

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

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

30 **Abstract**

31 An 8-week feeding trial was conducted to determine the optimal dietary docosahexaenoic
32 acid/eicosapentaenoic acid (DHA/EPA) ratio of mud crab (*Scylla paramamosain*) supplied with optimal n-3 LC-
33 PUFA at two dietary lipid levels. Eight isonitrogenous diets were formulated to contain 7% and 12% crude lipid,
34 each with DHA/EPA ratios of 0.6, 1.2, 2.3 and 3.2, respectively. Each diet was randomly assigned to triplicate
35 groups of 30 juvenile mud crabs (initial weight 20.9 ± 0.6 g) that were stocked in single crab cells. In crabs fed 7%
36 lipid, the diet with a DHA/EPA ratio of 2.3 showed significantly higher weight gain than crabs fed the other ratios
37 while in crabs fed 12% lipid, lower weight gain and specific growth rate were observed in crabs fed the diet with a
38 DHA/EPA ratio of 0.6 than crabs fed the other ratios. Lipid content in hepatopancreas significantly increased as
39 dietary DHA/EPA ratio increased from 1.2 to 2.3 in crabs fed 7% lipid, while no differences were observed among
40 crabs fed the diets with DHA/EPA ratios higher than 0.6 when fed 12% lipid. Total fatty acid and DHA contents
41 and DHA/EPA ratio showed increasing, and EPA decreasing, trends in muscle and hepatopancreas with increased
42 dietary DHA/EPA ratio, at both dietary lipid levels. The hemolymph triacylglycerol and total cholesterol contents
43 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
44 lipid, and lowest low and high-density lipoprotein cholesterol contents were observed in crabs fed DHA/EPA dietary
45 ratios of 0.6 and 3.2 at 7% and 12% lipid, respectively. The expression levels of *fas*, *aco3* and *fatp4* were
46 significantly up-regulated, and *cpt1*, *hsl* and *ldlr* were down-regulated, with increased dietary DHA/EPA ratio in
47 crabs fed 7% lipid. In crabs fed 12% lipid, the expression levels of *g6pd*, *6pgd*, *srebp-1*, *aco1* and *fatp4* were down-
48 regulated, and *fabp-1* was up-regulated, with increased dietary DHA/EPA ratio. The expression levels of *elovl4* and
49 *$\Delta 6$ fad* initially increased and then decreased as dietary DHA/EPA ratio increased from 0.6 to 3.2 in crabs fed both
50 7% and 12% lipid. Based on analysis of weight gain versus dietary DHA/EPA ratio, the optimal dietary DHA/EPA
51 ratios of mud crab *S. paramamosa* were estimated to be 2.2 and 1.2 when supplied with optimal n-3 LC-PUFA at
52 7% and 12% lipid, respectively.

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

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55 **1. Introduction**

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

63 Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are the major important n-
64 3 LC-PUFA, which are necessary for crustacean growth, molting and development. Specifically, DHA plays crucial
65 structural roles in bio-membranes, especially of neural tissues such as brain and eye, where it is a major component
66 of polar lipids (Wassall and Stillwell, 2008). Thus, it is expected that DHA requirements are high in fast growing
67 stages of development in order to satisfy the demands of rapidly forming tissues that accumulate DHA. In addition,
68 EPA has a major role as a precursor of highly bioactive regulatory compounds such as eicosanoids, and can also
69 partly satisfy DHA requirements in species that have the necessary fatty acyl elongase and desaturase activities to
70 convert EPA to DHA (Castro et al., 2016). It was reported that DHA and EPA in biomembrane phospholipids of
71 marine fish must be present in an appropriate ratio, and an imbalance resulted in reduced survival and stress
72 resistance capability (Copeman et al., 2002). Previous studies in fish also reported that overall n-3 LC-PUFA
73 requirement decreased with increased dietary DHA/EPA ratio in gilthead sea bream (*Sparus aurata* L.) (Rodriguez
74 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
75 DHA/EPA ratio of 1.0 for juvenile gilthead sea bream (Kalogeropoulos, et al., 1992). When dietary inclusion level
76 of n-3 LC-PUFA was increased to 1.9% of dry weight, juvenile gilthead sea bream required a higher dietary content
77 of EPA (1.0%) than DHA (0.5%) for maximum growth (Ibeas, et al., 1997). Therefore, the optimal dietary n-3 LC-
78 PUFA content and DHA/EPA ratio could be affected by each other and, therefore, the quantitative requirements for
79 n-3 LC-PUFA was reported to vary with stage of development, dietary lipid content, and the ratio of dietary LC-
80 PUFA (DHA/EPA) (NRC, 2011). Thus, it is clearly important to determine the appropriate dietary DHA/EPA ratio
81 in combination with dietary lipid and n-3 LC-PUFA levels in feed.

