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Dietary DHA/EPA ratio affects growth, tissue fatty acid profiles and expression of genes involved in lipid metabolism in mud crab *Scylla paramamosain* supplied with appropriate n-3 LC-PUFA at two lipid levels

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Abbreviations: WG, weight gain; SGR, Specific growth rate; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; ALP, alkaline phosphatase; TP, total protein; GLU, glucose; TAG, triacylglycerol; T-CHO, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *srebp-1*, sterol regulatory element binding protein-1; *fas*, fatty acid synthase; *acc*, acetyl-CoA carboxylase; *6pgd*, 6-phosphogluconate dehydrogenase; *g6pd,* glucose-6-phosphate dehydrogenase; *cpt*, carnitine palmitoyltransferase; *aco,* acyl-CoA oxidase; *hsl,* hormone-sensitive triglyceride lipase; *fabp*, fatty acid binding protein; *fatp*, fatty acid transport protein; *ldlr*, low-density lipoprotein receptor; *lrp*, low-density lipoprotein receptor-related protein; *srb*, scavenger receptor b; *fad*, fatty acyl desaturase; *elovl*, elongase of very long-chain fatty acids.

Abstract

An 8-week feeding trial was conducted to determine the optimal dietary docosahexaenoic acid/eisosapentaenoic acid (DHA/EPA) ratio of mud crab (*Scylla paramamosain*) supplied with optimal n-3 LC-PUFA at two dietary lipid levels. Eight isonitrogenous diets were formulated to contain 7% and 12% crude lipid, each with DHA/EPA ratios of 0.6, 1.2, 2.3 and 3.2, respectively. Each diet was randomly assigned to triplicate groups of 30 juvenile mud crabs (initial weight 20.9 ± 0.6g) that were stocked in single crab cells. In crabs fed 7% lipid, the diet with a DHA/EPA ratio of 2.3 showed significantly higher weight gain than crabs fed the other ratios while in crabs fed 12% lipid, lower weight gain and specific growth rate were observed in crabs fed the diet with a DHA/EPA ratio of 0.6 than crabs fed the other ratios. Lipid content in hepatopancreas significantly increased as dietary DHA/EPA ratio increased from 1.2 to 2.3 in crabs fed 7% lipid, while no differences were observed among crabs fed the diets with DHA/EPA ratios higher than 0.6 when fed 12% lipid. Total fatty acid and DHA contents and DHA/EPA ratio showed increasing, and EPA decreasing, trends in muscle and hepatopancreas with increased dietary DHA/EPA ratio, at both dietary lipid levels. The hemolymph triacylglycerol and total cholesterol contents were higher in crabs fed dietary DHA/PA ratios of 1.2 and 2.3 than those fed ratios of 0.6 and 3.2 at 7% dietary lipid, and lowest low and high-density lipoprotein cholesterol contents were observed in crabs fed DHA/EPA dietary ratios of 0.6 and 3.2 at 7% and 12% lipid, respectively. The expression levels of *fas, aco3* and *fatp4* were significantly up-regulated, and *cptI, hsl* and *ldlr* were down-regulated, with increased dietary DHA/EPA ratio in crabs fed 7% lipid. In crabs fed 12% lipid, the expression levels of *g6pd, 6pgd, srebp-1, aco1* and *fatp4* were down-regulated, and *fabp-1* was up-regulated, with increased dietary DHA/EPA ratio. The expression levels of *elovl4* and *Δ6 fad* initially increased and then decreased as dietary DHA/EPA ratio increased from 0.6 to 3.2 in crabs fed both 7% and 12% lipid. Based on analysis of weight gain versus dietary DHA/EPA ratio, the optimal dietary DHA/EPA ratios of mud crab *S. paramamosa* were estimated to be 2.2 and 1.2 when supplied with optimal n-3 LC-PUFA at 7% and 12% lipid, respectively.

Keywords: DHA/EPA; Growth; LC-PUFA biosynthesis; Lipid metabolism; *Scylla paramamosain*

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) are considered as essential fatty acids (EFA) for marine fish and crustaceans because they are generally unable to convert linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6) to n-3 and n-6 LC-PUFA, respectively, probably reflecting evolutionary adaptation to marine ecosystems being naturally rich in LC-PUFA (Tocher, 2003). Previous studies reported that dietary deficiency or excessive LC-PUFA could result in reduced survival, poor growth, and prolonged inter-molt periods of crustaceans (Suprayudi et al., 2004; Yang et al., 2013). Therefore, it is clear that dietary EFA must be at very precise levels to fulfil requirements for survival, optimum growth and development (NRC, 2011).

Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are the major important n-3 LC-PUFA, which are necessary for crustacean growth, molting and development. Specifically, DHA plays crucial structural roles in bio-membranes, especially of neural tissues such as brain and eye, where it is a major component of polar lipids (Wassall and Stillwell, 2008). Thus, it is expected that DHA requirements are high in fast growing stages of development in order to satisfy the demands of rapidly forming tissues that accumulate DHA. In addition, EPA has a major role as a precursor of highly bioactive regulatory compounds such as eicosanoids, and can also partly satisfy DHA requirements in species that have the necessary fatty acyl elongase and desaturase activities to convert EPA to DHA (Castro et al., 2016). It was reported that DHA and EPA in biomembrane phospholipids of marine fish must be present in an appropriate ratio, and an imbalance resulted in reduced survival and stress resistance capability (Copeman et al., 2002). Previous studies in fish also reported that overall n-3 LC-PUFA requirement decreased with increased dietary DHA/EPA ratio in gilthead sea bream (*Sparus aurata* L.) (Rodriguez et al., 1998). Best growth was obtained at a total n-3 LC-PUFA inclusion level of 0.9% of diet dry weight with a DHA/EPA ratio of 1.0 for juvenile gilthead sea bream (Kalogeropoulos, et al., 1992). When dietary inclusion level of n-3 LC-PUFA was increased to 1.9% of dry weight, juvenile gilthead sea bream required a higher dietary content of EPA (1.0%) than DHA (0.5%) for maximum growth (Ibeas, et al., 1997). Therefore, the optimal dietary n-3 LC-PUFA content and DHA/EPA ratio could be affected by each other and, therefore, the quantitative requirements for n-3 LC-PUFA was reported to vary with stage of development, dietary lipid content, and the ratio of dietary LC-PUFA (DHA/EPA) (NRC, 2011). Thus, it is clearly important to determine the appropriate dietary DHA/EPA ratio in combination with dietary lipid and n-3 LC-PUFA levels in feed.

The mud crab, *Scylla paramamosain*, is distributed widely throughout the coasts of China, Vietnam, Japan and Malaysia, and is a commercially important farmed species due to their short growth cycle, high adaptability and

84 nutritional value (Shi et al., 2018). In 2019, the yield of farmed mud crabs (mainly *S. paramamosain*) reached 160，
85 116 tons (China Fishery Statistical Yearbook, 2020), although there are relatively few studies on the nutritional
86 requirements of mud crab (Dong et al., 2017a, b; Wang et al., 2019; Xu et al., 2020; Zhao et al., 2015, 2016). Our
87 overarching aims were to determine n-3 LC-PUFA requirements of juvenile mud crab, and demonstrate the
88 relationship between n-3 LC-PUFA requirement and dietary lipid level. Our previous study demonstrated that the
89 optimum n-3 LC-PUFA requirement of juvenile mud crab was significantly affected by dietary lipid level, and
90 determined to be 20.1mg g^{-1} and 12.7mg g^{-1} of dry weight at 7% and 12% lipid, respectively, when the DHA/EPA
91 ratio was fixed at approximately 1 (Wang et al., 2020). The specific objective of the present study was to determine
92 the appropriate dietary DHA/EPA ratio when total n-3 LC-PUFA was supplied at optimal levels in diets with 7%
93 and 12% lipid, and evaluate the effects of dietary DHA/EPA ratio on growth performance, fatty acid profiles of
94 tissues, and expression of genes involved in lipid and fatty acid metabolism of juvenile mud crab, *S. paramamosain*.
95
96 **2. Materials and methods**
97 *2.1. Ethics statement*
98 All experimental procedures complied with the Standard Operation Procedures (SOPs) of the Guide for Use
99 of Experimental Animals of Ningbo University. The study was approved by the Scientific Ethics Committee for
100 Experiments on Animals of Ningbo University.
101 *2.2. Diet preparation*
102 Eight isonitrogenous purified diets were formulated to contain 7% and 12% crude lipid with total n-3 LC-
103 PUFA levels of 20.1mg g^{-1} and 12.7mg g^{-1} of dry weight, respectively, each with DHA:EPA ratios of 1:2, 1:1, 2:1
104 and 3:1 (Table 1). Palmitic acid was used to supply the bulk of the dietary lipid and maintain the 7% and 12% lipid
105 levels, with arachidonic acid (ARA, 20:4n-6) and cholesterol supplemented to all diets at the levels required to
106 support normal growth and molting according to data for *Portunus trituberculatus* and *Scylla serrata* (Sheen and
107 Wu, 1999; Yang, 2013). The analyzed fatty acid profiles of the experimental diets are presented as mg g^{-1} in Table
108 2, with the total dietary n-3 LC-PUFA levels in 7% and 12% lipid diets measured to be around 19.2mg g^{-1} and
109 11.9mg g^{-1} of dry weight, respectively. The DHA/EPA ratios were measured to be 0.6, 1.2, 2.3 and 3.2 at both 7%
110 and 12% dietary lipid levels, and the diets were named as L7R0.6, L7R1.2, L7R2.3, L7R3.2 and L12R0.6, L12R1.2,
111 L12R2.3, L12R3.2, respectively. All the ingredients were ground to fine powder with a particle size less than 177μm.

