A  P  V  Q  M  R  L  I
421   CCTGTGACTTACGCGAAGGACAGGACAGTGAGACTCTGCTCTCTCTCTCCCGCTCTGAGG
140    S  C  D  L  R  E  G  Q  D  S  E  T  L  L  S  L  S  R  S  E
481   GGGGCGCCATCTCTTTGTCACTGGGGCTGAAAACCAAGCGCCTCCCATCATCTCCCTGCC
160    G  G  A  I  S  L  S  L  G  L  K  T  K  R  L  P  S  S  P  C
541   TCCTGATTCACTTCCACGGGGGAGGATTTGTGGCCCAGACCTCCAAGTCAC
180    L  L  I  H  F  H  G  G  G  F  V  A  Q  T  S  K
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Figure 1 Partial nucleotide sequence and deduced amino acid sequence of Epinephelus coioides HSL cDNA.

Table 3 Real-time PCR primers used in the experiment

Target gene	Reference	Forward (5′–3′)	Reverse (5′–3′)
FAS	GenBank:FJ196231	CTCCCAACCTGCACTTCAA	TTCACTGCGTCCTCTGTCC
LPL	GenBank:EU683732	GGGAGAAGGATGCACTCAT	TTTACGGCTCATGTTGTCTT
HSL	GenBank: KF049203	AGAATTTGAACGCATCACC	GAGCCTCATCTGAACTGGA
β-actin	GenBank: AY510710	TACGAGCTGCCTGACGGACA	GGCTGTGATCTCCTTCTGCA

FAS, fatty acid synthase; LPL, lipoprotein lipase; HSL, hormone sensitive lipase.

according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The expression levels of the target genes were calculated followed the $2^{-\Delta\Delta t}$ method described by Yao, Kong, Wang, Ji, Liu, Cai and Han (2009).

Calculations and statistical analysis

The following calculations were performed:

$$\text{Specific growth rate}(\text{SGR}, \%\text{day}^{-1}) = (\ln W_t - \ln W_0) \times 100/\text{d}$$

Where W_t and W_0 were final and initial wet body weight respectively; d is experimental period in days.

$$\text{Survival rate}(\%) = \text{Nf}_t \times 100/\text{Nf}_i$$

Nf_i and Nf_t were the initial and final fish number during the feeding trail experiment respectively.

The results were presented as means ± SEM. Data from each treatment were subjected to a one-way ANOVA and correlation analysis where appropriate using SPSS 19.0 for Windows. Tukey's multiple range test was chosen as a multiple comparison test and the significance level of 5% were used.

Results

Survival and growth performance

Larvae fed the diet with 13.30% lipid had comparable survival with 17.71% lipid group, but significant higher than other groups (Table 4). Meanwhile, larvae fed the diet with 17.71% lipid had comparable specific growth rate (SGR) with 13.30% and 21.87% lipid groups but significant higher than other groups (Table 4). The broken-line regression curve, usually used to quantify the requirements of nutrients in aquatic species, was used to estimate the optimal lipid level for grouper larvae: $Y = 11.20 + 0.131\ (X - 15.978)$ ($R^2 = 0.916$) and $(x - 15.987) = 0$ when $x > 15.987$ (Fig. 2). This result indicated that the lipid requirement of larval grouper was estimated to be 15.99% based on SGR.

Body composition

Larvae fed the diet with 13.30% lipid had significant higher protein than 17.71% and 21.78% lipid groups but comparable with other groups. As dietary lipid increased from 5.94% to 17.71%, the crude lipid level increased significantly ($P < 0.05$) and thereafter decreased significantly ($P < 0.05$) (Table 5). The moisture of larvae fed

Table 4 Growth response and survival of larval grouper (29DAH) fed diets with graded levels of lipid for 4 weeks

	Dietary lipid content (%)				
Growth response	**5.94**	**9.92**	**13.3**	**17.71**	**21.87**
Initial body weight (mg)	71 ± 2	71 ± 2	71 ± 2	71 ± 2	71 ± 2
Final body weight (g)	1.07 ± 0.04^{a}	1.12 ± 0.04^{a}	1.40 ± 0.05^{b}	1.54 ± 0.05^{b}	1.46 ± 0.05^{b}
Final body length (cm)	4.10 ± 0.05	4.19 ± 0.20	4.37 ± 0.16	4.62 ± 0.06	4.43 ± 0.13
SGR (% d^{-1})	10.00 ± 0.15^{a}	10.17 ± 0.12^{a}	10.78 ± 0.13^{b}	11.35 ± 0.11^{b}	11.05 ± 0.23^{b}
Survival (%)	51.79 ± 2.23^{a}	53.08 ± 1.54^{a}	64.87 ± 3.45^{b}	56.41 ± 1.85^{ab}	48.97 ± 0.51^{a}

Mean values ($n = 3$) within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0.05$).