82 The mud crab, *Scylla paramamosain*, is distributed widely throughout the coasts of China, Vietnam, Japan and
83 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*.
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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 ($20.92 \pm 0.56\text{g crab}^{-1}$) were randomly allocated into 240 single crab cells ($0.33\text{m} \times 0.23\text{m} \times 0.15\text{m}$, length \times width
127 \times 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

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

147 *2.5. Biochemical analysis*

148 *2.5.1. Proximate composition and fatty acids*

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

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

162 *2.5.2. Haematological characteristics*

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

167 *2.5.3. Real-time quantitative PCR (RT-qPCR) analysis of fatty acid biosynthesis and lipid metabolism genes in* 168 *hepatopancreas*

169 Total RNA was extracted from hepatopancreas samples using Trizol reagent (Invitrogen, USA), and the
170 quantity and quality of total RNA assessed using a Nano DropND-1000 spectrophotometer (NanoDrop
171 Technologies, USA) and 1.2% denaturing agarose gel electrophoresis. The 260/280nm absorbance ratios of all
172 samples ranged from 1.86 to 2.00, indicating a satisfactory purity of the RNA samples. The RNA was dissolved in
173 30µL Recombinant DNase I (RNase-free) (Takara, Japan) and stored at -80°C until use. The cDNA was synthesized
174 for quantitative reverse-transcriptase polymerase chain reaction (qPCR) using the PrimeScript™ RT Reagent Kit
175 (Takara, Japan) according to the manufacturer's instructions.

176 Elongation factor-1α (*ef-1α*) was used as a house-keeping gene after the stability of its expression was
177 confirmed. Specific primers for elongase of very long-chain fatty acids 4, 5 and 6 (*elovl4*, *elovl5* and *elovl6*), delta-
178 6 and delta-9 fatty acyl desaturase (*Δ6 fad* and *Δ9 fad*), fatty acid synthase (*fas*), acetyl-CoA carboxylase (*acc*),
179 glucose-6-phosphate dehydrogenase (*g6pd*), 6-phosphogluconate dehydrogenase (*6pgd*), sterol regulatory element
180 binding protein-1 (*srebp-1*), hormone-sensitive triglyceride lipase (*hsl*), carnitine palmitoyltransferase I and II (*cptI*
181 and *cptII*), acyl-CoA oxidase 1 and 3 (*aco1* and *aco3*), fatty acid-binding protein 1 and 3 (*fabp-1* and *fabp-3*), fatty
182 acid transport protein 4 (*fatp-4*), low-density lipoprotein receptor (*ldlr*), low-density lipoprotein receptor-related
183 protein 2 (*lrp2*) and scavenger receptor b (*srb*) used for RT-qPCR were designed using Primer Premier 5.0 software
184 (Supplementary Table 1). The expression of mRNA was determined by RT-qPCR (Light Cycler 96; Roche,
185 Switzerland). The RT-qPCR was performed in a 20µL reaction volume containing 10µL of SYBR Green premix,
186 0.8µL of cDNA template, 0.4µL of each primer (10µM) and 8.4µL of diethyl pyrocarbonate-treated water. The RT-
187 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
188 data were optimized using the comparative Ct ($2^{-\Delta\Delta Ct}$) value method as described by Livak and Schmittgen (2001)
189 and then subjected to statistical analysis.

190 2.6. Calculations and statistical analysis

191 The parameters were calculated as follows:

$$192 \text{ Weight gain (WG, \%)} = 100 \times (W_t - W_i) / W_i$$

$$193 \text{ Specific growth rate (SGR, \% d}^{-1}\text{)} = 100 \times (\ln W_t - \ln W_i) / t,$$

$$194 \text{ Molting frequency (MF)} = 2 \times N_m / (\text{initial number of crabs} + \text{final number of crabs})$$

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

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

203

204 3. Results

205 3.1. Growth performance

206 The growth performance of crabs fed the different experimental diets is shown in Table 3. WG and SGR were
207 significantly impacted by dietary DHA/EPA ratio at both 7% and 12% dietary lipid levels. Crabs fed the diets with
208 a DHA/EPA of 0.6 at both 7% or 12% lipid had significantly lower WG than those fed the diets with higher
209 DHA/EPA ratios, but there were no differences in WG and SGR between crabs fed diets with DHA/EPA ratios of
210 1.2, 2.3 and 3.2 at 12% lipid. Two-slope broken-line and second-order polynomial regression analysis of WG
211 against dietary DHA/EPA ratio showed that the optimal ratios were 2.2 and 1.2 in crabs fed dietary lipid at 7% and
212 12%, respectively (Figure 1). MF was not significantly influenced by dietary DHA/EPA ratios at either 7% or 12%
213 lipid levels.

214 Insert Table 3 here.

215 Insert Figure 1 here.

216 3.2. Proximate compositions of muscle and hepatopancreas

217 As shown in Table 4, moisture content in muscle decreased with increased dietary DHA/EPA ratio, and
218 significantly higher lipid content was observed in crabs fed the diet with a DHA/EPA ratio of 2.3 than those fed
219 diets with DHA/EPA ratios of 1.2 and 3.2 at 12% lipid. Lipid content in hepatopancreas increased as dietary
220 DHA/EPA ratio increased from 0.6 to 2.3, but a marginal decreasing trend was found when dietary DHA/EPA ratio
221 was higher than 2.3 at both 7% and 12% lipid levels. The moisture contents of hepatopancreas decreased as dietary
222 DHA/EPA ratio increased from 0.6 to 3.2 in crabs fed 7% lipid. Hepatopancreas of crabs fed the diet with a
223 DHA/EPA ratio of 0.6 had significantly higher protein content than those fed the diet with a DHA/EPA ratio of 2.3
224 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
225 fed diets with DHA/EPA ratios of 0.6 and 1.2 at 12% lipid.