112 The micro-components including vitamin and mineral premixes were mixed using the progressive enlargement
113 method, and EPA, DHA, palmitic acid, soybean lecithin and distilled water (about 40%) were then added to the
114 premixed dry ingredients and mixed until homogenous in a Hobart-type mixer. Cold-extruded pellets were produced
115 (F-26, machine factory of South China University of Technology, Guangzhou, China), and the pellet strands cut
116 into two uniform pellet sizes (2.0mm diameter, 4.0mm length; 4mm diameter, 6.0mm length) using a granulating
117 machine (G-250, machine factory of South China University of Technology, Guangzhou, China), heated for 30min
118 at 90°C, and then air-dried to approximately 10% moisture. The dried diets were sealed in vacuum-packed bags and
119 stored at -20°C until used.

120 Insert Table 1 here.

121 Insert Table 2 here.

122 *2.3. Experimental crabs and feeding trial*

123 Juvenile mud crabs were obtained from Jia-Shun aquatic-cooperatives (Taizhou, China) and, prior to the
124 experiment, were acclimated and fed a commercial feed (45% crude protein, 8% crude lipid; Ningbo Tech- Bank
125 Corp., Ningbo, China) for 2 weeks in a cement pool. At the beginning of feeding trial, a total of 240 juvenile crabs
126 (20.92 ± 0.56g crab^{-1}) were randomly allocated into 240 single crab cells (0.33m × 0.23m × 0.15m, length ×
127 × height) (Zhao et al., 2015; Li et al., 2018), and three replicates (10 crabs per replicate) were randomly assigned to
128 each dietary treatment. Each cell was half filled with a continuous flow of seawater (300mL min^{-1}) and crabs were
129 fed once daily at 18:00 to apparent satiation with 6 - 8% of wet body weight during the feeding period (Unnikrishnan
130 and Paulraj, 2010). Feces and uneaten feed were removed daily from each cell. Any dead crabs were removed and
131 weighed as soon as being observed, and the number of molts were recorded daily.

132 During the experimental period, the temperature of flowing water in the crab cells was 26 - 30°C, salinity was
133 approximately 26 - 28g L^{-1}, pH was 7.7 - 8.0, ammonia nitrogen was lower than 0.05mg L^{-1}, and dissolved oxygen
134 was 6.5 - 7.0mg L^{-1}. Salinity, pH, ammonia nitrogen and dissolved oxygen in the pool were measured by the YSI
135 Pro plus (YSI, Yellow Springs, Ohio, USA). The feeding trial lasted for 8 weeks.

136 *2.4. Sample collection*

137 All the surviving crabs molted their shells at least once during the 8 weeks. At the end of the feeding trial, all
138 the crabs were starved for 24h and were counted and weighed to determine weight gain (WG), specific growth rate
139 (SGR) and molting frequency (MF), which were all calculated per replicate. In each replicate, hemolymph samples
140 from three crabs were taken from the pericardial cavity using a 1mL syringe, placed into 1.5mL microfuge tubes

and centrifuged at 956g for 10min at 4°C (Eppendorf centrifuge 5810R, Germany). The supernatant was collected and stored at -80°C until further analysis. Hepatopancreas and muscle samples were dissected from the same crabs that blood had been drawn, and were stored at -20°C prior to analyses of proximate composition and fatty acid profile. Hepatopancreas samples were taken from a further three crabs per replicate, and then frozen immediately in liquid nitrogen and stored at -80°C for gene expression analysis. Samples collected from the same replicate were pooled prior to analysis.

2.5. Biochemical analysis

2.5.1. Proximate composition and fatty acids

The crude protein, crude lipid, moisture and ash content of diets, muscle and hepatopancreas of the crabs were determined according to the method of the Association of Official Analytical Chemists (AOAC, 2006). The moisture content was determined by drying the samples to a constant weight at 105°C. The crude protein contents (N × 6.25) were assayed by the Dumas combustion method with a protein analyzer (FP-528, LECO, USA). Crude lipid was measured via the petroleum ether extraction method using a Soxtec System HT (SX360, OPSIS, Sweden), and the ash content was determined after incineration in a muffle furnace at 550°C for 8h.

Fatty acid compositions of diets, hepatopancreas and muscle were analyzed as described in detail previously (Gao et al., 2012). In brief, total lipid was extracted with chloroform/methanol (2:1 by vol.) and fatty acid methyl esters (FAME) were produced from total lipid by methanolic sulfuric acid with 0.01% butylated hydroxytoluene (BHT) as antioxidant. Methyl tricosanoate (23:0; Sigma Aldridge Trading Co., Ltd., Shanghai, China) was used as internal standard at 1.0mg mL^{-1} hexane. Gas chromatography (Agilent Technologies GC-MS 7890B-5977A, USA) was used to analysis FAME with fatty acids identified by reference to known standards and presented as percentages of area.

2.5.2. Haematological characteristics

Total protein (TP), glucose (GLU), triacylglycerol (TAG), total cholesterol (T-CHO), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) contents, and alkaline phosphatase (ALP) activity in the hemolymph were assayed by an automatic blood analyzer (Hitachi 7170A, Japan) using commercial assay kits purchased from Biosno Bio-Technology and Science Inc. (Beijing, China).

2.5.3. Real-time quantitative PCR (RT-qPCR) analysis of fatty acid biosynthesis and lipid metabolism genes in hepatopancreas

Total RNA was extracted from hepatopancreas samples using Trizol reagent (Invitrogen, USA), and the quantity and quality of total RNA assessed using a Nano DropND-1000 spectrophotometer (NanoDrop Technologies, USA) and 1.2% denaturing agarose gel electrophoresis. The 260/280nm absorbance ratios of all samples ranged from 1.86 to 2.00, indicating a satisfactory purity of the RNA samples. The RNA was dissolved in 30μL Recombinant DNase I (RNase-free) (Takara, Japan) and stored at -80°C until use. The cDNA was synthesized for quantitative reverse-transcriptase polymerase chain reaction (qPCR) using the PrimeScript™ RT Reagent Kit (Takara, Japan) according to the manufacturer's instructions.

Elongation factor-1α (*ef-1α*) was used as a house-keeping gene after the stability of its expression was confirmed. Specific primers for elongase of very long-chain fatty acids 4, 5 and 6 (*elovl4, elovl5 and elovl6*), delta-6 and delta-9 fatty acyl desaturase (*Δ6 fad* and *Δ9 fad*), fatty acid synthase (*fas*), acetyl-CoA carboxylase (*acc*), glucose-6-phosphate dehydrogenase (*g6pd*), 6-phosphogluconate dehydrogenase (*6pgd*), sterol regulatory element binding protein-1 (*srebp-1*), hormone-sensitive triglyceride lipase (*hsl*), carnitine palmitoyltransferase I and II (*cptI* and *cptII*), acyl-CoA oxidase 1 and 3 (*aco1* and *aco3*), fatty acid-binding protein 1 and 3 (*fabp-1* and *fabp-3*), fatty acid transport protein 4 *(fatp-4),* low-density lipoprotein receptor (*ldlr*), low-density lipoprotein receptor-related protein 2 (*lrp2*) and scavenger receptor b (*srb*) used for RT-qPCR were designed using Primer Premier 5.0 software (Supplementary Table 1). The expression of mRNA was determined by RT-qPCR (Light Cycler 96; Roche, Switzerland). The RT-qPCR was performed in a 20μL reaction volume containing 10μL of SYBR Green premix, 0.8μL of cDNA template, 0.4μL of each primer (10μM) and 8.4μL of diethyl pyrocarbonate-treated water. The RT-qPCR conditions were as follows: 95°C for 10min; 45 cycles of 95°C for 15s, 58°C for 15s and 72°C for 20s. The data were optimized using the comparative Ct ($2^{-\Delta\Delta Ct}$) value method as described by Livak and Schmittgen (2001) and then subjected to statistical analysis.

2.6. Calculations and statistical analysis

The parameters were calculated as follows:

Weight gain (WG, %) = $100 \times (W_t - W_i) / W_i$,

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

Molting frequency (MF) = $2 \times N_m$ / (initial number of crabs + final number of crabs)

Where W_t is the final body weight (g), W_i is the initial body weight (g), t is the experimental duration in days, N_m is the number of moltings.

Data were transformed before analysis as necessary and were first analyzed using one-way analysis of variance ANOVA to detect differences among all the treatments. When there were significant differences ($P < 0.05$), the group means were further compared using Tukey's multiple range tests. All the results are presented as means ± SEM (n = 3). The two-slope broken-line and second-order polynomial regression analysis was conducted to analyze the WG of mud crab in response to dietary DHA/EPA ratio (Figure 1). All statistical analyses were performed using SPSS 23.0 (SPSS, IBM, USA).

3. Results

3.1. Growth performance

The growth performance of crabs fed the different experimental diets is shown in Table 3. WG and SGR were significantly impacted by dietary DHA/EPA ratio at both 7% and 12% dietary lipid levels. Crabs fed the diets with a DHA/EPA of 0.6 at both 7% or 12% lipid had significantly lower WG than those fed the diets with higher DHA/EPA ratios, but there were no differences in WG and SGR between crabs fed diets with DHA/EPA ratios of 1.2, 2.3 and 3.2 at 12% lipid. Two-slope broken-line and second-order polynomial regression analysis of WG against dietary DHA/EPA ratio showed that the optimal ratios were 2.2 and 1.2 in crabs fed dietary lipid at 7% and 12%, respectively (Figure 1). MF was not significantly influenced by dietary DHA/EPA ratios at either 7% or 12% lipid levels.

