

1           **Lipidomic profiling reveals molecular modification of lipids in**  
2           **hepatopancreas of juvenile mud crab (*Scylla paramamosain*) fed with**  
3                           **different dietary DHA/EPA ratios**

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19

20 **Abstract**

21 Untargeted lipidomic analysis was conducted to explore how different dietary docosahexaenoic  
22 acid (DHA) / eicosapentaenoic acid (EPA) ratio and, specifically, how an optimal ratio (2.3)  
23 compared to a suboptimum ratio (0.6) impacted lipid molecular species and the positional  
24 distribution of fatty acids in hepatopancreas of mud crab. The results indicated that major category  
25 of lipid affected by dietary DHA/EPA ratio was glycerophospholipids (GPs). The optimum dietary  
26 DHA/EPA ratio increased the contents of DHA bound to the *sn*-2 and *sn*-3 positions of  
27 phosphatidylcholine (PC) and triacylglycerol, EPA bound to the *sn*-2 position of  
28 phosphatidylcholine and 18:2n-6 bound to the *sn*-2 position of phosphatidylethanolamine (PE).  
29 Increased dietary DHA/EPA ratio also led to competition between arachidonic acid (ARA) and  
30 18:2n-6 bound to esterified sites. Appropriate dietary DHA/EPA ratio can not only improve the  
31 growth performance and nutritional quality of mud crab, but also provide higher quality products  
32 for human consumers.

33

34 **Key words:** *Scylla paramamosain*, DHA/EPA, Untargeted lipidomics, Lipid molecules, Fatty acid  
35 composition, Positional distribution.

36

37 **1. Introduction**

38 Most animals cannot synthesize the polyunsaturated fatty acids (PUFA), linoleic acid (LA,  
39 18:2n-6) and  $\alpha$ -linolenic acid (LNA, 18:3n-3) from the precursor oleic acid (18:1n-9), and they have  
40 to be obtained in the diet. In addition, the metabolic conversion of LNA and LA to long-chain  
41 polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA, 20:4n-6), eicosapentaenoic  
42 acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is poor in marine animals, and hence  
43 dietary uptake is significantly more effective. However, marine animal species including fishes,  
44 shrimps, prawns, crabs and shellfish are rich in EPA and DHA. Usually, microalgae are the primary  
45 source of n-3 LC-PUFA for marine fish and shellfish. Thus, in mud crab *Scylla paramamosain*,  
46 PUFA represent 51.0% - 62.5% of total fatty acids with most being the n-3 LC-PUFA such as EPA  
47 and DHA (Li, Zhao, Li, Wang, Mu, Song, et al., 2019). The mud crab is widely distributed in coastal  
48 Malaysia, Vietnam, Japan and China, it has become the major marine crustacean farmed in China in  
49 recent years (Wang, Jin, Cheng, Luo, Jiao, Betancor, et al., 2021). According to the [China Fishery  
50 Statistical Yearbook \(2020\)](#), production of farmed mud crabs was over 160 thousand tons in 2019,  
51 mainly *S. paramamosain*. In Asia, the hepatopancreas and ovaries of the marine or freshwater crabs  
52 are prized for their delicious and unique taste.

53 The hepatopancreas in crustaceans is central to lipid metabolism and plays critical roles in  
54 growth and reproduction, especially during ovarian development. During the processes of molting  
55 and reproduction abundant lipids are accumulated and deposited in the hepatopancreas of  
56 crustaceans (Wang, Wu, Liu, Zheng, & Cheng, 2014). Lipids in crustaceans hepatopancreas not only  
57 supply energy, but also provide essential fatty acids to maintain the integrity of cell membranes and  
58 other metabolic roles, and cholesterol for the synthesis of molting hormones (Harrison, 1990).

59 Generally, the two predominant lipid classes in tissues are triacylglycerols (TGs) that are the major  
60 neutral lipid and perceived as an energy reserve, whereas glycerophospholipids (GPs) are important  
61 polar lipids that are the main components of biological membranes and implicated in a variety of  
62 cellular functions (Lykidis, 2007). The GPs are critical for lipid absorption, transportation and  
63 deposition and are a rich source of LC-PUFA, therefore, are also precursors of eicosanoids,  
64 diacylglycerol inositol phosphates and other highly biologically active mediators, which play  
65 important metabolic and physiological functions (Tocher, Bendiksen, Campbell & Bell, 2008).  
66 Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) are  
67 quantitatively the most important GPs in animal tissues which play key roles in regulating  
68 membrane structure, fluidity, signal transduction and lipid metabolism (Vance & Tasseva, 2013).  
69 While TG molecules consist of a glycerol backbone esterified with three fatty acids at *sn-1*, *sn-2*  
70 and *sn-3* positions, typical GP molecules such as PC, PE and PI share a common structure consisting  
71 of two fatty acids esterified at the *sn-1* and *sn-2* positions of the glycerol moiety, with phosphate  
72 and a base (e.g. choline, ethanolamine or inositol) esterified to *sn-3*. The location of fatty acids on  
73 the glycerol backbone is important for lipid and fatty acid utilization and hydrolysis (Liu, Jiao, Gao,  
74 Ning, Limbu, Qiao, et al., 2019; Xu, Wei, Xie, Lv, Dong, & Chen, 2018). Importantly, the fatty acid  
75 composition of lipids in tissues including hepatopancreas or liver usually reflects dietary fatty acid  
76 profiles (Unnikrishnan & Paulraj, 2010), which means that the fatty acid compositions of lipids in  
77 mud crab hepatopancreas can be modified by diet. However, there is little information about the  
78 impact that dietary DHA/EPA ratio could have in modifying composition and structure of lipid  
79 molecules in the hepatopancreas of mud crab.

80 High-resolution mass spectrometry (MS), such as quadrupole time-of-flight MS and

81 quadrupole Exactive Orbitrap (Q-Exactive Orbitrap), with extremely high resolution, sensitivity,  
82 and mass precision has recently been applied to the non-target lipid analysis of various food matrices  
83 such as milk, meat and fish (Li, Zhao, Zhu, Pang, Liu, Frew, et al., 2017; Mi, Shang, Li, Zhang, Liu,  
84 & Huang, 2019; Wang, Zhang, Song, Cong, Li, Xu, et al., 2019). The advanced analytical technique  
85 of MS combined with highly selective ultra-performance liquid chromatography (UPLC) enables  
86 hundreds of lipids to be separated and identified in an unbiased way (Li, Liang, Xue, Wang, & Wu,  
87 2019; Lim, Long, Mo, Dong, Cui, Kim, et al., 2017) and so lipidomics has developed rapidly in  
88 recent years. However, lipidomic studies on crustacean lipids are limited up to now, therefore, the  
89 objective of present study was conducted to use an MS-based lipidomic approach to investigate how  
90 dietary DHA/EPA ratio affects the abundance and structures of lipid molecules in hepatopancreas  
91 for mud crab. The overall aim is to provide novel insights into lipid nutrition and metabolism of  
92 crustaceans, and improve the culture and nutritional quality of farmed mud crab.

93

## 94 **2. Materials and methods**

### 95 *2.1. Ethics statement*

96 This study was conducted in strict compliance with the Standard Operation Procedures of the  
97 Guide for Use of Experimental Animals of Ningbo University. Specific protocols and procedures in  
98 the experiment were endorsed by the Institutional Animal Care and Use Committee of Ningbo  
99 University.

### 100 *2.2. Diets*

101 Four purified diets containing approximately 45% crude protein and 7% crude lipid were  
102 formulated with DHA:EPA ratios of approximately 1:2, 1:1, 2:1 and 3:1 and named D1, D2, D3 and  
103 D4, respectively (Wang, Jin, Cheng, Hu, Zhao, Yuan, et al., 2021). The experimental diets were

104 manufactured as described in detail previously (Wang, Jin, Cheng, Luo, Jiao, Betancor, et al., 2021),  
105 after which the air-dried diets were kept at -20 °C prior to use. The detailed formulations and  
106 proximate compositions of the diets are presented in Supplemental Table 1 and the fatty acid profiles  
107 are presented in Supplemental Table 2. The average total n-3 LC-PUFA content of the experimental  
108 diets was 19.2 mg g<sup>-1</sup> diet dry weight and ranged between 18.5 and 20.0 mg g<sup>-1</sup> with final DHA :  
109 EPA ratios of 0.6, 1.2, 2.3 and 3.2, respectively.

### 110 2.3. Feeding trial and sampling

111 A total of 120 healthy juvenile mud crabs ( $20.92 \pm 0.56$  g crab<sup>-1</sup>) were obtained from Jia-Shun  
112 Aquatic-Cooperatives (Taizhou, China) and the feeding trail carried out in single crab cells (0.33 m  
113 × 0.23 m × 0.15 m, length × width × height) at the Ningbo Marine and Fishery Science and  
114 Technology Innovation Base (Ningbo, China). Crabs were randomly divided into triplicate groups  
115 with 10 crabs per replicate and 3 replicates per dietary treatment to give 30 crabs per treatment. The  
116 conditions in cells were stable and as follows: temperature 26 - 30 °C, salinity 26 - 28 g L<sup>-1</sup>, pH 7.7  
117 - 8.0, dissolved oxygen 6.5 - 7.0 mg L<sup>-1</sup>, and ammonia nitrogen was lower than 0.05 mg L<sup>-1</sup>. Further  
118 details of the management of the 8-week feeding trial were presented previously (Wang, Jin, Cheng,  
119 Hu, Zhao, Yuan, et al., 2021).

