Aquaculture Research

Aquaculture Research, 2017, **48**, 767–779 doi: 10.1111/are.12921

Influence of dietary lipid on growth performance and some lipogenesis-related gene expression of tongue sole (*Cynoglossus semilaevis*) larvae

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Abstract

A 30-day feeding trial was conducted to investigate the effects of dietary lipid levels on growth performance, activities of digestive enzymes, fatty acid composition and some lipogenesis-related gene expression of half-smooth tongue sole (*Cynoglossus semilaevis*) larvae. Five isoproteic diets were formulated with graded lipid levels (6.68%, 9.84%, 13.47%, 17.89% and 21.88% dry weight) using fish oil as the main lipid source. Each diet was randomly allocated to triplicate groups of 150 larval tongue sole (35 DAH, 54 ± 1 mg). Fish were fed five times daily to apparent satiation during the feeding experiment. Results showed that, the survival rate (SR) of larvae increased significantly firstly, and thereafter decreased significantly. The specific growth rates (SGR) of larvae fed the diet with 13.47% lipid were significantly higher than other treatments. Larvae fed 9.84% or 13.47% dietary lipid showed higher trypsin, lipase, leucine aminopeptidase and alkaline phosphatase (AP) activities than other treatments, whereas amylase activity nearly showed reverse trend with them. The fatty acid composition of the tongue sole larvae was well correlated with dietary fatty acid profile. Expression of acetyl-CoA carboxylase alpha (ACC1) was found to be slightly negatively correlated with dietary lipid level, and high dietary lipid level depressed the expressions of acetyl-CoA carboxylase beta (ACC2) and fatty acid synthase (FAS) mRNA expression significantly, implying that larvae may cope with high dietary lipid mainly through down-regulating lipogenesis-related gene expression of FAS and ACC2. Besides, on the basis of SGR, the optimal dietary lipid level for larval tongue sole was estimated to be 13.56% using second-order polynomial curve.

Keywords: dietary lipid, growth performance, lipogenesis, genes expression, *Cynoglossus semilaevis*

Introduction

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a rare marine flatfish distributed in the coastal waters of northern China, promising for marine farming due to its high growth rates and market value (Liu, Xu, Ma, Jiang & Zhai 2004; Wan, Jiang & Zhuang 2004). Dietary lipids are the main source of energy, providing essential fatty acids, phospholipids, sterols and fat-soluble vitamins for larval fish species (Sargent, McEvoy, Estevez, Bell, Bell, Henderson & Tocher 1999). Moreover, it was recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe, Kitajima & Fujita 1983). Many studies have demonstrated that dietary lipid contributed to a better larval rearing (reviewed by Rainuzzo, Reitan & Olsen 1997; Coutteau, Geurden, Camara, Bergot & Sorgeloos 1997; Sargent *et al.* 1999; Izquierdo, Socorro, Arantzamendi & Hernández-Cruz 2000; Bell, McEvoy, Estevez, Shields & Sargent 2003). However, there is still a lack of information on optimal dietary lipid requirement for larval tongue sole.

In recent years, formulated micro-diet, compared with live prey, has drawn much attention in aquaculture research to reduce costs and increase predictability of juvenile production (Zheng, Zhu, Han, Yang, Lei & Xie 2010; Li, Mai, Xu, Yuan, Zhang, Zhou & Ai 2015). Nevertheless, at present, formulated diet substitution for live prey is still identified as a bottleneck in sustainable production of juvenile half-smooth tongue sole (Chen, Zhang, Zhang & Yu 2004; Chang, Liang, Wang, Chen, Zhang & Liu 2006). Considering the positive role of dietary lipid in larval rearing, research on lipid nutrition may make some contributions to successful weaning for larval tongue sole.

As the digestion and absorption of nutrient change during larval development, data obtained in juvenile fish are of little help when studying the requirements and mechanisms of larval stages. Thus, it is necessary to carry out corresponding research on fish larvae (Dabrowski 1984). The ability of larvae to assimilate the required nutrients is dependent on the composition of the diet and their capacity of modulating their digestive enzymes and metabolic processes (Cahu & Zambonino Infante 2001). Better knowledge of regulation by diet nutrients on the physiology and development of digestive tract is critical to replace live food with formulated diets.

During the last years, much attention has been paid to different aspects of larval lipid nutrition. Nevertheless, these studies mainly focused on aspects such as growth, survival, digestion, absorption and fatty acid composition (Izquierdo *et al.* 2000; Morais, Conceicao, Rønnestad, Koven, Cahu, Zambonino Infante & Dinis 2007). In particular, the requirements of polar lipid and highly unsaturated fatty acid and their effects on growth have been extensively studied in marine fish larvae (Salhi, Hernández-Cruz, Bessonart, Izquierdo & Fernández-Palacios 1999; Sargent *et al.* 1999; Bell *et al.* 2003). Relatively fewer studies have examined the effect of total lipid levels on transport and metabolism of dietary lipid (Izquierdo *et al.* 2000; Morais *et al.* 2007). Although there was an increasing evidence that dietary lipid levels have an impact on lipid metabolism in fish (Wang, Liu, Tian, Mai, Du, Wang & Yang 2005; Kolditz, Borthaire, Richard, Corraze, Panserat, Vachot, Lefe'vre & Me′dale 2008; Zheng *et al.* 2010; Han, Wen, Zheng & Li 2011; Wang, Wang & Li 2013), limited information was available on fish larvae.