226

Insert Table 4 here.

227 *3.3. Fatty acid profiles of muscle and hepatopancreas*

228 Principal component analysis (PCA) score plot based on the first component was used to present the fatty acid
229 compositions of muscle (Fig. 2A) and hepatopancreas (Fig. 2B) in crabs fed the different diets. The further the
230 components were separated, the greater the difference. Crabs fed the diets with DHA/EPA ratios of 2.3 and 3.2 at
231 12% dietary lipid showed similar muscle fatty acid profiles as their components overlapped in Fig. 2A, while the
232 components of crabs in other treatments were clustered and separated from others. No overlap was observed in Fig.
233 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,
234 and to crabs fed the diets with these DHA/EPA ratios at 12% dietary lipid. Fig. 2B showed that hepatopancreas fatty
235 acid profiles were affected by different dietary DHA/EPA ratios, but crabs fed the diets with DHA/EPA ratios of
236 2.3 and 3.2 showed similar hepatopancreas fatty acid profiles at 7% and 12% lipid. Complete fatty acid compositions
237 of muscle and hepatopancreas are provided in Supplementary Tables 2 and 3.

238 The saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), n-6 polyunsaturated fatty acid (PUFA),
239 n-3 PUFA and total fatty acid (TFA) contents of crab muscle were all significantly influenced by dietary DHA/EPA
240 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
241 and 2.3 showed significantly higher TFA, SFA, MUFA and n-6 PUFA contents than those fed other diets. The
242 lowest n-3 PUFA content was observed in crabs fed diet with a DHA/EPA ratio of 0.6, and the EPA content
243 significantly decreased when dietary DHA/EPA ratio increased from 1.2 to 2.3, while no differences were found in
244 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
245 12% lipid level, crabs fed the diet with a DHA/EPA of 0.6 had significantly lower TFA, SFA, and n-6 PUFA
246 contents than those fed the diets with higher DHA/EPA ratios, but there were no differences in TFA and SFA
247 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
248 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-
249 3 PUFA content was observed in crabs fed the diet with a DHA/EPA ratio of 2.3, and there no differences between
250 crabs fed the other diets. Muscle DHA/EPA ratio significantly increased with increased dietary DHA/EPA ratios,
251 while EPA content showed the opposite trend. The DHA content increased as dietary DHA/EPA ratio increased
252 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
253 12% lipid level.

254 In crabs fed 7% dietary lipid, the hepatopancreas MUFA, n-6 PUFA and TFA contents showed at first an
255 increase and then a marginal decreasing trend as dietary DHA/EPA ratio increased from 0.6 to 3.2, with highest
256 values observed when dietary DHA/EPA ratio was 2.3 (Table 6). The n-3 PUFA and DHA contents increased
257 significantly when dietary DHA/EPA ratio increased from 1.2 to 2.3, while no differences were found between
258 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
259 a DHA/EPA ratio of 0.6 was significantly lower than those fed the other diets, but there were no differences in SFA
260 between crabs fed diets with DHA/EPA ratios of 1.2, 2.3 and 3.2. The EPA content showed a negative correlation
261 with dietary DHA/EPA ratio. At 12% lipid level, the n-3 PUFA content was not affected by dietary DHA/EPA ratio
262 but the EPA content decreased significantly with increased dietary DHA/EPA ratio, and similar trends were
263 observed in SFA, MUFA and n-6 PUFA contents. The DHA content increased as dietary DHA/EPA ratio increased
264 from 0.6 to 2.3, but no differences were found when dietary DHA/EPA ratio was higher than 2.3. The DHA/EPA
265 ratio in hepatopancreas show a significantly positive correlation with dietary DHA/EPA ratio at both 7% and 12%
266 dietary lipid levels.

267 Insert Figure 2 here.

268 Insert Table 5 here.

269 Insert Table 6 here.

270 *3.4. Hematological enzyme activities and characteristics*

271 In crabs fed 7% dietary lipid, the TP, TAG and T-CHO contents first increased and then decreased with
272 increasing dietary DHA/EPA ratio, with highest values observed in crabs fed DHA/EPA ratios of 2.3 and/or 3.2.
273 The lowest GLU and LDL-C levels were observed in crabs fed the diet with a DHA/EPA ratio of 0.6, but there
274 were no differences among crabs fed the other ratios (Table 7). In crabs fed 12% dietary lipid, the lowest HDL-C
275 content was found in crabs fed the diet with a DHA/EPA ratio of 3.2, and there were no differences among crabs
276 fed the other ratios. Increasing trends with increasing dietary DHA/EPA ratio were observed in the ALP and TP
277 contents, but the GLU content showed at first an increase and then a decreasing trend as dietary DHA/EPA ratio
278 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
279 fed the DHA/EPA ratio of 3.2.

