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Research Article

Protein-Sparing Effect of α-Lipoic Acid in Diets with Different Protein/Carbohydrate Ratios for the Oriental River Prawn, *Macrobrachium nipponense*

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Carbohydrates are commonly used in aquaculture feed because they are the cheapest energy source. Promoting carbohydrate catabolism for energy production can increase the dietary protein utilization efficiency (i.e., a protein-sparing effect). Alpha-lipoic acid (α-LA) is a cofactor of some rate-limiting key enzymes (pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase) in carbohydrate metabolism. To determine whether α-LA could promote carbohydrate catabolism to have a protein-sparing effect, we investigated the growth, activities of key enzymes involved in carbohydrate metabolism, transcript levels of genes encoding enzymes involved in energy metabolism, and the hepatopancreas structure of *Macrobrachium nipponense* fed with diets containing different protein/carbohydrate ratios (P/C) with and without α-LA. Six experimental diets for *M. nipponense* were formulated with casein and fish meal as the protein sources, fish oil and soybean oil as the lipid sources, and corn starch as the carbohydrate source. The six diets consisted of three different P/Cs (P41/C18, P37/C24, and P33/C30) without or with α-LA (at 1300 mg/kg). Each diet had six replicates and was fed to prawns (initial weight 0.110 ± 0.010 g) twice daily to apparent satiation. Dietary supplementation with α-LA significantly increased the survival rate of prawns, regardless of the P/C ratio. In the low-P/C group (P33/C30), the weight gain did not vary between those that consumed α-LA and those that did not. The P/C ratio and α-LA significantly affected the activities of key enzymes involved in glycolysis and the citric acid cycle. In the P41/C18 group, the hexokinase (HK) and pyruvate dehydrogenase (PDH) activities were significantly higher in prawns that consumed α-LA than in those that did not. In both the P37/C24 and P33/C30 groups, the pyruvate kinase (PK) activity was significantly higher in prawns that consumed α-LA than in those that did not. Significant interactions between P/C and α-LA were found for the transcript levels of genes encoding adenine ribonucleotide-dependent protein kinase subunits (AMPK αand AMPK β), HK, glucose-6-phosphate dehydrogenase (G6PDH), acetyl-CoA carboxylase (ACC), and fatty acid binding protein 10 (FABP 10), all of which are involved in energy metabolism. In the low-P/C group, α-LA increased the transcript levels of genes encoding AMPK α, AMPK β, HK, phosphoenolpyruvate carboxykinase (PEPCK), G6PDH, and MDH, decreased the transcript level of the gene encoding ACC, and did not affect the transcript levels of genes encoding FABP10 and palmitoyl transferase 1 (CPT1). The P/C ratio and α-LA did not affect the overall morphology of the hepatopancreas, but at a low P/C, dietary supplementation of α-LA increased the number of hepatopancreatic B cells. In conclusion, supplementation with α-LA at 1300 mg/kg in a low-P/C diet of *M. nipponense* increased its carbohydrate utilization efficiency, energy metabolism, and the number of hepatopancreas B cells, thereby having a protein-sparing effect.

1. Introduction

Fish meal is an excellent protein source in aquafeeds because it has a high digestible protein content and a balanced essential amino acid profile [1]. However, animal proteins, including fish meal, are usually the most expensive ingredient, whether or not they are scarce. Therefore, it is important to find equally functional and economical alternatives to fish meal or other animal proteins to formulate diets for aquacultured animals. Omnivorous crustaceans digest grass more efficiently than carnivorous ones [2], so they can potentially utilize plant proteins/carbohydrates. Improving the efficiency of carbohydrate utilization may be one way to reduce the proportion of fish meal protein in feed for crustaceans.

Carbohydrates are the cheapest energy source and are often included in artificial diets for fish or crustaceans [3]. The protein-saving effect of carbohydrates in the diet of crustaceans has been demonstrated in several studies. For example, juvenile *Litopenaeus vannamei* fed protein (P) and carbohydrate (C) at ratios of P30/C25 and P34/C19 showed normal growth and the carbohydrate had a protein-sparing effect [4]. Carbohydrates in the diet of *Macrobrachium rosenbergii* also had a protein-sparing effect [5]. The addition of starch or dextrin to decrease the dietary protein content did not reduce the weight gain or the feed efficiency ratio of *Penaeus monodon* [6]. The addition of carbohydrates to aquafeed to save protein can decrease the excretion of nitrogenous compounds in aquatic ecosystems that lead to environmental pollution [7, 8]. Thus, increasing the proportion of digestible carbohydrates in aquafeeds is one of the most promising solutions for sustainable aquaculture production [9]. However, crustaceans have a limited ability to utilize carbohydrates and cannot adapt to a high carbohydrate intake [3]. Therefore, the increases in carbohydrate levels in the diet of crustaceans should be in the appropriate range to have a protein-saving effect, without negatively affecting the animal's growth and development.

Alpha-lipoic acid (α-LA) is a universal antioxidant and a cofactor of key enzymes in glycolysis (pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase). Therefore, it plays an important role in regulating energy metabolism [10]. Several studies have demonstrated its antioxidant role in a variety of aquatic animals. For example, the antioxidant capacity was altered in Pacific white shrimp (*Litopenaeus vannamei*) fed with a diet containing 196.4 ± 70.2 mg α-LA/kg feed [11], in northern snakehead (*Channa argus*) fed with a diet containing 300–1500 mg α-LA/kg feed [12], and catfish (*Corydoras paleatus*) fed with a diet containing 70 mg α-LA/kg body mass [13]. The regulatory function of α-LA in carbohydrate metabolism has been well studied in higher animals [14–16], but less so in aquatic animals, with the only studies so far focusing on carp (*Cyprinus carpio*) and oriental river prawn (*Macrobrachium nipponense*). The study on *C. carpio* showed that dietary α-LA at 1400 mg/kg feed regulated lipid, protein, and carbohydrate metabolism and improved the utilization efficiency of crude starch [17]. The study on *M. nipponense* showed that α-LA at 1000–2000 mg/kg feed improved its antioxidant status, corn starch metabolism, and hepatopancreas structure [18]. Therefore, we hypothesized that α-LA can enhance the protein-sparing effect of carbohydrates in the diet.