Besides, obtained from exogenous diet, lipid could also *de novo* synthesized from other carbon donors, such as carbohydrates or amino acids (Gélineau, Corraze, Boujard, Larroquet & Kaushik 2001). Acetyl-CoA carboxylase alpha (ACC1) and beta (ACC2), both the major rate-limiting enzyme in lipogenesis metabolism, catalyse the carboxylation of acetyl-CoA forming malonyl-CoA (Wakil 1958; Nugteren 1965; Baud, Guyon, Kronenberger, Wuillème, Miquel, Caboche, Lepiniec & Rochat 2003; Castle, Hara, Raymond, Garrett-Engele, Ohwaki, Kan, Kusunoki & Johnson 2009). In mammals, both of ACC1 and ACC2 are multifunctional enzymes, and ACC1 is believed to be the primary ACC involved in *de novo* fatty acid synthesis (Castle *et al.* 2009). In contrast, ACC2 has been reported to regulate fatty acid oxidation through malonyl-CoA-mediated inhibition of carnitine palmitoyltransferase-1 (Bianchi, Evans, Iverson, Nordlund, Watts & Witters 1990; McGarry & Brown 1997; Zhang, Joshi & Smith 2003). Fatty acid synthase (FAS) is also a key lipogenic enzyme, catalysing the nicotinamide adenine dinucleotide phosphate-dependent condensation of malonyl-CoA and acetyl-CoA to produce the 16-carbon saturated free fatty acid in the terminal steps of the *de novo* biogenesis of fatty acids (Kuhajda 2000; Menendez & Lupu 2007). Several investigations on mammals, such as cow and rat, reported the depressed activity of ACC and FAS by high dietary lipid level (Piperova, Teter, Bruckental, Sampugna, Mills, Yurawecz, Fritsche, Ku & Erdman 2000; Kang, Hong, Jang, Kim, Choue & Lim 2006). In coho salmon (Lin, Romsos, Tack & Leveille 1977) and channel catfish (Likimani & Wilson 1982), the activity of hepatic FAS was also reported to be depressed as the lipid level increased, whereas FAS activity in adipose tissue was not influenced. However, Regost, Arzel, Cardinal, Robin, Laroche and Kaushik (2001) and Gélineau *et al.* (2001) reported that the activity of ACC in turbot and FAS in rainbow trout did not show any clear change in response to dietary lipid content. These studies indicated that lipogenic enzymes were differently regulated by dietary lipid in different fish species and fish size. However, to our knowledge, little information was known about the regulation of lipogenesis metabolism in larval flatfish.

This study was conducted to determine the effects of dietary lipid levels on growth, survival, activities of digestive enzymes, fatty acid composition and expression of some lipogenic

metabolism-related genes in larval half-smooth tongue sole, and seeking appropriate lipid requirement in a formulated diet for tongue sole larvae.

Materials and methods

Feed ingredients and diet formulation

Five isoproteic experimental diets (56% crude protein) were formulated to contain increasing levels of lipid (6.68%, 9.84%, 13.47%, 17.89% and 21.88% respectively) by adding graded fish oil. Defatted fish meal, krill meal, squid meal and hydrolysed fish meal, together with casein were chosen as primary protein sources. Fish oil and phospholipids were used as lipid sources. Mineral premix and vitamin premix were supplemented in the basic diet. Ingredients and nutrient composition of the five experimental diets are given in detail (Tables 1 and 2).

Micro-diets were processed as follows: primary ingredients of micro-diets were grounded into fine powder through a 75-μm mesh. After that, ingredients of all the diets were blended manually and then the oil mixtures (fish oil and lecithin) were added to each diet to mix other ingredients thoroughly. Finally, water was incorporated 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, diets were broken and sieved to obtain two sizes of particle: 180–250 μm for the fish larvae between 35 and 50 days after hatching and 250–420 μm for the larval fish thereafter. All formulated diets were packed in separate silver bags and stored at −20°C until used.

Table 1 Formulation and proximate analysis of the experimental diets (% dry weight)

	Dietary lipid content (% dry matter)				
Ingredients	**6.68**	**9.84**	**13.47**	**17.89**	**21.88**
Defatted fish meal*	35	35	35	35	35
Casein*	10	10	10	10	10
Krill meal†	9	9	9	9	9
Squid meal‡	9	9	9	9	9
Hydrolysed fish meal‡	8	8	8	8	8
LT-Yeast	2	2	2	2	2
Soy lecithin	4	4	4	4	4
Fish oil	0	4	8	12	16
Wheat starch	16.1	12.1	8.1	4.1	0.1
Sodium alginate	1.5	1.5	1.5	1.5	1.5
Vitamin premix§	1.5	1.5	1.5	1.5	1.5
Mineral premix¶	1.5	1.5	1.5	1.5	1.5
Attractant**	2	2	2	2	2
Antioxidant	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.2	0.2	0.2	0.2	0.2
Mould inhibitor††	0.1	0.1	0.1	0.1	0.1
Proximate composition (n = 3)					
Crude protein	56.12	56.02	55.77	55.73	55.76
Crude lipid	6.68	9.84	13.47	17.89	21.88

*Defatted fish meal: 77.81% crude protein and 2.88% crude lipid. White fish meal were defatted with ethanol (fish meal: ethanol = 1:2 (w:v)) at 37°C for three times. Casein: crude protein 92.34% dry matter, crude lipid 0.89% dry matter. Qingdao Great Seven Bio-Tech, China.

