

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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Ethics statement

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

2.2. Diets

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

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

2.3. Feeding trial and sampling

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

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

treatment).

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

In brief, total lipid was extracted from approximately 100 g of diets and freeze-dried hepatopancreas samples using chloroform/methanol (2:1, v:v) according to [Bligh & Dyer, \(1959\)](#) and as described in previous study ([Rey, Alves, Melo, Domingues, Queiroga, Rosa, et al., 2015](#)). Approximately 10 mg of total lipid were placed in a glass test tube along with 1 mL of 23:0 internal standard solution (1 mg mL⁻¹, in HPLC grade hexane > 95%), and dried by Termovap TV10 sample concentrator (Ecom, Czech Republic). Three mL methanolic sulfuric acid solution (1 mL H₂SO₄ : 100 mL methanol : 0.05 g butylated hydroxytoluene [BHT; as antioxidant]) were added to the glass tubes that were then incubated in a water bath at 80 °C for 3 h to produce FAME. One mL hexane and distilled water were added and the mixture vortexed for 1 min and cooled to room temperature. The upper layer was filtered through a lipid phase filter (SCAA-104, ANPEL, China) and the solvent evaporated under a stream of nitrogen and FAME dissolved in 0.5 mL hexane. The FAME samples were analyzed on a GC (GC-MS 7890B-5977A, Agilent Technologies, USA) with the GC-MS operating conditions as described previously ([Yuan, Xu, Jin, Wang, Hu, Zhao, et al., 2021](#)). Fatty acids were identified by their retention time in comparison to a FAME standard solution (FAME 37 MIX, Supelco), and fatty acid concentrations calculated according to the peak area ratio of FAME/23:0 standard.

2.5. Lipid preparation and extraction

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

(SPLASH 330707, SPLASHTM Lipidomix Mass Spec Standard, Avanti Polar Lipids, USA), and then incubated at -20 °C for 1 h.. Following centrifugation at 25 000 rpm for 15 min at 4 °C, 600 µL of supernatant was concentrated by vacuum concentrator (Maxi Vacbeta, GENE COMPANY). The concentrates were resuspended in 200 µL of isopropanol/acetonitrile/H₂O (2:1:1, v:v:v), vortexed for 1 min and incubated at room temperature for 10 min, were stored into -80 °C until further analysis.

2.6. UPLC-MS method for lipidomics

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

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

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

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

The raw MS datasets were imputed into commercially available software, LipidSearch v.4.1 (Thermo Fisher Scientific, USA), to identify peaks on a single sample, and then perform peak alignment on all samples. As for identifying lipids, the mass tolerances for molecular precursors, product and fragment ions were all set at 5 ppm, the thresholds of m-score and c-score were set to 5.0 and 2.0. The adducts form were [M+H]⁺, [M+NH₄]⁺ and [M+Na]⁺ for ESI⁺ mode, and [M-H]⁻, [M-2H]⁻ and [M-HCOO]⁻ for ESI⁻ mode. Peak alignment was then performed on all the identified lipids, with deviation of retention time set at 0.1 min. The grade of identification levels were A, B, C and D where grade A represented the lipid category and all fatty acid chains completely determined, for grade B both class specific ions and fatty acid fragment ions could be detected, for grade C either class-specific ions or fatty acid fragment ions could be detected, and for grade D the lipid structure could not be recognized, such as dehydrated ions. The discovery of outlier and assessment of batch effects was carried out by principal component analysis (PCA) of the dataset being pre-processed ([Supplemental Figure 2](#)). The standardized peak data was further analyzed using metaX, the process of which included 1) removing lipids that were observed in less than 50% of QC or 80% of biological samples, 2) filling missing values using k-Nearest Neighbor algorithm,

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

2.8. Statistical analyses

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

3. Results and Discussion

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

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

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

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

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

Insert Figure 1 here

3.2. Composition of lipid classes in hepatopancreas

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

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) ≥ 1.20 or ≤ 0.83 ($\log_2\text{FC} \geq$
268 0.26 or ≤ -0.27), $P < 0.05$ and Variable Importance in Projection (VIP) ≥ 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 Insert Figure 3 here

3.4. Analysis of the predominant DLMs in hepatopancreas

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

PI, were analyzed further.