SGR, specific growth rate.

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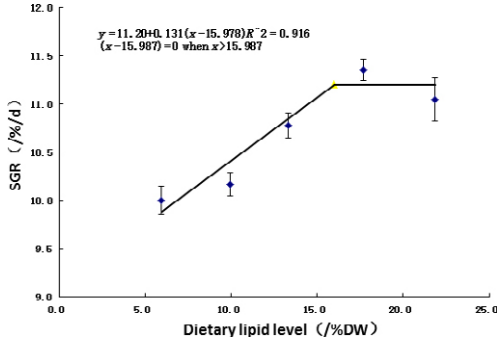

Figure 2 Relationship between dietary lipid levels and specific growth rates (SGRs) of larval grouper (29DAH) fed experimental diets for 4 weeks as fitted by broken-line regression analysis, and showed that the optimal dietary lipid level is 15.99%.

diets with 5.94% and 9.92% lipid was significantly higher than 13.30% and 17.71% lipid groups ($P < 0.05$), but comparable to 21.87% lipid group (Table 5).

Fatty acid composition

The fatty acid profiles of carcass, muscle and visceral mass reflected those of diets in the study (Tables 6–8). The ratio of saturated fatty acid (SFA) and 18:2n-6 to identified fatty acids in carcass, muscle and visceral mass decreased significantly with increasing dietary lipid ($P < 0.05$). However, the changing trend of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)/identified fatty acids were inverse relationship with SFA (Tables 6–8). The ratio of DHA to identified fatty acids in carcass and muscle, especially muscle, were higher than its dietary ratio. Meanwhile, the ratio of EPA to identified fatty acids in carcass, muscle and visceral mass were lower than its dietary ratio.

Activities of digestive enzymes

Larvae fed diets with 5.94% and 17.71% lipid had significantly higher activities of pepsin than other groups ($P < 0.05$). Meanwhile, the activity of pepsin in larvae fed the diet with 17.71% lipid was significant higher than 5.94% lipid group ($P < 0.05$). The activity of trypsin in larvae fed the diet with 9.92% lipid was comparable to 21.87% lipid group, but significantly higher than other groups ($P < 0.05$). However, the activity of amylase in larvae fed the diet with 17.71% lipid was significant lower than 21.87% lipid group which was also significant lower than other groups ($P < 0.05$). Larvae fed the diet with 13.30% lipid had higher activity of lipase than other groups, while significant difference was found only between treatments fed diets with 9.92% and 13.30% lipid (Table 9).

Lipid metabolism related gene expression

Relative mRNA expression of FAS, LPL and HSL in the visceral mass of grouper were affected significantly by dietary lipid (Fig. 3). The expression levels of FAS significantly decreased with increasing dietary lipid ($P < 0.05$) and the mRNA expression levels were decreased by approximately 0.11-fold, 0.30-fold, 0.50-fold and 0.79-fold in larvae fed diet with 9.92%, 13.30%, 17.71% and 21.87% lipid respectively. Meanwhile, LPL expression levels significantly increased to the maximum level ($P < 0.05$) when dietary lipid level increased from 5.94% to 17.71% and then decreased when lipid content increased to 21.87%. The expression level of LPL was up-regulated by aboult 0.27-fold, 1.02-fold, 0.79-fold and 0.03-fold in treatments with 9.92%, 13.30%, 17.71% and 21.87% lipid respectively. The expression levels of HSL and LPL showed a similar variation trend, while no

Table 5 Body composition (% wet weight) of grouper larvae (29DAH) fed diets with graded levels of lipid for 4 weeks

	Dietary lipid content (%)				
	5.94	**9.92**	**13.3**	**17.71**	**21.87**
Crude protein, %	15.41 ± 0.03^{bc}	15.33 ± 0.11^{bc}	15.59 ± 0.07^{c}	15.16 ± 0.03^{b}	14.75 ± 0.11^{a}
Crude lipid, %	3.23 ± 0.03^{a}	3.67 ± 0.02^{b}	5.13 ± 0.01^{d}	5.58 ± 0.05^{e}	4.92 ± 0.05^{c}
Moisture, %	78.00 ± 0.02^{b}	77.69 ± 0.02^{b}	75.97 ± 0.05^{a}	75.95 ± 0.41^{a}	77.04 ± 0.67^{ab}

Mean values ($n = 3$) within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0.05$).