120 At the end of the 8-week feeding period, 18 crabs were selected randomly from each dietary  
121 treatment (6 crabs per replicate). The hepatopancreas was dissected from each crab and pooled from  
122 3 crabs to provide a total of 6 samples per dietary treatment. Approximately 500 mg of each pooled  
123 sample was taken and stored at -80 °C prior to untargeted lipidomic analysis (n = 6 per dietary  
124 treatment). The remaining of the pooled hepatopancreas (6 per dietary treatment) was immediately  
125 frozen and stored at -20 °C prior to the analysis of fatty acid composition (n = 3 per dietary

126 treatment).

127 *2.4. Preparation of fatty acid methyl esters (FAME) and analysis of fatty acid composition by gas*  
128 *chromatography (GC)*

129 In brief, total lipid was extracted from approximately 100 g of diets and freeze-dried  
130 hepatopancreas samples using chloroform/methanol (2:1, v:v) according to [Bligh & Dyer, \(1959\)](#)  
131 and as described in previous study ([Rey, Alves, Melo, Domingues, Queiroga, Rosa, et al., 2015](#)).  
132 Approximately 10 mg of total lipid were placed in a glass test tube along with 1 mL of 23:0 internal  
133 standard solution (1 mg mL<sup>-1</sup>, in HPLC grade hexane > 95%), and dried by Termovap TV10 sample  
134 concentrator (Ecom, Czech Republic). Three mL methanolic sulfuric acid solution (1 mL H<sub>2</sub>SO<sub>4</sub> :  
135 100 mL methanol : 0.05 g butylated hydroxytoluene [BHT; as antioxidant]) were added to the glass  
136 tubes that were then incubated in a water bath at 80 °C for 3 h to produce FAME. One mL hexane  
137 and distilled water were added and the mixture vortexed for 1 min and cooled to room temperature.  
138 The upper layer was filtered through a lipid phase filter (SCAA-104, ANPEL, China) and the solvent  
139 evaporated under a stream of nitrogen and FAME dissolved in 0.5 mL hexane. The FAME samples  
140 were analyzed on a GC (GC-MS 7890B-5977A, Agilent Technologies, USA) with the GC-MS  
141 operating conditions as described previously ([Yuan, Xu, Jin, Wang, Hu, Zhao, et al., 2021](#)). Fatty  
142 acids were identified by their retention time in comparison to a FAME standard solution (FAME 37  
143 MIX, Supelco), and fatty acid concentrations calculated according to the peak area ratio of  
144 FAME/23:0 standard.

145 *2.5. Lipid preparation and extraction*

146 Briefly, 25 mg of hepatopancreas sample was defrosted at 4 °C in a microfuge tube,  
147 resuspended in 800 µL dichloromethane/methanol (3:1, v:v) and 10 µL internal standard stock

148 (SPLASH 330707, SPLASHTM Lipidomix Mass Spec Standard, Avanti Polar Lipids, USA), and  
149 then incubated at -20 °C for 1 h.. Following centrifugation at 25 000 rpm for 15 min at 4 °C, 600  
150 µL of supernatant was concentrated by vacuum concentrator (Maxi Vacbeta, GENE COMPANY).  
151 The concentrates were resuspended in 200 µL of isopropanol/acetonitrile/H<sub>2</sub>O (2:1:1, v:v:v),  
152 vortexed for 1 min and incubated at room temperature for 10 min, were stored into -80 °C until  
153 further analysis.

#### 154 *2.6. UPLC-MS method for lipidomics*

155 Lipidomic analysis was carried out using a Waters 2D UPLC (Waters, Milford, MA, USA)  
156 coupled to a Q-Exactive Orbitrap MS (Thermo Fisher Scientific, USA) with electrospray ionization  
157 (ESI). The lipids of hepatopancreas samples were separated on a UPLC charged surface hybrid C18  
158 column (2.1 × 100 mm, 1.7 µm; Waters) with a mobile phase consisting of a mixture of 10 mM  
159 ammonium formate in acetonitrile/water (60:40, v:v; A) with a gradient of 10 mM ammonium  
160 formate in isopropanol/acetonitrile (90:10, v:v; B) in ESI<sup>-</sup> mode. For ESI<sup>+</sup> mode, the same A and B  
161 solutions containing 0.1% formic acid were used. In both cases the mobile phase was delivered at a  
162 flow rate of 0.35 mL min<sup>-1</sup> with the column initially eluted with 60% A and 40% B. The proportion  
163 of B increased to 43% over 2 min, then rapidly to 50% (0.1 min), then linearly to 54% over 4.9 min,  
164 before rapidly increasing to 70% (0.1 min), before a final linear gradient to 99% over 5.9 min in the  
165 last section of the gradient. The percentage of B solution percentage decreased back to 40% (0.1  
166 min) and the column equilibrated for 2 min before the next sample injection.

167 Primary data were obtained using the full scan mass-to-charge ratio range of the Q-Exactive  
168 Orbitrap MS of 200 to 2000 with 70,000 of the primary resolution, 3e6 automatic gain control (AGC)  
169 and 100 ms maximum injection time. Secondary level data were acquired by fragmenting the top

170 three highest intensity precursor ions with 15, 30 and 45 eV of stepped normal collision energies,  
171 17,500 of the secondary resolution, 1e5 AGC and 50 ms maximum injection time. The parameters  
172 of ESI mode of MS were set as follows: 40 sheath gas flow rate, 10 auxiliary gas flow rate, 320 °C  
173 capillary temperature, 350 °C auxiliary gas heater temperature and 3.80 (3.20) spray voltage for  
174 ESI<sup>+</sup> (ESI<sup>-</sup>) mode. In order to determine the stability of data, a QC sample was run and analyzed  
175 every ten samples.

## 176 *2.7. High quality non-targeted metabolic profile acquisition and metabolite identification*

177 The raw MS datasets were imputed into commercially available software, LipidSearch v.4.1  
178 (Thermo Fisher Scientific, USA), to identify peaks on a single sample, and then perform peak  
179 alignment on all samples. As for identifying lipids, the mass tolerances for molecular precursors,  
180 product and fragment ions were all set at 5 ppm, the thresholds of m-score and c-score were set to  
181 5.0 and 2.0. The adducts form were [M+H]<sup>+</sup>, [M+NH<sub>4</sub>]<sup>+</sup> and [M+Na]<sup>+</sup> for ESI<sup>+</sup> mode, and [M-H]<sup>-</sup>,  
182 [M-2H]<sup>-</sup> and [M-HCOO]<sup>-</sup> for ESI<sup>-</sup> mode. Peak alignment was then performed on all the identified  
183 lipids, with deviation of retention time set at 0.1 min. The grade of identification levels were A, B,  
184 C and D where grade A represented the lipid category and all fatty acid chains completely  
185 determined, for grade B both class specific ions and fatty acid fragment ions could be detected, for  
186 grade C either class-specific ions or fatty acid fragment ions could be detected, and for grade D the  
187 lipid structure could not be recognized, such as dehydrated ions. The discovery of outlier and  
188 assessment of batch effects was carried out by principal component analysis (PCA) of the dataset  
189 being pre-processed ([Supplemental Figure 2](#)). The standardized peak data was further analyzed  
190 using metaX, the process of which included 1) removing lipids that were observed in less than 50%  
191 of QC or 80% of biological samples, 2) filling missing values using k-Nearest Neighbor algorithm,

192 3) normalizing data to gain relative peak area using the method of probabilistic quotient  
193 normalization, 4) deleting lipid molecules with a coefficient of variation of relative peak area > 30%.  
194 The high-resolution LC-MS/MS features were then identified using Progenesis QI 2.0 by searching  
195 in the public databases including Human Metabolome Database (HMDB), LIPID MAPS Structure  
196 Database (LMSD).

### 197 *2.8. Statistical analyses*

198 The data for growth performance and fatty acid compositions were presented as means  $\pm$  SEM  
199 ( $n = 3$ ). The lipidomic data were presented as means  $\pm$  SEM ( $n = 6$ ). Data were first analyzed using  
200 one-way analysis of variance (ANOVA) using SPSS 23.0 (SPSS, IBM, USA). Student's t-test was  
201 applied for comparison of the lipidomic data between the two treatments with dietary DHA/EPA  
202 ratios of 0.6 and 2.3. PCA and partial least squares discriminant analysis (PLS-DA) was processed  
203 by SIMCA-P + 14.0 software package (Umetrics, Umea, Sweden). Cluster heatmaps were  
204 performed using MultiExperiment viewer (MEV, version 4.9.0).