Insert Figure 4 here

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

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

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

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

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

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

conducted.

Insert Figure 5 here

Insert Figure 6 here

4. Conclusions

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

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CRedit authorship contribution statement

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

Declaration of competing interest

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

Supplementary data

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

Supplementary Table 2. Fatty acid contents (mg g⁻¹, dry matter) of the experimental diets

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

Supplementary Table 4. Fatty acid contents (mg g⁻¹, dry matter) of hepatopancreas of mud crabs fed with the experimental diets.

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

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

Supplementary Figure 3. The overlap plot of base peak chromatograms (BPC) acquired in positive (A) and negative (B) ionization mode of all the quality control samples.

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.

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

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

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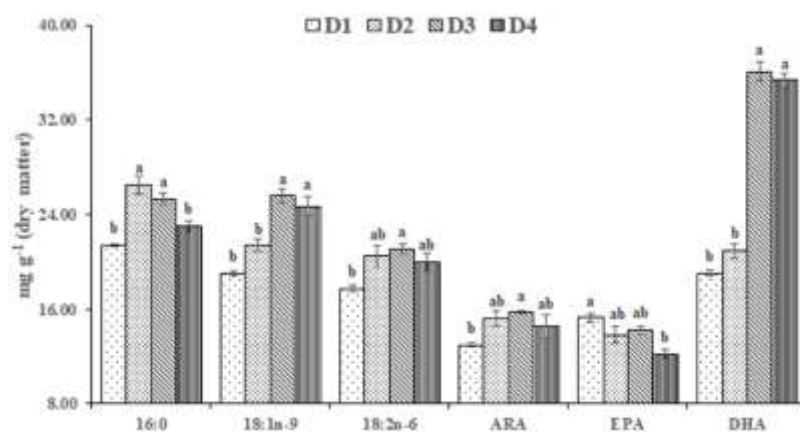


Figure 1. Absolute contents of fatty acids in hepatopancreas of mud crab fed with experiment diets.