†Krill meal: crude protein 52.99% dry matter, crude lipid 12.95% dry matter. Shandong Keruier Biological Products, Jinan, China.

‡Squid meal: crude protein 62.72% dry matter, crude lipid 3.5% dry matter; Hydrolysed fish meal: crude protein 75.98% dry matter, crude lipid 1.34% 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; $MgSO_4{\cdot}2H_2O$, 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.

††Mould inhibitor: 50% calcium propionic acid and 50% fumaric acid.

Experimental procedure

Larvae used in this study were obtained and reared at the hatchery of Haiyang Seafood Company of Yantai (Shandong, China). For this experiment, a total of 2700 larvae (35 DAH) were randomly distributed into 18 tanks (65 × 65 × 90 cm) with flat bottom, each tank was stocked with 150 individuals. About 200–400% of the water volume was renewed daily and each tank had an air stone. The feeding trial lasted for 30 days. At the beginning, the fish were fasted for 24 h and each diet was randomly assigned to triplicate groups of fish. Enriched *artemia nauplii* and micro-diet were used alternately to wean the larvae for 3 days before formal experiment, and thereafter, the five groups of fish were manually fed the corresponding experimental diets to apparent satiation five times daily

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

	Dietary lipid content (% dry matter)				
Fatty acid	**6.68**	**9.84**	**13.47**	**17.89**	**21.88**
C 14: 0	3.15	4.16	4.48	4.70	4.84
C 16: 0	20.83	19.09	18.16	17.72	17.64
C 18: 0	0.11	0.18	0.22	0.23	0.26
C 20: 0	4.12	3.99	3.80	3.67	3.58
∑SFA	28.20	27.42	26.67	26.31	26.31
C 16: 1	5.01	6.56	7.29	7.24	7.42
C 18: 1n-9	3.42	3.02	2.27	2.30	1.45
C 18: 1n-7	17.18	19.43	20.36	20.52	21.70
∑MUFA	25.61	29.00	29.92	30.06	30.57
C 18: 2n-6	22.52	18.74	15.30	13.76	12.86
C 20: 4n-6	0.28	0.73	0.69	0.70	0.77
∑n-6 PUFA	22.80	19.47	15.99	14.46	13.63
C 18: 3n-3	2.96	2.77	2.61	2.54	2.56
C 20: 5n-3	4.95	5.70	6.23	6.49	6.35
C 22: 6n-3	6.95	7.49	8.61	9.06	9.31
∑n-3 PUFA	14.86	15.96	17.45	18.09	18.22
∑n-3/∑n-6 PUFA	0.65	0.82	1.09	1.25	1.34
C 18: 1n-9/∑n-3PUFA	0.23	0.19	0.13	0.13	0.08
DHA/EPA	1.40	1.32	1.38	1.40	1.46

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; n-3 PUFA, n-3 polyunsaturated fatty acids; DHA/EPA, C 22:6n-3/C 20:5n-3.

(06:00, 9:00, 15:00, 18:00 and 21:00 hours). During the rearing period, water temperature was kept at 23 ± 1°C, pH was 8.0 ± 0.2 and salinity was 30 ± 3‰. Light intensity was about 7.9 WM^{-2} during daytime at the water surface. The surface water was skimmed with a polyvinyl chloride tube to remove the suspended waste in time. Also, accumulations of feed and faeces at the tank bottoms were siphoned after feeding 40 min later.

Sampling and dissection

Before the experiment, initial wet body weight and body length of 50 randomly sampled larvae were measured. At the termination of the experiment, 30 fish were randomly sampled in each tank to determine wet body weight and body length. All fish were deprived of food for 24 h before sampling. Survival was determined by counting individuals remaining in each tank. All larvae were anaesthetised with eugenol (1:10 000; Shanghai Reagent, Shanghai, China) and rinsed in distilled water before further treatment, and larvae collected for further assays were immediately frozen in liquid nitrogen then stored at −80°C.

Enzymatic assays were determined on the pancreatic segment (PS) and intestinal segment (IS) of digestive tract. Larvae were dissected on a glass plate maintained at 0°C according to the methods described previously by Ribeiro, Zambonino-Infante, Cahu and Dinis (1999) for sole. Digestive tracts were separated at the junction of the oesophagus and the pyloric sphincter to obtain the PS and IS. Dissected samples were refrozen at −80°C for enzymatic assays. Purified brush border membranes (BBM) from the IS homogenate were obtained following the method developed for intestinal scrapings by Crane, Boge and Rigal (1979) and Zambonino Infante, Cahu and Péres (1997).

Analytical methods

The chemical composition of diets was determined according to the standard procedures (AOAC 1995). The samples of diets were dried to a constant weight at 105°C to determine the dry matter content. Crude protein was determined by digestion using the Kjeldahl method ($N \times 6.25$); Crude lipid was measured by ether extraction using Soxhlet method.