Insert Table 3 here.

Insert Figure 1 here.

3.2. Proximate compositions of muscle and hepatopancreas

As shown in Table 4, moisture content in muscle decreased with increased dietary DHA/EPA ratio, and significantly higher lipid content was observed in crabs fed the diet with a DHA/EPA ratio of 2.3 than those fed diets with DHA/EPA ratios of 1.2 and 3.2 at 12% lipid. Lipid content in hepatopancreas increased as dietary DHA/EPA ratio increased from 0.6 to 2.3, but a marginal decreasing trend was found when dietary DHA/EPA ratio was higher than 2.3 at both 7% and 12% lipid levels. The moisture contents of hepatopancreas decreased as dietary DHA/EPA ratio increased from 0.6 to 3.2 in crabs fed 7% lipid. Hepatopancreas of crabs fed the diet with a DHA/EPA ratio of 0.6 had significantly higher protein content than those fed the diet with a DHA/EPA ratio of 2.3 at 7% lipid, while protein contents were higher in crabs fed the diets with DHA/EPA ratios of 2.3 and 3.2 than those fed diets with DHA/EPA ratios of 0.6 and 1.2 at 12% lipid.

Insert Table 4 here.

3.3. Fatty acid profiles of muscle and hepatopancreas

Principal component analysis (PCA) score plot based on the first component was used to present the fatty acid compositions of muscle (Fig. 2A) and hepatopancreas (Fig. 2B) in crabs fed the different diets. The further the components were separated, the greater the difference. Crabs fed the diets with DHA/EPA ratios of 2.3 and 3.2 at 12% dietary lipid showed similar muscle fatty acid profiles as their components overlapped in Fig. 2A, while the components of crabs in other treatments were clustered and separated from others. No overlap was observed in Fig. 2B, but the components of crabs fed DHA/EPA ratios of 2.3 and 3.2 at 7% dietary lipid were close to each other, and to crabs fed the diets with these DHA/EPA ratios at 12% dietary lipid. Fig. 2B showed that hepatopancreas fatty acid profiles were affected by different dietary DHA/EPA ratios, but crabs fed the diets with DHA/EPA ratios of 2.3 and 3.2 showed similar hepatopancreas fatty acid profiles at 7% and 12% lipid. Complete fatty acid compositions of muscle and hepatopancreas are provided in Supplementary Tables 2 and 3.

The saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), n-6 polyunsaturated fatty acid (PUFA), n-3 PUFA and total fatty acid (TFA) contents of crab muscle were all significantly influenced by dietary DHA/EPA ratio at both 7% and 12% lipid levels (Table 5). At the 7% lipid level, crabs fed diets with DHA/EPA ratios of 1.2 and 2.3 showed significantly higher TFA, SFA, MUFA and n-6 PUFA contents than those fed other diets. The lowest n-3 PUFA content was observed in crabs fed diet with a DHA/EPA ratio of 0.6, and the EPA content significantly decreased when dietary DHA/EPA ratio increased from 1.2 to 2.3, while no differences were found in EPA content and DHA/EPA ratio between crabs fed diets with DHA/EPA ratios of 0.6 and 1.2, and 2.3 and 3.2. At 12% lipid level, crabs fed the diet with a DHA/EPA of 0.6 had significantly lower TFA, SFA, and n-6 PUFA contents than those fed the diets with higher DHA/EPA ratios, but there were no differences in TFA and SFA between crabs fed diets with DHA/EPA ratios of 1.2, 2.3 and 3.2. The MUFA content in crabs fed the diet with a DHA/EPA of 0.6 was significantly lower than those fed diets with DHA/EPA ratios of 1.2 and 3.2. The highest n-3 PUFA content was observed in crabs fed the diet with a DHA/EPA ratio of 2.3, and there no differences between crabs fed the other diets. Muscle DHA/EPA ratio significantly increased with increased dietary DHA/EPA ratios, while EPA content showed the opposite trend. The DHA content increased as dietary DHA/EPA ratio increased from 0.6 to 2.3, but no differences were found when dietary DHA/EPA ratio was higher than 2.3 at either 7% or 12% lipid level.

In crabs fed 7% dietary lipid, the hepatopancreas MUFA, n-6 PUFA and TFA contents showed at first an increase and then a marginal decreasing trend as dietary DHA/EPA ratio increased from 0.6 to 3.2, with highest values observed when dietary DHA/EPA ratio was 2.3 (Table 6). The n-3 PUFA and DHA contents increased significantly when dietary DHA/EPA ratio increased from 1.2 to 2.3, while no differences were found between crabs fed the diets with DHA/EPA ratios of 0.6 and 1.2, and 2.3 and 3.2. The SFA content in crabs fed the diet with a DHA/EPA ratio of 0.6 was significantly lower than those fed the other diets, but there were no differences in SFA between crabs fed diets with DHA/EPA ratios of 1.2, 2.3 and 3.2. The EPA content showed a negative correlation with dietary DHA/EPA ratio. At 12% lipid level, the n-3 PUFA content was not affected by dietary DHA/EPA ratio but the EPA content decreased significantly with increased dietary DHA/EPA ratio, and similar trends were observed in SFA, MUFA and n-6 PUFA contents. The DHA content increased as dietary DHA/EPA ratio increased from 0.6 to 2.3, but no differences were found when dietary DHA/EPA ratio was higher than 2.3. The DHA/EPA ratio in hepatopancreas show a significantly positive correlation with dietary DHA/EPA ratio at both 7% and 12% dietary lipid levels.

Insert Figure 2 here.

Insert Table 5 here.

Insert Table 6 here.

3.4. Hematological enzyme activities and characteristics

In crabs fed 7% dietary lipid, the TP, TAG and T-CHO contents first increased and then decreased with increasing dietary DHA/EPA ratio, with highest values observed in crabs fed DHA/EPA ratios of 2.3 and/or 3.2. The lowest GLU and LDL-C levels were observed in crabs fed the diet with a DHA/EPA ratio of 0.6, but there were no differences among crabs fed the other ratios (Table 7). In crabs fed 12% dietary lipid, the lowest HDL-C content was found in crabs fed the diet with a DHA/EPA ratio of 3.2, and there were no differences among crabs fed the other ratios. Increasing trends with increasing dietary DHA/EPA ratio were observed in the ALP and TP contents, but the GLU content showed at first an increase and then a decreasing trend as dietary DHA/EPA ratio increased from 0.6 to 3.2, with the value in crabs fed the DHA/EPA ratio of 2.3 being significantly higher than those fed the DHA/EPA ratio of 3.2.

Insert Table 7 here.

3.5. Expression of genes related to lipid metabolism in hepatopancreas

Among genes related to lipogenesis and lipolysis, the expression of *fas* increased significantly with increased dietary DHA/EPA ratios in crabs fed 7% dietary lipid, while the expression of *hsl* was down regulated by increasing dietary DHA/EPA ratios (Figure 3). The expression levels of *6gpd*, *g6pd, acc* and *srebp-1* showed a tendency to first increase and then decrease as dietary DHA/EPA ratio increased from 0.6 to 3.2. In crabs fed diets with 12% lipid, the lowest expression of *fas* was observed in crabs fed the diet with a DHA/EPA ratio of 2.3, while crabs fed the diet with a DHA/EPA ratio of 1.2 showed the highest expression level of *hsl*, and the expression levels of *6gpd*, *g6pd* and *srebp-1* all showed decreasing trends with increased dietary DHA/EPA ratios.

With regards to genes related to β-oxidation, in crabs fed 7% dietary lipid, the expression level of *cptI* decreased with increased dietary DHA/EPA ratio, and *cptII* expression was significantly higher in crabs fed the diet with a DHA/EPA ratio of 0.6 than in crabs fed the diet with a ratio of 1.2. Highest expression levels of *aco1* and *aco3* were observed in crabs fed the diets with DHA/EPA ratios of 2.3 and 3.2, respectively. In crabs fed 12% dietary lipid, the expression levels of *cptI, cptII* and *aco3* showed similar trends with increasing dietary DHA/EPA ratio, with highest expression levels observed in crabs fed the diets with DHA/EPA ratios of 1.2 and/or 2.3, and the expression level of *aco1* showed an increasing trend with increasing dietary DHA/EPA ratio.

The expression level of *fatp1* in hepatopancreas was higher when dietary DHA/EPA ratio was higher than 0.6/1.2 at 7% or 12% lipid. Highest *fabp3* expression levels were observed in crabs fed diets with DHA/EPA ratios of 2.3 and 1.2 at 7% and 12% lipid, respectively. The expression level of *fabp4* showed an increasing trend at 7% lipid, but decreased as dietary DHA/EPA ratio increasing from 1.2 to 3.2 at 12% lipid. In crabs fed 7% lipid, the expression level of *ldlr* significantly decreased with increased dietary DHA/EPA ratio, and expression of *lrp2* significantly decreased when dietary DHA/EPA ratio increased from 2.3 to 3.2, while the expression level of *srb* was not affected by dietary DHA/EPA ratio. In crabs fed 12% dietary lipid, *ldlr*, *lrp2* and *srb* expression levels showed similar trends with increased dietary DHA/EPA ratio, with highest expression levels observed when dietary DHA/EPA ratios were 1.2 and/or 2.3.

Insert Figure 3 here.