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

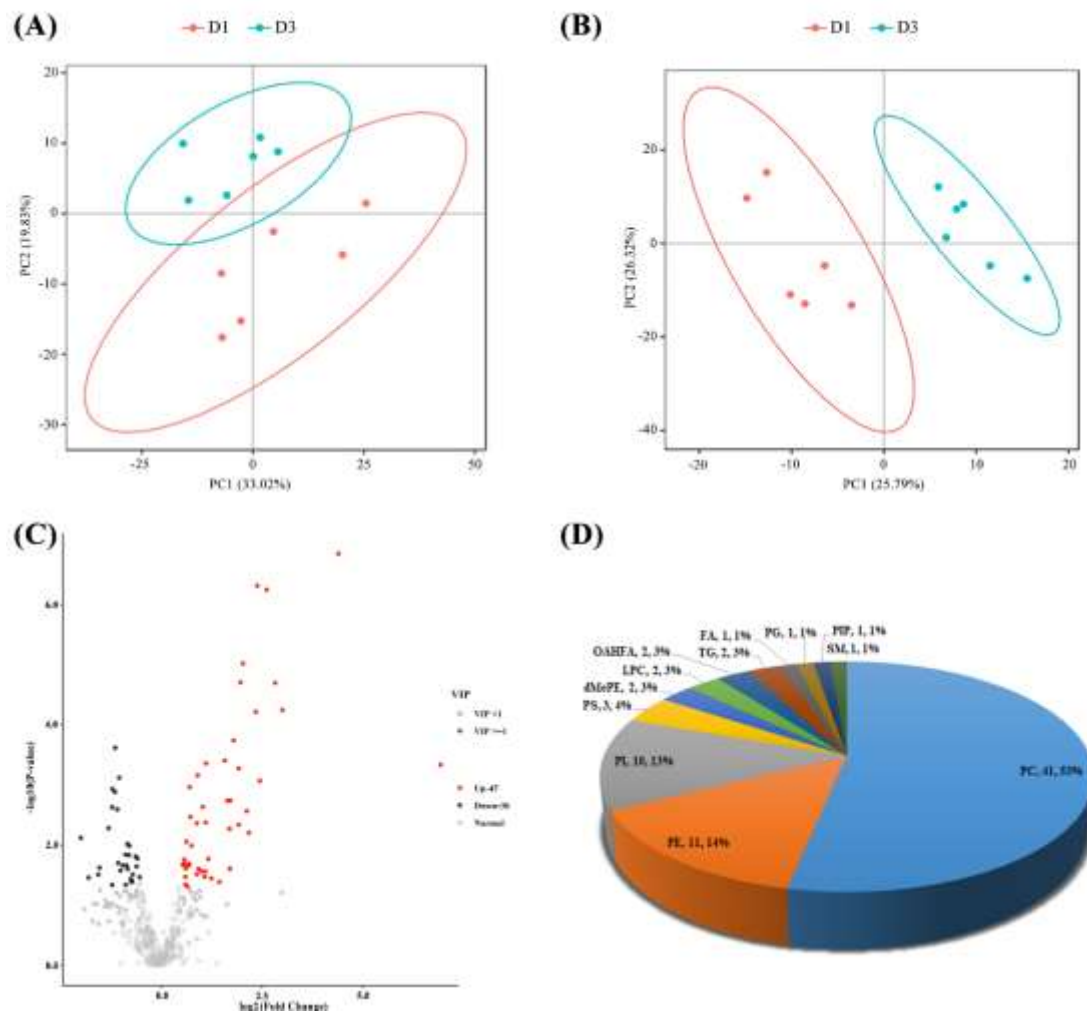


Figure 3. Composition of different lipid metabolites in the hepatopancreas of *S. paramamosain* fed with diet D1 and D3 (DHA/EPA = 0.6 and 2.3, respectively). (A) and (B) in principal component analysis, PCA (A) and partial least squares method-discriminant analysis, PLS-DA (B) plots represent diets D1 and D3, respectively. In volcano plot (C), * and • represent down-regulated and up-regulated lipid metabolites with $\log_2(\text{fold change}) \geq 0.26$ or ≤ -0.27 and $P < 0.05$, circle and × show lipid metabolites with Variable Importance in Projection (VIP) ≥ 1 and VIP < 1 , respectively. (D) the composition of different lipid metabolites (number, percentage).

