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Research Article

Study on the Optimal Phospholipid Addition Levels for Button-Sized Juvenile Chinese Mitten Crab (*Eriocheir sinensis*): Survival, Growth, Physiological Parameters, and *Neverland* Expression

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A 50-day feeding experiment was performed to investigate the effects of dietary phospholipid (PL) addition on the survival, growth, serum, and hepatopancreas lipid indexes, calcium content, and neverland (*Nvd*) gene expression in button-sized juvenile Chinese mitten crab (*Eriocheir sinensis*). Five experimental diets were formulated with increasing addition of PL (0%, 1%, 2%, 3%, and 4%), which were named PL0, PL1, PL2, PL3, and PL4, respectively. Each diet was randomly assigned to four tanks of juvenile *E. sinensis* (initial weight: 5.00 ± 0.55 g), with eight crabs held in each tank. The results showed that PL addition significantly increased the survival rate (SR) but just slightly increased the weight gain rate (WGR) of juvenile *E. sinensis*. As the PL addition level increased, SR and WGR first increased and then decreased, with the highest values observed in the PL3 group. As the PL addition level increased, the activities of trypsin and cellulase significantly increased and then decreased, with the highest value observed in the PL2 group and PL3 group, respectively. As PL addition level increased from 1% to 2%, lipase activity increased sharply and then kept constant with a further increase of dietary PL. As PL addition level increased from 0% to 3%, the calcium content in the serum significantly increased from 7.96 to 12.43 mmol/L and then significantly decreased to 11.36 mmol/L with a further increase of PL addition. PL addition just slightly promoted the calcium content in the hepatopancreas compared with the control group. As PL addition level increased from 0% to 3%, the expression of *Nvd* in the hepatopancreas significantly increased by 1.47-fold and then decreased to basal level with a further increase of PL addition. In conclusion, 2%−3% addition of the optimal PL addition level was estimated to be 2%−3% for juvenile *E. sinensis* based on their survival, growth performance, and digestive enzyme activities. PL addition at a relatively higher level (3%) increased the calcium content and *Nvd* expression, the latter possibly facilitating cholesterol metabolism into vitamin D.

1. Introduction

Chinese mitten crab (*Eriocheir sinensis*) is known as the hairy crab or river crab, which is popular among East Asian consumers due to its nutritious value and excellent taste [1, 2]. In decades of the last century, the population of wild *E. sinensis* has seriously declined due to the ever-increasing demand and subsequent overfishing activities [3]. The intensive culture has proved to be an effective way to decrease the reliance on wild capture of *E. sinensis* [4, 5]. Up to now, it has become one of the most important economical culture species in China, with the annual output of *E. sinensis* reaching 808,000 tons in 2021 [6]. *E. sinensis* used to be fed on trash fish, pumpkins, corns, and soybeans [7]. However, there are some defects in these live foods, such as imbalanced nutrition, unstable supply and quality, and inconvenient storage, which greatly limited their extensive use for crab aquaculture. In contrast, the formulated feeds were more and more acceptable because of their complete nutrition, stable supply and quality, and relatively low waste [8].

Phospholipid (PL) is usually referred to as a lipid that contains phosphorus, including phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine [9, 10]. PL plays an important role in promoting growth, immunity, stress-resistant capacity, gonad development, and reproductive performance of aquatic animals [9, 11–16]. The PL requirement of *E. sinensis* is affected by PL sources, growth stage, physiological state, environmental factors, and quantity of other nutrients such as cholesterol [17]. Wang et al. [18] found that 2%−4% soy lecithin in the diets effectively improved the molting efficiency and growth performance of juvenile *E. sinensis* (initial body weight: 1.23 ± 0.36 g). Lin et al. [19] found that dietary supplementation of phosphatidylcholine at the addition level of 2.89%–2.95% improved the growth rate, antioxidant capacity, and the transportation of lipids from hepatopancreas to the muscle of juvenile *E. sinensis* (initial body weight: 0.52 ± 0.01 g). Lin et al. [20] reported that the best growth promotion effects on juvenile *E. sinensis* (initial body weight: 0.26 ± 0.01 g) were achieved by the supplementation of krill oil, followed by yolk PL, and worst by soybean lecithin. However, to the best of our knowledge, little information is available about the optimal PL requirement for button-sized juvenile *E. sinensis* with higher body weight (appropriately 5.0 g), which are the crab seeds for the rice-crab symbiotic culture in East Northern China. Rice-crab symbiotic culture is a successful practice of the integration of crab and rice culture in China, which is characterized by a series of advantages, such as improving soil fertility, reducing weed emergence and pest reduction, and increasing rice and crab yield [21]. Under the rice-crab coculture, Chinese mitten crabs are cultured for 2 years, which grow from early juvenile crabs to button-sized crabs in the first year and grow from button-sized crabs to market-sized crabs in the second year [21]. In the last decades, rice-crab coculture has been promoted by government and nongovernment agencies with significant success achieved among small-scale farms in northeast China [22].