3.6. *Expression of genes involved in LC-PUFA biosynthesis in hepatopancreas*

In crabs fed 7% dietary lipid, the expression level of *Δ6 fad* increased and then decreased as dietary DHA/EPA ratio increased from 0.6 to 2.3 and from 2.3 to 3.2 (Figure 4), while the expression level of *Δ9 fad* showed an increasing trend as dietary DHA/EPA ratio increased from 1.2 to 3.2. In crabs fed 12% lipid, the expression level of *Δ6 fad* was significantly lower in crabs fed a DHA/EPA ratio of 0.6 than those fed other ratios, and there was no

311 differences among crabs fed the DHA/EPA ratios of 1.2, 2.3 and 3.2, while the expression level of *Δ9 fad* was
312 significantly higher in crabs fed a dietary DHA/EPA ratio of 0.6 compared to those fed the ratio of 3.2. The
313 expression level of *elovl4* showed similar trends with increased dietary DHA/EPA ratio at both 7% and 12% lipid,
314 with highest expression levels observed when dietary DHA/EPA ratios were 1.2 and 2.3, respectively.
315 Insert Figure 4 here.
316 **4. Discussion**
317 As vertebrates and most invertebrate species cannot synthesize PUFA from monounsaturated fatty acids *de*
318 *novo*, they have an absolute dietary requirement for certain specific n-3 and/or n-6 PUFA (NRC, 2011). Early studies
319 indicated there was a hierarchy of effectiveness of LC-PUFA and PUFA to satisfy EFA requirements of kuruma
320 shrimp (*Marsupenaeus japonicus*) according to the following order: EPA > DHA > 18:3n-3 > 18: 2n-6 (Kanazawa
321 et al., 1979a, b). Some studies have also demonstrated LC-PUFA, particularly EPA, were more biologically active
322 and elicited significantly higher growth rates than PUFA (NRC, 2011). However, Merican and Shim (1997) found
323 that DHA had the highest EFA activity measured by WG in marine tiger shrimp (*Penaeus monodon*). These results
324 also suggested that EFA requirements might not only be a function of the total amount of these fatty acids in the
325 diet, but also of the relative proportions of essential LC-PUFA such as DHA and EPA (NRC, 2011). In the present
326 study, the values of WG and MF agreed with our previous study on the optimal n-3 LC-PUFA requirement of mud
327 crab at 7% and 12% dietary lipid levels (Wang et al., 2020). This may reflect the fact that the two studies shared the
328 same dietary ingredients and similar initial weight of crab. The two-slope broken-line and second-order polynomial
329 regression analysis of WG against dietary DHA/EPA ratio indicated that the optimal DHA/EPA ratios were 2.2 and
330 1.2 at 7% and 12% dietary lipid levels, respectively. In terms of absolute levels, the results in mud crab were higher
331 than those reported in juvenile *P. trituberculatus*, where the optimal DHA/EPA ratio was estimated to be 0.7 - 0.8
332 at 11% dietary lipid (Hu et al., 2017). However, the optimum dietary DHA/EPA ratio for swimming crab at the
333 stage of ovarian developmental was 2.0 at 11% lipid, and lower or higher ratios could lead to hepatopancreas
334 albinism (Feng, 2011). Base on growth performance and resistance to hypoxia stress, the optimal DHA/EPA ratio
335 of Chinese mitten crab (*Eriocheir sinensis*) was 2 - 3 at 7.5% lipid (Zhao et al., 2013). The differences between
336 reported optimal DHA/EPA ratios and requirements among different crustacean and fish species are likely related
337 to culture species, developmental and physiological stage, dietary formulation, lipid level and sources, and
338 experimental conditions (Glencross et al., 2011). Combined with the result of our previous study that determined
339 how the optimal n-3 LC-PUFA requirement of mud crab varied with dietary lipid content(Wang et al., 2020), the

present study showed that the optimal DHA/EPA ratio was 2.2 at 7% lipid with a total n-3 LC-PUFA level of 19mg g^{-1} of diet, and was 1.2 at 12% lipid with a total n-3 LC-PUFA level of 12mg g^{-1} of diet. Therefore, the present study confirmed that dietary lipid level significantly affected both the optimum dietary n-3 LC-PUFA level and the optimum DHA/EPA ratio of juvenile mud crab. Similar studies have been reported in other species. The n-3 LC-PUFA requirements of juvenile gilthead bream (*S. aurata*) were estimated to be 0.9% of diet when the DHA/EPA ratio was 1.0 at a dietary lipid level of 13% (Kalogeropoulos et al., 1992), whereas it was 1.9% when the DHA/EPA ratio was 0.5 and dietary lipid level was 8% (Ibeas et al., 1994). Another study showed that n-3 LC-PUFA requirement was about 3% when sea bream fed a diet with a DHA/EPA ratio of 1.0 and 22% dietary lipid (Houston et al., 2017).

In the present study, the proximate composition of muscle was not affected by dietary DHA/EPA ratio, but lipid content in hepatopancreas increased with increased dietary DHA/EPA ratio in crabs fed 7% dietary lipid, similar to results observed in *E. sinensis* and *P. trituberculatus.* Hepatopancreas is an important tissue for the deposition of lipid and energy storage in crustaceans (Cavalli et al., 2000; Johnston et al., 2003). It was notable that the hepatopancreas lipid content in crabs fed diets with DHA/EPA ratios of 0.6 - 1.2 at 7% dietary lipid ranged from 28.4% to 28.8%, significantly lower than those fed the diets with higher ratios. Meanwhile, hepatopancreas protein content decreased as dietary DHA/EPA ratio increased from 0.6 to 2.3 in crabs fed 7% dietary lipid. This may be due to protein (as well as lipid) in the hepatopancreas being used to supply energy, when dietary lipid level was lower than the optimum level (9.5%) (Zhao et al., 2015). No significant difference was found in hepatopancreas lipid contents among crabs fed diets with 7% lipid and DHA/EPA ratios higher than 1.2. In addition, muscle lipid content and hepatopancreas lipid and protein contents initially increased and then decreased as dietary DHA/EPA ratio increased in crabs fed 12% lipid. Based on these results, we speculate that dietary DHA/EPA ratio could improve energy storage while preventing excess lipid deposition in hepatopancreas and, thus, play an important role in lipid metabolism, which was supported by data on the expression of genes related to lipid anabolism and catabolism. Therefore, energy and protein metabolism may also be affected by dietary DHA/EPA ratio in mud crab, and so this requires further study.

It was demonstrated that the fatty acid compositions of fish and crustacean tissues generally reflect dietary fatty acid profiles (Nasopoulou and Zabetakis, 2012; Unnikrishnan and Paulraj, 2010; Zhang et al., 2019b). In the present study, the fatty acid compositions of hepatopancreas and muscle showed similar results, with increased DHA content and DHA/EPA ratio and decreased EPA content in both tissues as dietary DHA/EPA ratio increased,

irrespective of dietary lipid level. The DHA/EPA ratios in hepatopancreas were similar to those of the diets and higher than those of muscle, which indicated that LC-PUFA may be preferentially deposited in hepatopancreas rather than muscle in mud crab. These results also suggested a selective retention of DHA over EPA or other fatty acids in mud crab *S. paramamosain* underpinning its greater biological value as EFA, as reported in other marine species (Carvalho et al., 2018; Izquierdo, 1996). Based on the higher DHA/EPA ratio in hepatopancreas, we speculated that *S. paramamosain* may also synthesize DHA from EPA or shorter chain PUFA, albeit the capacity may be low. A recent study indicated that *Litopenaeus vannamei* had the potential ability to convert linolenic acid to EPA and DHA (Chen et al., 2014a; b), which supports the speculation in the present study. The SFA, MUFA, n-6 PUFA, n-3 PUFA and total fatty acid contents increased and then decreased or marginally decreased in muscle as dietary DHA/EPA ratio increased at both dietary lipid levels, while hepatopancreas showed a similar trend at 7% lipid but opposite at 12% lipid, which may indicate differences in deposition and utilization of fatty acids in the different tissues (Izquierdo et al., 2003). It should be noted that these data reflect differences in the lipid contents of the tissues as the fatty acid compositions were presented in absolute quantitative terms in the present study.

Previously, Elovl4, Elovl5 and Δ6 Fad were reported to be key enzymes in the LC-PUFA biosynthesis pathway (Zhang et al., 2019a). Elovl5 elongates 18:4n-3 and 18:3n-6 to 20:5n-3 and 20:4n-6, respectively (Zuo et al., 2012) and Elovl4 could effectively elongate C_{22} PUFA to C_{24} PUFA and have the potential to participate in the production of DHA (Li et al., 2017a, b). The Δ6 Fad is the first enzyme involved in the bioconversion of C_{18} PUFA to longer and more unsaturated fatty acids and is involved in the synthesis of DHA from EPA via the "Sprecher pathway" (Monroig et al., 2011). Additionally, it is known that DHA biosynthesis through the "Sprecher pathway" is also catalysed by ACO in peroxisomes (Sprecher, 2000). In the present study, the expression levels of *elovl4* and *Δ6 fad* showed similar trends with increased DHA/EPA ratio at both 7% and 12% lipid levels, initially increasing and then decreasing, and a similar result was also observed in the expression level of *elovl5* in crabs fed 7% lipid, which was consistent with the expression levels of *aco3* and *srebp-1*, and the contents of DHA in hepatopancreas and muscle. These data were further evidence suggesting that mud crab require high DHA to maintain basic functions, and some capacity for the *in vivo* synthesis of DHA from EPA via "Sprecher pathway". An increase in the expression of *elovl4-like*, *elovl5-like* and *Δ6 fad* were also observed in liver and brain of juvenile golden pompano (*Trachinotus ovatus*) fed a diet with a higher DHA/EPA ratio (Zhang et al., 2019a). Additionally, the underlying regulatory mechanisms demonstrated that the transcription levels of Elovl4, Elovl5 and Δ6 Fad were positively mediated by *lxrα* directly or indirectly through the regulation of *srebp-1* transcription (Chen et al., 2019; Dong et al., 2017c; Li

et al., 2017b). The results of the present study were generally consistent with this, however, the function of these enzymes and the underlying mechanisms by which their expression is regulated in mud crab is still unknown and requires further study.