The oriental river prawn, *M. nipponense*, is an important freshwater aquaculture species in China, Japan, and other South-East Asian countries. The farmed prawns have a high protein requirement and there is a strong dependence on fish meal to provide protein in the diet [19]. Considering these facts, this study aimed to evaluate the protein-sparing effect of α-LA in the diet of *M. nipponense* under different protein/carbohydrate (P/C) ratios. We determined the effects of different diets on prawn growth, activities of key enzymes in carbohydrate metabolism, transcript levels of genes related to energy metabolism, and hepatopancreas structure. The results of this study demonstrate that α-LA can promote carbohydrate catabolism, ultimately having a protein-sparing effect.

2. Materials and Methods

2.1. Experimental Diets. Six isolipidic and isonitrogenous diets for *M. nipponense* were formulated with casein and fish meal as the protein sources, fish oil and soybean oil as the lipid sources, and corn starch as the carbohydrate source. The diets had three different P/C ratios (P41/C18, P37/C24, and P33/C30) without or with α-LA at 1300 mg/kg (Table 1). The calculation of P/C ratios was based on the crude protein and corn starch contents in the diet. The α-LA was obtained from the Shanghai Yuanye Biotechnology Co., Ltd, and the concentration of α-LA used was based on our previous study [20]. The dry raw materials were thoroughly pulverized, sieved through a 212-μm sieve, and weighed accurately according to each recipe. After all dry materials were mixed, fish oil and soybean oil were added, and the mixture was made into a dough by adding an appropriate amount of distilled water. The dough was formed into pellets using an F-26 twin-screw extruder (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China), and the pellets were dried in a forced-air oven at 40°C. The final pellets were about 1.5 mm in size and were stored at −20°C until use.

2.2. Experimental Animals and Feeding Trial. Healthy prawns were obtained from a local breeding farm (Huzhou, China). Before the trial, prawns were reared in a 300-L tank and fed with a commercial diet (39% protein, 8% lipid) for 1 week to acclimatize to these experimental conditions. At the beginning of the feeding trial, healthy prawns (average initial weight 0.110 ± 0.010 g) were randomly selected and distributed among 36 tanks (six groups, six replicates). Each tank had a 300-L volume and held 40 prawns. The prawns were fed to apparent satiation twice daily (8:00 h, 17:00 h), and the feeding trial lasted for 50 days. During the experimental period, one-third of the water in each tank was replaced daily. The water quality parameters were as follows: temperature 25–28°C, dissolved oxygen > 6.5 mg·L^{-1}, and ammonia and nitrate levels < 0.1 mg·L^{-1}.

Table 1: Composition and nutrient level of experimental feeds (air-dried basis, %).

Ingredients	Composition of diet (%)					
	0 mg/kg α-lipoic acid			1300 mg/kg α-lipoic acid		
	P41/C18	P37/C24	P33/C30	P41/C18	P37/C24	P33/C30
Casein	21	21	21	21	21	21
Fish meal	32	26	20	32	26	20
Corn starch	18	24	30	18	24	30
α-lipoic acid	0	0	0	0.13	0.13	0.13
Fish oil: Soybean oil (2 : 1)	3.4	3.9	4.4	3.4	3.9	4.4
Soybean lecithin	0.5	0.5	0.5	0.5	0.5	0.5
Cholesterol	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix[(1)]	2	2	2	2	2	2
Mineral premix[(2)]	3	3	3	3	3	3
Attractant[(3)]	3	3	3	3	3	3
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5
Cellulose	14.1	13.6	13.1	13.97	13.47	12.97
Sodium carboxymethylcellulose	2	2	2	2	2	2
Total	100	100	100	100	100	100
Nutrient level [(4)]						
Dry matter	94.28	94.26	94.46	94.33	94.44	94.47
Crude protein	40.87	36.76	32.94	40.59	37.02	33.12
Crude lipid	6.42	6.45	6.52	6.43	6.48	6.51
Ash	7.43	6.51	5.65	7.47	6.56	5.66

[(1)]Vitamin premix contained the following (per kg): vitamin A 4,200,000 IU, vitamin C 60 g, α-tocopherol acetate 20 g, vitamin D3 1,200,000 IU, vitamin K 10 g, vitamin B_1 7.5 g, vitamin B_2 10 g, vitamin B_6 16 g, vitamin B_{12} 20 mg, nicotinic acid 50 g, folic acid 4 g, inositol 60 g, biotin 100 mg, calcium pantothenate 35 g. [(2)]Mineral premix contained the following (per kg): KCl 28 g, $MgSO_4 \cdot 7H_2O$ 100 g, NaH_2PO_4 215 g, KH_2PO_4 100 g, Ca $(H_2PO_4)_2 \cdot H_2O$ 265 g, $CaCO_3$ 105 g, $C_6H_{10}CaO_6 \cdot 5H_2O$ 165 g, $FeC_6H_5O_7 \cdot 5H_2O$ 12 g, $ZnSO_4 \cdot 7H_2O$ 4.76 g, $MnSO_4 \cdot H_2O$ 1.07 g, $AlCl_3 \cdot 6H_2O$ 0.15 g, $CuCl_2 \cdot 2H_2O$ 0.24 g, $CoCl_2 \cdot 6H_2O$ 1.4 g, KI 0.23 g, α-cellulose 2.15 g. [(3)]Attractant: alanine 0.6%, glycine 0.6%, glutamic acid 0.6%, betaine 1.2%. [(4)]Crude protein, crude lipid, ash, and dry matter are measured values.

2.3. Sample Collection. Prawns were fasted for 24 h before sampling. The prawns were counted and weighed to determine the growth performance in each tank. The hepatopancreas was dissected from cephalothorax with sterilized tweezers and scissors. Some hepatopancreas samples were fixed in Bouin's solution for 24 h before histological analysis, and some were stored in labeled tubes at −80°C until subsequent analyses of relevant indicators.

2.4. Analytical Indicators and Methods

2.4.1. Proximate Composition Analysis. The moisture, crude lipid, and crude protein contents of the diets were determined using standard procedures [21]. Moisture was determined by drying the sample (105°C) to a constant weight. Crude protein was measured by the combustion method, using an Elementar rapid N exceed analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Crude lipid was determined by ether extraction using a Soxtec System (Soxtec Avanti 2055; Foss Tecator, Höganäs, Sweden).