Molting activity is one of the indicators of the growth in the developmental stage of crustaceans. The crustaceans need more calcium during molting than in other periods of growth to sustain normal molting and skeletal malformations [23]. Although the beneficial effects of PL addition on growth and molting have been widely reported in crustaceans, the underlying mechanisms remain not fully understood. Digestive enzymes play an important role in the digestion and absorption of nutrients. Their activities directly reflect digestive capacity and feed conversion efficiency and affect the growth rate of animals [24]. Neverland (*Nvd*) gene was first found in silkworm and Drosophila. It belongs to the evolutionarily conserved *Rieske* domain protein family. The researchers found that the abnormal traits that limit the expression of this gene can be restored by the addition of 7-dehydrocholesterol. Thus, it is believed that this gene plays a role in the transformation of cholesterol to 7-dehydrocholesterol [25–28]. *Nvd* is verified to exert regulating effects by attaching to cholesterol 7-desaturase that dehydrogenates cholesterol to form 7-dehydrogenated cholesterol (the provitamin D), which is successively catalyzed by cytochrome P450 (CYP) 105A1, and cytochrome P450 (CYP) 2R1 [29–32] to form 1α, 25-dihydroxy vitamin D_3 commonly known as vitamin D. Vitamin D can promote the absorption of calcium and phosphorus of crustaceans and benefits their molting and growth [33–35]. However, it remains unclear about the effects of PL addition on the expression of *Nvd* in *E. sinensis*.

Table 1: Ingredient and formulation of experimental diets (% dry weight).

Ingredients	Phospholipid levels (%)				
	0%	1%	2%	3%	4%
Fish meal[1]	12	12	12	12	12
Soybean meal[2]	28	28	28	28	28
Wheat flour[3]	22	22	22	22	22
Casein	12	12	12	12	12
Gluten	5	5	5	5	5
Cholesterol	0.5	0.5	0.5	0.5	0.5
Palm oil	5.5	4.5	3.5	2.5	1.5
Soybean lecithin	0	1	2	3	4
Cellulose	4.69	4.69	4.69	4.69	4.69
Beer yeast[4]	5	5	5	5	5
Mineral premix[5]	2	2	2	2	2
Vitamin premix[6]	2	2	2	2	2
Choline chloride[7]	0.2	0.2	0.2	0.2	0.2
Calcium dihydrogen phosphate[7]	1	1	1	1	1
Calcium propionate[7]	0.1	0.1	0.1	0.1	0.1
Ethoxyquinine[7]	0.01	0.01	0.01	0.01	0.01

[1]Fish meal: crude protein 68.10%, crude lipid 10.20%, purchased from Qingdao Qihao Biotechnology Company (Qingdao, Shandong Province, China). [2]Soybean meal: crude protein 43.40%, crude lipid 1.90%, purchased from Qingdao Qihao Biotechnology Company (Qingdao, Shandong Province, China). [3]Wheat meal: crude protein 11.20%, crude lipid 0.60%, purchased from Xingtai Hualongnongzhuang Wheat Meal Company (Xingtai, Hebei Province, China). [4]Beer yeast: crude protein 42.60%, crude lipid 1.00%, purchased from Jinan Hualong Feedstuff Company (Jinan, Shandong Province, China). [5]Mineral premix (mg or g/kg diet): $CuSO_4 \cdot 5H_2O$, 10 mg; Na_2SeO_3 (1%), 25 mg; $ZnSO_4 \cdot H_2O$, 50 mg; $CoCl_2 \cdot 6H_2O$ (1%), 50 mg; $MnSO_4 \cdot H_2O$, 60 mg; $FeSO_4 \cdot H_2O$, 80 mg; Ca $(IO_3)_2$, 180 mg; $MgSO_4 \cdot 7H_2O$, 1200 mg; zeolite, 18.35 g. [6]Vitamin premix (mg or g/kg diet): vitamin D, 5 mg; vitamin K, 10 mg; vitamin B12, 10 mg; vitamin B6, 20 mg; folic acid, 20 mg; vitamin B1, 25 mg; vitamin A, 32 mg; vitamin B2, 45 mg; pantothenic acid, 60 mg; biotin, 60 mg; niacin acid, 200 mg; α-tocopherol, 240 mg; inositol, 800 mg; ascorbic acid, 2,000 mg; microcrystalline cellulose, 16.47 g. [7]These additives were bought from Weifang Zhongtian Feed Techonology Co. Ltd. (Weifang, Shandong Province, China).