Acc is a cytosolic enzyme producing alonyl-CoA, the first step in the biosynthesis of long-chain fatty acids (Yu et al., 2015). 6Gpd and G6pd are key enzymes related to the production of NADPH (Chen et al., 2013; Zheng et al., 2013), essential for *de novo* fatty acid biosynthesis catalyzed by Fas (Chen et al., 2013; Zheng et al., 2013), while Hsl is involved in lipolysis (Ma et al., 2013). Additionally, Srebp-1 is a transcription factor regulating fatty acid, lipid and cholesterol biosynthesis pathways (Minghetti et al., 2011; Zheng et al., 2013). Previous studies have reported that dietary fatty acid profile could affect gene expression or activity of these enzymes involved in the mechanisms of lipogenesis and lipolysis (Jin et al., 2017; Kim et al., 1999; Morais et al., 2011, 2012; Panserat et al., 2008; Peng et al., 2014). In the present study in crabs fed 7% dietary lipid, the expression level of *fas* significantly increased with increased dietary DHA/EPA ratio, while the expression level of *hsl* was decreased. The expression levels of *6gpd*, *g6pd* and *srebp-1* showed similar trends to each other, initially increased and then decreased as dietary DHA/EPA ratio increased. These results showed that, at 7% dietary lipid, increasing dietary DHA/EPA ratio improved lipogenesis and inhibited lipolysis in mud crab, and that hepatopancreas of mud crab may require a certain level of lipid to maintain energy supply energy and basic functions. At 12% dietary lipid, while the expression of *acc* was not affected by dietary DHA/EPA ratio, the expression levels of *6gpd*, *g6pd* and *srebp-1* decreased with increased dietary DHA/EPA ratio, whereas lowest expression levels of *fas* were observed in crabs fed the diets with dietary DHA/EPA ratios of 2.3, and the highest expression levels of *hsl* were found in crabs fed diets with DHA/EPA ratios of 1.2. These results indicated that dietary DHA/EPA ratio played an important role in the inhibition of lipogenesis in mud crabs fed high-lipid diets, which may prevent excess lipid deposition in hepatopancreas.

It was demonstrated that β-oxidation in the mitochondrial matrix and peroxisome are main pathways of fatty acid catabolism (Lu et al., 2014). Cpt I catalyzes the conversion of fatty acid-CoAs to fatty acid-carnitines for entering the mitochondrial matrix, with the fatty acyl group transferred back to CoA by Cpt II (Kerner and Hoppel, 2000; Li et al., 2019), while Aco is the rate-limiting enzyme for fatty acid β-oxidation in peroxisomes (Lu et al., 2014). In the present study, the expression level of *cptI* decreased with increased dietary DHA/EPA ratio at 7% dietary lipid, which suggested reduced long-chain fatty acid transport into the mitochondrial matrix, leading to reduced β-oxidation and increased lipid deposition. At 7% dietary lipid, the highest expression of *aco1* was observed

in crabs fed diet with a DHA/EPA ratio of 2.3, while highest expression levels of *aco3* were observed in crabs fed the diets with dietary DHA/EPA ratios of 3.2 and 2.3 at 7% and 12% lipid, respectively, consistent with the lipid and DHA contents of hepatopancreas. At 12% dietary lipid, the expression levels of *cptI,* and *cptII* showed similar trends with increased dietary DHA/EPA ratios, initially increasing and then decreasing, with highest expression levels observed in crabs fed diets with DHA/EPA ratios of 1.2 and/or 2.3. Overall, the results indicated that dietary DHA/EPA ratio affected the relative gene expression levels of *cptI, cptII, aco1* and *aco3*, influencing fatty acid oxidation and lipid content in mud crab. Thus, increased dietary DHA/EPA ratio promoted the β-oxidation of fatty acids and reduced lipid deposition.

FATP promote the transport of long-chain fatty acids and are expressed in tissues with active fatty acid metabolism (Jeppesen et al., 2012; Nickerson et al., 2009). In mice, the transport rates of LC-PUFA varied among members of the FATP family with relative rates of 8, 5, 2, 13, 2, 0 for FATP-1 to FATP-6. FABP bind fatty acids with different specificities and play important roles in the uptake, transport and metabolic regulation of long-chain fatty acids in organelles within cell (Storch and Thumser, 2000). For example, FABP-1 has a close relationship with the transport and uptake of LC-PUFA in general (Mcarthur et al., 1999), while FABP-3 has high affinity to especially EPA (Tan et al., 2015). In the present study, the expression level of *fabp-1* was up-regulated by increased dietary DHA/EPA ratio irrespective of dietary lipid level, while the highest expression levels of *fabp-3* were observed in crabs fed diets with DHA/EPA ratios of 1.2 and 2.3 at 7% and 12% dietary lipid, respectively. The expression of *fabp-3* showed a positive relationship to hepatopancreas LC-PUFA content, which agreed with a previous study (Tan et al., 2015). It was reported that FABP can transport fatty acids for not only lipogenesis but also β-oxidation (Ockner et al., 1972). Therefore, the expression levels of *fabp* in the present study suggested an activation of fatty acid metabolism with increasing dietary DHA/EPA ratio. In contrast, the expression levels of *fatp-4* were up-regulated by dietary DHA/EPA ratios at 7% lipid, but down-regulated at 12% lipid, which reflected a similar trend with hepatopancreas total fatty acid content and supported our speculation on the impact of dietary DHA/EPA ratio on hepatopancreas lipid content.

Lipids in blood are transported to peripheral tissues by lipoproteins (Weil et al., 2013), where LDLR and LRP2 can identify and promote clearance of lipoproteins (Magkos, 2009). Studies in human reported that LRP6 and LDLR could promote the dissolution of LDL-C in lysosomes, thereby reducing LDL-C levels in the blood (Voros et al., 1996) while SRBI is an HDL receptor that participates in the reverse transport of cholesterol (Viñals et al., 2003). In the present study, the expression levels of *ldlr* and *lrp2* were down-regulated by increased dietary DHA/EPA

ratio in crabs fed 7% lipid, and thus increased the T-CHO and LDL-C contents in hemolymph. At 12% dietary lipid, the expression levels of *ldlr*, *lrp2* and *srb* showed similar trends as dietary DHA/EPA ratio increased, initially increasing and then decreasing, which may lead to decreased HDL-C content in hemolymph. It is well known that hematological components such as hemoglobin, hematocrit, red blood cells and leucocytes, as well as serum components such as TP, TAG, CHO and GLU, are correlated with health and immune response (Zhou et al., 2015). Moreover, ALP is involved in the regulation of immune functions in fish and crustacean (Meyran and Graf, 1986), and the activity of ALP significantly increased with increased dietary DHA/EPA ratio in crabs fed 12% lipid in the present study. TAG and CHO levels also reflect lipid metabolism and deposition in crustaceans (Zhang et al., 2019b), which was supported in the present study as the trends in TAG and T-CHO contents with increased dietary DHA/EPA ratio were consistent with hepatopancreas lipid content in crabs fed 7% dietary lipid. These results also indicated that dietary DHA/EPA ratio significantly affected hematological components suggesting that an increased ratio could improve the health of mud crab.

5. Conclusion

In summary, the present study is the first to measure lipid anabolism and catabolism genes to explore mechanisms related to the physiological effects of dietary DHA/EPA ratio in mud crab. Dietary DHA/EPA ratio influenced energy storage and prevented excess lipid deposition in hepatopancreas by regulating genes related to lipogenesis, lipolysis, β-oxidation, fatty acid uptake and lipoprotein receptors. Mud crabs require a higher level of DHA than EPA. Based on WG, the optimal dietary DHA/EPA ratios of mud crab were estimated to be 2.2 and 1.2 when n-3 LC-PUFA was supplied appropriately at 7% and 12% dietary lipid levels, respectively.

Author contribution

X. X. W. formulated the research question, designed the study, carried out the study, analyzed the data and wrote the manuscript. M. J designed the study and assisted in the data analysis. X. C. was involved into feeding trial. X. Y. H. was involved in blood biochemical analysis. M. M. Z. was involved into fatty acids analysis. Y. Y. participated in statistical analysis P.S. was involved in data analysis. L. F. J. revised the manuscript. M. B. B. formulated the research question, designed the study. D. R. T. formulated the research question, designed the study and revised the manuscript. Q. Z. formulated the research question, designed the study, and revised the manuscript. All the authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

Table 1. Real-time quantitative PCR primers for fatty acid biosynthesis and lipid metabolism related genes and *ef1-α* of mud crab *S. paramamosain* in the study.

Table 2. Fatty acid profile of muscle of mud crab fed the different experimental diets.

Table 3. Fatty acid profile of hepatopancreas of mud crab fed the different experimental diets.