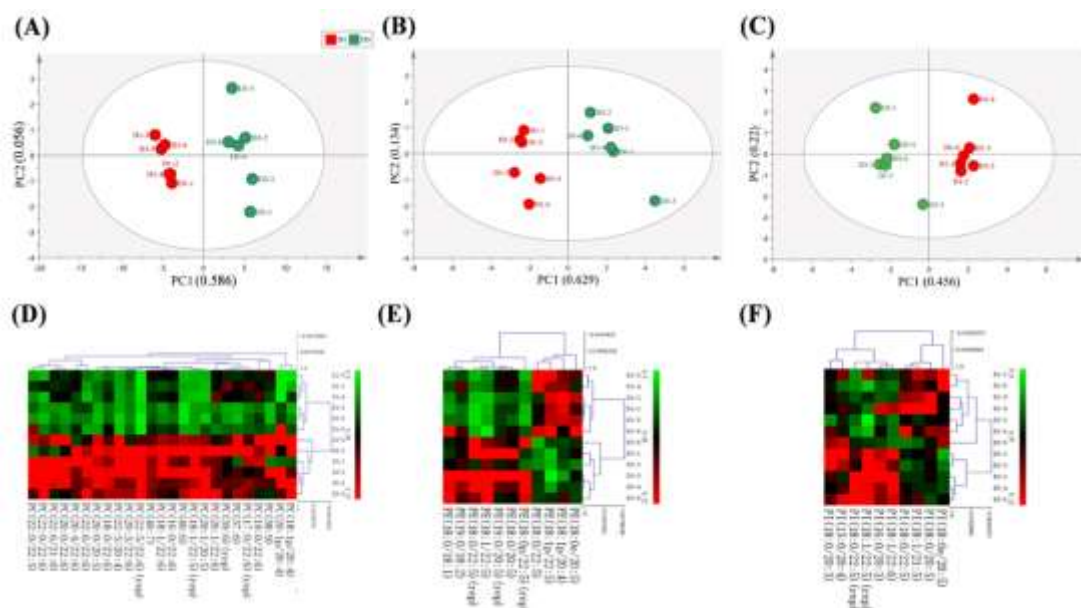


Figure 4. Composition and clustering heatmap of significantly different phosphatidylcholine, PC (A, D), phosphatidylethanolamine, PE (B, E) and phosphatidylinositol, PI (C, F) molecules in the hepatopancreas of *S. paramamosain* fed with diet D1 and D3. ● and ● in PLS-DA plots A, B and C, represent diets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

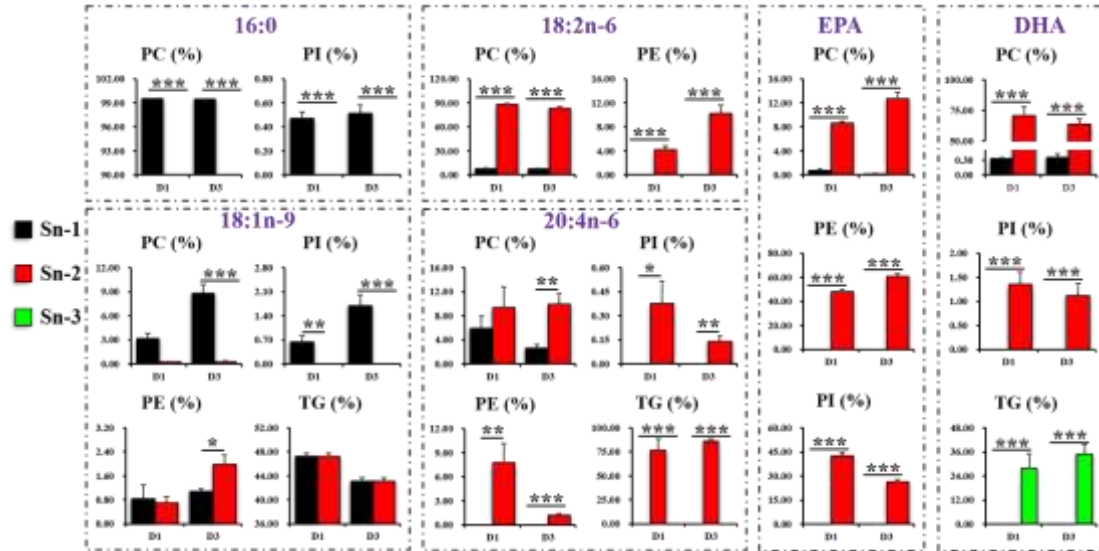


Figure 5. The positional distributions of key fatty acids in phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and triacylglycerol (TG) molecules in hepatopancreas of *S. paramamosain* fed with diet D1 and D3. Values are means \pm SEM (n = 6). *, ** and *** represent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