Amylase and trypsin activities were assayed using starch(S-9765; Métais & Bieth 1968) and Na-Benzoyl-DL-arginine-p-nitroanilide (B-4875; Holm, Hanssen, Krogdahl & Florholmen 1988) as substrates respectively. Both in the PS and IS, lipase activities were assayed using polyvinylpyrrolidone-olive oil emulsion as substrates according to the methods of Brockman (1981). Alkaline phosphatase (AP) activity was measured using p-nitrophenylphosphate (106850; Merck, Darmstadt, Germany) and $MgCl_2$ as the substrates (Bessey, Lowry & Brock 1946), and leucine alanine peptidase (LAP) activity was measured using leucine-p-nitroanilide (L-9125; Sigma, St. Louis, MO, USA) as the substrate (Nicholson & Kim 1975). Protein was determined according to Bradford (1976) using bovine serum albumin (A-2153; Sigma) as a standard. Enzyme activities are expressed as specific activity (mU mg $protein^{-1}$ or U mg $protein^{-1}$).

The procedures for analysis of the fatty acid profiles were analysed using the procedure described by Metcalfe, Schmitz and Pelka (1996) with some modification (Ai, Zhao, Mai, Xu, Tan, Ma & Liufu 2008). Fatty acid methyl esters were separated and quantified using HP6890 gas chromatograph equipment (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 to 200°C at a rate of 15°C min^{-1}, from 200°C to 270°C at a rate of 2°C min^{-1}, then kept steady 10 min. Injector and detector temperature was 270°C respectively.

RNA extraction and reverse transcription

Total RNA was extracted from larval visceral mass using Trizol Reagent (Invitrogen, Paisley, UK) according to the instructions of the manufacturer, and electrophoresed on a 1.2% denaturing agarose gel to test the integrity. The purity and the concentration of total RNA were determined on NanoDrop® ND-1000 (Wilmington, DE, USA). The ratios of absorption (260/280 nm) for all samples were approximately 2.0. Then First strand cDNA was synthesized using PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan) following the instructions. The resulting product was used as a template for polymerase chain reaction (PCR) amplification.

Partial cloning of lipogenesis related genes

In order to obtain fragments of ACC1, ACC2 and FAS genes, three pairs of degenerate PCR primers (Table 3) were designed in highly conserved regions on the basis of available sequences of other fish (cobia, zebrafish and Atlantic salmon) in Genbank and were synthesized by Biosune Biotech (Shanghai, China). The PCR programme was carried out in Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and the PCR conditions were: 2 min at 94°C; 35cycles of 30 s at 94°C, 30 s at Annealing Temperature, 40 s (change according to the length of target gene) 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 microliters of each ligation reaction were transformed into the competent cells of *Escherichia coli* TOP10, and then plated on LB agar plates. The positive clones in each PCR fragment were sequenced in Sangon Biotech (Shanghai, China). Sequence alignment and analysis were conducted using the BLAST sequence analysis service of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/). Multiple alignments of ATGL were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/).

This study obtained the partial nucleotide sequences of ACC1, ACC2 and FAS cDNA, and the nucleotide sequence data of these genes have been deposited in the GenBank nucleotide sequence database. The sequence of ACC1 (GenBank accession number: KJ459042) revealed a fragment of 661 bp highly homologous to *Xiphophorus maculatus* (84%), *Oreochromis niloticus* (84%) and zebrafish (82%). The sequence of ACC2 (GenBank accession number: KJ459043) revealed a fragment of 732 bp highly homologous to *Oreochromis niloticus* (88%), Neolamprologus brichardi (87%) and Maylandia zebra (87%). The sequence of FAS (GenBank accession number: KJ459041) revealed a fragment of 505 bp highly homologous to *Epinephelus coioides* (78%) and *Megalobrama amblycephala* (76%).

Real-time quantitative PCR

The quantitative PCR (qPCR) primer pairs (Table 4) were designed by Primer Primier 5.0 based on the obtained nucleotide sequences of ACC1, ACC2 and FAS. β-2 microglobulin of half-smooth tongue sole (GenBank: FJ965561) was chosen as housekeeping gene. Real-time qPCR was carried out in a quantitative thermal cycler (Mastercycler ep realplex; Eppendorf). The amplification was performed in a total volume of 25 μL, containing 1 μL of each primer (10 μM), 1 μL of

Table 3 PCR primers for targeted genes cloning used in the experiment

Target gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
ACC1	GTGAAATGCATGCGCTCC	GCTGCATGACAAAAATGG	570
ACC2	CCGCTGGTCCTATGAAAT	TGGATGGAGCAGTCTCGT	680
FAS	GG(C/T)TC(T/C/A)ACCAAGTCCAACAT	CCCACTGT(T/G)T(T/C/G)CCCATACCT	535

ACC1, acetyl-CoA carboxylase alpha; ACC2, acetyl-CoA carboxylase beta; FAS, fatty acid synthase.