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712 **Table 1**

713 Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	Dietary DHA/EPA ratios							
	7% lipid level				12% lipid level			
	0.6	1.2	2.3	3.2	0.6	1.2	2.3	3.2
Casein [a]	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Soy protein concentrate [b]	27.61	27.61	27.61	27.61	27.61	27.61	27.61	27.61
Wheat flour	25.26	25.26	25.26	25.26	25.26	25.26	25.26	25.26
DHA-enriched oil [c]	0.00	1.28	2.57	3.20	0.00	0.85	1.71	2.13
EPA-enriched oil [d]	2.96	2.22	1.48	1.11	1.97	1.48	0.99	0.74
ARA-enriched oil [e]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Palmitic acid [f]	1.40	0.86	0.31	0.05	7.39	7.03	6.66	6.49
Soybean lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cholesterol	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Betaine (98%)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix [g]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix [g]	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Cellulose	8.97	8.97	8.97	8.97	3.97	3.97	3.97	3.97
Sodium alginate	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition								
Moisture	7.59	7.81	7.13	6.91	7.92	7.50	8.80	7.60
Crude protein	45.69	44.85	45.03	45.08	46.73	45.17	45.03	45.70
Crude lipid	7.43	7.85	7.51	7.51	12.00	12.50	12.10	12.02
Ash	6.62	6.15	6.26	6.11	6.04	6.75	6.19	6.55

714 [a] Casein, 89.55% crude protein and 0.2% crude lipid.

715 [b] Soy protein concentrate, 69.88% crude protein and 0.51% crude lipid.

716 [c] DHA–enriched oil, DHA content, 406.5mg g^{-1} oil.

717 [d] EPA–enriched oil, EPA content, 462.5mg g^{-1} oil; DHA content, 235.6mg g^{-1} oil.

718 [e] ALA–enriched oil, ALA content, 468.0mg g^{-1} oil.

719 [f] Palmitic acid, Palmitic acid content, 97% of total fatty acids, in the form of methylester; Shanghai Yiji Chemical

720 Co., Ltd., China.

721 [g] Vitamin premix and Mineral premix were based on Jin et al.(2015)

722

Table 2

Fatty acid compositions of the experimental diets (mg g^{-1}, dry matter).

Fatty acids	7% lipid level				12% lipid level			
	DHA/EPA ratio							
	0.6	1.2	2.3	3.2	0.6	1.2	2.3	3.2
14:0	0.56	0.58	0.63	0.65	0.79	0.77	0.77	0.81
16:0	10.99	9.70	8.23	7.66	28.75	28.28	27.46	26.60
18:0	2.02	2.10	2.12	2.27	2.04	2.14	2.27	2.31
20:0	0.20	0.23	0.24	0.26	0.19	0.19	0.22	0.23
∑SFA [a]	13.78	12.61	11.22	10.84	31.78	31.38	30.72	29.94
16:1n-7	0.20	0.21	0.24	0.25	0.18	0.20	0.23	0.24
18:1n-9	5.23	5.84	6.28	6.83	4.99	5.31	5.70	6.02
20:1n-9	0.15	0.11	0.11	0.10	0.12	0.10	0.10	0.10
22:1n-11	0.05	0.05	0.04	0.04	0.03	0.03	0.03	0.02
∑MUFA [b]	5.63	6.21	6.67	7.22	5.33	5.64	6.07	6.37
18:2n-6	7.27	7.19	6.90	7.20	6.96	6.97	6.96	6.84
18:3n-6	0.23	0.21	0.23	0.24	0.22	0.21	0.24	0.22
20:2n-6	0.11	0.08	0.09	0.09	0.07	0.07	0.06	0.06
20:4n-6	2.19	2.24	2.12	2.15	2.11	2.07	2.02	2.02
22:4n-6	0.16	0.29	0.09	0.07	0.06	0.09	0.06	0.06
∑n-6 PUFA [c]	9.97	10.02	9.43	9.75	9.42	9.41	9.35	9.20
18:3n-3	1.04	1.02	1.00	1.04	0.95	0.90	0.91	0.92
18:4n-3	0.42	0.35	0.24	0.28	0.25	0.19	0.18	0.16
20:4n-3	0.42	0.38	0.39	0.42	0.24	0.24	0.23	0.23
EPA [d]	10.37	8.18	5.48	4.57	6.65	5.06	3.49	2.74
22:5n-3	1.28	1.02	0.68	0.54	0.77	0.62	0.42	0.34
DHA [e]	6.45	9.92	12.33	14.49	4.13	5.93	7.90	8.79
∑n-3 PUFA [f]	19.98	20.87	20.12	21.35	12.99	12.93	13.13	13.17
n-3/n-6 PUFA	2.00	2.08	2.13	2.19	1.38	1.37	1.40	1.43
DHA/EPA	0.62	1.21	2.25	3.17	0.62	1.17	2.26	3.21
∑n-3 LC-PUFA [g]	18.53	19.50	18.88	20.03	11.79	11.83	12.03	12.10

[a] SFA, saturated fatty acids: 14:0, 16:0, 18:0, 20:0.

[b] MUFA, monounsaturated fatty acids: 16:1n-7, 18:1n-9, 20:1n-9.

[c] n-6 PUFA, n-6 polyunsaturated fatty acids: 18:2n-6, 18:3n-6, 20:2n-6, 20:4n-6, 22:4n-6.

[d] EPA, 20:5n-3.

[e] DHA, 22:6n-3.

[f] n-3 PUFA, n-3 polyunsaturated fatty acids: 18:3n-3, 18:4n-3, 20:4n-3, EPA, 22:5n-3, DHA.

[g] n-3 LC-PUFA, n-3 long-chain polyunsaturated fatty acids: 20:4n-3, EPA, 22:5n-3, DHA.

732 **Table 3**

733 Growth performance and feed utilization of mud crab fed the experimental diets.

Lipid levels	DHA/EPA ratio	Initial weight (g)	WG [a] (%)	SGR [b] (% d^{-1})	MF [d]
7%	0.6	20.62±1.09	44.26±2.83^{c}	0.65±0.04^{b}	0.63±0.19
	1.2	21.68±1.08	52.85±1.29^{b}	0.75±0.01^{ab}	0.65±0.05
	2.3	23.38±1.17	62.41±0.49^{a}	0.81±0.01^{a}	1.03±0.10
	3.2	20.05±1.45	55.80±1.65^{ab}	0.73±0.02^{ab}	0.75±0.11
12%	0.6	20.12±1.15	45.17±0.96^{B}	0.66±0.02^{B}	0.67±0.10
	1.2	21.63±1.38	57.51±0.98^{A}	0.79±0.02^{A}	0.80±0.05
	2.3	18.08±0.97	56.77±1.90^{A}	0.79±0.02^{A}	0.52±0.09
	3.2	21.83±1.82	56.59±2.66^{A}	0.78±0.02^{A}	0.81±0.07

734 Data are presented as means ± SEM (n = 3). Values in the same column with different superscripts are significantly

735 different ($P < 0.05$).

736 [a] WG: weight gain;

737 [b] SGR: specific growth rate;

738 [c] MF: molting frequency.

Table 4

Proximate compositions of muscle and hepatopancreas of mud crab fed the different experimental diets (dry matter).

Lipid levels	DHA/EPA ratio	Muscle			Hepatopancreas		
		Moisture (%)	Lipid (%)	Protein (%)	Moisture (%)	Lipid (%)	Protein (%)
7%	0.6	80.73±0.78	14.43±0.23	86.69±0.02	76.25±1.72^{a}	28.38±3.95^{b}	48.08±1.09^{a}
	1.2	80.07±0.94	15.32±0.31	84.92±0.75	74.57±0.93^{ab}	28.79±1.56^{b}	44.92±0.02^{ab}
	2.3	79.04±0.54	13.46±0.70	84.13±0.53	68.88±1.69^{bc}	38.73±0.41^{a}	43.64±1.39^{b}
	3.2	79.49±0.40	14.45±0.25	85.28±0.81	67.87±0.24^{c}	37.14±0.08^{ab}	45.40±0.52^{ab}
12%	0.6	82.13±0.16^{A}	14.75±0.34^{AB}	86.63±1.00	78.10±1.75	35.42±1.47^{B}	45.70±1.38^{B}
	1.2	81.38±0.23^{AB}	14.35±0.23^{B}	86.17±0.52	74.00±0.26	36.77±0.54^{AB}	44.05±0.88^{B}
	2.3	81.42±0.29^{AB}	15.65±0.03^{A}	84.46±0.62	77.20±1.22	41.18±1.58^{A}	51.22±1.98^{A}
	3.2	80.65±0.21^{B}	14.28±0.19^{B}	85.96±0.31	76.70±0.81	40.05±0.29^{AB}	48.77±1.30^{A}

Data are presented as means ± SEM (n = 3). Values in the same column with different superscripts are significantly different ($P < 0.05$).

Table 5

Fatty acid compositions of muscle of mud crab fed the different experimental diets (mg g^{-1}, dry matter).