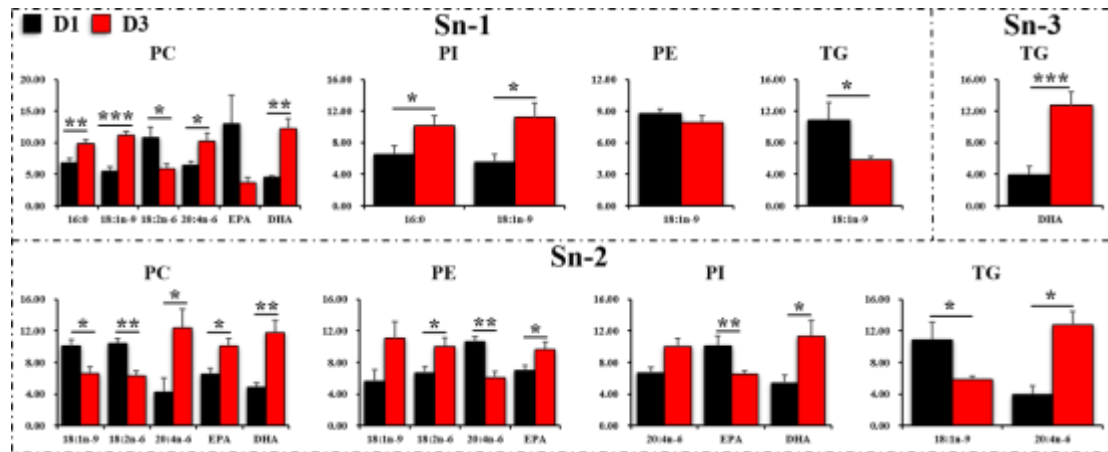


Figure 6. Effects of dietary DHA/EPA ratio on positional distributions of key fatty acids in PC, PE, PI and TG molecules in hepatopancreas of *S. paramamosain* fed with diet D1 and D3. Values are means \pm SEM (n = 6). *, ** and *** represent significant differences with $P < 0.05$, $P < 0.01$ and $P < 0.001$.

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

Ingredients	D1	D2	D3	D4
Casein ¹	25.00	25.00	25.00	25.00
Soy protein concentrate ²	27.61	27.61	27.61	27.61
Wheat flour	25.26	25.26	25.26	25.26
DHA-enriched oil ³	0.00	1.28	2.57	3.20
EPA-enriched oil ⁴	2.96	2.22	1.48	1.11
ARA-enriched oil ⁵	0.50	0.50	0.50	0.50
Palmitic acid ⁶	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 ⁷	1.00	1.00	1.00	1.00
Mineral premix ⁷	1.50	1.50	1.50	1.50
Ca(H ₂ PO ₄) ₂	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

¹ Casein: 89.6% crude protein and 0.2% crude lipid.

² Soy protein concentrate: 69.9% crude protein and 0.5% crude lipid.

³ DHA-enriched oil: extracted from marine microalgae, DHA content, 406.5 mg g⁻¹ oil (Changsha Kenan Biotechnology Co., Ltd., China).

⁴ EPA-enriched oil: extracted from marine microalgae, EPA content, 462.5 mg g⁻¹ oil, DHA content, 235.6 mg g⁻¹ oil (Changsha Kenan Biotechnology Co., Ltd., China).

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

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

⁷ Vitamin and mineral premixes were based on Jin et al. (2015).

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

589 **Supplementary Table 2.** Fatty acid contents (mg g⁻¹, 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 ¹	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 ²	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 ³	2.19	2.24	2.12	2.15
22:4n-6	0.16	0.29	0.09	0.07
Total n-6 PUFA ⁴	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 ⁵	10.37	8.18	5.48	4.57
22:5n-3	1.28	1.02	0.68	0.54
DHA ⁶	6.45	9.92	12.33	14.49
Total n-3 PUFA ⁷	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 ⁸	18.53	19.50	18.88	20.03

590 Data are means of duplicate analyses.

591 ¹ SFA, saturated fatty acids: 14:0, 16:0, 18:0, 20:0.

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

593 ³ ARA, 20:4n-6

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

595 ⁵ EPA, 20:5n-3. ⁶ DHA, 22:6n-3.

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

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

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

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

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

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

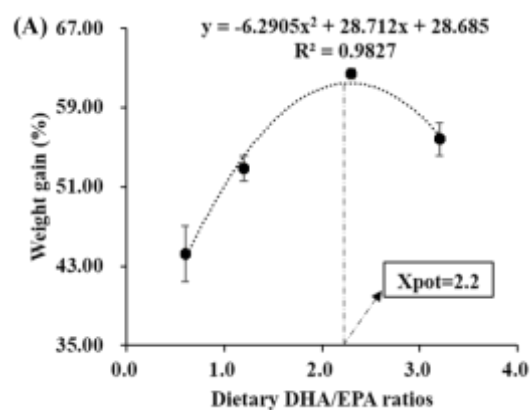
MF, molting frequency; SGR, specific growth rate; WG, weight gain.