2.4.2. Growth Performance and Activities of Enzymes Involved in Carbohydrate Metabolism. The weight gain, specific growth rate, and survival rate of *M. nipponense* were calculated as described previously. To detect enzyme activities, each hepatopancreas sample was homogenized with precooled saline (0.86% w/v) and then the mixture was centrifuged at 1500 g using an Eppendorf 5810 centrifuge (Eppendorf, Hamburg, Germany) at 4°C for 20 min. The supernatant was transferred to a 1.5-mL microtube for further enzyme activity determinations.

The activities of hexokinase (HK), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), and pyruvate dehydrogenase (PDH) in the hepatopancreas were determined as indicators of carbohydrate metabolism and analyzed using kits purchased from the Suzhou Comin Biotechnology Co. Ltd. (Suzhou, China) according to the manufacturer's instructions. Details of the methods used for these analyses have been described by Liu et al. [22]. The absorbance values of reaction mixtures were determined using a Thermo Multiskan Spectrum spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.4.3. Cell Morphology in the Hepatopancreas. To observe cell morphology, the fixed hepatopancreas samples from prawns were dehydrated using an alcohol gradient. Dehydrated tissues were embedded in paraffin and the blocks were cut into continuous 5-μm sections. To reveal changes in the hepatopancreas, the 5-μm sections were stained with hematoxylin and eosin and observed under a microscope. The micrographs were analyzed using CaseViewer software (3DHISTECH Ltd., Budapest, Hungary).

2.4.4. Analysis of Gene Transcript Profiles. Total RNA was extracted using the Trizol method, and the RNA concentration and purity were determined using a Thermo NanoDrop 2000 nucleic acid and protein analyzer (Thermo

Table 2: Sequences of primers used for qRT-PCR analyses of *Macrobrachium nipponense*.

Gene	Primer	Product length (bp)	ID
β-actin	S: 5′- CTGTGCCCATCAACGAGG-3′ A: 5′- GCGGTGGTAGTGAAGGTGTA-3′	127	KY780298.1
AMPK α	S: 5′- ACAGGGACAAAGGTAGCG-3′ A: 5′- ATGTGTGGATGGCGAAAC-3′	116	MG792549.1
AMPK β	S: 5′- TCGTCTCGTAGGGTGTAGG-3′ A: 5′- TAGGCTGATGGTAGGTGGA-3′	191	MG792548.1
AMPK γ	S: 5′- CCTGGCATCCGACAATCCT-3′ A: 5′- GCTGGTGGTGGTGGTAGTAA-3′	188	MG792547.1
HK	S: 5′- GAAGCCTAATGGGAGAAGTT-3′ A: 5′-ATTATGGTTGGACCAGGAG-3′	105	KY270495.1
PEPCK	S: 5′- CCTGCTGCTCATCCCAACT-3′ A: 5′- GCTGCTCCTACCATTACGC-3′	185	MW815348.1
G6PDH	S: 5′-CCCTCCTTCGGTGTTCG-3′ A: 5′-GGTAGTGGTCAATGCGGTA-3′	182	MK307768.1
ACC	S: 5′-GCAGCATTGGAGGTGTATGT-3′ A: 5′-GGATGAGATGATGGCAGCAG-3′	125	KP690138.1
FABP 10	S: 5′- CCGAATGGCTTTCTGCT-3′ A: 5′- GAGGCTGCTTCCGCTAA-3′	224	JN995589.1
CPT1	S: 5′-CCGTCCAATGCTGTATTCCT-3′ A: 5′- GCATAGGCCTCATCATCCAT-3′	120	KP690136.1

Scientific). Total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Takara, Otsu, Japan) and stored at −20°C until use.

The transcript levels of genes encoding enzymes involved in carbohydrate, lipid, and energy metabolism in the hepatopancreas were examined by quantitative real-time polymerase chain reaction (qRT-PCR). We determined the transcript levels of genes encoding hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), phosphoenolpyruvate carboxykinase (PEPCK), acetyl-CoA carboxylase (ACC), palmitoyl transferase 1 (CPT1), fatty acid-binding protein 10 (FABP 10), and three AMP-activated protein kinase (AMPK) subunits (AMPKα, AMPKβ, and AMPKγ). Each qRT-PCR reaction mixture had a volume of 20 μL and consisted of 10 μL 2 × SYBR Green Premix Ex Taq, 0.2 μL each primer (10 μM), 2 μL cDNA, and 7.6 μL ddH_2O. The thermal cycling conditions for qPCR were as follows: 10 min at 95°C, followed by 40 cycles of 10 s at 94°C, 30 s at 58°C, and 32 s at 72°C. The melting curve (from 65°C to 95°C, increasing by 0.5°C per 5 s) was plotted after the PCR reaction to verify the identity of the amplified product. The reference gene was *β-Actin*. The sequences of primers used to amplify genes are shown in Table 2, and the relative gene transcript levels were calculated using the $2^{-\Delta\Delta CT}$ quantitative method [23].

2.5. Statistical Analyses. Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA), and the data are expressed in figures and tables as mean ± standard deviation (SD). GraphPad Prism9.0 was used to draw graphs. Two-way analysis of variance (ANOVA) was used to determine whether there was a significant interaction between α-LA and P/C in the diet. At the same α-LA level, one-way analysis of variance (ANOVA) was used to evaluate the effects of different P/C on *M. nipponense* after testing for normality and homogeneity of variance. Within the same P/C group, independent samples *t*-test was used to detect statistically significant differences between the two groups (with or without α-LA in the diet). A P value less than 0.05 was considered significant; and $P < 0.01$ was considered extremely significant.

3. Results

3.1. Growth Performance of Prawns. The two-factor analysis revealed a significant interaction between the P/C ratio and α-LA concentration in terms of the survival rate, weight gain, and specific growth rate of prawns ($P < 0.05$) (Figure 1). At all P/C ratios, the survival rate of prawns was significantly higher for those that consumed α-LA than for those that did not ($P < 0.05$) (Figure 1(a)). In the high- and medium-P/C groups (P41/C18 and P37/C24), the weight gain (Figure 1(b)) and specific growth rate (Figure 1(c)) were extremely significantly lower for prawns that consumed α-LA than for those that did not ($P < 0.01$). However, in the low-P/C group (P33/C30), there was no significant difference in the weight gain rate and specific growth rate between prawns that consumed α-LA and those that did not (Figures 1(b) and 1(c)).