Table 4 qPCR primers

Primer	Forward (5′–3′)	Reverse (5′–3′)	Reference
ACC1	CCGAAGACCTAAAAGCCAATGC	TAGGGTTCTCTGAGGCGTGAC	GenBank: KJ459042
ACC2	TGGGGTCATGCCTCAGAAAATC	ATGGTAGAGTGGGAATGTCAGC	GenBank: KJ459043
FAS	ACCTGCTGCACTTCTGTGAC	TACAGAGGAGGCGGTGAAGA	GenBank: KJ459041
β-2 microglobulin	TTGGCTCGTGTTCGTCGTTC	TCAGGGTGTTGGGCTTGTTG	GenBank: FJ965561

ACC1, acetyl-CoA carboxylase alpha; ACC2, acetyl-CoA carboxylase beta; FAS, fatty acid synthase.

the diluted first strand cDNA product, 12.5 μL of 2× SYBR® Premix Ex Taq™ (Takara) and 9.5 μL of sterilized double-distilled water. The qPCR programme was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 20 s. At the end of each PCR reaction, melting curve analysis was performed to confirm that only one PCR product was present in these reactions. Standard curves were made with six different dilutions (in triplicate) of the cDNA samples and amplification efficiency was analysed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$. The primer amplification efficiencies were 0.9576, 1.0499, 1.0459 for ACC1, ACC2 and FAS, respectively, and 1.0507 for β-2 microglobulin. The expression levels of the target genes were calculated followed the $2^{-\Delta\Delta t}$ method described by Livak and Schmittgen (2001).

Calculations and statistical analysis

The following variables were calculated:

$$\text{Specific growth rate}(\text{SGR}, \%\text{day}^{-1}) = [(\ln W_f - \ln W_i)/d] \times 100$$

where W_f = final wet body weight, W_i = initial wet body weight, d = experimental days.

$$\text{Survival rate}(\text{SR}, \%) = 100 \times N_f/N_i$$

N_i and N_f were the initial and final fish number in the experiment respectively.

All statistical evaluations were performed by using the software SPSS 19.0 (SPSS, Chicago, IL, USA). Data from each treatment were subjected to a one-way ANOVA according to Tukey's multiple range test. The level of significance was chosen at $P < 0.05$ and the results are presented as means ± SEM. The second-order polynomial curve was used to estimate the optimal dietary lipid level for tongue sole larvae.

Result

Survival and growth

The survival rates (SR) and specific growth rates (SGR) of tongue sole larvae were shown in Table 5. The SR of larvae significantly increased with increasing dietary lipid level from 6.68% to 13.47% and then decreased significantly ($P < 0.05$). Specific growth rates followed the same pattern with SR. The second-order polynomial curve was used to estimate the optimal dietary lipid content for tongue sole larvae: $Y = -0.0201X^2 + 0.545X + 1.6319$ ($R^2 = 0.8098$; Fig. 1). The result indicated that the optimal dietary lipid requirement of larval tongue sole was estimated to be 13.56% based on SGR.

Activities of digestive enzymes

Activities of trypsin in PS significantly increased with dietary lipid level from 6.68% to 13.47% and then decreased significantly ($P < 0.05$). Meanwhile, activity of trypsin in IS increased with increasing dietary lipid from 6.68% to 17.89% and then decreased significantly ($P < 0.05$; Table 6).

Lipase activities of PS in larvae fed the diet with 13.47% lipid was significant higher than the 21.88% group, but comparable with other treatments. In IS, activity of lipase increased significantly with dietary lipid from 6.68% to 13.47% and thereafter declined significantly ($P < 0.05$; Table 6).

Amylase activities of PS in larvae fed the diet with 21.88% lipid was significantly higher than other treatments ($P < 0.05$). Meanwhile, amylase activity of IS in larvae fed diets with 6.68% and 9.84% lipid was significantly higher than other treatments (Table 6).

Table 5 Growth responses and survival of larval tongue sole fed diets with graded levels of lipid

	Dietary lipid content					ANOVA		
Growth response	**6.68**	**9.84**	**13.47**	**17.89**	**21.88**	**Pooled SEM**	***F*-values**	***P*-values**
Initial body weight (mg)	54	54	54	54	54			
Final body weight (g)	0.23^{ab}	0.26^{b}	0.34^{c}	0.25^{b}	0.21^{a}	0.13	48.727	0.000
SGR (% d^{-1})	4.40^{ab}	4.86^{b}	5.71^{c}	4.64^{b}	4.06^{a}	0.15	35.970	0.000
Survival (%)	30.26^{ab}	38.39^{c}	40.51^{c}	37.95^{bc}	26.50^{a}	1.58	11.963	0.000

Data with the same superscript letter in the same row are not significantly different determined by Tukey's test ($P > 0.05$).
SEM, standard error of means; SGR, specific growth rate.

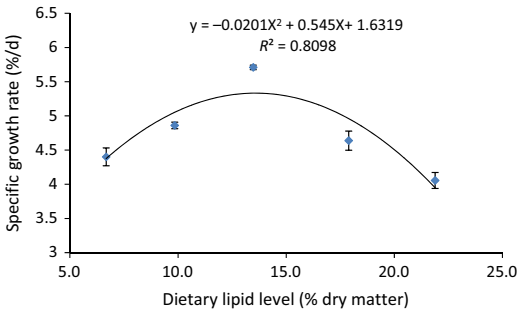

Fig. 1 Requirement of dietary lipid based on specific growth rate (SGR) of larval tongue sole, *Cynoglossus semilaevis*.