Supplementary Table 4. Fatty acid contents (mg g⁻¹, dry matter) of hepatopancreas of mud crabs
fed with the experimental diets

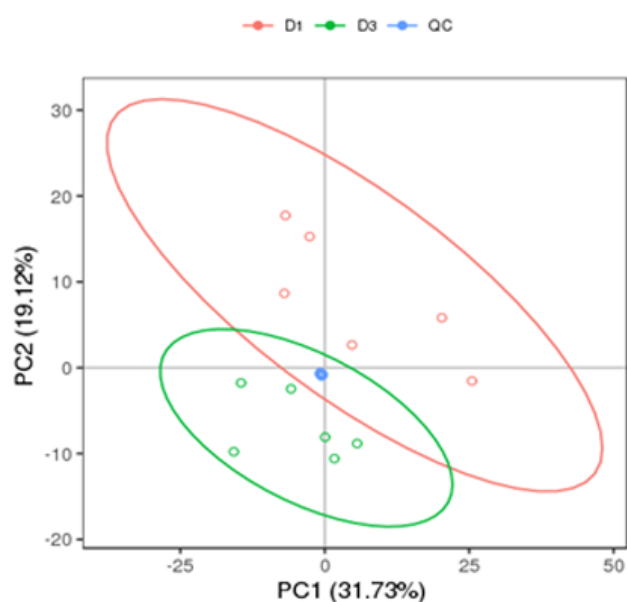
Fatty acids	Diet			
	D1	D2	D3	D4
14:0	1.43±0.08 ^b	1.62±0.04 ^b	2.06±0.05 ^a	1.95±0.05 ^a
16:0	21.42±0.11 ^b	26.55±0.76 ^a	25.36±0.45 ^a	23.01±0.48 ^b
18:0	8.53±0.02 ^b	10.11±0.08 ^a	10.22±0.16 ^a	10.00±0.41 ^a
20:0	1.09±0.05 ^b	1.32±0.00 ^a	1.43±0.03 ^a	1.39±0.04 ^a
Total SFA	32.48±0.12 ^c	39.6±0.88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

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

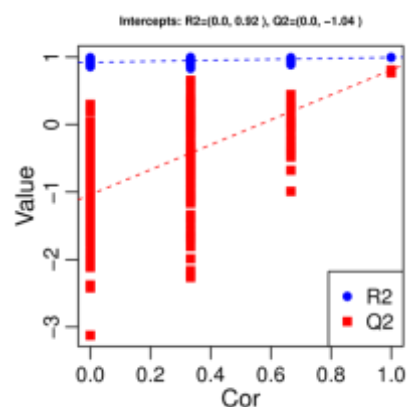
ARA, 20:4n-6; DHA, 22:6n-3; EPA, 20:5n-3; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TFA, Total fatty acids.



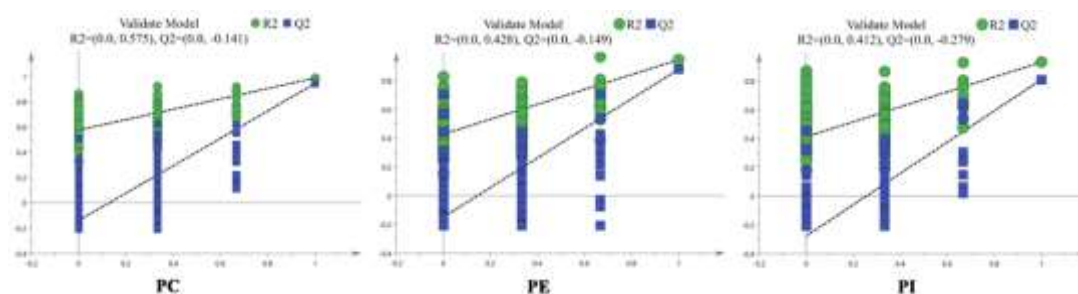
Supplementary Figure 1. Optimum dietary DHA/EPA requirement of mud crab fed with 7% lipid.



Supplementary Figure 2. Principal component analysis (PCA) plots for quality control (QC) samples. The horizontal axis represents the first principal component, the ordinate axis represents the second principal component, the numbers are the score of the principal component indicating the ability of the principal component to explain the entire model.



Supplementary Figure 5. Cross-validation plot of the PLS-DA model of all the lipid species. R^2 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 than 0.



Supplementary Figure 6. Cross-validation plot of the PLS-DA model of PC, PE and PI. R^2 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 than 0. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