Table 6 Fatty acid composition (% identified fatty acids) in the carcass of larval grouper (29DAH) fed the diets with graded lipid for 4 weeks*

	Dietary lipid content (%)				
Fatty acid	**5.94**	**9.92**	**13.3**	**17.71**	**21.87**
14:0	3.54 ± 0.14^{a}	3.69 ± 0.10^{a}	4.02 ± 0.12^{ab}	4.22 ± 0.04^{b}	4.25 ± 0.11^{b}
16:0	21.26 ± 0.12^{c}	19.57 ± 0.22^{b}	17.32 ± 0.13^{a}	17.85 ± 0.16^{a}	17.33 ± 0.31^{a}
18:0	4.58 ± 0.21	4.22 ± 0.08	4.25 ± 0.12	4.28 ± 0.19	4.35 ± 0.19
20:0	2.42 ± 0.05^{a}	2.71 ± 0.04^{b}	2.75 ± 0.06^{b}	2.59 ± 0.03^{ab}	2.61 ± 0.05^{ab}
∑SFA	31.80 ± 0.38^{c}	30.19 ± 0.41^{b}	28.34 ± 0.31^{a}	28.94 ± 0.12^{a}	28.53 ± 0.28^{a}
16:1	5.90 ± 0.29^{ab}	5.59 ± 0.25^{a}	6.71 ± 0.10^{bc}	6.50 ± 0.22^{abc}	7.00 ± 0.08^{c}
18:1	23.18 ± 0.31	23.46 ± 0.09	24.05 ± 0.15	24.15 ± 0.33	23.89 ± 0.41
∑MUFA	29.09 ± 0.19^{ab}	29.05 ± 0.26^{a}	30.76 ± 0.08^{c}	30.64 ± 0.52^{bc}	30.90 ± 0.43^{c}
18:2n-6	17.20 ± 0.39^{a}	14.35 ± 0.25^{b}	13.08 ± 0.24^{c}	11.97 ± 0.12 ^{cd}	11.19 ± 0.05^{d}
20:4n-6	0.64 ± 0.00^{a}	0.76 ± 0.00^{b}	0.66 ± 0.02^{a}	0.69 ± 0.03^{ab}	0.85 ± 0.02^{c}
∑n-6PUFA	17.76 ± 0.46^{d}	15.06 ± 0.20^{c}	13.74 ± 0.25^{b}	12.67 ± 0.15^{ab}	12.05 ± 0.03^{a}
18:3n-3	2.40 ± 0.12	2.26 ± 0.05	2.19 ± 0.06	2.19 ± 0.07	2.22 ± 0.04
20:5n-3	4.10 ± 0.26^{a}	4.77 ± 0.14^{ab}	5.25 ± 0.21^{bc}	5.79 ± 0.09^{c}	5.81 ± 0.03^{c}
22:6n-3	6.25 ± 0.08^{a}	7.89 ± 0.22^{b}	7.96 ± 0.20^{b}	8.99 ± 0.18^{c}	9.44 ± 0.16^{c}
∑n-3PUFA	13.16 ± 0.06^{a}	14.92 ± 0.21^{b}	15.40 ± 0.47^{b}	16.98 ± 0.23^{c}	17.46 ± 0.20^{c}
n-3/n-6PUFA	0.68 ± 0.04^{a}	0.99 ± 0.03^{b}	1.12 ± 0.02^{c}	1.34 ± 0.02^{d}	1.45 ± 0.02^{d}
n-3LC-PUFA	10.44 ± 0.15^{a}	12.66 ± 0.21^{b}	13.22 ± 0.41^{b}	14.78 ± 0.20^{c}	15.25 ± 0.16^{c}
ARA/EPA	0.14 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.12 ± 0.00	0.15 ± 0.00
DHA/EPA	1.37 ± 0.08^{a}	1.66 ± 0.08^{b}	1.52 ± 0.02^{ab}	1.55 ± 0.04^{ab}	1.63 ± 0.03^{ab}

Mean values (n = 3) within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0.05$).

*Some fatty acids, of which the contents are 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, were not listed in the table.

SFAs, saturated fatty acids; MUFAs, mono-unsaturated fatty acids; n-6 PUFAs, n-6 poly-unsaturated fatty acids; n-3 PUFAs, n-3 poly-unsaturated fatty acids; n-3 LC-PUFAs, n-3 long chain-polyunsaturated fatty acids; ARA/EPA, 20:4n-6/20:5n-3; DHA/EPA, 22:6n-3/20:5n-3.

significant difference were found among fish fed diets with 9.92%, 13.30% and 17.71% lipid. The HSL expression levels were increased by about 0.73-fold, 0.72-fold and 0.87-fold in larvae fed diets with 9.92%, 13.30% and 17.71% while decreased by about 0.23-fold in larvae fed diets with 21.87% lipid.