Activities of LAP and AP in both IS and BBM segments increased first and then decreased. Meanwhile, larvae fed diets with 9.84% and 13.47% lipid had significantly ($P < 0.05$) higher activity of LAP in IS than other treatments, whereas the activity of LAP in BBM increased first with increasing dietary lipid from 6.68% to 17.89% and then decreased significantly. However, no significant differences existed in AP activities among all treatments (Table 6).

Fatty acid composition

The fatty acid composition of larval whole body generally reflected the fatty acid profile of the diets (Table 7). Contents of saturated fatty acid (SFA; % total fatty acid) and n-6 poly unsaturated fatty acid (PUFA) in whole body were lower than dietary contents, whereas monounsaturated fatty acid (MUFA), DHA, n-3/n-6 PUFA and DHA/EPA had reversed trends. The contents of 16:1, 20:0 and n-6 PUFA in whole body decreased significantly ($P < 0.05$) with increasing dietary lipid, and whole-body DHA and EPA contents of larvae fed 13.47% and 17.89% dietary lipid were higher than larvae fed diets with 6.68%, 9.84% and 21.88% lipid level, but no significant difference ($P > 0.05$) was found in all treatments. The n-3/n-6 PUFA showed increasing tendency with dietary lipid level varying from 6.68% to 21.88%, and larvae fed the diet with 13.47% lipid had the lowest 18: 1n-9/n-3 PUFA ratio ($P < 0.05$). Nevertheless, DHA/EPA did not show significant difference ($P > 0.05$) in all treatments.

Lipogenesis-related gene expression

Relative mRNA expression levels of lipogenesis-related genes (ACC1, ACC2 and FAS) in the visceral mass of larvae fed diets with different lipid levels was presented in Figure 2. Among different dietary lipid treatments, no significant difference ($P > 0.05$) was observed in relative expression of ACC1 (Fig. 2A), whereas the relative expression of ACC2 was significantly ($P < 0.05$) repressed by high dietary lipid level (Fig. 2B). Also, FAS showed a declining expression trend with increasing dietary lipid (Fig. 2C).

Discussion

An appropriate dietary lipid level is critical for better growth performance of larval fish, whereas excessive dietary lipid reduced fish growth or produced fatty fish (Han *et al.* 2011). In the present study, larvae fed the diet with 13.47% lipid had the highest SGR and SR, suggesting that the optimal dietary lipid level was about 13.47% dry matter. Meanwhile, larvae fed the diet with 9.84% and 17.89% lipid levels showed comparable SR with 13.47% lipid treatment. This might indicate that 9.84% dietary lipid could meet the minimum requirement of tongue sole larvae to maintain normal physiological function, and larval tongue sole seemingly could tolerate higher dietary lipid (17.89%). However, larval tongue sole fed 21.88%

Table 6 Digestive enzymes activities of larval tongue sole fed diets with graded levels of lipid

	Dietary lipid content (% dry matter)					ANOVA		
Digestive enzymes	**6.68**	**9.84**	**13.47**	**17.89**	**21.88**	**Pooled SEM**	***F*-values**	***P*-values**
Trypsin (mU mg Pro^{-1})								
PS	7.45^{a}	10.01^{c}	13.08^{d}	9.90^{bc}	8.02^{ab}	0.55	28.630	0.000
IS	15.95^{ab}	16.12^{ab}	19.70^{ab}	22.62^{b}	12.96^{a}	1.11	4.671	0.022
Lipase (U mg Pro^{-1})								
PS	1.59^{ab}	1.79^{ab}	2.03^{b}	1.60^{ab}	1.43^{a}	0.07	3.400	0.053
IS	3.71^{c}	9.87^{d}	12.27^{e}	1.38^{b}	0.55^{a}	1.22	3027.042	0.000
Amylase (mU mg Pro^{-1})								
PS	3.98^{a}	3.83^{a}	3.64^{a}	3.43^{a}	5.09^{b}	0.17	9.126	0.002
IS	4.18^{b}	7.17^{b}	1.29^{a}	2.58^{a}	1.95^{a}	0.40	24.436	0.000
LAP (mU mg Pro^{-1})								
IS	62.33^{a}	68.56^{b}	68.95^{b}	61.54^{a}	65.48^{ab}	0.89	13.069	0.001
BBM	103.77^{b}	107.30^{bc}	114.02^{c}	127.81^{d}	88.33^{a}	3.52	64.984	0.000
AP (mU mg Pro^{-1})								
IS	352.63	424.57	593.89	365.34	363.57	31.85	3.411	0.053
BBM	116.04	133.59	144.00	133.50	121.97	3.59	2.858	0.081

Data with the same superscript letter in the same row are not significantly different determined by Tukey's test ($P > 0.05$).
SEM, standard error of means; PS, pancreatic segment; IS, intestinal segment; BBM, brush border membrane; LAP, leucine aminopeptidase; AP, alkaline phosphatase.