Therefore, this study was conducted to explore the effects of dietary PL addition on the survival, growth, and digestive enzyme activities, as well as the expression of *Nvd* in button-sized juvenile *E. sinensis*. The objectives of this study were as follows: (1) quantify the precise PL requirement for button-sized juvenile *E. sinensis* with an initial body weight above 5.0 g; (2) improve understanding of the mechanisms about the beneficial effects of PL on the growth of *E. sinensis*.

2. Materials and Methods

2.1. Experimental Feed. Five diets with graded levels of PL (0% (control group), 1%, 2%, 3%, and 4%) were formulated by including palm oil and soybean lecithin as primary lipid sources, and fish meal, soybean meal, and casein as the primary protein sources. The feed formula composition is shown in Table 1. The feed manufacture procedures have been previously described by

Zuo et al. [36]. First, all solid feed stuffs were fully ground and sifted through an 80-mesh sieve. Then, the powders were mixed according to the feed formulation in a step-by-step enlargement principle. After that, the palm oil and soybean lecithin were added into the powder and mixed evenly. Then, pure water was added to 35% of the feed ingredients and mixed again evenly. At last, feed ingredients were pressed into 1.5 mm diameter granules using a twin-screw automatic granulator (Jinan Dingrun Machinery Co., Jinan, China). The prepared feeds were dried in the oven at 55°C, sealed in a packing bag after cooling, and kept in the refrigerator at −20°C to prevent lipid peroxidation.

2.2. Feeding Experiment and Daily Management. The feeding experiment was performed at the experimental base of the Panjin Guanghe Crab Co., Ltd. (Panjin, China). The crabs used in the experiment were purchased from Panjin Guanghe Crab Co., Ltd. (Panjin, China). They were temporarily kept in indoor cement ponds for a 2-week acclimation. During the acclimation, experimental animals were fed experimental feed (0% PL) twice daily (7:00 and 19:00). After that, healthy juvenile crabs with similar body size (5.00 ± 0.55 g) and complete appendages were chosen out and randomly assigned to 20 plastic tanks (60 cm × 40 cm × 40 cm) with each tank holding eight individuals. Each experimental diet was allocated to four replicates of crabs. Plastic pipes and double layers of plastic mesh were placed in each tank as shelters to avoid cannibalism.

The feeding experiment lasted for 50 days. During the feeding experiment, the crabs were fed twice daily (7:00 and 19:00). The crabs were monitored, and the dead individuals were removed from the tank daily. The residual feeds and feces were removed by siphoning 2 hr after each feeding. In total, 50% of the water in the tank was replaced with clean, aerated water every other day. During the feeding experiment, the pH was between 7.6 and 7.8, the temperature was between 23 and 26°C, and the dissolved oxygen was kept above 8 mg/L. The concentration of ammonia nitrogen and nitrite was controlled below 0.05 and 0.07 mg/L, respectively.

2.3. Sample Collection. When the feeding experiment ended, all *E. sinensis* were starved for 24 hr before they were counted and weighed individually in each tank. After that, two or three *E. sinensis* were removed from each tank and anesthetized for 5 min in a foam tank filled with ice. Then, blood was individually drawn and transferred into enzyme-free centrifuge tubes with an anticoagulant. After that, the hepatopancreas was individually dissected and stored into the enzyme-free centrifuge tubes. All the tubes with samples were quickly frozen in liquid nitrogen and stored in the refrigerator at −80°C.

2.4. Biochemical Analysis

2.4.1. Determination of Each Component in Serum. Hemolymph samples were taken from the −80°C refrigerator and placed at 4°C for 24 hr, then centrifuged at 4°C for 10 min. Then, the supernatant of the homogenate was separated for test. The contents of triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), and calcium in serum were determined by Nanjing Jiancheng Biotechnology Institute.

Table 2: Primers used for quantitative PCR.

Gene	Primer sequence 5′–3′	Length
Neverland (Nvd)	F:GGCGTGGTGTACCTGTACTTCAAC	24
	R:GTGCGGGACGAGAAGAACTGATG	24
β-Actin	F:GCATCCACGAGACCACTTACA	21
	R:CTCCTGCTTGCTGATCCACATC	22

2.4.2. Determination of Components in Hepatopancreas. The hepatopancreas was mixed with phosphate buffer solution and homogenized to get homogenates. Then, the homogenates were centrifuged at 4°C for 30 min to separate the supernatant from other parts of the homogenate. The contents of TG, TC, calcium, and the activities of lipase, cellulase, trypsin, and amylase in the supernatant were determined by using commercial kits (Nanjing Jiancheng Biotechnology Institute, Nanjing, China). The detailed procedures can be found in the instructions of corresponding commercial kits.