205

## 206 **3. Results and Discussion**

### 207 *3.1. Growth performance, fatty acid composition in hepatopancreas and expression of genes related* 208 *to LC-PUFA biosynthesis*

209 The growth performance of mud crabs fed with diets containing different DHA/EPA ratios is  
210 presented in [Supplementary Table 3](#). Molting frequency (MF) was not influenced significantly by  
211 dietary DHA/EPA ratios although it was numerically higher in crabs fed with diet containing 2.3 of  
212 dietary DHA/EPA ratio. Crabs fed with diets containing 0.6 and 2.3 of DHA/EPA ratios respectively  
213 showed the lowest and highest weight gain (WG) and specific growth rate (SGR) among all

214 treatments. Based on second-order polynomial regression analysis of WG against dietary DHA/EPA  
215 ratio, 2.2 was determined to be the optimum ratio for mud crab fed with 7% lipid ([Supplementary](#)  
216 [Fig. 1](#)). This was higher than that reported previously in juvenile *P. trituberculatus*, where 0.7 - 0.8  
217 was estimated to be the optimum dietary DHA/EPA ratio although this was with a diet containing  
218 11% lipid ([Hu, Wang, Han, Li, Jiang, & Wang, 2017](#)). However, 2.0 was also reported to be the  
219 optimum dietary DHA/EPA ratio for swimming crab fed with 11% lipid during ovarian development  
220 ([Feng, 2011](#)). In addition, 2.0 - 3.0 was determined to be the optimum range for this ratio in Chinese  
221 mitten crab (*Eriocheir sinensis*) fed with 7.5% lipid ([Zhao, 2013](#)). These studies indicated that a  
222 precise dietary DHA/EPA ratio is essential to fulfil requirements for growth and development but  
223 that the ratio varied with culture species, diet formulation and lipid content, and developmental stage  
224 ([NRC, 2011](#)).

225       The fatty acid content and relative expression of genes involved to LC-PUFA biosynthesis in  
226 hepatopancreas are presented in [Figure 1](#) and [Supplementary Table 4](#). In crustaceans, while the  
227 hepatopancreas is the center of lipid metabolism, fatty acid composition generally reflects that of  
228 diet. In the present study, significantly higher contents of total FA, total SFA, total MUFA and total  
229 n-6 PUFA were observed in crabs fed with diet D2 and D3 than in crabs fed with diet D1 and D4.  
230 The lowest content of total n-3 PUFA was shown in crabs fed with diet D1, and the content of DHA  
231 and the DHA/EPA ratio in hepatopancreas both increased significantly as dietary DHA/EPA ratio  
232 increased. This may simply be a consequence of the greater retention of DHA compared to other  
233 fatty acids including EPA ([Tocher et al., 2010](#)), but may also reflect the greater biological value of  
234 DHA ([Carvalho, Peres, Saleh, Fontanillas, Rosenlund, Oliva-Teles, et al., 2018](#)). Thus, compared to  
235 crabs fed with the lowest dietary DHA/EPA ratio (D1), higher dietary DHA/EPA ratios up to 2.3

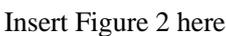
236 significantly promoted the growth of mud crab and improved the nutritional value of hepatopancreas  
237 by increasing DHA and overall n-3 LC-PUFA contents.

238 Insert Figure 1 here

### 239 3.2. Composition of lipid classes in hepatopancreas

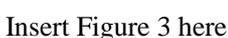
240 Based on weight gain, a dietary ratio of DHA/EPA of approximately 2 was estimated to be  
241 optimum for juvenile mud crab fed with 7% lipid diet ([Supplemental Figure 1](#)) ([Wang, Jin, Cheng,](#)  
242 [Hu, Zhao, Yuan, et al., 2021](#)). Therefore, in the present study, samples of hepatopancreas from crabs  
243 fed with diet D1 (lowest growth) and D3 (optimal growth) were used to evaluate the effects of  
244 dietary DHA/EPA ratio on the distribution and composition of lipid using untargeted lipidomics.  
245 [Supplementary Figures 3](#) and [4](#) show representative Q-Exacte plus mass spectra in ESI<sup>+</sup> and ESI<sup>-</sup>  
246 modes of QC and experimental samples, respectively, which confirmed the high stability of the  
247 system and reliability of the data. The number and content (%) of lipid categories and classes in  
248 hepatopancreas are shown in [Figure 2](#). After removal duplicate molecules, a total of 390 unique  
249 lipid molecular species belonging to a total of 22 lipid classes in 4 major categories (GPs, fatty acyls,  
250 glycerolipids and sphingolipids) were identified. Specifically, 15 lipid classes represented by 336  
251 lipid species (i.e. 86% of all identified species) were GPs including 144 PCs, 76 PEs, 28 PSs, 27  
252 PIs, 18 LPCs and others. Thus, the major lipid metabolites found in hepatopancreas of mud crab fed  
253 with diet D1 and D3 containing 0.6 and 2.3 of dietary DHA/EPA ratios were GPs, which was similar  
254 to the results reported for hepatopancreas of *P. trituberculatus* and muscle of *Sagmariasus verreauxi*  
255 fed with different lipid sources ([Shu-Chien, Han, Carter, Fitzgibbon, Simon, Kuah, et al., 2017;](#)  
256 [Yuan, et al., 2021](#)). The relative contents of lipid classes in hepatopancreas were generally not  
257 affected by the diet, however the absolute contents of fatty acids were significantly affected by

258 dietary DHA/EPA ratio, therefore it was necessary to detect the different lipid metabolites (DLMs).

259 

### 260 3.3. Identification of DLMs

261 Principal component analysis was used to observe clustering trends simultaneously to identify  
262 and exclude outliers in the data (Figure 3). In addition, PLS-DA was used for building a discriminant  
263 model with validity and potential over-fitting of the model checked by performing 200 permutation  
264 tests and visualization using a validation plot (Supplementary Figure 6). A Volcano plot was used  
265 to show the difference in lipid metabolites (Figure 3). The components of lipid species in  
266 hepatopancreas of crabs fed with diet D1 and D3 were clustered as two groups and separated from  
267 each other with no overlap. The lipid metabolites with fold change (FC)  $\geq 1.20$  or  $\leq 0.83$  ( $\log_2\text{FC} \geq$   
268  $0.26$  or  $\leq -0.27$ ),  $P < 0.05$  and Variable Importance in Projection (VIP)  $\geq 1$  were defined as DLMs.  
269 Compared to crabs fed with diet containing a dietary DHA/EPA ratio 0.6, a total of 77 DLMs with  
270 47 up-regulated and 30 down-regulated were identified in hepatopancreas of crabs fed with diet D3  
271 containing an optimum DHA/EPA ratio of 2.3 (Figure 3C). Figure 3D showed that PC (54%) species  
272 contributed greatly to the difference between D1 and D3, followed by PE (14%) and PI (13%)  
273 species. Previously it was reported that the predominant DLMs in hepatopancreas of *P.*  
274 *trituberculatus* and fillets of Nile tilapia (*Oreochromis niloticus*) fed with different lipid sources  
275 were TG, PC and PE (Liu, et al., 2019; Yuan, et al., 2021). Differences in the predominant DLMs  
276 between the studies may be due to crab species, diet formulation including dietary lipid content and  
277 composition, and further analysis of DLMs in different species and trials should be conducted to  
278 further evaluate the importance and role of dietary changes to lipid metabolites.

279 

280 *3.4. Analysis of the predominant DLMs in hepatopancreas*

281 In order to further investigate the effects of dietary DHA/EPA ratio on the distribution of lipid  
282 metabolites, PLS-DA and clustering heatmap were used to discriminate the composition of all the  
283 predominant DLMs and analyze the variation in these lipid species identified in hepatopancreas of  
284 crabs fed with diet D1 and D3. In the present study, the PLS-DA plot (Figures 4A, B and C and  
285 Supplementary Figure 6) showed the distinctions and trends in PC, PE and PI molecules. All the  
286 samples were within the 95% Hotelling T<sup>2</sup> ellipse, and samples from the two diets were separated  
287 from each other, indicating the significant difference between metabolites in hepatopancreas of  
288 crabs fed with diet D1 and D3 containing suboptimal and optimal DHA/EPA ratio, respectively.  
289 Cluster heatmap analysis showed more comprehensive and intuitive distribution pattern and  
290 relationship of lipid metabolites between the samples of the two dietary groups, which was helpful  
291 to evaluate the rationality of different lipid metabolites. The clustering heatmap (Figures 4D, E and  
292 F) showed the variation in the PC, PE and PI molecular species directly, which confirmed the result  
293 of PLS-DA. The heatmap also showed that the 6 replicates of the same group and lipid metabolites  
294 containing the same fatty acid were well clustered. For example, PC molecules containing DHA  
295 were positively correlated with the dietary ratio of DHA/EPA. The results indicated that the  
296 molecular compositions of PC, PE and PI of crab hepatopancreas were significantly affected by  
297 dietary DHA/EPA ratio. However, TG also plays a key role in cellular biology, organ function and  
298 lipid metabolism and is the main form for deposition and storage of fatty acids (Goldberg, 2012;  
299 Stubhaug, Tocher, Bell, Dick, & Torstensen, 2005). Considering that the location of fatty acids on  
300 the glycerol backbone of lipid metabolites is important for lipid and fatty acid utilization and  
301 hydrolysis, the compositions of TG and the predominant glycerophospholipid classes, PC, PE and