Table 7 Whole-body fatty acid composition of larval tongue sole fed diets with graded levels of lipid (% total fatty acid; means with pooled SEM)

	Dietary lipid content (% dry matter)					ANOVA		
Fatty acid	**6.68**	**9.84**	**13.47**	**17.89**	**21.88**	**Pooled SEM**	***F*-values**	***P*-values**
14: 0	1.24^{bc}	0.77^{a}	1.93^{d}	1.32^{c}	0.86^{ab}	0.12	28.596	0.000
16: 0	18.86	17.05	18.04	18.14	18.48	0.24	2.238	0.138
18: 0	0.65^{ab}	0.79^{b}	0.90^{b}	0.44^{a}	0.88^{b}	0.05	7.532	0.005
20: 0	2.15^{c}	2.16^{c}	2.10^{c}	1.54^{b}	1.14^{a}	0.11	34.518	0.000
∑SFA	22.91	20.77	22.97	21.45	21.20	0.33	3.068	0.069
16: 1	5.90^{c}	3.68^{a}	5.46^{bc}	4.13^{ab}	3.36^{a}	0.30	10.109	0.002
18: 1n-9	9.01^{b}	11.09^{c}	6.81^{a}	8.79^{ab}	12.04^{c}	0.52	21.449	0.000
18: 1n-7	22.02^{a}	24.30^{ab}	24.48^{ab}	25.54^{b}	24.74^{ab}	0.41	3.714	0.042
∑MUFA	36.93	39.06	36.76	38.46	40.14	0.51	2.113	0.154
18: 2n-6	10.85^{c}	6.36^{a}	9.46^{bc}	7.98^{ab}	5.93^{a}	0.53	15.854	0.000
20: 4n-6	0.26^{a}	0.30^{ab}	0.31^{b}	0.31^{b}	0.32^{b}	0.01	7.397	0.005
∑n-6 PUFA	11.11^{c}	6.65^{a}	9.72^{bc}	7.92^{ab}	6.24^{a}	0.54	12.306	0.001
18: 3n-3	1.38	1.78	1.55	1.61	2.17	0.11	2.093	0.157
20: 5n-3	2.71^{a}	2.70^{a}	3.28^{b}	3.22^{b}	2.96^{ab}	0.08	5.749	0.011
22: 6n-3	9.26	10.09	10.64	10.66	9.74	0.25	1.238	0.355
∑n-3 PUFA	13.36	14.57	15.47	15.49	14.87	0.28	3.147	0.064
∑n-3/∑n-6 PUFA	1.20^{a}	2.25^{bc}	1.59^{ab}	1.98^{bc}	2.41^{c}	0.13	11.866	0.001
18: 1n-9/∑n-3PUFA	0.68^{bc}	0.76^{bc}	0.44^{a}	0.57^{ab}	0.82^{c}	0.04	9.563	0.002
DHA/EPA	3.41	3.73	3.25	3.33	3.29	0.07	1.920	0.184

Data are means of triplicate. Means in the same row sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$). Some fatty acids, of which the contents are minor, trace amount or not detected, were not listed in the table.
SEM, standard error of means; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acids; n-3 PUFA, n-3 polyunsaturated fatty acids; DHA/EPA, C 22:6n-3/C 20:5n-3.

dietary lipid had the lower SGR and SR than all other treatments, which revealed the negative effects of excessive dietary lipid on larval fish. This was consistent with previous study on turbot (Kjørsvik, Olsen, Rosenlund & Vadstein 1991), gilthead seabream (Pousão-Ferreira, Morais, Dores & Narciso 1999), white sturgeon (Gawlicka, Herold, Barrows, de la Noüe & Hung 2002) and cobia

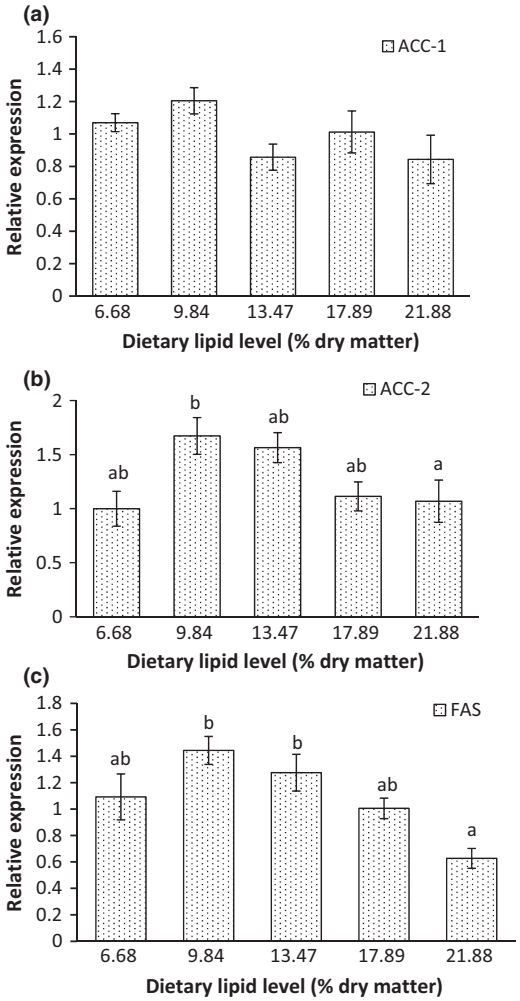

Fig. 2 Relative mRNA expression of lipogenesis related genes (ACC1 (a), ACC2 (b) and FAS (c)) in visceral mass of larval tongue sole, *Cynoglossus semilaevis* fed graded levels of lipid. Relative mRNA expression was evaluated by real-time quantitative PCR. Values are means ± SEM (n = 3). Bars of the same gene bearing with the same letter are not significantly different determined by Tukey's test ($P > 0.05$).