2.5. Quantitative PCR (qPCR). The expression of *Nvd* in hepatopancreas was detected with qPCR. The hepatopancreas was ground with a tissue grinder (Wuhan Xavier Biotechnology Co., Ltd.). Trizol universal reagent (DP424, Tiangen) was used for extracting total RNA from the hepatopancreas of juvenile crabs. Then, the integrity was tested by agarose-electrophoresis, and the concentration was measured by microspectrophotometer with the Agilent2100 bioanalyzer. After that, total RNA was reverse transcribed to get cDNA by using the Prime ScriptTM Real-time PCR Kit (TaKaRa, Beijing, China). Finally, the cDNA templates were diluted by five times before they were used for qPCR. The qPCR was performed with the SYBR Premix Ex Taq Kit (TaKaRa, Dalian, China) by following the instructions. LightCycler®96 (Roche Group, Basel, Switzerland) was used to perform the qPCR. The reaction conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s; 95°C for 10 s; 65°C for 60 s; and 97°C for 1 s. The primer sequences for qPCR are listed in Table 2. The primer amplification efficiency of *Nvd* and *β-actin* was 0.94 and 1.03, respectively. The relative expression of *Nvd* was calculated by using the method of $2^{-\Delta\Delta CT}$ [37].

2.6. Calculation and Statistical Analysis.

$$\text{Survival rate (SR, \%)} = S_f/S_i \times 100, \quad (1)$$

$$\text{Weight gain rate (WGR, \%)} = (W_f - W_i)/W_i \times 100. \quad (2)$$

In the formula, S_i and S_f were the initial and final number of *E. sinensis* in each tank, respectively. W_i and W_f were the initial and final average body weights of *E. sinensis* in each group, respectively.

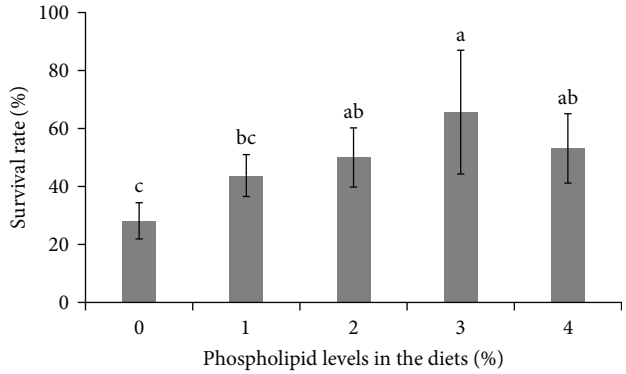

FIGURE 1: Effects of phospholipid levels on the survival rate of *Eriocheir sinensis*. Mean values of bars bearing with different letters are significantly different at $P<0.05$.

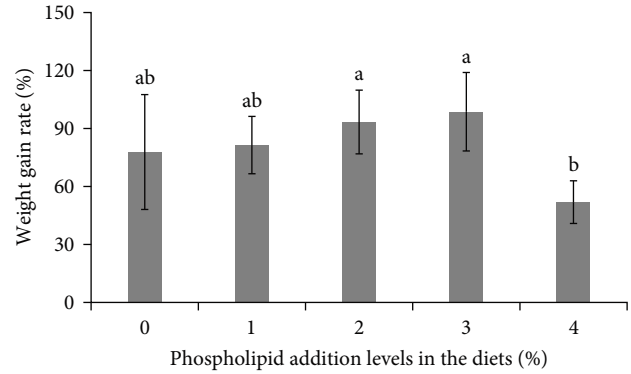

FIGURE 2: Effects of phospholipid addition levels on the weight gain rate of *Eriocheir sinensis*. Mean values of bars bearing with different letters are significantly different at $P<0.05$.

All data were checked for normality and homogeneity of variance and then statistically analyzed by using a one-way analysis of variance in SPSS 26 software. Duncan multiple comparison method was used to test the significance of differences between groups. $P<0.05$ was considered the appearance of a significant difference. All statistical data was expressed as mean ± standard deviation (mean ± SD).

3. Results

3.1. Survival and Growth. Compared with the control group, PL addition significantly increased the survival rate (SR) of juvenile *E. sinensis*. As PL addition level increased from 1% to 3%, the SR increased from 43.8% to 65.6% ($P=0.02$) and then decreased to 53.1% with further increase of PL addition ($P>0.05$). SR in the PL3 group was comparable to that in the PL2 and PL4 groups ($P>0.05$) but was significantly higher than that in the other groups ($P<0.05$). In addition, the SR in the PL2 and PL4 groups was significantly higher than that in the PL0 group ($P<0.05$) (Figure 1).

As PL addition level increased from 0% to 3%, the weight gain rate (WGR) slightly increased from 81.8% to 98.9% ($P=0.14$), and then significantly decreased to 43.9% with a further increase of PL addition ($P<0.05$). WGR in the PL4 group was comparable to that in the PL0 and PL1 groups ($P>0.05$) but was significantly lower than that in the other groups ($P<0.05$) (Figure 2).