Discussion

Optimal dietary lipid is required to maintain high growth and survival of marine fish larvae. In this study, 13.30% lipid could meet the minimum requirement of grouper larvae based on SGR. Insufficient digestible energy and deficiencies in essential fatty acids may account for lower growth and survival of larval grouper fed diets with 5.94% and 9.92% lipid. Meanwhile, the survival of larvae fed the diet with 21.87% lipid was lower markedly than those fed 13.30% lipid diet. That is to say, high dietary lipid level caused the increasing mortality of grouper larvae. Similar results have been reported in darkbarbel catfish larvae (Zheng *et al.* 2010), large yellow croaker larvae (Ai *et al.* 2008) and juvenile cobia (Wang, Liu, Tian, Mai, Du, Wang & Yang 2005). This may be partial due to increased malonaldehyde in oxidized lipid, which is harmful to fish (Baker & Davies 1997). High dietary lipid level could also influence fatty acid digestion and absorption (Diaz, Guyot, Vigierand & Connes 1997), lead to fat accretion (Wang *et al.* 2005), impair neurogenesis and induce some metabolic disease (Fraulob, Ogg-Diamantino, Fernandes-Santos, Aguila & Mandarim-de-Lacerda 2010; Park, Park, Choi, Park, Chung & Lee 2010; Kirpich, Gobejishvili, Homme, Waigel, Cave, Arteel, Barve, McClain & Deaciuc 2011), which may also result in lower survival.

The optimal dietary lipid level of grouper larvae was estimated to be 15.99% on basis of SGR in this study through broken-line method, which has usually been used to quantify the requirements of nutrients in aquatic species (Shiau & Huang 2001). However, the dietary lipid requirement of

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Table 7 Fatty acid composition (% identified fatty acids) in the muscle of larval grouper (29DAH) fed the diets with graded lipid for 4 weeks*

	Dietary lipid content (%)				
Fatty acid	**5.94**	**9.92**	**13.3**	**17.71**	**21.87**
14:0	3.05 ± 0.29^{b}	2.30 ± 0.13^{ab}	1.94 ± 0.05^{a}	2.94 ± 0.21^{b}	2.69 ± 0.01^{ab}
16:0	25.71 ± 0.83^{c}	22.35 ± 0.66^{b}	20.31 ± 0.20^{ab}	20.84 ± 0.48^{ab}	18.92 ± 0.24^{a}
18:0	6.28 ± 0.06^{d}	6.88 ± 0.03^{c}	6.68 ± 0.16^{c}	5.89 ± 0.04^{b}	4.79 ± 0.04^{a}
20:0	1.47 ± 0.02^{a}	1.83 ± 0.05^{ab}	2.20 ± 0.19bc	2.62 ± 0.11c	2.39 ± 0.06c
∑SFA^{b}	36.51 ± 1.04^{d}	33.36 ± 0.87^{c}	31.13 ± 0.22^{ab}	32.30 ± 0.54^{b}	28.80 ± 0.16^{a}
16:1	6.26 ± 0.36^{bc}	4.71 ± 0.21^{a}	4.92 ± 0.09^{a}	5.76 ± 0.29^{ab}	7.11 ± 0.16^{c}
18:1	26.35 ± 0.26	25.98 ± 0.12	25.81 ± 0.08	25.88 ± 0.42	25.93 ± 0.35
∑MUFA^{c}	32.61 ± 0.11^{ab}	30.68 ± 0.33^{a}	30.73 ± 0.01^{a}	31.64 ± 0.71^{ab}	33.04 ± 0.51^{b}
18:2n-6	19.73 ± 0.08^{d}	14.89 ± 0.07^{c}	12.42 ± 0.15^{b}	11.50 ± 0.22^{a}	11.19 ± 0.10^{a}
20:4n-6	0.71 ± 0.01^{a}	1.28 ± 0.00^{b}	1.37 ± 0.03^{b}	1.11 ± 0.04^{c}	1.16 ± 0.02^{c}
∑n-6PUFA^{d}	20.44 ± 0.07^{d}	16.17 ± 0.06^{c}	13.79 ± 0.18^{b}	12.62 ± 0.19^{a}	12.34 ± 0.09^{a}
18:3n-3	1.68 ± 0.03^{b}	1.50 ± 0.03^{a}	1.43 ± 0.05^{a}	1.50 ± 0.03^{a}	1.74 ± 0.01^{b}
20:5n-3	5.16 ± 0.09^{a}	5.73 ± 0.08^{b}	6.65 ± 0.09^{c}	6.73 ± 0.07^{c}	6.84 ± 0.06^{c}
22:6n-3	5.83 ± 0.22^{a}	10.60 ± 0.34^{b}	12.73 ± 0.13^{c}	12.01 ± 0.44^{bc}	11.65 ± 0.46^{bc}
∑n-3PUFA^{e}	12.67 ± 0.34^{a}	17.83 ± 0.45^{b}	20.81 ± 0.01^{c}	20.24 ± 0.47^{c}	20.23 ± 0.51^{c}
n-3/n-6PUFA	0.62 ± 0.02^{a}	1.10 ± 0.03^{b}	1.51 ± 0.02^{c}	1.61 ± 0.06^{c}	1.64 ± 0.05^{c}
n-3LC-PUFA^{f}	11.00 ± 0.31^{a}	16.33 ± 0.42^{b}	19.38 ± 0.04^{c}	18.74 ± 0.51^{c}	18.49 ± 0.52^{c}
ARA/EPA^{g}	0.14 ± 0.00^{a}	0.22 ± 0.00^{b}	0.21 ± 0.00^{c}	0.17 ± 0.00^{d}	0.17 ± 0.00^{d}
DHA/EPA^{h}	1.13 ± 0.02^{a}	1.85 ± 0.03^{bc}	1.91 ± 0.04^{c}	1.78 ± 0.05^{bc}	1.70 ± 0.05^{b}

Mean values (n = 3) within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0.05$).