3.2. Activities of Enzymes Involved in Carbohydrate Metabolism. The two-factor analysis revealed a significant interaction between the P/C ratio and α-LA concentration in terms of HK, PEPCK, and PDH activities ($P < 0.05$) (Figure 2). The P/C ratio was the main factor affecting the HK activity (Figure 2(a)). In the high-PC group (P41/C18), HK and PDH activities were significantly higher in the prawns that consumed α-LA than in those that did not ($P < 0.01$) (Figures 2(a) and 2(d)). In the medium- and low-P/C groups (P37/C24 and P33/C30), the PK activity (Figure 2(b)) was significantly higher ($P < 0.05$) in prawns that consumed α-LA than in those that did not. Neither α-LA nor the dietary P/C ratio significantly affected the PEPCK activity (Figure 2(c)).

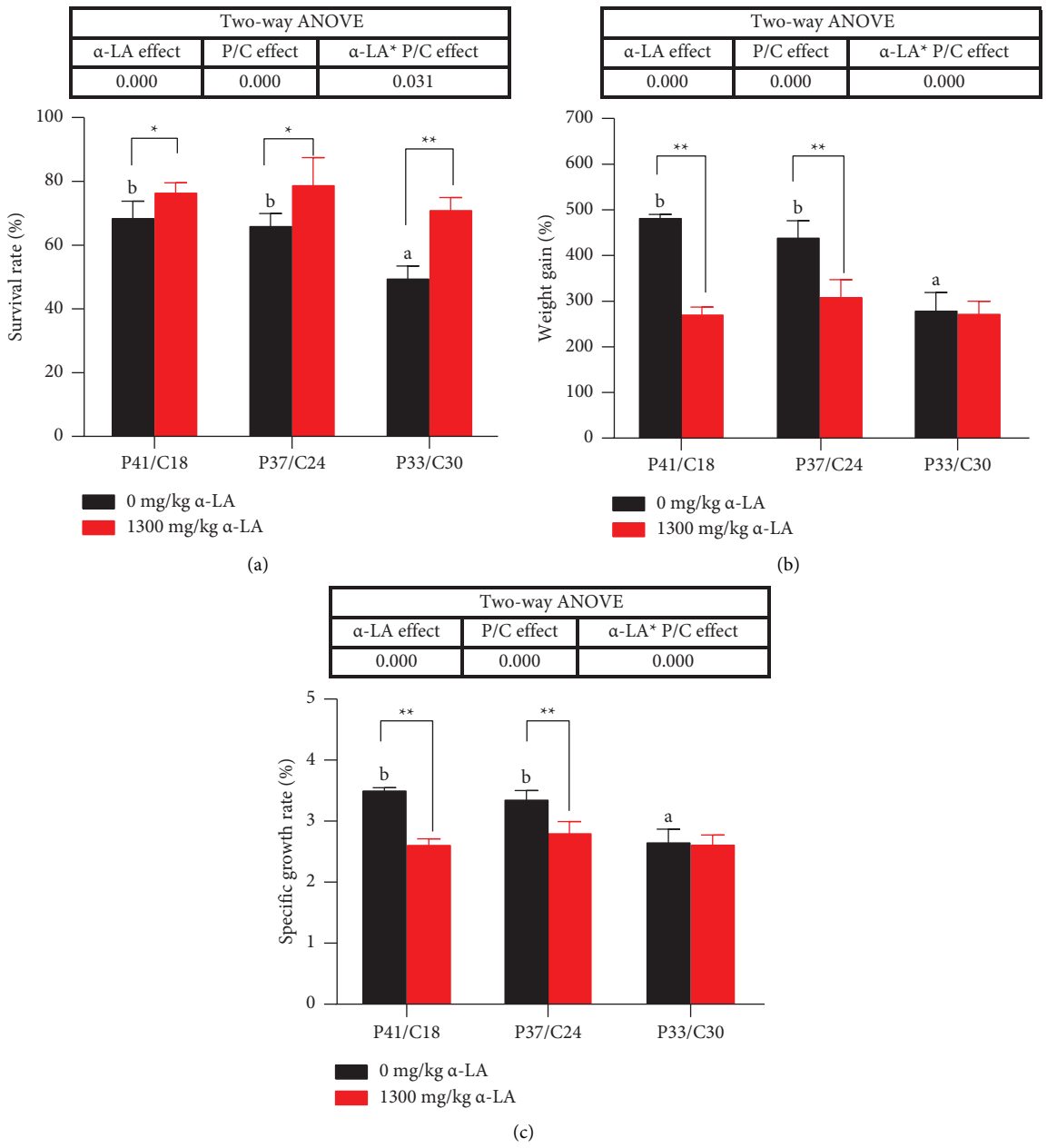

Figure 1: Effects of α -LA in diets with three different protein/carbohydrate (P/C) ratios on the growth performance of *Macrobrachium nipponense*. Data are mean ± SD. Different superscript letters indicate significant difference at $P < 0.05$: lowercase letters denote significant differences among P/C groups without α-LA supplementation; capital letters denote significant differences among P/C groups fed with a diet containing α-LA at 1300 mg/kg. *denotes significant difference within each P/C group without and with α-LA. ** denotes extremely significant difference within each P/C group without and with α-LA.

3.3. Transcript Levels of Genes Encoding Enzymes Involved in Carbohydrate, Lipid, and Energy Metabolism in the Hepatopancreas. The results of two-factor ANOVA showed that there was an extremely significant interaction between the P/C ratio and α-LA concentration in terms of the transcript levels of *AMPK α*, *AMPK β*, *HK*, *G6PDH*, *ACC*, and *FABP 10* ($P < 0.01$) (Figures 3 and 4). In the low-P/C group (P33/C30), the transcript levels of *AMPK α*, *AMPK β*, *HK*, *PEPCK*, and *G6PDH* in the hepatopancreas were significantly higher in prawns that consumed α-LA than in those that did not ($P < 0.01$). In contrast, the transcript level of *ACC* was significantly lower in prawns that consumed α-LA than in those that did not. Dietary supplementation with α-LA did not affect the transcript levels of *FABP10* or *CPT1* (Figure 4).