280 Insert Table 7 here.

281 *3.5. Expression of genes related to lipid metabolism in hepatopancreas*

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

289 With regards to genes related to β -oxidation, in crabs fed 7% dietary lipid, the expression level of *cptI* decreased
290 with increased dietary DHA/EPA ratio, and *cptIII* expression was significantly higher in crabs fed the diet with a
291 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
292 observed in crabs fed the diets with DHA/EPA ratios of 2.3 and 3.2, respectively. In crabs fed 12% dietary lipid, the
293 expression levels of *cptI*, *cptIII* and *aco3* showed similar trends with increasing dietary DHA/EPA ratio, with highest
294 expression levels observed in crabs fed the diets with DHA/EPA ratios of 1.2 and/or 2.3, and the expression level
295 of *aco1* showed an increasing trend with increasing dietary DHA/EPA ratio.

296 The expression level of *fatp1* in hepatopancreas was higher when dietary DHA/EPA ratio was higher than
297 0.6/1.2 at 7% or 12% lipid. Highest *fabp3* expression levels were observed in crabs fed diets with DHA/EPA ratios
298 of 2.3 and 1.2 at 7% and 12% lipid, respectively. The expression level of *fabp4* showed an increasing trend at 7%
299 lipid, but decreased as dietary DHA/EPA ratio increasing from 1.2 to 3.2 at 12% lipid. In crabs fed 7% lipid, the
300 expression level of *ldlr* significantly decreased with increased dietary DHA/EPA ratio, and expression of *lrp2*
301 significantly decreased when dietary DHA/EPA ratio increased from 2.3 to 3.2, while the expression level of *srb*
302 was not affected by dietary DHA/EPA ratio. In crabs fed 12% dietary lipid, *ldlr*, *lrp2* and *srb* expression levels
303 showed similar trends with increased dietary DHA/EPA ratio, with highest expression levels observed when dietary
304 DHA/EPA ratios were 1.2 and/or 2.3.

305 Insert Figure 3 here.

306 3.6. Expression of genes involved in LC-PUFA biosynthesis in hepatopancreas

307 In crabs fed 7% dietary lipid, the expression level of $\Delta 6$ *fad* increased and then decreased as dietary DHA/EPA
308 ratio increased from 0.6 to 2.3 and from 2.3 to 3.2 (Figure 4), while the expression level of $\Delta 9$ *fad* showed an
309 increasing trend as dietary DHA/EPA ratio increased from 1.2 to 3.2. In crabs fed 12% lipid, the expression level
310 of $\Delta 6$ *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 fat* 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

340 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
341 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
342 confirmed that dietary lipid level significantly affected both the optimum dietary n-3 LC-PUFA level and the
343 optimum DHA/EPA ratio of juvenile mud crab. Similar studies have been reported in other species. The n-3 LC-
344 PUFA requirements of juvenile gilthead bream (*S. aurata*) were estimated to be 0.9% of diet when the DHA/EPA
345 ratio was 1.0 at a dietary lipid level of 13% (Kalogeropoulos et al., 1992), whereas it was 1.9% when the DHA/EPA
346 ratio was 0.5 and dietary lipid level was 8% (Ibeas et al., 1994). Another study showed that n-3 LC-PUFA
347 requirement was about 3% when sea bream fed a diet with a DHA/EPA ratio of 1.0 and 22% dietary lipid (Houston
348 et al., 2017).

349 In the present study, the proximate composition of muscle was not affected by dietary DHA/EPA ratio, but
350 lipid content in hepatopancreas increased with increased dietary DHA/EPA ratio in crabs fed 7% dietary lipid,
351 similar to results observed in *E. sinensis* and *P. trituberculatus*. Hepatopancreas is an important tissue for the
352 deposition of lipid and energy storage in crustaceans (Cavalli et al., 2000; Johnston et al., 2003). It was notable that
353 the hepatopancreas lipid content in crabs fed diets with DHA/EPA ratios of 0.6 - 1.2 at 7% dietary lipid ranged from
354 28.4% to 28.8%, significantly lower than those fed the diets with higher ratios. Meanwhile, hepatopancreas protein
355 content decreased as dietary DHA/EPA ratio increased from 0.6 to 2.3 in crabs fed 7% dietary lipid. This may be
356 due to protein (as well as lipid) in the hepatopancreas being used to supply energy, when dietary lipid level was
357 lower than the optimum level (9.5%) (Zhao et al., 2015). No significant difference was found in hepatopancreas
358 lipid contents among crabs fed diets with 7% lipid and DHA/EPA ratios higher than 1.2. In addition, muscle lipid
359 content and hepatopancreas lipid and protein contents initially increased and then decreased as dietary DHA/EPA
360 ratio increased in crabs fed 12% lipid. Based on these results, we speculate that dietary DHA/EPA ratio could
361 improve energy storage while preventing excess lipid deposition in hepatopancreas and, thus, play an important role
362 in lipid metabolism, which was supported by data on the expression of genes related to lipid anabolism and
363 catabolism. Therefore, energy and protein metabolism may also be affected by dietary DHA/EPA ratio in mud crab,
364 and so this requires further study.