*Some fatty acids, of which the contents are 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, were not listed in the table.

SFAs, saturated fatty acids; MUFAs, mono-unsaturated fatty acids; n-6 PUFAs, n-6 poly-unsaturated fatty acids; n-3 PUFAs, n-3 poly-unsaturated fatty acids; n-3 LC-PUFAs, n-3 long chain-polyunsaturated fatty acids; ARA/EPA, 20:4n-6/20:5n-3; DHA/EPA, 22:6n-3/20:5n-3.

larvae was higher than that estimated in grouper juvenile (10.0%) (Luo, Liu, Mai, Tian, Liu *et al.* 2005), which confirmed that fish larvae require more lipid as sources of energy and essential fatty acids than both juvenile and adult to maintain quick growth performance and development (Rainuzzo, Reitan & Olsen 1997; Sargent *et al.* 1999). This agrees well with previous study on large yellow croaker. The optimal dietary lipid level of large yellow croaker was estimated to be 17.2% and 17.7% (Ai *et al.* 2008), while Duan, Mai, Zhong, Si and Wang (2001) found 12.0–14.0% was the appropriate lipid level for juvenile large yellow croaker. Compared with juvenile, fish larvae required much more n-3 long chain polyunsaturated fatty acids (LC-PUFA), especially DHA and EPA, to maintain normal growth and physiological functions (Benítez-Santana, Masuda, Juárez Carrillo, Ganuza, Valencia, Hernández-Cruz & Izquierdo 2007). Moreover, DHA is necessary for normal development and functioning of nervous system and sensory organs (Benítez-Santana *et al.* 2007). The appropriate proportion of DHA and EPA is as important as the total n-3LC-UFA due to the low enzyme activities involved in PUFA biosynthetic pathway (Sargent, Tocher & Bell 2002), which has been demonstrated in some marine fish larvae (Mourente, Rodriguez, Tocher & Sargent 1993). The content of n-3 LC-PUFA and ratio of DHA to EPA increased with increasing dietary lipid, which may be the major reason for higher lipid requirement of fish larvae.

Fatty acid composition of the grouper in this study was highly reflective of dietary fatty acid profile. However, specific fatty acids (i.e. DHA) were selectively retained in grouper, since the ratio of certain FA to identified FAs was higher than its dietary ratio. Similar result has also been found in gilthead sea bream (Fountoulaki, Vasilaki, Hurtado, Grigorakis, Karacostas, Nengas, Rigos, Kotzamanis, Venou & Alexis 2009), European sea bass (Montero, Robaina, Caballero, Ginés & Izquierdo 2005; Mourente & Bell 2006), rainbow trout and turbot (Caballero, Obach,

Table 8 Fatty acid composition (% total fatty acids) in the visceral mass of larval grouper (29DAH) fed the diets with graded lipid for 4 weeks*