302 PI, were analyzed further.

303 Insert Figure 4 here

### 304 3.5. The distribution of key fatty acids in TG, PC, PE and PI in hepatopancreas

305 [Figure 5](#) shows the positional distribution of 16:0, 18:1n-9, 18:2n-6, ARA, EPA and DHA in  
306 different TG, PC, PE and PI. Although, TG (18:0/20:4/22:6) and TG (18:1/18:1/22:5) were the only  
307 two neutral glycerolipid species in DLMs found in the present study, the contents of 18:1n-9 and  
308 ARA combined were significantly higher in TG than in PC, PE and PI ([Figure 5](#)), indicating TG  
309 was the main class for deposition of these fatty acids in mud crab hepatopancreas. The deposition  
310 of 18:1n-9 at the *sn-1* position was lower than that at the *sn-2* position of PE molecules in crabs fed  
311 with diet D3, while the opposite trends were observed in the deposition of 16:0 and 18:1n-9 in PC  
312 and PI molecules in crabs fed with both diet D1 and D3. It was reported that an excess intake of  
313 lipid containing 16:0 at the *sn-2* position may increase the risk of atherogenesis, whereas fatty acids  
314 at the *sn-1/3* positions are preferred for pancreatic lipase and are readily lipolyzed ([Mattson &](#)  
315 [Volfenhein, 1962](#)). Monounsaturated fatty acid, especially 18:1n-9 is generally regarded as  
316 "healthy" fat in human nutrition ([Grundy, 1989](#)). It has been shown that 18:1n-9, especially bound  
317 to TG, is better for supplying energy via  $\beta$ -oxidation than n-3 LC-PUFA ([Du, Araujo, Stubhaug, &](#)  
318 [Frøyland, 2010](#)). [Figure 6](#) shows that the content of 16:0 bound at the *sn-1* position of PC was higher,  
319 and 18:1n-9 bound at *sn-2* position of PC and *sn-1* and *sn-2* positions of TG were lower in crabs fed  
320 with diet D3 containing optimal DHA/EPA ratio than crabs fed with diet D1 containing a suboptimal  
321 ratio. This suggested that 18:1n-9 could be a good supply of energy and that SFA and MUFA tend  
322 to bind the *sn-1* position of lipid molecules and, most importantly in terms of the present study, that  
323 mud crabs fed with the diet containing optimum DHA/EPA ratio (2.3) are recommended for

324 consumption compared to crabs fed with a suboptimum ratio (0.6).

325       The positional distribution of other fatty acids in DLMs were similar, with the contents of  
326 18:2n-6, ARA, EPA and DHA higher at the *sn-2* position than at *sn-1* position of lipid molecules in  
327 hepatopancreas of crabs fed with both diet D1 and D3, other than ARA in PC in crabs fed with diet  
328 D1 where no difference was observed in the deposition between *sn-1* and *sn-2* (Figure 5). It has  
329 been long accepted that fatty acids bound at the *sn-2* position are more stable than those at *sn-1/3*  
330 and so the results indicate *sn-2* is the predominant binding site for LC-PUFA in hepatopancreas of  
331 *S. paramamosain* irrespective of diet contributing to its high nutritional value, and similar results  
332 were reported previously in a lipidomic study on *O. niloticus* fed with different dietary oils (Liu, et  
333 al., 2019). However, significantly different (DLM) TG had DHA preferentially bound at *sn-3* rather  
334 than *sn-1* and *sn-2* positions, suggesting sufficient DHA may be bound at the *sn-2* position of TG in  
335 crabs fed with diet D1 and D3, and higher intake of DHA in crabs fed with diet D3 was bound at  
336 the *sn-3* position. A previous study reported that seal oil had high anti-inflammatory effects due to  
337 EPA and DHA being predominantly bound at the *sn1/3* positions (Christensen et al., 1995). The  
338 present study may also indicate that mud crabs fed with diet containing optimal dietary DHA/EPA  
339 ratio may have increased anti-inflammatory capacity for humans consuming the crabs. In contrast,  
340 18:2n-6 at the *sn-1/3* positions could be more easily hydrolyzed and might lead to inflammation  
341 (Naughton, Mathai, Hryciw, & McAinch, 2016). In the present study, the content of 18:2n-6 at the  
342 *sn-1* and *sn-2* position of PC was lower and at *sn-2* position of PE was higher in hepatopancreas of  
343 crabs fed with diet D3 containing optimal DHA/EPA ratio than crabs fed with diet D1 containing  
344 suboptimal ratio. Combined with the higher 18:2n-6 content in crabs fed with diet D3, these results  
345 showed that the *sn-2* position of PE was the predominant binding site for 18:2n-6. Similarly, 18:2n-

346 6 was specifically deposited at the *sn*-2 position of PC and PE in *O. niloticus* (Liu, et al., 2019).  
347 Moreover, the risk of inflammation for consumption of mud crab hepatopancreas may be low, and  
348 could be further decreased by feeding an optimum dietary DHA/EPA ratio (Figure 6).

349 Importantly, the contents of ARA at *sn*-1 and *sn*-2 positions of PC were significantly higher in  
350 crabs fed with diet D3 than crabs fed with diet D1, whereas the content of ARA at the *sn*-2 position  
351 of PE showed the opposite (Figure 6). Interestingly, the opposite trends were also observed between  
352 contents of ARA and 18:2n-6 at the same binding site of PC and PE, which may indicate competition  
353 between ARA and 18:2n-6 for esterification sites and/or the biosynthesis of ARA from 18:2n-6 in *S.*  
354 *paramamosain*, both worthwhile topics for further study. The contents of EPA at *sn*-1 in PC and *sn*-  
355 2 in PE were significantly lower, and at *sn*-2 in PC significantly higher, in crabs fed with diet D3  
356 than crabs fed with diet D1. It was reported previously that LC-PUFA at the *sn*-2 positions of PC  
357 and PE increase the fluidity of cell membranes (van der Veen, Kennelly, Wan, Vance, Vance, &  
358 Jacobs, 2017) and the present study has demonstrated that an optimal dietary DHA/EPA ratio  
359 promoted this function in mud crab. The contents of DHA in PC, PI and TG molecules were higher  
360 in hepatopancreas of crabs fed with diet D3 than in crabs fed with diet D1 (Figure 6), moreover, the  
361 content of DHA bound at the *sn*-2 position of PC was higher than that bound at the *sn*-3 position of  
362 TG (Figure 5). When taking the changes of ARA, EPA and DHA contents in lipid molecules and  
363 hepatopancreas into consideration (Figure 1 and Supplementary Table 4), we speculate that ARA,  
364 EPA and DHA are preferentially stored at the *sn*-2 position of PC molecules in hepatopancreas of  
365 mud crab. In contrast, a previous study demonstrated that DHA was preferentially deposited in PE  
366 molecules in the muscle of largemouth bass (*Micropterus salmoides*) (Zhang, 2019). Given that the  
367 species are different and relevant research in crustaceans are few, further studies still require to be

368 conducted.

369 Insert Figure 5 here

370 Insert Figure 6 here

#### 371 **4. Conclusions**

372 In the present study, the results of lipidomic analysis revealed that the major lipids in  
373 hepatopancreas affected by dietary DHA/EPA ratios were glycerophospholipids (GPs). Irrespective  
374 of diet, ARA, EPA and DHA were preferentially located at the *sn*-2 position PC molecules while  
375 SFA and MUFA tended to be bound at the *sn*-1 position of lipid molecules in hepatopancreas of  
376 mud crab. The *sn*-2 position of PE was the predominant binding site for 18:2n-6. The optimum  
377 dietary DHA/EPA ratios increased the contents of ARA, EPA and DHA bound to the *sn*-2 position  
378 of PC molecules, the content of 18:2n-6 bound to the *sn*-2 position of PE molecules, and the content  
379 of DHA bound to the *sn*-3 position of TG, potentially improve the anti-inflammatory properties of  
380 mud crab hepatopancreas when consumed. Increased dietary DHA/EPA ratio may lead to  
381 competition between ARA and 18:2n-6 bound to esterified sites. To the best of our knowledge, this  
382 is the first study investigated the impact of dietary DHA/EPA ratio in modifying the species  
383 composition of lipid molecules in hepatopancreas. Overall, optimal dietary DHA/EPA ratio could  
384 not only improve the culture of *S. paramamosain* potentially providing economic benefits, but also  
385 increase the nutritive value of farmed crab providing health benefits for human consumers.

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394

#### 395 **CRedit authorship contribution statement**

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397 Project administration, Validation, Visualization, Writing-original draft. **Min Jin:** Formal analysis,  
398 Resources, Supervision, Writing-review & editing. **Xin Cheng:** Investigation, Methodology.  
399 **Xiaoying Hu:** Investigation, Methodology. **Mingming Zhao:** Investigation, Methodology. **Ye Yuan:**  
400 Software, Visualization. **Peng Sun:** Software, Visualization. **Lefei Jiao:** Software, Visualization.  
401 **Douglas R. Tocher:** Formal analysis, Writing-review & editing. **Mónica B. Betancor:** Writing-  
402 review & editing. **Qicun Zhou:** Conceptualization, Formal analysis, Funding acquisition,  
403 Resources, Supervision, Writing-review.

404 **& editing.**

405

#### 406 **Declaration of competing interest**

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

409

#### 410 **Supplementary data**

411 **Supplementary Table 1.** Formulations and proximate compositions of the experimental diets.

412 **Supplementary Table 2.** Fatty acid contents (mg g<sup>-1</sup>, dry matter) of the experimental diets

413 **Supplementary Table 3.** Growth performance of mud crabs fed with the experimental diets.