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

369 irrespective of dietary lipid level. The DHA/EPA ratios in hepatopancreas were similar to those of the diets and
370 higher than those of muscle, which indicated that LC-PUFA may be preferentially deposited in hepatopancreas
371 rather than muscle in mud crab. These results also suggested a selective retention of DHA over EPA or other fatty
372 acids in mud crab *S. paramamosain* underpinning its greater biological value as EFA, as reported in other marine
373 species (Carvalho et al., 2018; Izquierdo, 1996). Based on the higher DHA/EPA ratio in hepatopancreas, we
374 speculated that *S. paramamosain* may also synthesize DHA from EPA or shorter chain PUFA, albeit the capacity
375 may be low. A recent study indicated that *Litopenaeus vannamei* had the potential ability to convert linolenic acid
376 to EPA and DHA (Chen et al., 2014a; b), which supports the speculation in the present study. The SFA, MUFA, n-
377 6 PUFA, n-3 PUFA and total fatty acid contents increased and then decreased or marginally decreased in muscle as
378 dietary DHA/EPA ratio increased at both dietary lipid levels, while hepatopancreas showed a similar trend at 7%
379 lipid but opposite at 12% lipid, which may indicate differences in deposition and utilization of fatty acids in the
380 different tissues (Izquierdo et al., 2003). It should be noted that these data reflect differences in the lipid contents of
381 the tissues as the fatty acid compositions were presented in absolute quantitative terms in the present study.

382 Previously, Elov14, Elov15 and $\Delta 6$ Fad were reported to be key enzymes in the LC-PUFA biosynthesis pathway
383 (Zhang et al., 2019a). Elov15 elongates 18:4n-3 and 18:3n-6 to 20:5n-3 and 20:4n-6, respectively (Zuo et al., 2012)
384 and Elov14 could effectively elongate C₂₂ PUFA to C₂₄ PUFA and have the potential to participate in the production
385 of DHA (Li et al., 2017a, b). The $\Delta 6$ Fad is the first enzyme involved in the bioconversion of C₁₈ PUFA to longer
386 and more unsaturated fatty acids and is involved in the synthesis of DHA from EPA via the “Sprecher pathway”
387 (Monroig et al., 2011). Additionally, it is known that DHA biosynthesis through the “Sprecher pathway” is also
388 catalysed by ACO in peroxisomes (Sprecher, 2000). In the present study, the expression levels of *elov14* and *$\Delta 6$ fad*
389 showed similar trends with increased DHA/EPA ratio at both 7% and 12% lipid levels, initially increasing and then
390 decreasing, and a similar result was also observed in the expression level of *elov15* in crabs fed 7% lipid, which was
391 consistent with the expression levels of *aco3* and *srebp-1*, and the contents of DHA in hepatopancreas and muscle.
392 These data were further evidence suggesting that mud crab require high DHA to maintain basic functions, and some
393 capacity for the *in vivo* synthesis of DHA from EPA via “Sprecher pathway”. An increase in the expression of
394 *elov14-like*, *elov15-like* and *$\Delta 6$ fad* were also observed in liver and brain of juvenile golden pompano (*Trachinotus*
395 *ovatus*) fed a diet with a higher DHA/EPA ratio (Zhang et al., 2019a). Additionally, the underlying regulatory
396 mechanisms demonstrated that the transcription levels of Elov14, Elov15 and $\Delta 6$ Fad were positively mediated by
397 *lxra* directly or indirectly through the regulation of *srebp-1* transcription (Chen et al., 2019; Dong et al., 2017c; Li

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

401 *Acc* is a cytosolic enzyme producing alanyl-CoA, the first step in the biosynthesis of long-chain fatty acids
402 (Yu et al., 2015). *6Gpd* and *G6pd* are key enzymes related to the production of NADPH (Chen et al., 2013; Zheng
403 et al., 2013), essential for *de novo* fatty acid biosynthesis catalyzed by *Fas* (Chen et al., 2013; Zheng et al., 2013),
404 while *Hsl* is involved in lipolysis (Ma et al., 2013). Additionally, *Srebp-1* is a transcription factor regulating fatty
405 acid, lipid and cholesterol biosynthesis pathways (Minghetti et al., 2011; Zheng et al., 2013). Previous studies have
406 reported that dietary fatty acid profile could affect gene expression or activity of these enzymes involved in the
407 mechanisms of lipogenesis and lipolysis (Jin et al., 2017; Kim et al., 1999; Morais et al., 2011, 2012; Panserat et al.,
408 2008; Peng et al., 2014). In the present study in crabs fed 7% dietary lipid, the expression level of *fas* significantly
409 increased with increased dietary DHA/EPA ratio, while the expression level of *hsl* was decreased. The expression
410 levels of *6gpd*, *g6pd* and *srebp-1* showed similar trends to each other, initially increased and then decreased as
411 dietary DHA/EPA ratio increased. These results showed that, at 7% dietary lipid, increasing dietary DHA/EPA ratio
412 improved lipogenesis and inhibited lipolysis in mud crab, and that hepatopancreas of mud crab may require a certain
413 level of lipid to maintain energy supply energy and basic functions. At 12% dietary lipid, while the expression of
414 *acc* was not affected by dietary DHA/EPA ratio, the expression levels of *6gpd*, *g6pd* and *srebp-1* decreased with
415 increased dietary DHA/EPA ratio, whereas lowest expression levels of *fas* were observed in crabs fed the diets with
416 dietary DHA/EPA ratios of 2.3, and the highest expression levels of *hsl* were found in crabs fed diets with
417 DHA/EPA ratios of 1.2. These results indicated that dietary DHA/EPA ratio played an important role in the
418 inhibition of lipogenesis in mud crabs fed high-lipid diets, which may prevent excess lipid deposition in
419 hepatopancreas.