3.2. Digestive Enzyme Activities in Hepatopancreas. As PL addition level increased, the activities of trypsin and cellulase first increased and then decreased, with the highest value observed in the PL2 group and PL3 group, respectively. The trypsin activity in the PL2 group was only significantly higher than that in the PL1 group ($P=0.04$). The cellulase activity in the PL3 group was only significantly higher than that in the PL0 group ($P<0.05$). The activity of amylase showed a similar tendency with that of trypsin and cellulose. However, no statistical significance was observed in amylase activity between dietary groups ($P>0.05$). As PL addition level increased, lipase activity increased in a stepwise way ($P>0.05$), with the break point observed in the PL2 group (Figure 3).

3.3. Calcium Content in the Serum and Hepatopancreas. As PL addition level increased from 0% to 3%, the calcium content in the serum significantly increased from 7.96 to 12.43 mmol/L ($P=0.04$) and then decreased to 11.36 mmol/L with further increase of PL addition ($P<0.05$). PL addition just slightly promoted the calcium content in the hepatopancreas compared with the control group ($P>0.05$) (Figure 4).

3.4. TG and Total Cholesterol Contents (TC) in the Serum and Hepatopancreas. As PL addition level increased from 0% to 2%, the TG content in the serum significantly increased from 0.35 to 0.59 mmol/L ($P>0.05$) and then decreased to 0.21 mmol/L with further increase of PL addition ($P<0.05$). PL addition just slightly promoted the TG content in the hepatopancreas compared with the control group ($P>0.05$) (Table 3).

As PL addition level increased from 0% to 2%, the TC content in the serum significantly increased from 0.5 to 1.01 mmol/L ($P=0.03$) and then decreased to 0.77 mmol/L with further increase of PL addition ($P>0.05$). The TC in the hepatopancreas showed a similar tendency with that in the serum, with no statistical significance observed between dietary groups ($P>0.05$) (Table 3).

3.5. LDL and HDL in the Serum. As PL addition level increased from 1% to 4%, the LDL contents in the serum fluctuated between 0.33 mmol/L and 0.45 mmol/L ($P>0.05$). The LDL contents in the PL3 and PL4 groups were significantly higher than that in the control group ($P<0.05$). PL addition did not significantly affect the HDL contents in the serum of experimental animals ($P>0.05$). As PL addition level increased, the HDL/LDL significantly decreased from 0.57 to 0.26 in a stepwise way ($P<0.05$). The HDL/LDL in PL1 and PL2 groups was significantly lower than that in the PL0 group but was significantly higher than that in PL3 and PL4 groups ($P<0.05$) (Table 3).

3.6. Expression of Nvd in Hepatopancreas. As PL addition level increased from 0% to 3%, the expression of *Nvd* in the hepatopancreas significantly increased by 1.47-fold and then decreased to basal level with further increase of PL addition ($P<0.05$). The *Nvd* expression in the PL2 was significantly lower than that in the PL3 but was significantly