(Wang *et al.* 2005). It was proposed that high dietary lipid content might result in reduced digestion and absorption efficiency and eventually reduced growth and survival of larval fish (Morais *et al.* 2007).

In addition to the changes in the growth performance, activities of digestive enzymes of larvae were also influenced by dietary lipid. To some extent, activities of digestive enzymes could reflect the development of larval digestive tract, and the ability of larvae to assimilate the required nutrients generally depends on the capacity of digestive tract to modulate digestive enzymes and metabolic processes (Cahu & Zambonino Infante 2001). In the present study, larvae fed 9.84%, 13.47% and 17.89% dietary lipid showed higher trypsin activities in both PS and IS than other treatments, whereas amylase activity in both PS and IS nearly showed reverse trend with trypsin. This indicated a better maturation and functionality of the pancreas in larvae fed moderate dietary lipid level, because the increase in trypsin and decline of amylase are observed during the normal maturation process of fish larvae (Segner, Storch, Reinecke, Kloas & Hanke 1994; Péres, Zambonino Infante & Cahu 1998; Zambonino Infante & Cahu 2001). In fish species, lipase is secreted by the hepatopancreas mainly in response to the presence of triglycerides in the lumen (Zambonino Infante & Cahu 2007). In the present study, with the elevation of dietary lipid level, lipase activity in IS significantly increased to the highest in 13.47% group and then decreased to the lowest, which may indicate that high dietary lipid might depress the development of larval digestive tract. Meanwhile, lipase in PS seemed to be less sensitive to dietary lipid level compared with lipase in IS, which might be related to the fact that lipase mainly play its role in lumen. Leucine alanine peptidase and AP, two intestinal enzymes, are located in the BBM of enterocytes (Cahu & Zambonino Infante 2001). In the present study, the increase in LAP and AP in intestinal BBM demonstrated the normal maturation of the enterocytes in developing larval tongue sole (Henning 1987). The data of digestive enzymes activities might partially explain the difference in growth performance of different treatments in the present study.

Lipogenesis represents the final common fate for surplus non-fat energy and contributes to the macronutrient energy balance (Hellerstein, Christiansen, Kaempfer, Kletke, Wu, Reid, Mulligan, Hellerstein & Shackleton 1991). Both ACC and FAS are key enzymes controlling lipogenesis metabolism, ACC catalyses the carboxylation of acetyl-CoA to form malonyl-CoA, which is the first step in the fatty acid biosynthetic process (Guillevic, Kouba & Mourot 2009). Its two isoforms, ACC1 and ACC2, are regulated by similar short-term mechanisms of allosteric activation by citrate and reversible phosphorylation and inactivation (Munday 2002). As for FAS, it plays a crucial role in catalysing all the reaction steps in the conversion

of acetyl-CoA and malonyl-CoA to palmitic acid (16:0; Sargent 1989). In the present study, the different expression pattern of ACC1 and ACC2 might demonstrate that, in fish species, ACC1 and ACC2 also played distinct roles in lipid metabolism just as findings on mammals (Ha, Lee, Kim, Witters & Kim 1996; Abu-Elheiga, Matzuk, Kordari, Oh, Shaikenov, Gu & Wakil 2005). Research on mammals revealed that ACC2 was associated to the mitochondria, and it might play an essential role in fatty acid oxidation and regulation of energy expenditure (Abu-Elheiga, Oh, Kordari & Wakil 2003). Hence, in the present study, the higher ACC2 mRNA expression in 9.84% and 13.47% lipid treatments might indicate an increased fatty acid oxidation and that more protein was spared, which was coincident to the better growth in these two treatments.

At present, the inhibitory effects of high dietary lipid level on activities of lipogenic enzymes have been reported in several mammals (Kang *et al.* 2006; Guillevic *et al.* 2009) and teleost species (Lin *et al.* 1977; Likimani & Wilson 1982; Wang *et al.* 2005). In the present study, expression of ACC1 was found to be slightly negatively correlated with dietary lipid level. However, high dietary lipid level significantly depressed the expressions of ACC2 and FAS mRNA expression, which may indicate that the larvae may cope with high dietary lipid mainly through down-regulating lipogenesis-related genes expression of FAS and ACC2. In conclusion, the dietary lipid did influence the survival, growth, digestive enzymes activities and fatty acid composition of tongue sole larvae significantly. Meanwhile, high dietary lipid level depressed the expressions of ACC2 and FAS mRNA expression significantly. The optimal dietary lipid level for larval tongue sole was estimated to be 13.56% on the basis of SGR, and appropriate dietary lipid could promote the development of digestive tract, survival and growth of larval tongue sole. Although the transcription of lipogenesis-related genes in response to dietary lipid have been investigated, the regulation mechanism should be further confirmed.

Acknowledgments

This study was financially supported by the China Agriculture Research System (CARS-50-G08), and China National Natural Science Foundation grants (31372541 and 31072222), the National Basic Research Program of China (973 Program) (2014CB138600), and Ph.D. Programs Foundation of Ministry of Education of China (20120132110007). We thank Y.X. Li, K. Lu, Y.L. Liu, P. Yang and J. Yan for their help in diet manufacture, and gratitude also is given to S. Zhang, B. Han and J.T. Guan for their help during the sampling. We also thank X.J. Dong for their help during the analytical experiment.

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