	Dietary lipid content (%)				
Fatty acid	**5.94**	**9.92**	**13.3**	**17.71**	**21.87**
14:0	4.03 ± 0.15^{a}	4.56 ± 0.07^{b}	4.60 ± 0.06^{b}	4.72 ± 0.04^{bc}	4.91 ± 0.06^{c}
16:0	20.93 ± 0.28^{d}	18.74 ± 0.06^{c}	16.81 ± 0.02^{b}	16.74 ± 0.03^{ab}	15.98 ± 0.26^{a}
18:0	9.48 ± 0.07^{c}	9.03 ± 0.06^{b}	8.91 ± 0.01^{b}	7.84 ± 0.01^{a}	7.73 ± 0.14^{a}
20:0	2.61 ± 0.26	2.94 ± 0.05	2.93 ± 0.01	2.80 ± 0.00	2.75 ± 0.01
∑SFA	37.39 ± 0.42^{d}	35.27 ± 0.06^{c}	33.26 ± 0.03^{b}	32.10 ± 0.02^{a}	31.37 ± 0.30^{a}
16:1	6.32 ± 0.41^{a}	6.78 ± 0.14^{ab}	7.49 ± 0.17^{bc}	7.68 ± 0.07^{bc}	7.83 ± 0.12^{c}
18:1	17.59 ± 0.39	17.46 ± 0.18	17.13 ± 0.02	17.94 ± 0.07	17.68 ± 0.19
∑MUFA	23.91 ± 0.14^{a}	24.24 ± 0.29^{a}	24.61 ± 0.19^{a}	25.62 ± 0.00^{b}	25.51 ± 0.21^{b}
18:2n-6	19.48 ± 0.44^{d}	15.95 ± 0.11^{c}	14.23 ± 0.05^{b}	12.43 ± 0.02^{a}	12.10 ± 0.02^{a}
20:4n-6	0.37 ± 0.02^{a}	0.45 ± 0.00^{b}	0.51 ± 0.01^{c}	0.53 ± 0.00^{c}	0.61 ± 0.01^{d}
∑n-6PUFA	19.85 ± 0.42^{d}	16.40 ± 0.11^{c}	14.74 ± 0.06^{b}	12.96 ± 0.02^{a}	12.70 ± 0.02^{a}
18:3n-3	2.85 ± 0.08^{b}	2.60 ± 0.06^{a}	2.63 ± 0.02^{ab}	2.45 ± 0.01^{a}	2.64 ± 0.05^{ab}
20:5n-3	4.60 ± 0.40^{a}	4.87 ± 0.10^{ab}	5.61 ± 0.05^{bc}	6.31 ± 0.01^{c}	6.22 ± 0.21^{c}
22:6n-3	4.78 ± 0.10^{a}	6.44 ± 0.06^{b}	7.22 ± 0.06^{c}	8.01 ± 0.04^{d}	8.21 ± 0.08^{d}
∑n-3PUFA	12.22 ± 0.38^{a}	13.92 ± 0.10^{b}	15.47 ± 0.03^{c}	16.78 ± 0.05^{d}	17.07 ± 0.26^{d}
n-3/n-6PUFA	0.62 ± 0.01^{a}	0.85 ± 0.01^{b}	1.05 ± 0.00^{c}	1.29 ± 0.01^{d}	1.34 ± 0.02^{e}
n-3LC-PUFA	9.37 ± 0.30^{a}	11.31 ± 0.15^{b}	12.84 ± 0.05^{c}	14.33 ± 0.04^{d}	14.43 ± 0.24^{d}
ARA/EPA	0.08 ± 0.01	0.09 ± 0.00	0.09 ± 0.00	0.08 ± 0.00	0.10 ± 0.00
DHA/EPA	1.06 ± 0.10^{a}	1.32 ± 0.02^{b}	1.29 ± 0.02^{ab}	1.27 ± 0.00^{ab}	1.32 ± 0.04^{b}

Mean values (n = 3) within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0.05$).

*Some fatty acids, of which the contents are 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, were not listed in the table.

SFAs, saturated fatty acids; MUFAs, mono-unsaturated fatty acids; n-6 PUFAs, n-6 poly-unsaturated fatty acids; n-3 PUFAs, n-3 poly-unsaturated fatty acids; n-3 LC-PUFAs, n-3 long chain-polyunsaturated fatty acids; ARA/EPA, 20:4n-6/20:5n-3; DHA/EPA, 22:6n-3/20:5n-3.

Table 9 Digestive enzymes activities of larval grouper (29DAH) fed diets with graded levels of lipid for 4 weeks

	Dietary lipid content (%)				
Digestive enzymes (U/mg pro)	**5.94**	**9.92**	**13.3**	**17.71**	**21.87**
Pepsin	111.04 ± 2.07^{d}	89.28 ± 1.14^{b}	80.11 ± 0.73^{a}	122.07 ± 1.17^{e}	98.51 ± 1.52^{c}
Trypsin	33.8 ± 0.39^{c}	45.81 ± 1.49^{d}	20.57 ± 1.00^{a}	27.9 ± 0.75^{b}	29.35 ± 1.52^{bc}
Amylase	1.92 ± 0.02^{a}	1.78 ± 0.00^{b}	1.87 ± 0.02^{a}	1.06 ± 0.02^{c}	1.19 ± 0.02^{d}
Lipase	0.48 ± 0.01^{ab}	0.42 ± 0.02^{a}	0.55 ± 0.02^{b}	0.49 ± 0.01^{ab}	0.49 ± 0.02^{ab}

Mean values (n = 3) within a row with a common superscript letter are not significantly different from the other dietary groups ($P > 0.05$).