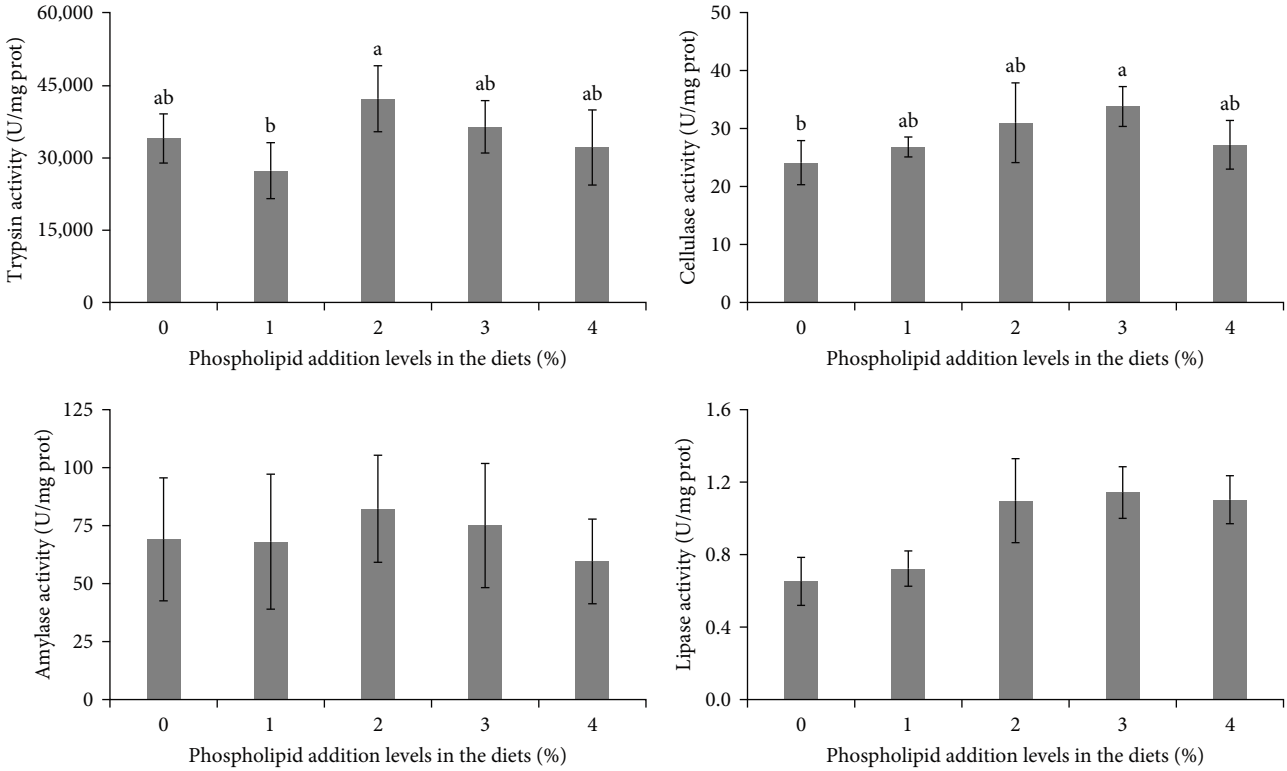

Figure 3: Effects of phospholipid addition levels on the digestive enzyme activities in the hepatopancreas of *Eriocheir sinensis*. Mean values of bars bearing with different letters are significantly different at $P < 0.05$.

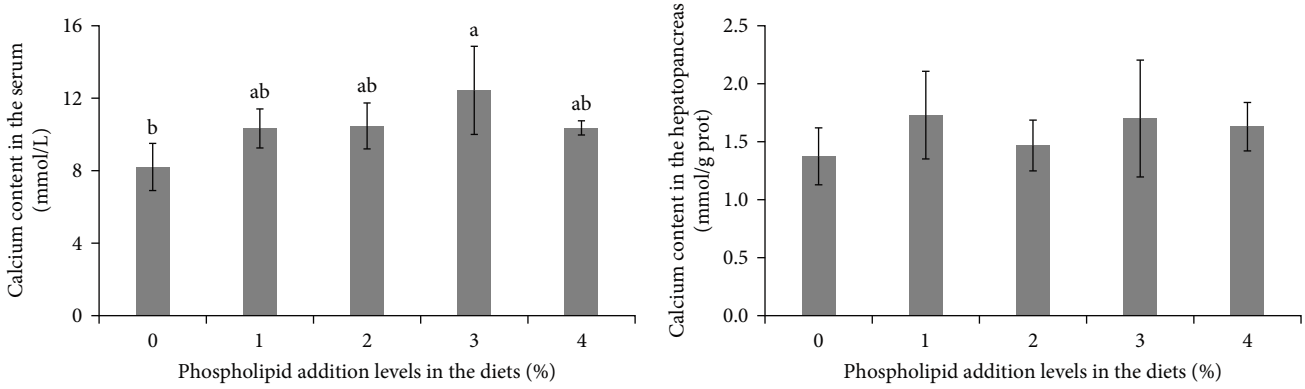

Figure 4: Effects of phospholipid addition levels on the calcium content in the serum and hepatopancreas of *Eriocheir sinensis*. Mean values of bars bearing with different letters are significantly different at $P < 0.05$.

higher than that in the other groups ($P < 0.05$). The *Nvd* expression in the PL2 and PL3 groups was increased by 0.88-fold and 1.47-fold than that in the PL0 group, respectively. However, there were no significant differences in the *Nvd* expression between PL0, PL1, and PL4 groups ($P < 0.05$) (Figure 5).

4. Discussion

In this study, the optimal PL requirement was estimated to be 2%–3% for *E. sinensis* from the perspective of survival and growth performance. This was consistent with the findings on *E. sinensis* [20], *Portunculus tritatus* [12], and *Scylla paramamosain* [38, 39] but was inconsistent with the findings on *P. tritatus* [13, 40], *Penaeus monodon* [41], *Penaeus penicillatus* [42], and *gilthead seabream* [43], which found that the optimal PL was 1%–2%. In addition, the type of PL also influences the growth of *E. sinensis*. Lin et al. [20] found that krill oil showed the best growth-promoting effects than yolk PL and soybean lecithin in juvenile *E. sinensis*. Thus, the specific requirement for different PL sources under the same experimental conditions should be ascertained for the juvenile *E. sinensis* in the following studies. In this study, the SR of *E. sinensis* is lower than 80% in all dietary

Table 3: Effect of phospholipid addition levels on serum and hepatopancreas biochemical indexes of *Eriocheir sinensis*.