420 It was demonstrated that β -oxidation in the mitochondrial matrix and peroxisome are main pathways of fatty
421 acid catabolism (Lu et al., 2014). *Cpt I* catalyzes the conversion of fatty acid-CoAs to fatty acid-carnitines for
422 entering the mitochondrial matrix, with the fatty acyl group transferred back to CoA by *Cpt II* (Kerner and Hoppel,
423 2000; Li et al., 2019), while *Aco* is the rate-limiting enzyme for fatty acid β -oxidation in peroxisomes (Lu et al.,
424 2014). In the present study, the expression level of *cptI* decreased with increased dietary DHA/EPA ratio at 7%
425 dietary lipid, which suggested reduced long-chain fatty acid transport into the mitochondrial matrix, leading to
426 reduced β -oxidation and increased lipid deposition. At 7% dietary lipid, the highest expression of *acoI* was observed

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

435 FATP promote the transport of long-chain fatty acids and are expressed in tissues with active fatty acid
436 metabolism (Jeppesen et al., 2012; Nickerson et al., 2009). In mice, the transport rates of LC-PUFA varied among
437 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
438 with different specificities and play important roles in the uptake, transport and metabolic regulation of long-chain
439 fatty acids in organelles within cell (Storch and Thumser, 2000). For example, FABP-1 has a close relationship with
440 the transport and uptake of LC-PUFA in general (Mcarthur et al., 1999), while FABP-3 has high affinity to
441 especially EPA (Tan et al., 2015). In the present study, the expression level of *fabp-1* was up-regulated by increased
442 dietary DHA/EPA ratio irrespective of dietary lipid level, while the highest expression levels of *fabp-3* were
443 observed in crabs fed diets with DHA/EPA ratios of 1.2 and 2.3 at 7% and 12% dietary lipid, respectively. The
444 expression of *fabp-3* showed a positive relationship to hepatopancreas LC-PUFA content, which agreed with a
445 previous study (Tan et al., 2015). It was reported that FABP can transport fatty acids for not only lipogenesis but
446 also β -oxidation (Ockner et al., 1972). Therefore, the expression levels of *fabp* in the present study suggested an
447 activation of fatty acid metabolism with increasing dietary DHA/EPA ratio. In contrast, the expression levels of
448 *fatp-4* were up-regulated by dietary DHA/EPA ratios at 7% lipid, but down-regulated at 12% lipid, which reflected
449 a similar trend with hepatopancreas total fatty acid content and supported our speculation on the impact of dietary
450 DHA/EPA ratio on hepatopancreas lipid content.

451 Lipids in blood are transported to peripheral tissues by lipoproteins (Weil et al., 2013), where LDLR and LRP2
452 can identify and promote clearance of lipoproteins (Magkos, 2009). Studies in human reported that LRP6 and LDLR
453 could promote the dissolution of LDL-C in lysosomes, thereby reducing LDL-C levels in the blood (Voros et al.,
454 1996) while SRBI is an HDL receptor that participates in the reverse transport of cholesterol (Viñals et al., 2003).
455 In the present study, the expression levels of *ldlr* and *lrp2* were down-regulated by increased dietary DHA/EPA

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

468

469 **5. Conclusion**

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

476

477 **Author contribution**

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

485 **Declaration of competing interest**

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

488

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496

497 **Supplementary data**

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

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

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

502

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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 ₂ PO 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)

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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

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^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.

739 **Table 4**

740 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

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

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743 **Table 5**

744 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±0.03 ^{bB}	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

745 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,

746 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

747 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.

748

749 **Table 6**750 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

751 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, 16:0,752 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-753 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.