Rosenlund, Montero, Gisvold & Izquierdo 2002). However, the ratio of EPA to identified FAs in carcass, muscle and visceral mass were much lower than its DHA ratio, indicating a priority utilization of EPA than DHA, which may be caused by a higher peroxisomal beta-oxidation of EPA when compared with DHA (Madsen, Frøyland, Dyrøy, Helland & Berge 1998). The ratio of DHA to identified FAs in muscle is higher than that of visceral, which denoted that DHA tend to be reserved in muscle than visceral mass. As dietary lipid increased, the ratio of DHA and EPA to identified FAs also increased significantly, which may indicate that the content of DHA and EPA also increased due to the increasing lipid content in larvae. This may also account for the relative higher SGR in larvae fed diets with higher lipid contents. Similar to DHA, the ratio of ARA to identified FAs in muscle was higher than its dietary ratios, which may also indicate its important

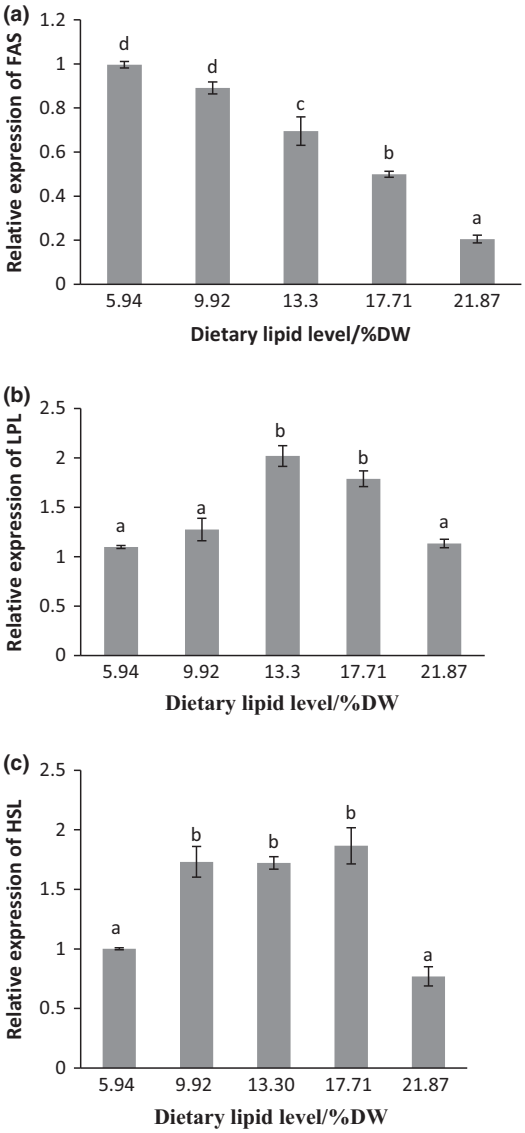

Figure 3 Relative mRNA expression of FAS (a), LPL (b) and HSL (c) in the visceral mass of grouper larvae (29DAH) fed with graded dietary lipid for 4 weeks. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± SEM ($n = 3$). Bars of the same gene bearing with different letters are significantly different by Tukey's test ($P < 0.05$).

role as essential fatty acid for proper function of the cell (Fountoulaki *et al.* 2009).

Activities of digestive enzymes could also reflect growth performance of fish larvae, because the ability of larvae to assimilate nutrients depends on their capacity to modulate activities of digestive enzymes (Cahu & Zambonino Infante 2001). In the present study, dietary lipid influenced activities of digestive enzymes significantly. Many studies have demonstrated that increasing dietary lipid has a protein-sparing effect (Chou & Shiau 1996; Du, Liu, Tian, Wang, Wang & Liang 2005). When dietary lipid content is lower, more protein would be catabolized to provide energy to maintain fish normal development, which may account for higher activities of pepsin and trypsin in first two treatments. The increase in dietary lipid level was made at the expense of wheat starch in this study. Previous study has found that high content of dietary carbohydrate could depress the amylase activity of seabass larvae (Péres, Cahu, Infante, Le Gall & Quazuguel 1996). The decreasing carbohydrate level in 17.71% and 21.87% lipid group may result in its lower amylase activity. A decline of amylase is observed during the normal maturation process (Péres, Infante & Cahu 1998), which can be considered as an indicator of the maturation of the exocrine pancreas (Cahu & Infante 1994; Ma, Cahu, Zambonino, Yu, Duan, Le Gall & Mai 2005). The development of digestive tract may also account for the variation in amylase activity.

Besides influencing activities of digestive enzymes, dietary lipid level also significantly influenced mRNA expression of FAS, HSL and LPL. FAS plays a crucial role in *de novo* lipogenesis by converting acetyl-CoA and malonyl-CoA into the final end product, palmitate, which is subsequently esterified into TAG and stored in adipose tissue (Wakil 1989). The tissue concentration of FAS, which is affected by a number of hormonal and dietary factors, is a key determinant for the maximal capacity of a tissue to synthesize fatty acids by the *de novo* pathway (Clarke 1993). Down regulation of FAS is an approach for reducing fat accumulation (Clarke 1993). In the present study, the mRNA expression of FAS decreased significantly with increasing dietary lipid, which indicated the decrease in lipid endogenous synthesis with increasing dietary lipid. In the study of rainbow trout (Gélineau, Corraze, Boujard, Larroquet & Kaushik 2001) and turbot (Regost, Arzel, Cardinal, Robin, Laroche & Kaushik 2001), high dietary lipid level could depress the activity of FAS, which was consistent with the variation expression of FAS in the present study. Besides, Clarke, Armstrong and Jump (1990) also found that dietary PUFA could decrease mRNA abundance of FAS, which could also account for the deficiency mRNA expression of FAS in the present study. Besides dietary lipid level, the content of dietary carbohydrate