Items	Phospholipid levels				
	0%	1%	2%	3%	4%
Serum					
TG[1] (mmol/L)	0.35 ± 0.07^{ab}	0.49 ± 0.05^{ab}	0.59 ± 0.3^{a}	0.30 ± 0.32^{ab}	0.21 ± 0.05^{b}
TC[2] (mmol/L)	0.50 ± 0.25^{b}	0.85 ± 0.14^{ab}	1.01 ± 0.72^{a}	0.93 ± 0.49^{ab}	0.77 ± 0.13^{ab}
HDL[3] (mmol/L)	0.09 ± 0.02	0.13 ± 0.04	0.08 ± 0.02	0.10 ± 0.04	0.11 ± 0.03
LDL[4] (mmol/L)	0.20 ± 0.12^{b}	0.40 ± 0.08^{ab}	0.33 ± 0.2^{ab}	0.43 ± 0.11^{a}	0.45 ± 0.1^{a}
HDL/LDL	0.57 ± 0.32^{a}	0.33 ± 0.12^{b}	0.37 ± 0.27^{b}	0.24 ± 0.07^{c}	0.26 ± 0.07^{c}
Hepatopancreas					
TG[1] (mmol/g prot)	2.90 ± 0.94	2.69 ± 0.65	2.90 ± 0.09	3.59 ± 1.05	2.92 ± 1.2
TC[2] (mmol/mg prot)	0.11 ± 0.04	0.16 ± 0.07	0.17 ± 0.03	0.14 ± 0.05	0.10 ± 0.04

Mean values with different superscript letters in the same row are significantly different at $P < 0.05$. [1]TG: triglyceride; [2]TC: total cholesterol; [3]HDL: high-density lipoprotein; [4]LDL: low-density lipoprotein.

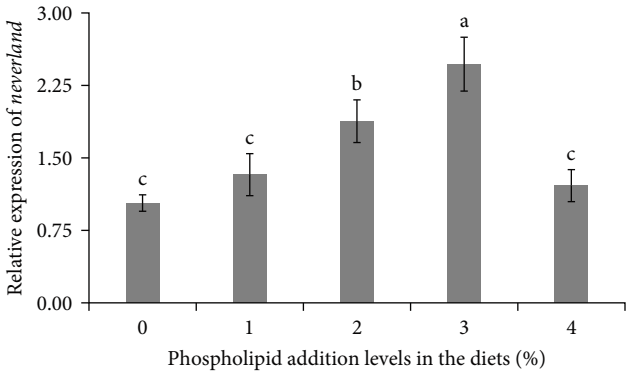

Figure 5: Effects of phospholipid addition levels on the expression of *neverland* in the hepatopancreas of *Eriocheir sinensis*. Mean values of bars bearing with different letters are significantly different at $P < 0.05$.

groups. On the one hand, it could be due to the limited amounts of fish meal and the scarcity of fish oil in the diets. It was previously found that the SR of *E. sinensis* was markedly decreased when fish oil was replaced with soybean oil [44]. On the other hand, the higher initial body weight (appropriately 5.0 g) used in this study means more serious cannibalism between individuals. Yang et al. [10] found that PL addition could enhance the molting process of *P. tritatus* by activating the molting signal pathway. Apart from sufficient substances, molt hormone (MH) is important for the molting process for crustaceans, including *E. sinensis* [45]. Cholesterol in Y-organ is converted into ecdysterone, the precursor of MH. Then, ecdysterone secreted by Y-organ enters the blood and binds to 20-hydroxyecdysterone to form the MH [46]. MH was first found in the prothymus of insects [47]. Later, more kinds of MH were found in the probranchial cavity of crustaceans [48]. The effect of PL addition on ecdysone will be paid attention to in the following studies.

In our study, the addition of PL at an appropriate level (2%) promoted lipase activity in the hepatopancreas and increased lipid content in the serum. Shields et al. [49] found a correlation between lipase activity and the fatty acid composition of the diet. Wang et al. [10] also observed that increasing levels of dietary linoleic acid (LA), EPA, and DHA promoted lipase activity in *E. sinensis*, with LA showing a higher feed coefficient than linolenic acid. As PL contains a significant amount of LA, it may explain the promotion of lipid digestion and absorption with the increase in PL levels. In this study, it was also found that an appropriate level of PL increased cellulase activity but did not significantly affect amylase activity. This suggests that the carbohydrate content of the feed was similar. The increase in cellulase activity may be related to the overall activity of the liver organ, which was stimulated by the increase in PLs. Since there are few studies on cellulase in crustaceans, this could be a new avenue for investigating sugar metabolism in *E. sinensis*. Also, lipase activity was significantly higher in groups with PL levels equal to or higher than 2% compared to those in the PL0 and PL1 groups. This suggests that an adequate amount of lipids can be absorbed from the intestine and utilized for energy supply, leading to a decreased reliance on carbohydrates such as starch and cellulose [50]. Wen et al. [51] have also demonstrated that a high ratio of carbohydrate to lipid is not conducive to the growth of juvenile *E. sinensis*. Furthermore, PL can increase the transportation of energy-supplying lipids, such as TG, to muscle or enhance the transfer efficiency of lipids into mitochondria for oxidation. This may explain the beneficial effects of increasing PL levels on lipid oxidation, which could lead to a decreased reliance on carbohydrates for energy supply [52]. This is reflected by the decreased cellulase activity in the hepatopancreas of the PL3 group.