3.4. Cell Morphology in the Hepatopancreas. The P/C ratio and α-LA concentration affected the number of blister cells (B cells) and resorptive cells (R cells) in the hepatopancreas. Among the diets without α-LA, the low-P/C group had fewer hepatopancreas B cells compared to the medium- and high-P/C groups (Figures 5(a), 5(c) and 5(e)). In the high- and

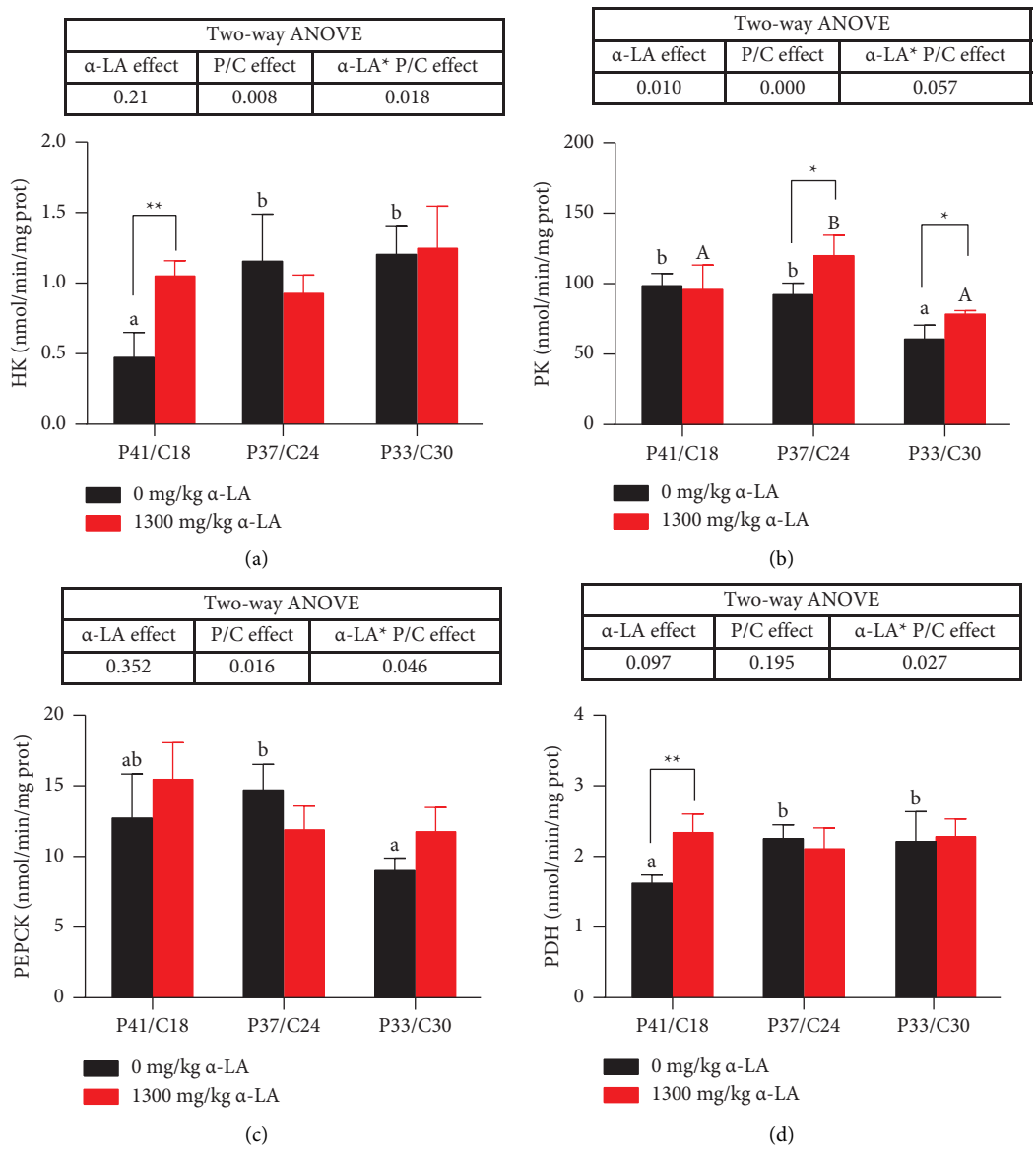

FIGURE 2: Effects of α-LA in diets with three different P/C ratios on activities of carbohydrate metabolic enzymes in the hepatopancreas of *M. nipponense*. Data are mean ± SD. Different superscript letters indicate significant difference at $P < 0.05$: lowercase letters denote significant differences among P/C groups without α-LA supplementation; capital letters denote significant differences among P/C groups fed with a diet containing α-LA at 1300 mg/kg. *denotes significant difference within each P/C group without and with α-LA. Hexokinase, HK; pyruvate kinase, PK; phosphoenolpyruvate carboxykinase, PEPCK; pyruvate dehydrogenase, PDH. ** denotes extremely significant difference within each P/C group without and with α-LA.

medium-P/C groups, the number of hepatopancreas B cells was lower in prawns that consumed α-LA than in those that did not (Figures 5(a)–5(d)). However, in the low-P/C group, the number of hepatopancreas B cells was higher in prawns that consumed α-LA than in those that did not (Figures 5(e) and 5(f)). In the medium-P/C group, the number of hepatopancreas R cells was higher in prawns that consumed α-LA than in those that did not (Figures 5(c) and 5(d)).