could also affect the activity and expression of FAS. Previous studies have found that high dietary carbohydrate-to-lipid ratios could depress the activity of FAS in juvenile Senegalese sole (Dias, Rueda-Jasso, Panserat, da Conceição, Gomes & Dinis 2004). Kim and Freake (1996) have found that the high carbohydrate diet induced the expression of FAS in rats. The decreasing dietary carbohydrate level may also account for the decreasing expression of FAS in the present study. Both LPL and HSL are key enzymes in lipolysis (Eckel 1989; Haemmerle *et al.* 2002). Preiss-Landl, Zimmermann, Hämmerle and Zechner (2002) found that the expression of LPL in a given tissue might be a rate-limiting factor for the uptake of TG-derived fatty acids. The role of LPL is to hydrolyse TG circulating in the TG-rich lipoprotein particles in order to deliver fatty acids to the tissue and regulate the disposition of dietary fatty acids (Fielding & Frayn 1998). The activity of LPL is regulated by nutritional state in a tissue-specific manner according to the needs of the tissue for fatty acids. The increasing expression of LPL in larvae fed diets with lipid from 9.92% to 13.30% may indicate that higher lipid could promote the expression of LPL, and more TG-rich lipoproteins were hydrolysed to TG and free fatty acid. In mammals, Khan, Minihane, Talmud, Wright, Murphy, Williams and Griffin (2002) also found that dietary n-3 LC-PUFA in fish oil stimulate the expression of LPL in human adipose tissue, which may account for the changing of LPL mRNA expression. Meanwhile, the LPL expression in larvae fed the diet 21.87% lipid was significant lower than 13.30% group. Similar changing trend of LPL expression was also found in tilapia (Han *et al.* 2011) and darkbarbel catfish larvae (Zheng *et al.* 2010). However, to our knowledge, the regulatory mechanism of lipid level on LPL expression was unclear. In mammals, higher lipid content may cause insulin resistance (von Eynatten, Schneider, Humpert, Rudofsky, Schmidt, Barosch, Hamann, Morcos, Kreuzer, Bierhaus, Nawroth & Dugi 2004), which may also account for the lower expression of LPL in larvae fed the diet with 21.87% lipid. Further study about the regulatory mechanism of LPL should be conducted. HSL is a crucial enzyme known to hydrolyse TG in adipose tissue and produces fatty acid for energy production (Zimmermann, Strauss, Haemmerle, Schoiswohl, Birner-Gruenberger, Riederer, Lass, Neuberger, Eisenhaber & Hermetter 2004) and is considered as the rate-limiting enzyme (Holm, Østerlund, Laurell & Contreras 2000; Lampidonis, Rogdakis, Voutsinas & Stravopodis 2011). The results in the present study show that increasing dietary lipid could promote the expression of HSL to avoid the accumulation of TG in adipose tissue. Meanwhile, similar to LPL, larvae fed diets with 21.87% lipid had significant lower HSL expression. Jocken, Langin, Smit, Saris, Valle, Hul, Hom, Arner and Blaak (2007) found that the expression of HSL was depressed in insulin resistance state, which may also explain the changing of HSL expression in 21.87% lipid group. In the present study, we could draw a conclusion that dietary lipid could influence not only *de novo* lipogenesis but also lipolysis in transcriptional level. Although increasing dietary lipid could regulate the expression of lipid metabolism related genes, the crude lipid content increased significantly ($P < 0.05$). The increasing dietary lipid might result in excessive fat deposition in visceral cavity, liver and muscle, which consequently increased the whole body lipid content (Tocher 2003). Morais, Narciso, Dores and Pousão-Ferreira (2004) have found that higher amounts of lipid droplets was noticeable in the posterior intestine epithelia and in the hepatocytes of larvae fed *Artemia* enriched with higher lipid doses and the accumulation of large lipid droplets in the enterocytes might depress lipid absorption efficiency and reduce growth of larvae (Morais, Rojas-Garcia, Conceição & Rønnestad 2005).

In conclusion, optimal dietary lipid could improve the survival, growth and digestive enzymes activities of grouper larvae. The optimal dietary lipid level was estimate to be 15.99% on basis of SGR. Dietary lipid could influence both *de novo* lipogenesis and lipolysis in transcriptional level, and the regulation mechanism should be further investigated.

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