754

755 **Table 7**

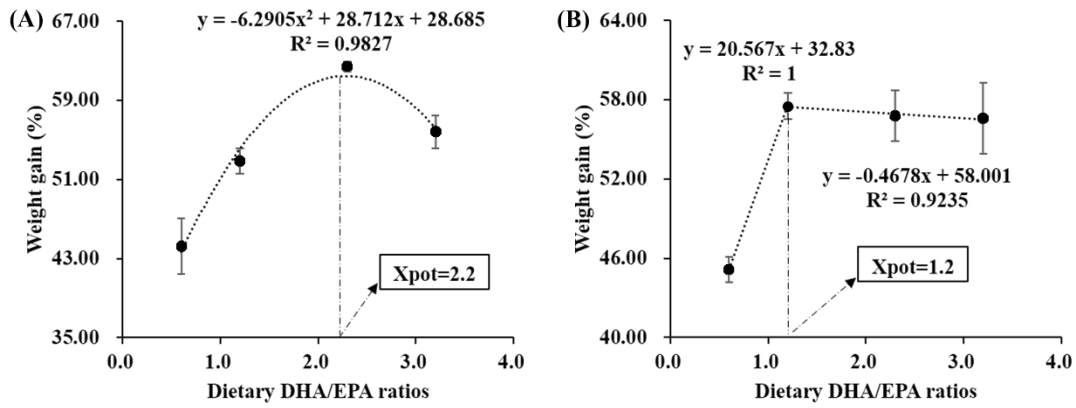
756 Hematological indices of mud crab fed the experimental diets.

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

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

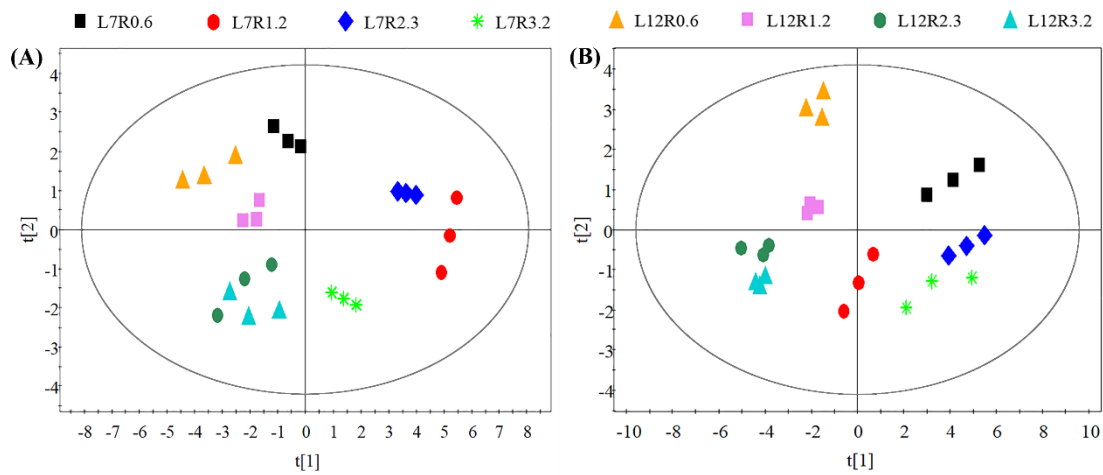
758 ^a ALP, alkaline phosphatase; ^b TP, total protein; ^c GLU, glucose; ^d TAG, triacylglycerol; ^e T-CHO, total cholesterol; ^f HDL-C, high-density lipoprotein cholesterol; ^g LDL-C, low-