414 **Supplementary Table 4.** Fatty acid contents (mg g<sup>-1</sup>, dry matter) of hepatopancreas of mud crabs

415 fed with the experimental diets.

416 **Supplementary Figure 1.** Optimal dietary DHA/EPA requirement of mud crab fed with 7% lipid.

417 **Supplementary Figure 2.** Principal component analysis (PCA) plots for QC samples.

418 **Supplementary Figure 3.** The overlap plot of base peak chromatograms (BPC) acquired in positive

419 (A) and negative (B) ionization mode of all the quality control samples.

420 **Supplementary Figure 4.** The base peak chromatograms (BPC) acquired in positive (A) and

421 negative (B) ionization mode of hepatopancreas of mud crab fed with diet D1 and D3.

422 **Supplementary Figure 5.** Cross-validation plot of the PLS-DA model of all lipid species.

423 **Supplementary Figure 6.** Cross-validation plot of PLS-DA model of PC, PE and PI.

424

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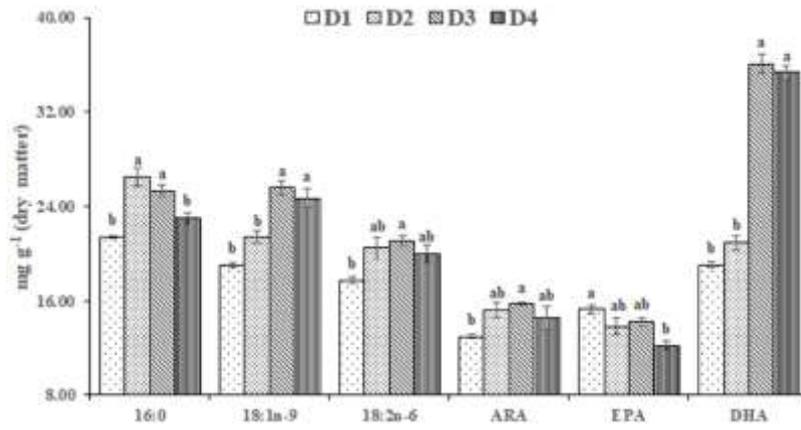
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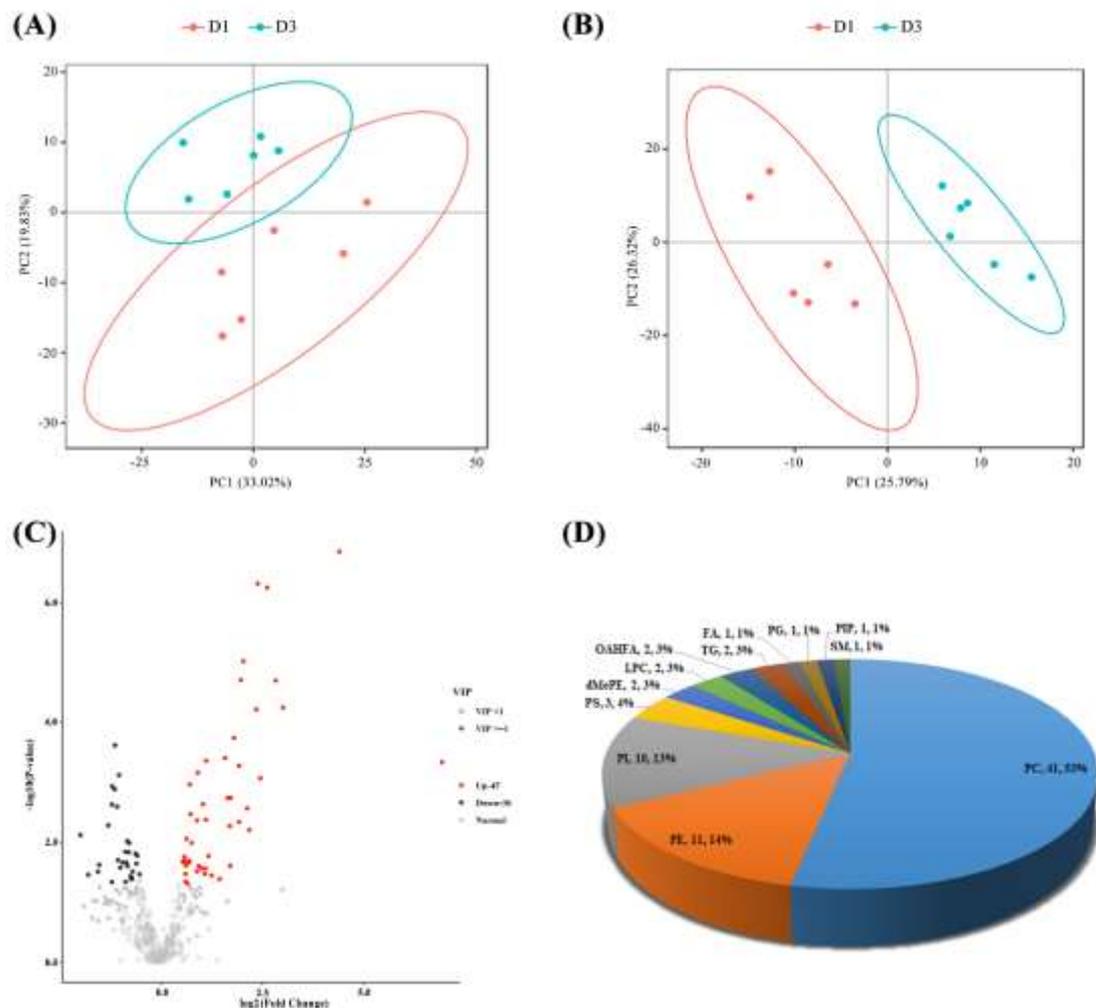
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531 **Figure 1.** Absolute contents of fatty acids in hepatopancreas of mud crab fed with experiment diets.

532 Values are means  $\pm$  SEM (n = 3).

533





547

548 **Figure 3.** Composition of different lipid metabolites in the hepatopancreas of *S. paramamosain* fed

549 with diet D1 and D3 (DHA/EPA = 0.6 and 2.3, respectively). (A) ● and ● in principal

550 component analysis, PCA (A) and partial least squares method-discriminant analysis, PLS-DA (B)

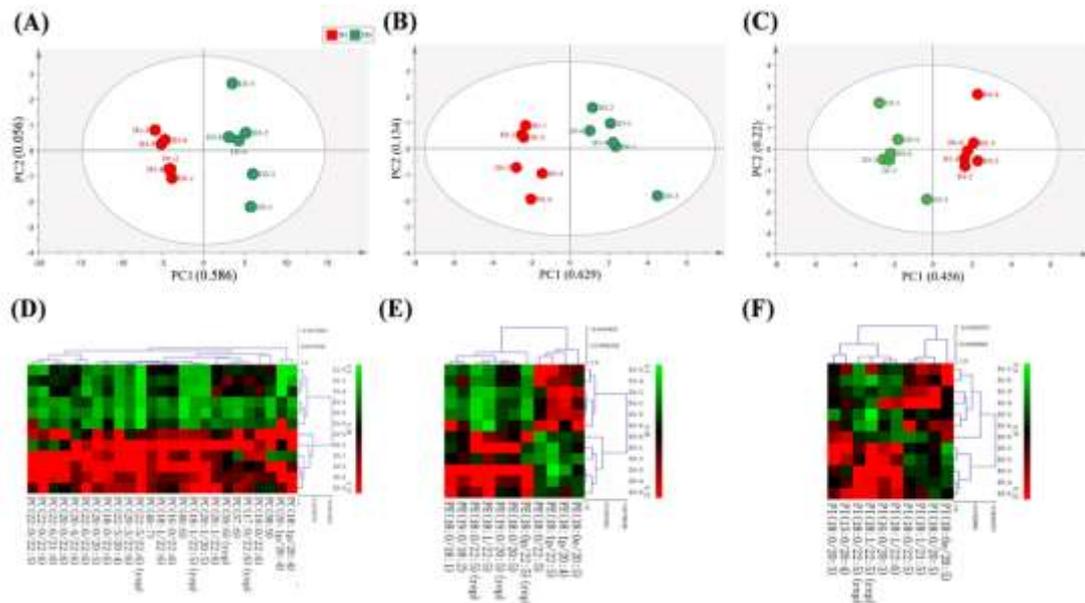
551 plots represent diets D1 and D3, respectively. In volcano plot (C), ● and ● represent down-

552 regulated and up-regulated lipid metabolites with  $\log_2(\text{fold change}) \geq 0.26$  or  $\leq -0.27$  and  $P < 0.05$ ,

553 circle and × show lipid metabolites with Variable Importance in Projection (VIP)  $\geq 1$  and VIP  $< 1$ ,

554 respectively. (D) the composition of different lipid metabolites (number, percentage).