4. Discussion

In the present study, in the high- and medium-P/C groups, the weight gain of *M. nipponense* was lower for those that consumed α-LA than for those that did not. As the P/C ratio decreased from P37/C24 to P33/C30, the weight gain of prawns decreased significantly, similar to the results reported in previous studies on the carnivorous freshwater fish, *Salmo salar* [24] and *Diplodus cervinus* [25], and the herbivorous freshwater fish, grass carp *Ctenopharyngodon idella* [26] and *L. vannamei* [4, 27]. Although eating habits differ between carnivorous fish and herbivorous prawns, it seems that carbohydrates can maximize performance when included at appropriate levels in the diet. Studies on surubim, Pseudoplatystoma reticulatum × P. corruscans [28], found that increasing the amount of dietary carbohydrate and decreasing the amount of dietary protein did not reduce

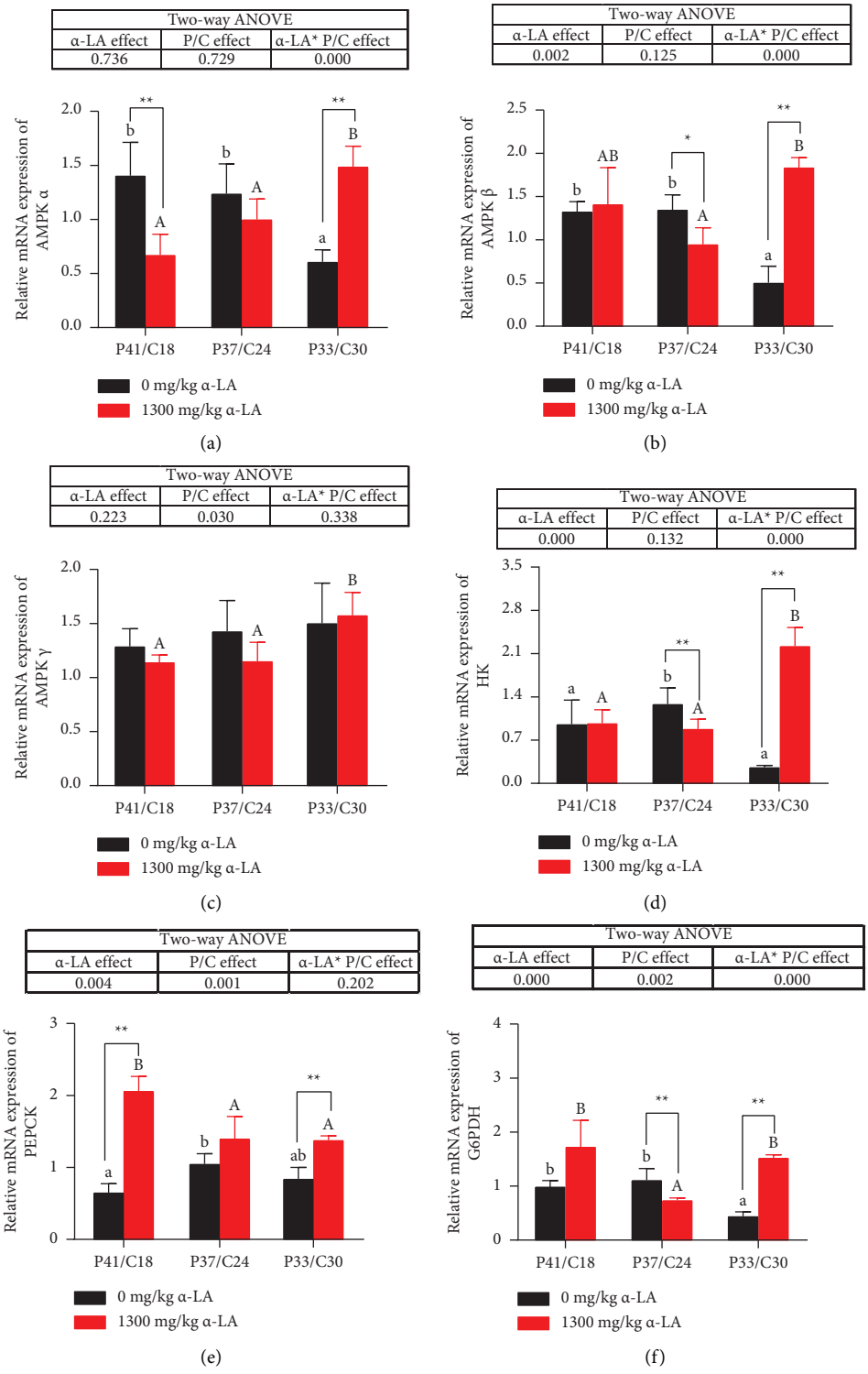

FIGURE 3: Effects of α-LA in diets with three different P/C ratios on transcript levels of genes encoding enzymes involved in carbohydrate and energy metabolism in *M. nipponense*. Data are mean ± SD. Different superscript letters indicate significant difference at $P < 0.05$: lowercase letters denote significant differences among P/C groups without α-LA supplementation; capital letters denote significant differences among P/C groups fed with a diet containing α-LA at 1300 mg/kg. *denotes significant difference within each P/C group without and with α-LA. Adenine ribonucleotide dependent protein kinase subunit α, AMPK α; adenine ribonucleotide dependent protein kinase subunit β, AMPK β; adenine ribonucleotide dependent protein kinase subunit γ, AMPK γ; hexokinase, HK; glucose-6-phosphate dehydrogenase, G6PDH. ** denotes extremely significant difference within each P/C group without and with α-LA.