Lipid levels	DHA/EPA ratio	∑SFA [a]	∑MUFA [b]	∑n-6 PUFA [c]	∑n-3 PUFA [d]	EPA [e]	DHA [f]	DHA/EPA	∑TFA [g]
7%	0.6	4.87 ± 0.05^{b}	2.13 ± 0.02^{c}	3.30 ± 0.06^{c}	7.96 ± 0.02^{c}	4.12 ± 0.03^{a}	3.53 ± 0.01^{c}	0.86 ± 0.01^{b}	18.26 ± 0.02^{c}
	1.2	5.45 ± 0.01^{a}	2.68 ± 0.06^{a}	4.15 ± 0.02^{a}	8.29 ± 0.03^{ab}	4.06 ± 0.02^{a}	3.92 ± 0.02^{b}	0.96 ± 0.01^{b}	20.57 ± 0.07^{a}
	2.3	5.38 ± 0.06^{a}	2.81 ± 0.01^{a}	4.02 ± 0.06^{a}	8.64 ± 0.18^{a}	3.69 ± 0.12^{b}	4.66 ± 0.09^{a}	1.27 ± 0.04^{a}	20.86 ± 0.19^{a}
	3.2	4.96 ± 0.01^{c}	2.44 ± 0.06^{b}	3.58 ± 0.04^{b}	8.44 ± 0.01^{b}	3.64 ± 0.05^{b}	4.55 ± 0.04^{a}	1.25 ± 0.03^{a}	19.43 ± 0.09^{b}
12%	0.6	4.66 ± 0.02^{B}	2.06 ± 0.01^{B}	3.2 ± 0.01^{C}	6.56 ± 0.02^{B}	3.56 ± 0.03^{A}	2.72 ± 0.01^{C}	0.76 ± 0.01^{D}	16.48 ± 0.03^{B}
	1.2	5.27 ± 0.09^{A}	2.31 ± 0.04^{A}	3.46 ± 0.06^{AB}	6.72 ± 0.02^{B}	3.33 ± 0.03^{B}	3.10 ± 0.00^{B}	0.93 ± 0.01^{C}	17.76 ± 0.16^{A}
	2.3	5.00 ± 0.04^{A}	2.18 ± 0.03^{AB}	3.56 ± 0.03^{A}	7.08 ± 0.04^{A}	3.27 ± 0.03^{B}	3.55 ± 0.00^{A}	1.08 ± 0.01^{B}	17.82 ± 0.1^{A}
	3.2	5.11 ± 0.08^{A}	2.27 ± 0.05^{A}	$3.38\pm0.03b^{B}$	6.57 ± 0.08^{B}	2.85 ± 0.02^{C}	3.45 ± 0.05^{A}	1.21 ± 0.01^{A}	17.33 ± 0.24^{A}

Data are presented as means ± SEM (n = 3). Values in the same column with different superscripts are significantly different ($P < 0.05$). [a] SFA, saturated fatty acids: 14:0, 16:0, 18:0, 20:0; [b] MUFA, monounsaturated fatty acids: 16:1n-7, 18:1n-9, 20:1n-9; [c] n-6 PUFA, n-6 polyunsaturated fatty acids: 18:2n-6, 20:2n-6, 20:4n-6, 22:4n-6; [d] n-3 PUFA, n-3 polyunsaturated fatty acids: 18:3n-3, EPA, 22:5n-3, DHA. [e] EPA, 20:5n-3; [f] DHA, 22:6n-3; [g] TFA, total fatty acid.

Table 6

Fatty acid compositions of hepatopancreas of mud crab fed the different experimental diets (mg g^{-1}, dry matter).

Lipid levels	DHA/EPA ratio	∑SFA [a]	∑MUFA [b]	∑n-6 PUFA [c]	∑n-3 PUFA [d]	EPA [e]	DHA [f]	DHA/EPA	∑TFA [g]
7%	0.6	32.48±0.12^{b}	21.15±0.15^{c}	32.52±0.47^{b}	42.00±1.03^{b}	15.28±0.34^{a}	19.04±0.29^{b}	1.25±0.01^{d}	128.15±4.01^{c}
	1.2	39.60±0.88^{a}	24.86±0.48^{b}	38.26±1.62^{ab}	40.33±1.74^{b}	13.84±0.72^{ab}	20.96±0.59^{b}	1.52±0.04^{c}	143.05±2.17^{b}
	2.3	39.06±0.69^{a}	29.02±0.75^{a}	39.16±0.57^{a}	56.67±1.26^{a}	14.25±0.33^{ab}	36.08±0.82^{a}	2.53±0.00^{b}	163.92±3.27^{a}
	3.2	36.35±0.92^{a}	27.97±0.87^{a}	36.86±1.85^{ab}	52.95±1.09^{a}	12.18±0.38^{b}	35.36±0.57^{a}	2.91±0.06^{a}	154.13±4.65^{ab}
12%	0.6	41.41±1.01^{A}	21.52±0.50^{A}	29.66±0.58^{A}	31.74±0.36	13.46±0.30^{A}	12.76±0.25^{C}	0.95±0.03^{D}	124.33±2.14^{A}
	1.2	39.50±0.55^{A}	20.14±0.04^{AB}	29.14±0.18^{A}	32.09±0.33	10.72±0.05^{B}	16.66±0.36^{B}	1.55±0.04^{C}	120.86±0.96^{AB}
	2.3	38.86±0.58^{AB}	17.91±0.59^{C}	26.18±0.03^{B}	31.76±0.77	8.14±0.08^{C}	19.96±0.56^{A}	2.45±0.04^{B}	114.71±1.26^{BC}
	3.2	36.28±0.04^{B}	19.25±0.09^{BC}	25.60±0.37^{B}	31.51±0.36	6.72±0.03^{D}	21.51±0.29^{A}	3.20±0.05^{A}	112.64±0.63^{C}

Data are presented as means ± SEM (n = 3). Values in the same column with different superscripts are significantly different ($P < 0.05$). [a] SFA, saturated fatty acids: 14:0, 16:0, 18:0, 20:0; [b] MUFA, monounsaturated fatty acids: 16:1n-7, 18:1n-9, 20:1n-9, 22:1n-11; [c] n-6 PUFA, n-6 polyunsaturated fatty acids: 18:2n-6, 18:3n-6, 20:2n-6, 20:4n-6, 22:4n-6; [d] n-3 PUFA, n-3 polyunsaturated fatty acids: 18:3n-3, 18:4n-3, 20:4n-3, EPA, 22:5n-3, DHA. [e] EPA, 20:5n-3; [f] DHA, 22:6n-3; [g] TFA, total fatty acid.

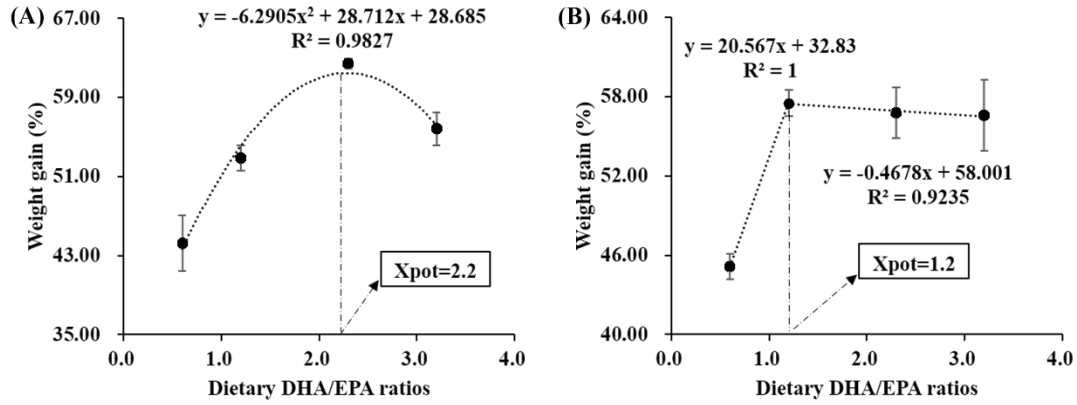
Table 7

Hematological indices of mud crab fed the experimental diets.

Lipid levels	DHA/EPA ratio	ALP [a] ($U\ L^{-1}$)	TP [b] ($g\ L^{-1}$)	GLU [c] ($mmol\ L^{-1}$)	TAG [d] ($mmol\ L^{-1}$)	T-CHO [e] ($mmol\ L^{-1}$)	HDL-C [f] ($mmol\ L^{-1}$)	LDL-C [g] ($mmol\ L^{-1}$)
7%	0.6	157.49±28.63	$35.54±1.13^{c}$	$1.11±0.05^{b}$	$0.11±0.00^{d}$	$0.19±0.00^{b}$	0.91±0.00	$0.53±0.01^{b}$
	1.2	162.69±27.12	$43.85±0.92^{b}$	$2.04±0.08^{a}$	$0.26±0.01^{a}$	$0.34±0.00^{a}$	0.93±0.01	$0.94±0.03^{a}$
	2.3	104.37±4.15	$50.45±1.38^{a}$	$1.65±0.09^{a}$	$0.20±0.00^{b}$	$0.31±0.01^{a}$	0.92±0.01	$0.93±0.01^{a}$
	3.2	138.31±16.65	$31.79±1.11^{c}$	$1.86±0.12^{a}$	$0.16±0.01^{c}$	$0.18±0.01^{b}$	0.93±0.01	$0.88±0.01^{a}$
12%	0.6	$113.92±12.83^{C}$	$24.07±2.64^{C}$	$1.37±0.03^{C}$	0.11±0.01	0.15±0.01	$0.96±0.02^{a}$	0.87±0.01
	1.2	$67.85±2.77^{C}$	$33.69±1.06^{AB}$	$1.71±0.06^{AB}$	0.11±0.00	0.16±0.01	$0.99±0.04^{a}$	0.89±0.01
	2.3	$233.44±24.55^{B}$	$32.30±0.47^{BC}$	$1.82±0.08^{A}$	0.10±0.01	0.13±0.02	$0.93±0.01^{a}$	0.89±0.01
	3.2	$383.96±24.29^{A}$	$42.54±2.94^{A}$	$1.50±0.02^{BC}$	0.10±0.01	0.13±0.01	$0.61±0.01^{b}$	0.91±0.01