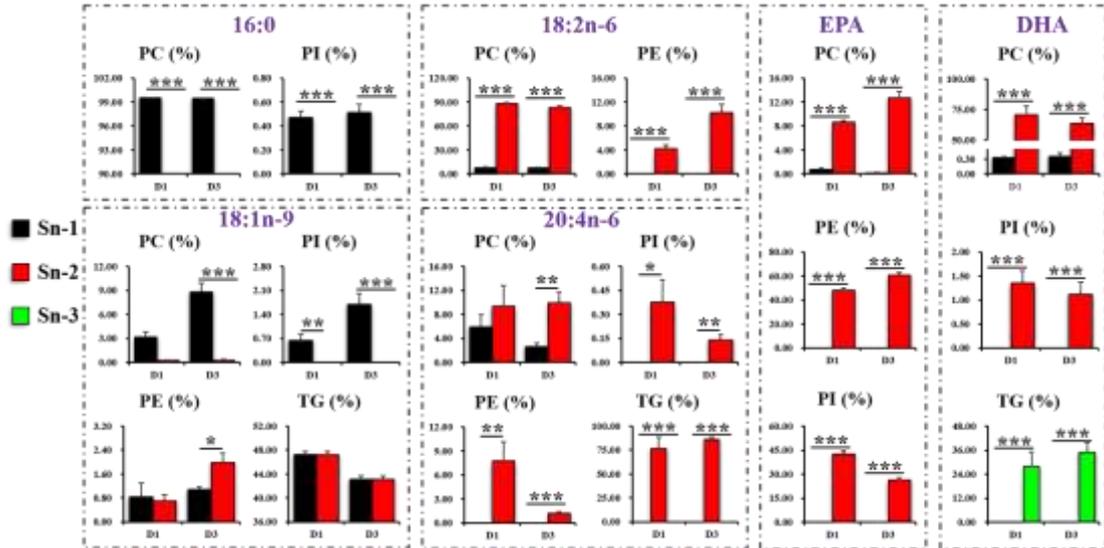
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556

557 **Figure 4.** Composition and clustering heatmap of significantly different phosphatidylcholine, PC  
 558 (A, D), phosphatidylethanolamine, PE (B, E) and phosphatidylinositol, PI (C, F) molecules in the  
 559 hepatopancreas of *S. paramamosain* fed with diet D1 and D3. ● and ● in PLS-DA plots A, B and  
 560 C, represent diets D1 and D3, respectively (n = 6). In heatmap plots D, E and F, columns represent  
 561 different samples; rows represent different lipid molecules, and color change from green to red  
 562 shows the increase of relative intensity of the lipid molecules.

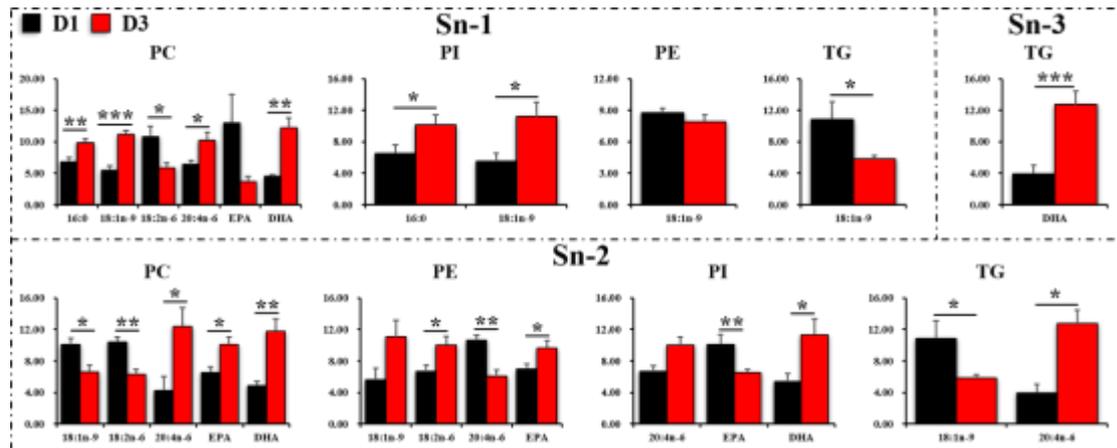
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564

565 **Figure 5.** The positional distributions of key fatty acids in phosphatidylcholine (PC),  
 566 phosphatidylethanolamine (PE), phosphatidylinositol (PI) and triacylglycerol (TG) molecules in  
 567 hepatopancreas of *S. paramamosain* fed with diet D1 and D3. Values are means  $\pm$  SEM (n = 6). \*,  
 568 \*\* and \*\*\* represent significant differences with  $P < 0.05$ ,  $P < 0.01$  and  $P < 0.001$ .

569



570

571 **Figure 6.** Effects of dietary DHA/EPA ratio on positional distributions of key fatty acids in PC, PE,

572 PI and TG molecules in hepatopancreas of *S.paramamosain* fed with diet D1 and D3. Values are

573 means  $\pm$  SEM (n = 6). \*, \*\* and \*\*\* represent significant differences with  $P < 0.05$ ,  $P < 0.01$  and  $P$

574  $< 0.001$ .

575

576 **Supplementary Table1.** Formulations and proximate compositions of the experimental diets.

Ingredients	D1	D2	D3	D4
Casein <sup>1</sup>	25.00	25.00	25.00	25.00
Soy protein concentrate <sup>2</sup>	27.61	27.61	27.61	27.61
Wheat flour	25.26	25.26	25.26	25.26
DHA-enriched oil <sup>3</sup>	0.00	1.28	2.57	3.20
EPA-enriched oil <sup>4</sup>	2.96	2.22	1.48	1.11
ARA-enriched oil <sup>5</sup>	0.50	0.50	0.50	0.50
Palmitic acid <sup>6</sup>	1.40	0.86	0.31	0.05
Soybean lecithin	1.00	1.00	1.00	1.00
Cholesterol	0.50	0.50	0.50	0.50
Betaine (98%)	0.10	0.10	0.10	0.10
Vitamin premix <sup>7</sup>	1.00	1.00	1.00	1.00
Mineral premix <sup>7</sup>	1.50	1.50	1.50	1.50
Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	2.00	2.00	2.00	2.00
Choline chloride	0.20	0.20	0.20	0.20
Cellulose	8.97	8.97	8.97	8.97
Sodium alginate	2.00	2.00	2.00	2.00
Total	100.00	100.00	100.00	100.00
Proximate composition				
Moisture	7.59	7.81	7.13	6.91
Crude protein	45.69	44.85	45.03	45.08
Crude lipid	7.43	7.85	7.51	7.51
Ash	6.62	6.15	6.26	6.11

577 <sup>1</sup> Casein: 89.6% crude protein and 0.2% crude lipid.

578 <sup>2</sup> Soy protein concentrate: 69.9% crude protein and 0.5% crude lipid.

579 <sup>3</sup> DHA-enriched oil: extracted from marine microalgae, DHA content, 406.5 mg g<sup>-1</sup> oil (Changsha  
580 Kenan Biotechnology Co., Ltd., China).

581 <sup>4</sup> EPA-enriched oil: extracted from marine microalgae, EPA content, 462.5 mg g<sup>-1</sup> oil, DHA content,  
582 235.6 mg g<sup>-1</sup> oil (Changsha Kenan Biotechnology Co., Ltd., China).

583 <sup>5</sup> ARA-enriched oil: extracted from *Mortierella alpine* (a yeast), ARA content, 468.0 mg g<sup>-1</sup> oil  
584 (Changsha Kenan Biotechnology Co., Ltd., China).

585 <sup>6</sup> Palmitic acid: Palmitic acid content 97% of total fatty acids, in the form of methyl ester (Shanghai  
586 Yiji Chemical Co., Ltd., China).

587 <sup>7</sup> Vitamin and mineral premixes were based on [Jin et al. \(2015\)](#).

588 ARA, 20:4n-6; DHA, 22:6n-3; EPA, 20:5n-3.

589 **Supplementary Table 2.** Fatty acid contents (mg g<sup>-1</sup>, dry matter) of the experimental diets.

Fatty acids	D1	D2	D3	D4
14:0	0.56	0.58	0.63	0.65
16:0	10.99	9.70	8.23	7.66
18:0	2.02	2.10	2.12	2.27
20:0	0.20	0.23	0.24	0.26
Total SFA <sup>1</sup>	13.78	12.61	11.22	10.84
16:1n-7	0.20	0.21	0.24	0.25
18:1n-9	5.23	5.84	6.28	6.83
20:1n-9	0.15	0.11	0.11	0.10
22:1n-11	0.05	0.05	0.04	0.04
Total MUFA <sup>2</sup>	5.63	6.21	6.67	7.22
18:2n-6	7.27	7.19	6.90	7.20
18:3n-6	0.23	0.21	0.23	0.24
20:2n-6	0.11	0.08	0.09	0.09
ARA <sup>3</sup>	2.19	2.24	2.12	2.15
22:4n-6	0.16	0.29	0.09	0.07
Total n-6 PUFA <sup>4</sup>	9.97	10.02	9.43	9.75
18:3n-3	1.04	1.02	1.00	1.04
18:4n-3	0.42	0.35	0.24	0.28
20:4n-3	0.42	0.38	0.39	0.42
EPA <sup>5</sup>	10.37	8.18	5.48	4.57
22:5n-3	1.28	1.02	0.68	0.54
DHA <sup>6</sup>	6.45	9.92	12.33	14.49
Total n-3 PUFA <sup>7</sup>	19.98	20.87	20.12	21.35
n-3/n-6 PUFA	2.00	2.08	2.13	2.19
DHA/EPA	0.62	1.21	2.25	3.17
Total n-3 LC-PUFA <sup>8</sup>	18.53	19.50	18.88	20.03

590 Data are means of duplicate analyses.

591 <sup>1</sup> SFA, saturated fatty acids: 14:0, 16:0, 18:0, 20:0.