759 density lipoprotein cholesterol

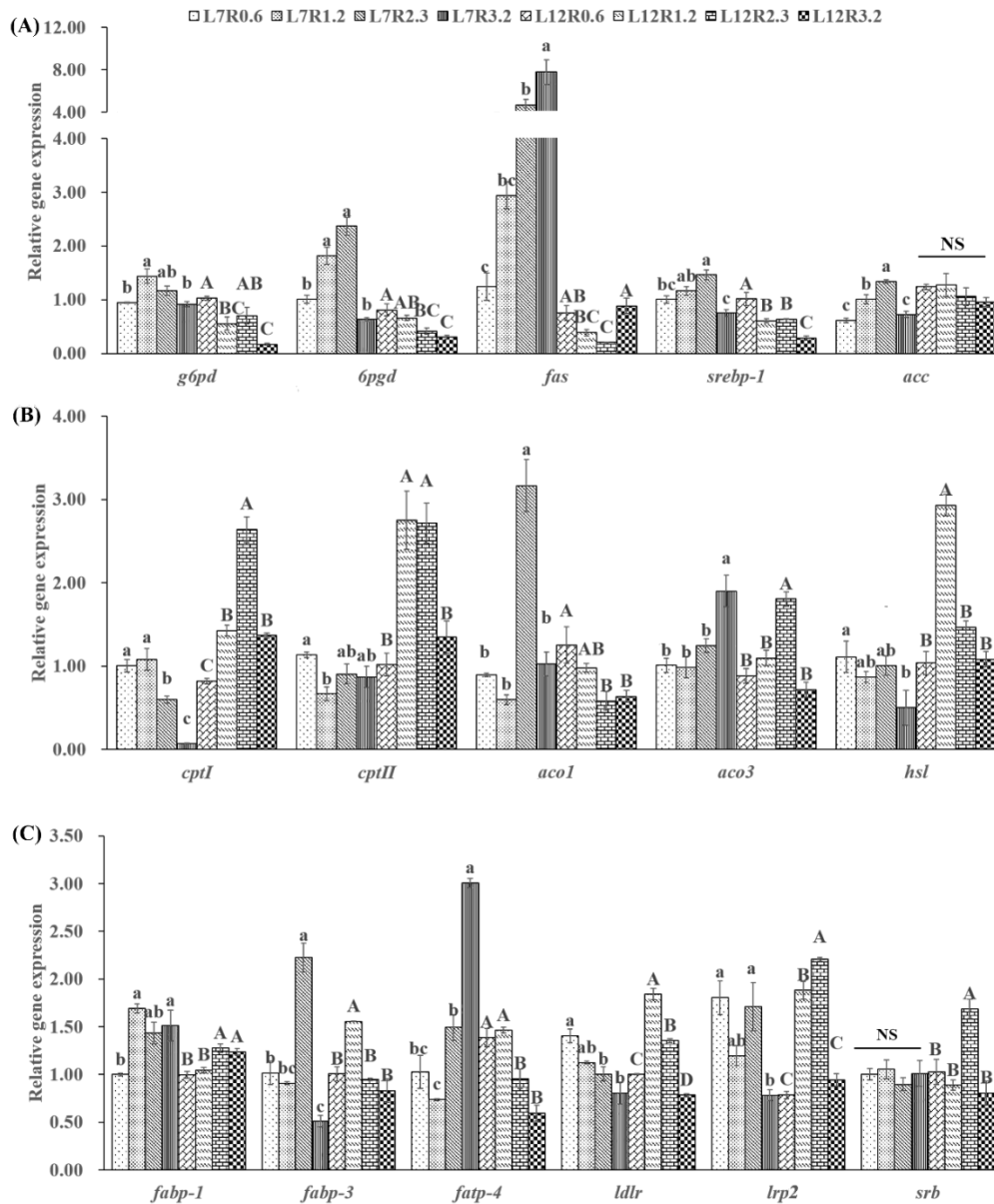


760
 761 **Figure 1.** The linear broken-line model and quadratic broken-line model for the relationship between
 762 dietary DHA/EPA ratio and WG of juvenile mud crab fed diets with 7% (A) and 12% (B) lipid. The
 763 horizontal axis represents the measured dietary DHA/EPA ratios. The Xpot represents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

765

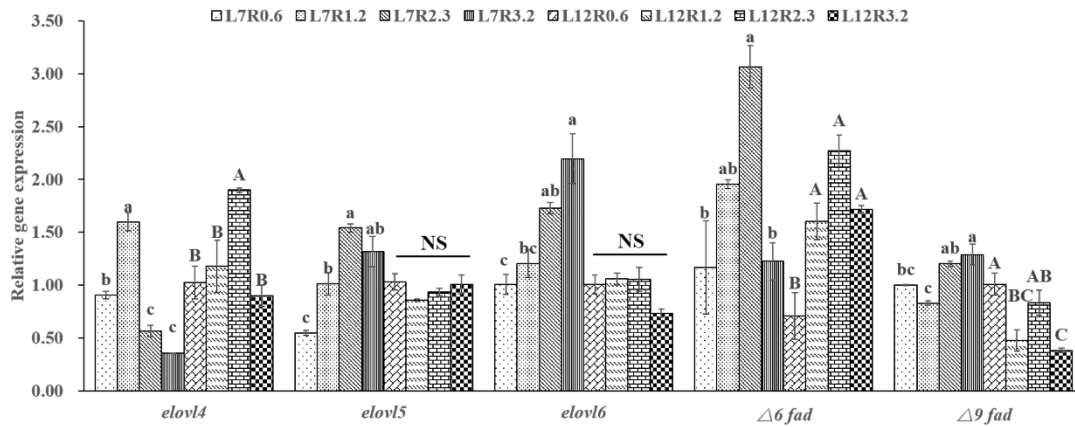


766
 767 **Figure 2.** Principal component analysis (PCA) score plots based on fatty acid profiles of muscle (A) and
 768 hepatopancreas (B) of crab fed the different experimental diets. For example, L7R0.6: dietary lipid level
 769 and DHA/EPA ratio were 7% and 0.6.



770

771 **Figure 3.** Effects of DHA/EPA ratio on relative mRNA expression levels of genes involved in lipid
 772 anabolism (A), lipid catabolism (B) and fatty acid and lipid transport (C) in the hepatopancreas of *S.*
 773 *Paramamosain* at 7% and 12% dietary lipid levels. Values are means \pm SEM (n = 3), and bars bearing
 774 different letters are significantly different by Tukey's test ($P < 0.05$). *srebp-1*, sterol regulatory element
 775 binding protein-1; *fas*, fatty acid synthase; *acc*, acetyl-CoA carboxylase; *6pgd*, 6-phosphogluconate
 776 dehydrogenase; *g6pd*, glucose-6-phosphate dehydrogenase; *cpt*, carnitine palmitoyltransferase; *aco*, acyl-
 777 CoA oxidase; *hsl*, hormone-sensitive triglyceride lipase; *fabp*, fatty acid binding protein; *fatp*, fatty acid
 778 transport protein; *ldlr*, low-density lipoprotein receptor; *lrp*, low-density lipoprotein receptor-related
 779 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 \pm 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.