The hepatopancreas of crustaceans play a crucial role in lipid storage and metabolism, which has similar functions with the fatty bodies of insects and the livers of vertebrates [53]. This organ regulates lipid transformation and transport, hormone, and enzyme release, and participates in various physiological regulatory mechanisms in crabs. The lipids stored in the hepatopancreas are transported to muscle tissue and gonads to promote body growth and gonad development [54]. The lipids stored in the hepatopancreas are transported to muscle tissue and gonads to promote body growth and gonad development. The hepatopancreas metabolizes and converts lipids, such as cholesterol, into hormones, such as ecdysone, ecdysone inhibiting hormone, and other steroid hormones, to regulate ecdysone and osmotic pressure

balance [54]. In this study, we found that the addition of PL significantly increased the contents of TG and TC in the serum of juvenile *E. sinensis* but did not significantly affect those in the hepatopancreas. This may be due to the increase in HDL/LDL. Lipoproteins are formed by the combination of apolipoprotein with cholesterol and PL, and their primary function is lipid transport. Among them, HDL can transport lipids from extrahepatic tissues to the liver for metabolism, while LDL participates in the transportation of lipids from the liver to the extrahepatic tissues of the body [55, 56]. Our study showed that decreasing HDL/LDL led to more lipids being transported out of the liver. This suggests that PL promotes the transport of lipids from the liver to extrahepatic tissues. The growth-promoting effect of PL may be due to its promotion of lipid absorption, inhibition of lipid accumulation in the liver, and transportation of lipids from the liver to extrahepatic tissues for growth and development of the body [57–61].

Nvd is verified to exert regulating effects by attaching to cholesterol 7-desaturase that dehydrogenates cholesterol to form 7-dehydrogenated cholesterol (the provitamin D), which was then catalyzed by cytochrome P450 (CYP) 105A1, and cytochrome P450 (CYP)2R1 to form vitamin D [29–32]. Vitamin D can promote the absorption of calcium and phosphorus of crustaceans and benefits their molting and growth [33–35]. Calcium is essential for osmotic regulation, molting regulation, and shell construction in crustaceans [62–64]. In this experiment, we observed that the calcium content in the serum increased with increasing PL addition levels, with the highest calcium content found in the 3% PL addition. Consistently, the expression of *Nvd* gene showed a similar changing tendency with that of calcium content. Therefore, the expression level of the *Nvd* gene may represent the amount of newly synthesized vitamin D to some extent. In addition to endogenous synthesis, vitamin D can also be obtained from the diet. Liu et al. [33] found that adequate lipid intake could help the body absorb more vitamin D since it is a fat-soluble vitamin. Our study showed that PL addition promoted the absorption of lipids and vitamin D in *E. sinensis*. Since vitamin D participates in calcium absorption, the increased calcium in the serum could be due to the elevated synthesis and absorption of vitamin D. However, we were unable to determine the contents of vitamin D due to insufficient samples, and further studies are needed to confirm this hypothesis.

5. Conclusion

In conclusion, our study demonstrates that the optimal PL addition level was estimated to be 2%−3% for juvenile *E. sinensis* based on their survival, growth performance, and digestive enzyme activities. Moreover, the 3% PL addition increased the calcium content and *Nvd* expression, the latter possibly facilitating cholesterol metabolism into vitamin D. These findings suggest that the addition of PL to the diet of *E. sinensis* could enhance their overall health and growth, providing a promising strategy for aquaculture practices.

Data Availability

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

Conflicts of Interest

The authors declare they have no conflicts of interest.

Authors' Contributions

Conceptualization was done by Rantao Zuo and Rong Yuan. Experimental analysis was done by Qilin Yi and Shu Huang. Data curation was done by Weishuai Shi and Changhong Tao. Funding acquisition and administration were done by Rantao Zuo, Yanming Su, and Yusheng Jiang. Writing—original draft was done by Rong Yuan and Rantao Zuo. Writing—review and editing were done by Yanming Su, Yusheng Jiang, Qilin Yi, and Shu Huang.

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