592 <sup>2</sup> MUFA, monounsaturated fatty acids: 16:1n-7, 18:1n-9, 20:1n-9.

593 <sup>3</sup> ARA, 20:4n-6

594 <sup>4</sup> n-6 PUFA, n-6 polyunsaturated fatty acids: 18:2n-6, 18:3n-6, 20:2n-6, 20:4n-6, 22:4n-6.

595 <sup>5</sup> EPA, 20:5n-3. <sup>6</sup> DHA, 22:6n-3.

596 <sup>7</sup> n-3 PUFA, n-3 polyunsaturated fatty acids: 18:3n-3, 18:4n-3, 20:4n-3, EPA, 22:5n-3, DHA.

597 <sup>8</sup> n-3 LC-PUFA, n-3 long-chain polyunsaturated fatty acids: 20:4n-3, EPA, 22:5n-3, DHA.

598 **Supplementary Table 3.** Growth performance of mud crabs fed with the experimental diets.

Diet	Initial Weight (g)	WG (%)	SGR (% d <sup>-1</sup> )	MF
D1	20.62±1.09	44.26±2.83 <sup>c</sup>	0.65±0.04 <sup>b</sup>	0.63±0.19
D2	21.68±1.08	52.85±1.29 <sup>b</sup>	0.75±0.01 <sup>ab</sup>	0.65±0.05
D3	23.38±1.17	62.41±0.49 <sup>a</sup>	0.81±0.01 <sup>a</sup>	1.03±0.10
D4	20.05±1.45	55.80±1.65 <sup>ab</sup>	0.73±0.02 <sup>ab</sup>	0.75±0.11

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

600 letters are significantly different ( $P < 0.05$ ).

601 MF, molting frequency; SGR, specific growth rate; WG, weight gain.

602

603 **Supplementary Table 4.** Fatty acid contents (mg g<sup>-1</sup>, dry matter) of hepatopancreas of mud crabs

604 **fed with** the experimental diets

Fatty acids	Diet			
	D1	D2	D3	D4
14:0	1.43±0.08 <sup>b</sup>	1.62±0.04 <sup>b</sup>	2.06±0.05 <sup>a</sup>	1.95±0.05 <sup>a</sup>
16:0	21.42±0.11 <sup>b</sup>	26.55±0.76 <sup>a</sup>	25.36±0.45 <sup>a</sup>	23.01±0.48 <sup>b</sup>
18:0	8.53±0.02 <sup>b</sup>	10.11±0.08 <sup>a</sup>	10.22±0.16 <sup>a</sup>	10.00±0.41 <sup>a</sup>
20:0	1.09±0.05 <sup>b</sup>	1.32±0.00 <sup>a</sup>	1.43±0.03 <sup>a</sup>	1.39±0.04 <sup>a</sup>
Total SFA	32.48±0.12 <sup>c</sup>	39.6±0.88 <sup>ab</sup>	39.06±0.69 <sup>ab</sup>	36.35±0.92 <sup>b</sup>
16:1n-7	1.37±0.10 <sup>b</sup>	2.30±0.08 <sup>a</sup>	2.44±0.15 <sup>a</sup>	2.27±0.15 <sup>a</sup>
18:1n-9	19.02±0.19 <sup>b</sup>	21.35±0.54 <sup>b</sup>	25.59±0.56 <sup>a</sup>	24.70±0.85 <sup>a</sup>
20:1n-9	0.59±0.05 <sup>b</sup>	0.90±0.01 <sup>a</sup>	0.78±0.04 <sup>a</sup>	0.80±0.03 <sup>a</sup>
22:1n-11	0.16±0.01 <sup>c</sup>	0.31±0.00 <sup>a</sup>	0.22±0.00 <sup>b</sup>	0.20±0.01 <sup>bc</sup>
Total MUFA	21.15±0.15 <sup>c</sup>	24.86±0.48 <sup>b</sup>	29.02±0.75 <sup>a</sup>	27.97±0.87 <sup>a</sup>
18:2n-6	17.76±0.31 <sup>b</sup>	20.48±0.93 <sup>ab</sup>	21.09±0.44 <sup>a</sup>	19.97±0.76 <sup>ab</sup>
18:3n-6	0.47±0.07	0.65±0.06	0.74±0.01	0.71±0.07
20:2n-6	1.10±0.09 <sup>b</sup>	1.53±0.03 <sup>a</sup>	1.26±0.02 <sup>ab</sup>	1.32±0.06 <sup>ab</sup>
ARA	12.97±0.16 <sup>b</sup>	15.23±0.65 <sup>ab</sup>	15.72±0.10 <sup>a</sup>	14.57±0.95 <sup>ab</sup>
22:4n-6	0.23±0.02 <sup>c</sup>	0.37±0.00 <sup>a</sup>	0.35±0.00 <sup>ab</sup>	0.29±0.03 <sup>bc</sup>
Total n-6 PUFA	32.52±0.47 <sup>bc</sup>	38.26±1.62 <sup>a</sup>	39.16±0.57 <sup>a</sup>	36.86±1.85 <sup>ab</sup>
18:3n-3	1.88±0.13 <sup>b</sup>	2.09±0.13 <sup>ab</sup>	2.41±0.11 <sup>a</sup>	2.20±0.08 <sup>ab</sup>
18:4n-3	0.36±0.05 <sup>b</sup>	0.51±0.03 <sup>a</sup>	0.42±0.01 <sup>ab</sup>	0.33±0.01 <sup>b</sup>
20:4n-3	1.00±0.07 <sup>b</sup>	1.24±0.08 <sup>ab</sup>	1.29±0.03 <sup>a</sup>	1.18±0.05 <sup>ab</sup>
EPA	15.28±0.34 <sup>a</sup>	13.84±0.72 <sup>ab</sup>	14.25±0.33 <sup>ab</sup>	12.18±0.38 <sup>b</sup>
22:5n-3	3.84±0.15 <sup>a</sup>	2.30±0.17 <sup>b</sup>	2.21±0.03 <sup>bc</sup>	1.69±0.07 <sup>c</sup>
DHA	19.04±0.29 <sup>b</sup>	20.96±0.59 <sup>b</sup>	36.08±0.82 <sup>a</sup>	35.36±0.57 <sup>a</sup>
Total n-3 PUFA	42.00±1.03 <sup>b</sup>	40.33±1.74 <sup>b</sup>	56.67±1.26 <sup>a</sup>	52.95±1.09 <sup>a</sup>
DHA/EPA	1.25±0.01 <sup>c</sup>	1.52±0.04 <sup>d</sup>	2.53±0.00 <sup>c</sup>	2.91±0.06 <sup>b</sup>
TFA	128.15±4.01 <sup>c</sup>	143.05±2.17 <sup>b</sup>	163.92±3.27 <sup>a</sup>	154.13±4.65 <sup>ab</sup>

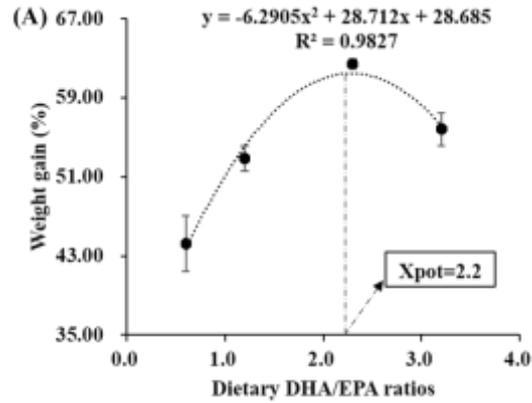
605 Data are presented as means ± SEM (n = 3). Values in the same row with different superscript letters

606 are significantly different ( $P < 0.05$ ).

607 ARA, 20:4n-6; DHA, 22:6n-3; EPA, 20:5n-3; MUFA, monounsaturated fatty acid; PUFA,

608 polyunsaturated fatty acid; SFA, saturated fatty acid; TFA, Total fatty acids.

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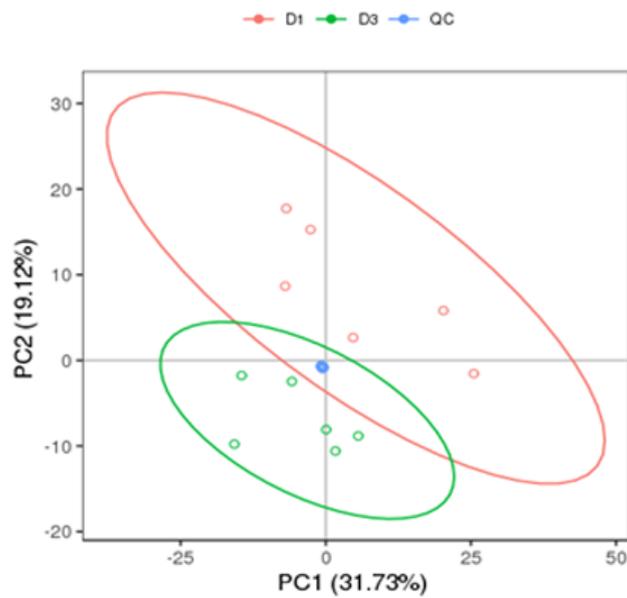
611 **Supplementary Figure 1.** Optimum dietary DHA/EPA requirement of mud crab fed with 7% lipid.