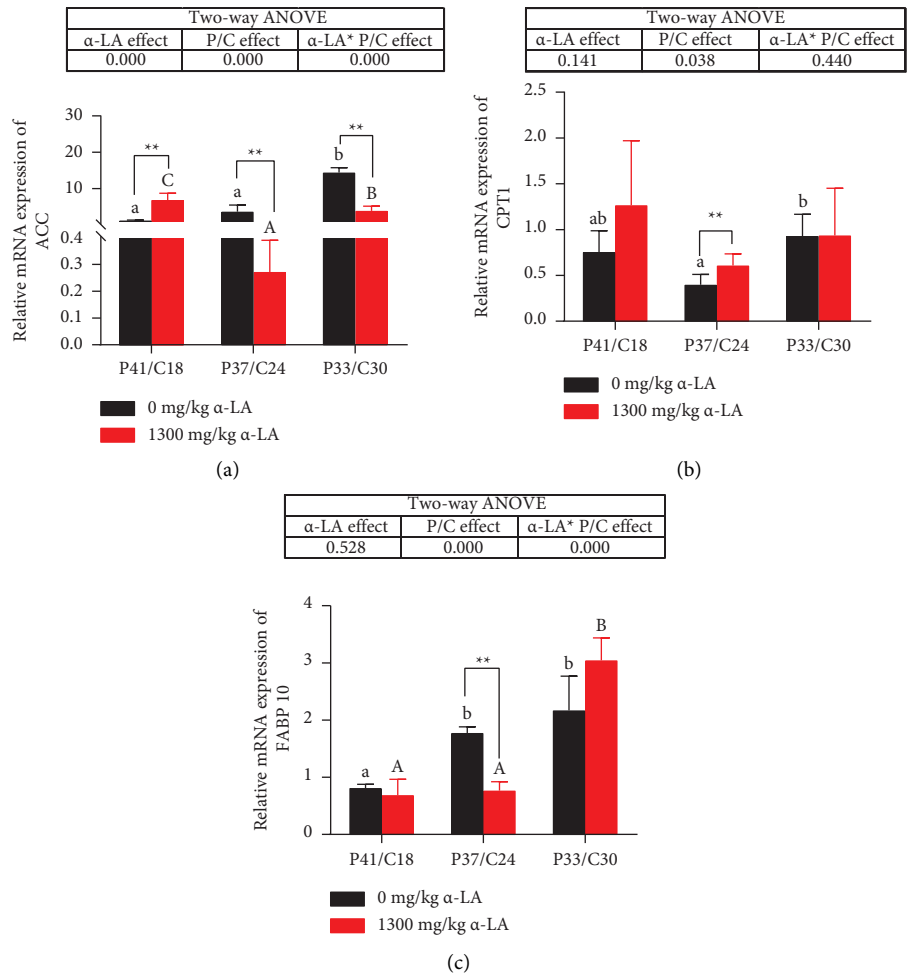

FIGURE 4: Effects of α-LA in diets with three different P/C ratios on transcript levels of genes encoding enzymes involved in lipid metabolism in *M. nipponense*. Data are mean ± SD. Different superscript letters indicate significant difference at $P < 0.05$: lowercase letters denote significant differences among P/C groups without α-LA supplementation; capital letters denote significant differences among P/C groups fed with a diet containing α-LA at 1300 mg/kg. *denotes significant difference within each P/C group without and with α-LA. Acetyl-CoA carboxylase, ACC; fatty acid binding protein 10, FABP 10; carnitine palmityl transferase, CPT1. ** denotes extremely significant difference within each P/C group without and with α-LA.

the weight gain of those animals. This may be due to differences in carbohydrate utilization efficiency among different aquatic animals. The inclusion of carbohydrates at an appropriate level in the diet can reduce protein degradation and amino acid oxidation for energy production, resulting in improved growth [29]. Besides, for carbohydrates to have a protein-sparing effect, the appropriate protein/carbohydrate ratio must be determined for each species [28, 30, 31] and dietary protein should be at a suboptimum level [32]. The differences in the dietary protein/carbohydrate ratios in experimental diets in the above studies may be one of the factors affecting the growth performance of each studied species. However, it is interesting to find that once adding α-LA to the diet of prawns, there was no significant difference for the weight gain under different P/C ratios. This is probably related to the energy modulating function of α-LA to carbohydrate utilization when protein level decreased, so protein-sparing effect by carbohydrate occurred in prawns fed α-LA in the three P/C ratio groups.

In this study, the addition of α-LA to the diet negatively affected the weight gain of prawns in the high- and medium-P/C groups, possibly because of increased expenditure of energy from the diet under α-LA supplementation [33]. A similar phenomenon was observed in our previous study and was attributed to the effect of α-LA to promote energy metabolism [18]. Interestingly, in the low-P/C group, growth was not significantly different between the prawns that consumed α-LA and those that did not. This suggests that the energy provided by the low-P/C diet likely countered the effect of α-LA to enhance energy expenditure. The survival rate decreased as the P/C ratio decreased from P37/C24 to P33/C30, suggesting that a low protein/high carbohydrate diet affected the health of this prawn. However, at all three P/C ratios, supplementation with α-LA improved the survival rate, possibly because of its effect to increase the antioxidant capacity. An increase in the antioxidant capacity of prawns by dietary supplementation with α-LA at an appropriate level was confirmed in our previous study [20].

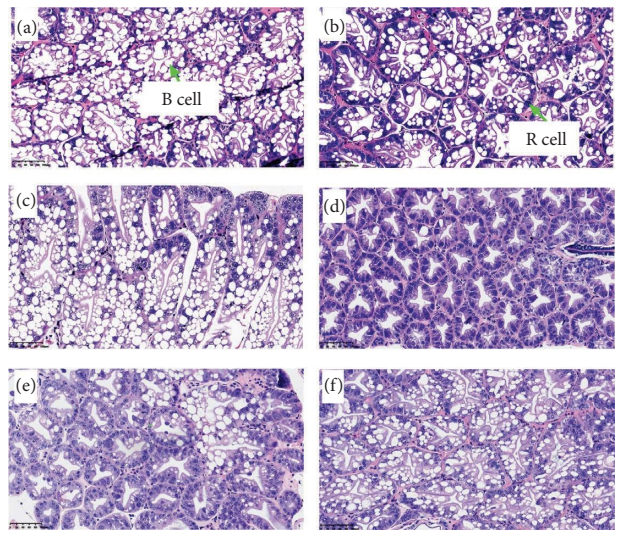

FIGURE 5: Effects of α-LA in diets with three different P/C ratios on hepatopancreas morphology of *M. nipponense*. (a, c, e) Hepatopancreas from prawns fed with high-medium-, and low-P/C diets (P41/C18, P37/C24, and P33/C30) without α-LA, respectively. (b, d, f) Hepatopancreas from prawns fed with high-, medium-, and low-P/C diets supplemented with 1300 mg/kg α-LA, respectively. R-cell, resorptive cell; B-cell, blister cell.