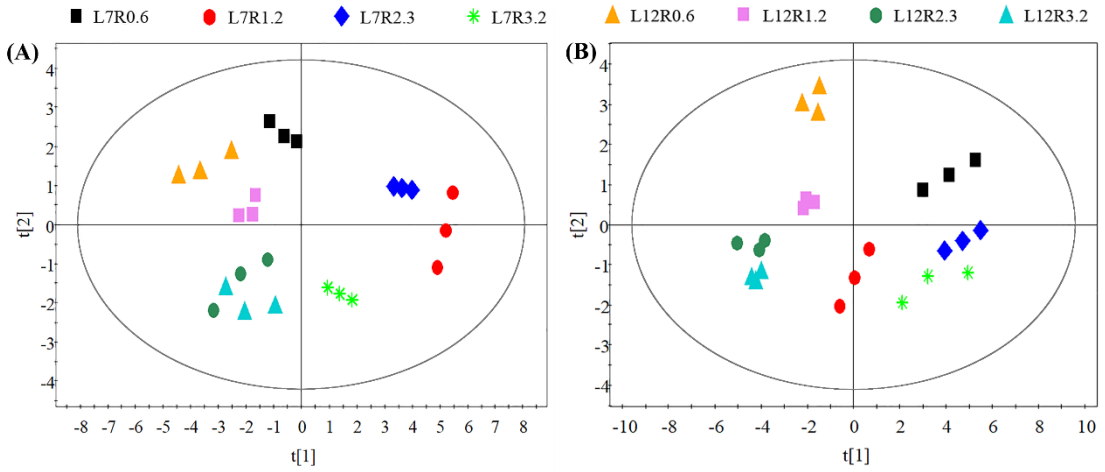
Data are presented as means ± SEM (n = 3). Values in the same column with different superscripts are significantly different ($P < 0.05$).

[a] ALP, alkaline phosphatase; [b] TP, total protein; [c] GLU, glucose; [d] TAG, triacylglycerol; [e] T-CHO, total cholesterol; [f] HDL-C, high-density lipoprotein cholesterol; [g] LDL-C, low-density lipoprotein cholesterol



760
761 **Figure 1.** The linear broken-line model and quadratic broken-line model for the relationship between
762 dietary DHA/EPA ratio and WG of juvenile mud crab fed diets with 7% (**A**) and 12% (**B**) lipid. The
763 horizontal axis represents the measured dietary DHA/EPA ratios. The Xpot represents the optimal dietary
764 DHA/EPA ratio for the maximum WG of *S. Paramamosain*.

765



766
767 **Figure 2.** Principal component analysis (PCA) score plots based on fatty acid profiles of muscle (**A**) and
768 hepatopancreas (**B**) of crab fed the different experimental diets. For example, L7R0.6: dietary lipid level
769 and DHA/EPA ratio were 7% and 0.6.

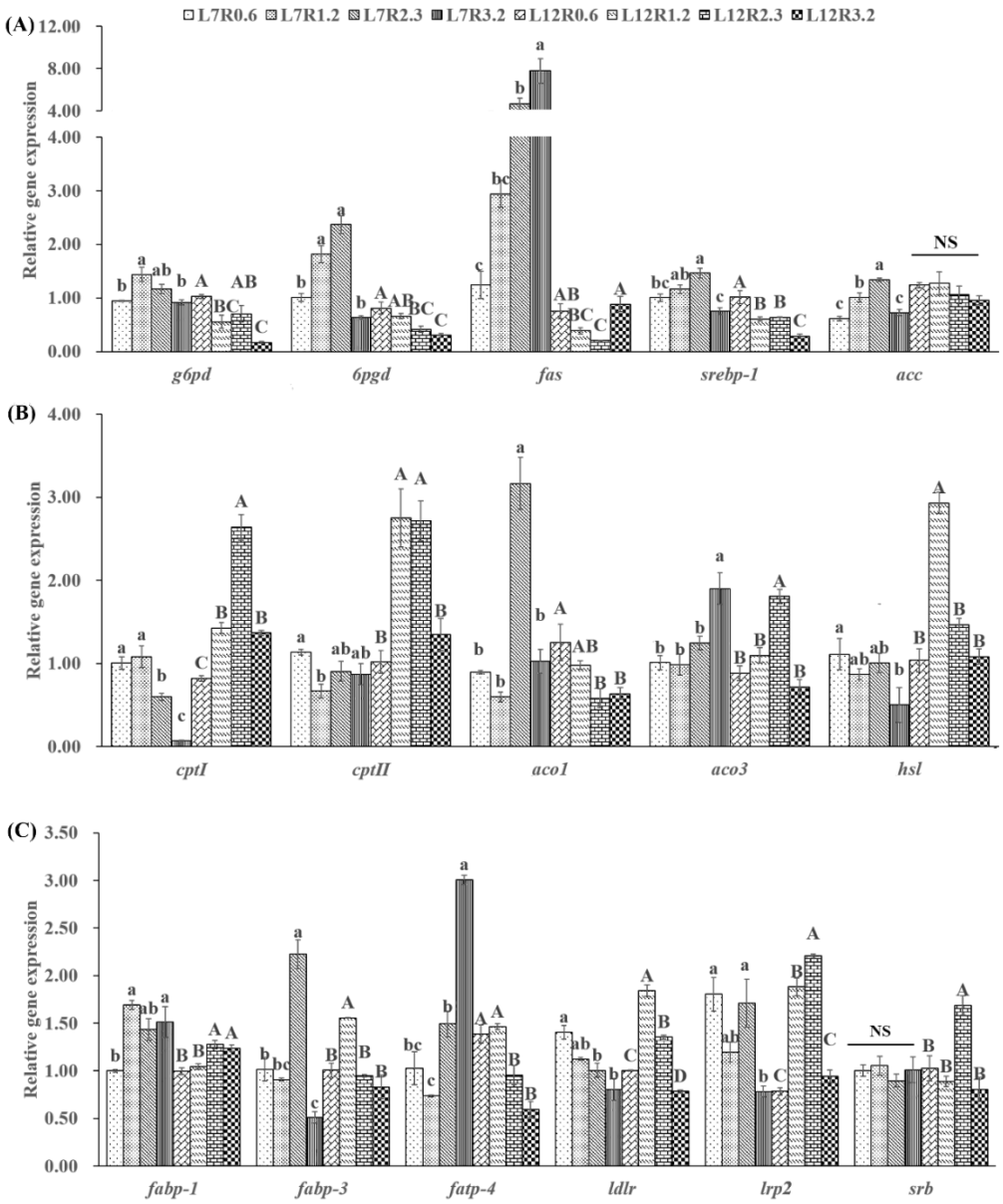
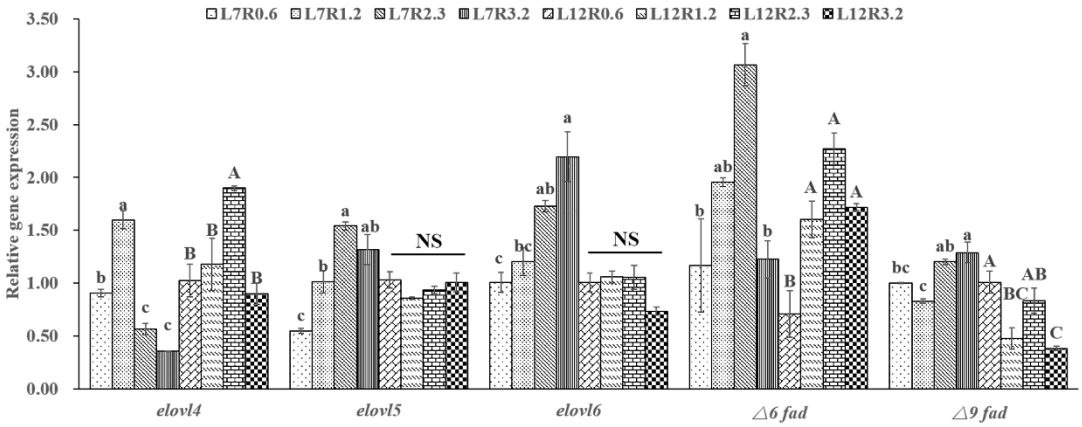


Figure 3. Effects of DHA/EPA ratio on relative mRNA expression levels of genes involved in lipid anabolism (**A**), lipid catabolism (**B**) and fatty acid and lipid transport (**C**) in the hepatopancreas of *S. Paramamosain* at 7% and 12% dietary lipid levels. Values are means ± SEM (n = 3), and bars bearing different letters are significantly different by Tukey's test ($P < 0.05$). *srebp-1*, sterol regulatory element binding protein-1; *fas*, fatty acid synthase; *acc*, acetyl-CoA carboxylase; *6pgd*, 6-phosphogluconate dehydrogenase; *g6pd,* glucose-6-phosphate dehydrogenase; *cpt*, carnitine palmitoyltransferase; *aco,* acyl-CoA oxidase; *hsl,* hormone-sensitive triglyceride lipase; *fabp*, fatty acid binding protein; *fatp*, fatty acid transport protein; *ldlr*, low-density lipoprotein receptor; *lrp*, low-density lipoprotein receptor-related protein; *srb*, scavenger receptor b; NS, no significance.



780

781 **Figure 4.** Effects of DHA/EPA ratio on relative mRNA expression levels of genes involved in fatty acid

782 biosynthesis in hepatopancreas of *S. Paramamosain* at 7% and 12% lipid levels. Values are means ± SEM

783 (n = 3), and bars bearing different letters are significantly different by Tukey's test ($P < 0.05$). *fad*, fatty

784 acyl desaturase; *elovl*, elongase of very long-chain fatty acids; NS, no significance.