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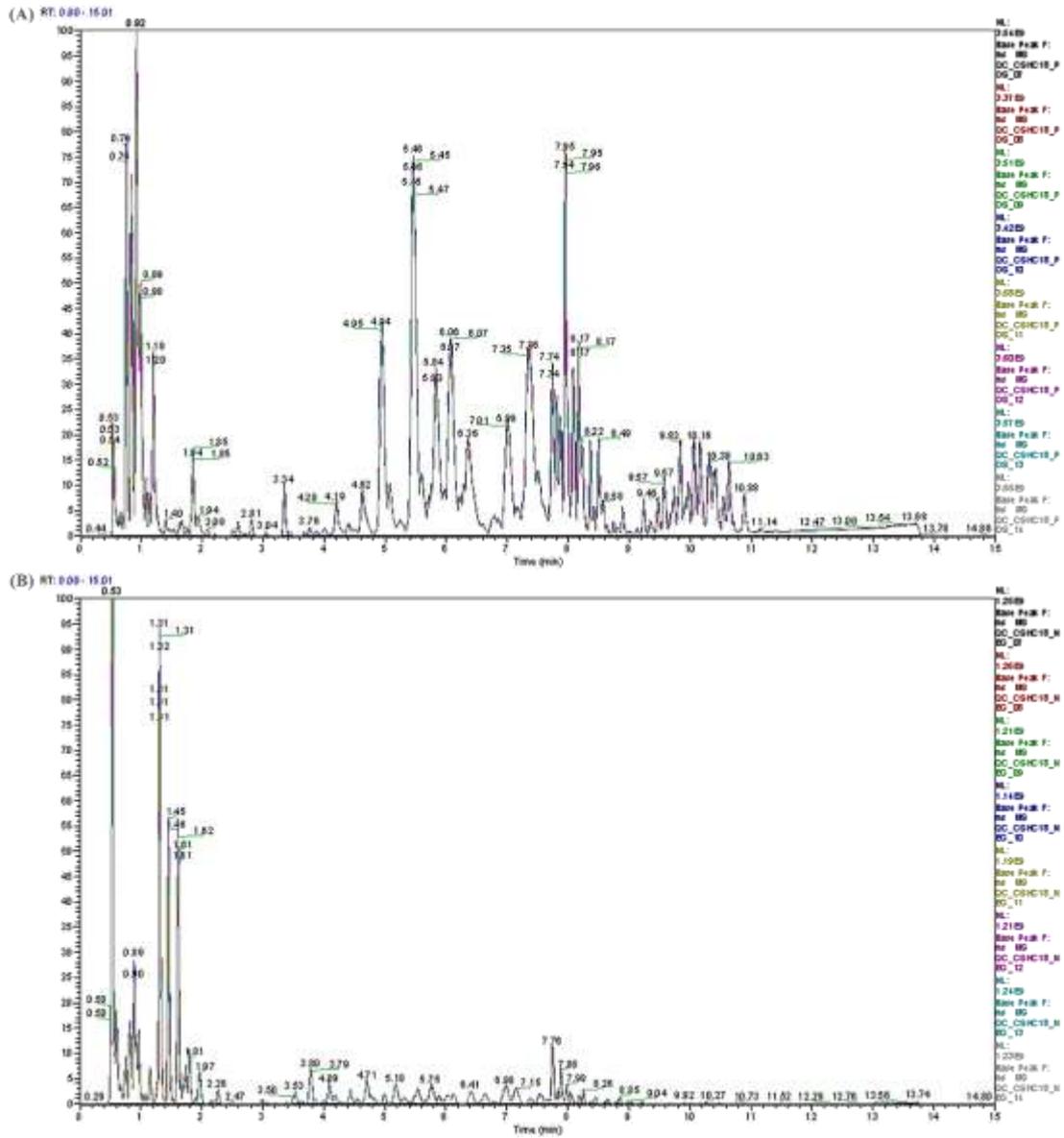
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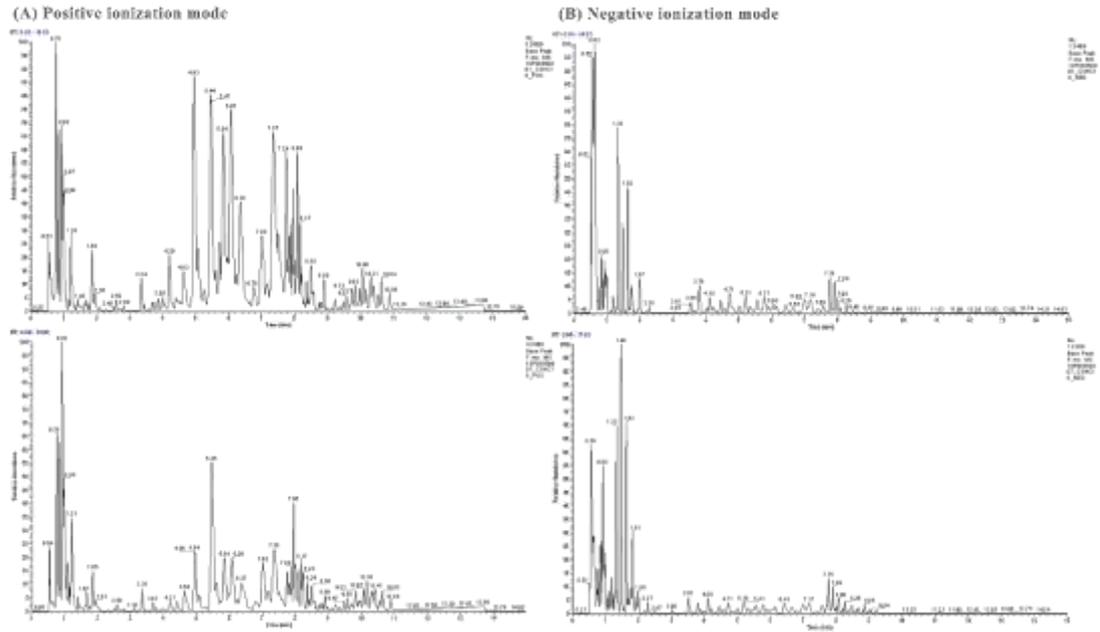
617 **Supplementary Figure 2.** Principal component analysis (PCA) plots for quality control (QC)

618 samples. The horizontal axis represents the first principal component, the ordinate axis represents

619 the second principal component, the numbers are the score of the principal component indicating

620 the ability of the principal component to explain the entire model.





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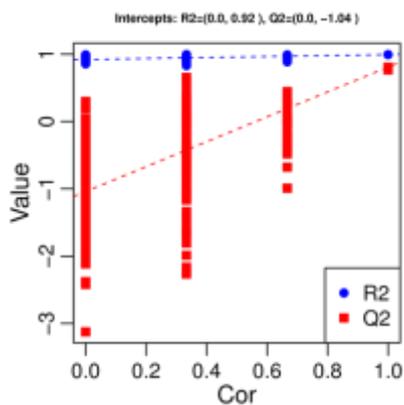
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**Supplementary Figure 4.** The base peak chromatograms (BPC) acquired in positive (A) and negative (B) ionization mode of hepatopancreas of mud crab fed with diet D1 and D3. The spectra of each ionization mode are diets D1 (upper panels) and D3 (lower panels)

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632 **Supplementary Figure 5.** Cross-validation plot of the PLS-DA model of all the lipid species.  $R^2$

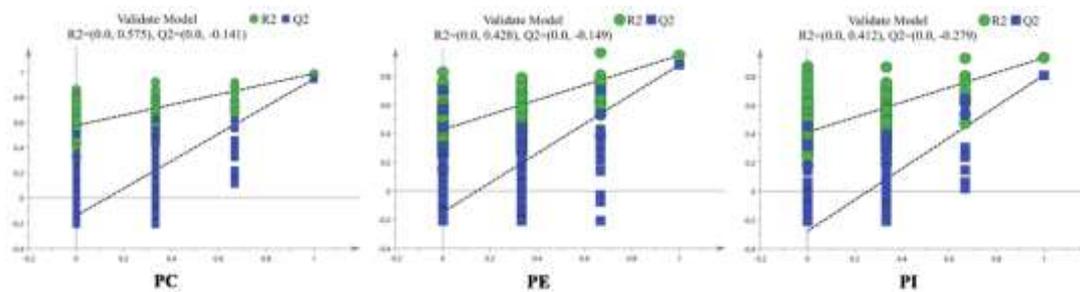
633 and  $Q^2$  are the intercepts of the ordinate axis of the regression lines of  $R^2$  and  $Q^2$ ,  $Q^2$  should be less

634 than 0.

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639 **Supplementary Figure 6.** Cross-validation plot of the PLS-DA model of PC, PE and PI.  $R^2$  and  $Q^2$

640 are the intercepts of the ordinate axis of the regression lines of  $R^2$  and  $Q^2$ ,  $Q^2$  should be less than 0.

641 PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

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