To further explore the effects of α-LA on energy expenditure in *M. nipponense* fed with diets with different P/C ratios, we analyzed the activities of key enzymes involved in carbohydrate, lipid, and energy metabolism and the transcript profiles of their encoding genes. Glycolysis is a key process in which carbohydrate is degraded into pyruvate, while gluconeogenesis is the conversion of simple non-glycogenic precursors into carbohydrate [34]. The rate-limiting enzymes in glycolysis are HK and PK [35]. The first rate-limiting enzyme in the gluconeogenesis pathway is PEPCK, which catalyzes the conversion of oxaloacetate into phosphoenolpyruvate [36]. The activities of the glycolytic key enzymes HK and PK and the gluconeogenic key enzyme PEPCK are indicators of the efficiency of carbohydrate metabolism. The decarboxylation of pyruvate to produce acetyl-CoA is catalyzed by PDH [37]. In the present study, dietary supplementation with α-LA enhanced PK, HK, and PDH activities in prawns, but did not affect the PEPCK activity, indicating that α-LA enhanced glycolysis and the conversion of pyruvate into acetyl-CoA without affecting gluconeogenesis.

To verify this result, we determined the transcript levels of genes encoding these enzymes. We found that dietary supplementation with α-LA increased the transcript levels of HK, providing further evidence that α-LA enhanced glycolysis under these conditions. Interestingly, although dietary supplementation with α-LA did not affect the activity of PEPCK, it did increase the transcript level of its encoding gene, *PEPCK*. Transcription and translation are complicated biological processes, and the correlation between gene transcript levels and the abundance of the encoded protein vary among different biological categories of genes [38]. In the low-P/C group, the transcript level of *G6PDH*, which encodes a key enzyme in the pentose phosphate pathway [39], was upregulated in prawns that consumed α-LA, suggesting that α-LA may promote the pentose phosphate pathway as well as glycolysis. These results show that the addition of α-LA to the diet of prawns can improve glycolysis and promote aerobic oxidation under different P/C ratios. The increase in aerobic oxidation of carbohydrate can reduce damage caused by oxidative stress [40], which may be another reason for the increased survival rate of prawns that consumed α-LA.

The enzyme at the center of energy metabolism is AMPK, which maintains energy metabolic homeostasis by regulating carbohydrate, lipid, and protein metabolism [41–43]. AMPK is composed of three subunits: AMPK-α, a catalytic subunit; AMPK-β, a scaffolding subunit; and AMPK-γ, a regulatory subunit [44]. Studies in rats have identified that the regulatory role of α-LA in carbohydrate and lipid metabolism may be related to the AMPK pathway [15, 45]. Therefore, we detected the transcript levels of the genes encoding the three subunits of AMPK. Dietary supplementation with α-LA affected the transcript levels of *AMPK α, AMPK β,* and *AMPK γ*. In the low-P/C group, *AMPK α* and *AMPK β* were significantly upregulated in the prawns that consumed α-LA. These results indicate that the mechanism of energy regulation by α-LA in *M. nipponense* may be similar to that in higher animals. For example, the α-LA-induced improvement of insulin sensitivity was found to be mediated via the activation of AMPK and reduced triglyceride accumulation in skeletal muscle [15].

To investigate whether the effect of α-LA on the AMPK pathway was related to lipid metabolism in the low-P/C group, we determined the transcript levels of *ACC, CPT1,* and *FABP 10*, which encode enzymes involved in fatty acid biosynthesis, fatty acid β-oxidation, and intracellular fatty acid transport, respectively [46, 47]. Although dietary supplementation with α-LA did not affect the transcript levels of *FABP 10* and *CPT1*, it increased the transcript level of *ACC* in the high-P/C group and decreased the *ACC* transcript levels in the medium- and low-P/C groups. In

other words, α-LA may affect the AMPK pathway in prawns fed with lower P/C diets by upregulating carbohydrate metabolism and inhibiting lipid synthesis. Together, our results suggest that α-LA regulates carbohydrate and lipid metabolism by modulating the AMPK pathway, thereby improving the carbohydrate utilization efficiency in low-P/C diets and sparing fishmeal protein in the diet of *M. nipponense*.

In arthropods, the hepatopancreas is an important organ for nutrient absorption and storage [48]. The hepatopancreas contains four types of epithelial cells: blister cells (B cells), resorptive cells (R cells), embryo cells (E cells), and fibrillar cell (F cells) [49]. The R cells are responsible for lipid and glycogen storage in the hepatopancreas gland [49]. In this study, in the medium-P/C group, there were more hepatopancreas R cells in prawns that consumed α-LA than in those that did not, which could be indicative of greater energy reserve requirements in the hepatopancreas. It seemed that there was enough energy to meet the normal physical requirements of prawns in the medium-P/C group when α-LA was included in the diet. In the hepatopancreas, B cells are responsible for the synthesis of digestive enzymes [50]. A previous study suggested that increased numbers of hepatopancreatic B cells can promote the synthesis and excretion of digestive enzymes, thereby providing shrimps with more energy [50]. In this study, in the absence of α-LA supplementation, the number of hepatopancreas B cells in prawns decreased as the dietary P/C ratio decreased, which may indicate that less energy was obtained from the diet. However, supplementation with α-LA in the low-P/C group (P33/C30) increased the number of hepatopancreas B cells, indicating that the addition of α-LA to low-P/C diets may increase the synthesis and secretion of enzymes in the hepatopancreas to satisfy the physical requirements of prawns.

It is worth mentioning that, corn starch was used in this study to detect a protein-sparing effects of α-LA because our previous studies proved that corn starch was a better source of carbohydrate compared to other carbohydrate sources (dextrin, maltose, glucose, and cellulose) [51]. However, some aquatic animals have other suitable sources of carbohydrate [52, 53]; so related research is required to determine whether the protein-sparing effects of α-LA supplementation are the same if other sources of carbohydrate are used in other aquatic animals.

5. Conclusions

The addition of α-LA (at 1300 mg/kg feed) increased the survival rate of prawns and had a protein-sparing effect in the low-P/C diet. This effect was achieved by increasing the number of hepatopancreatic B cells, promoting glycolysis, and increasing the transcript levels of genes related to pentose phosphate pathways and energy metabolism.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Additional Points

Highlights. (i) Prawns consumed diets with different protein/carbohydrate ratios (P/C) with/without α-LA. (ii) At a low P/C, α-LA at 1300 mg/kg improved carbohydrate and energy metabolism. (iii) Dietary supplementation with α-LA promoted carbohydrate metabolism in *M. nipponense*, thereby having a protein-sparing effect.

Ethical Approval

The protocols of animal culture and treatment performed in this study strictly complied with the relevant national guidelines of China.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Shanshan Li and Junbao Wang contributed equally to this work.

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