Research Article

Effects of Dietary Phospholipid and Cholesterol Levels on Growth, Molting Performance, and Ovary Development in Female Juvenile Crayfish (*Procambarus clarkii*)

Shouquan Hou,¹ Shaicheng Zhu,¹ Jinghao Li,¹ Jin Huang,¹ Jiayao Li^{1,2,3} , and Yongxu Cheng^{1,2,3}

¹Key Laboratory of Freshwater Aquatic Genetic Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China

²Centre for Research on Environmental Ecology and Fish Nutrition (CREEFN) of the Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China

³National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai 201306, China

Correspondence should be addressed to Jiayao Li; jy-li@shou.edu.cn and Yongxu Cheng; chengyongxucrab@163.com

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The interaction between phospholipid (PL) and cholesterol (CH) on growth performance, molting rate, hepatopancreas fatty acid composition, and ovary development of the female red swamp crayfish (*Procambarus clarkii*) was investigated. Nine diets containing three PL levels (0, 20, and 60 g/kg) and three CH levels (0, 5, and 10 g/kg) were included in the study. Female crayfish (initial weight: 5.92 ± 0.03 g) were reared for 60 days. There was no significant interaction between dietary PL and CH levels ($P > 0.05$). However, the best growth performance and ovarian development were observed in crayfish receiving the 60 g/kg PL and 10 g/kg CH diet. High PL levels promoted CH transport in the hepatopancreas, and long-chain unsaturated fatty acid (C20:4n-6, C20:5n-3, and C22:6n-3) content of the hepatopancreas increased with the addition of PL in the diet. The molting rate increased with increasing CH levels in the diet containing 60 g/kg PL. Real-time quantitative polymerase chain reaction revealed that dietary CH enhanced the molting rate by improving the ecdysone receptor genes at transcription. This study showed that crayfish fed a diet containing 10 g/kg CH and 60 g/kg PL obtained maximum growth, molting rate, and ovary development.

1. Introduction

The red swamp freshwater crayfish (*Procambarus clarkii*) belongs to the family Cambaridae. This species is widely produced in China because of its strong environmental adaptability, delicious taste, and high nutritional value [1]. Crayfish production reached more than 2 million tons in 2019, according to the China Fishery Statistical Yearbook [2]. Currently, molting performance and ovarian development have become two important factors affecting the sustainability of the crayfish farming industry [3, 4].

Therefore, an artificial diet that improves molting and ovarian development needs to be developed by the industry.

Phospholipids (PL) and cholesterol (CH) are lipids that are the critical components of membranes and lipoproteins [5]. Most crustaceans cannot synthesize PL or CH sufficiently to meet their requirements during juvenile stages [4, 6]. Hence, appropriate PL and CH levels in the feed of crustaceans are required. PL are polar lipids that provide energy for the life activities of crustaceans and are conducive for facilitating the absorption and transport of lipids via emulsification [7, 8]. PL can also improve feed palatability

TABLE 1: Feed formulations and approximate compositions of the experiment diets (g/kg dry matter).

Ingredients	Diets (phospholipid-cholesterol level (g/kg))								
	Diet1 (0/0)	Diet2 (20/0)	Diet3 (60/0)	Diet4 (0/5)	Diet5 (20/5)	Diet6 (60/5)	Diet7 (0/10)	Diet8 (20/10)	Diet9 (60/10)
Casein	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0	300.0
Gelatin	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
Corn starch	385.0	385.0	385.0	385.0	385.0	385.0	385.0	385.0	385.0
Soybean oil	70.0	50.0	10.0	65.0	45.0	5.0	60.0	40.0	0.0
Soybean lecithin	0.0	20.0	60.0	0.0	20.0	60.0	0.0	20.0	60.0
Cholesterol	0.0	0.0	0.0	5.0	5.0	5.0	10.0	10.0	10.0
Cellulose	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Choline chloride	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Betaine	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin premix ^a	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Mineral premix ^b	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
CMC-Na	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Yeast extract	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
Proximate composition (dry matter (g/kg))									
Crude protein	401.1	409.9	403.8	405.1	409.6	400.1	403.4	409.9	409.2
Crude lipid	72.1	71.1	71.9	70.9	72.1	71.7	71.7	71.6	72.0
Moisture	105.5	101.2	101.0	103.7	103.9	104.6	103.4	104.9	103.3
Ash	49.1	51.3	53.2	48.8	51.8	53.7	49.2	50.6	56.4

^aVitamin premix provided the following per kg of diets: VA 62500 IU, VD₃ 15000 IU, VE 1.05 g, VK₃ 35.4 mg, VB₁ 100 mg, VB₂ 150 mg, VB₆ 150 mg, VB₁₂ 0.2 mg, VC 700 mg, biotin 4 mg, D-calcium pantothenate 250 mg, folic acid 25 mg, and nicotinamide 300 mg. ^bMineral premix provided the following per kg of diets: FeSO₄·H₂O 200 mg, CuSO₄·5H₂O 96 mg, ZnSO₄·H₂O 360 mg, MnSO₄·H₂O 120 mg, MgSO₄·H₂O 240 mg, KH₂PO₄ 4.2 g, NaH₂PO₄ 0.5 g, KI 5.4 mg, CoCl₂·6H₂O 2.1 mg, and Na₂SeO₃ 3 mg.

[9]. The PL level added is approximately 20 g/kg in commercial crustacean feeds, satisfying the requirements of crustaceans [10–13]. However, several studies have shown that the PL in feed is not necessary for the growth performance of some crustaceans [14–16]. Song et al. [13] reported that excessive PL levels (60 g/kg) compared to low PL levels reduced the growth performance of female *Portunus trituberculatus*. Optimal dietary PL levels for juvenile shrimp have been reported to be between 1.2% and 1.5% [17–19]. The reasons for the different optimal PL levels in feed are attributed to its different sources and components. CH acts as a precursor of steroid hormones involved in reproduction and molting [20, 21]. The optimal levels of dietary CH in shrimp diets range from 0 g/kg to 10 g/kg [5, 22–24]. The recommended level of CH is approximately 5 g/kg in crayfish feeds [25–27].

The underlying reasons for molt death syndrome are related to inadequate content of nutrients (e.g., high unsaturated fatty acids, PL, CH, and total lipids), which are stored in the hepatopancreas [11, 28–30]. Previous studies have shown that CH transported from the hepatopancreas results in the variation of the ecdysteroid signal transduction and changes the molting performance in crustacean [4, 31, 32].

Studies have revealed that PL promotes the transfer of lipids from the hepatopancreas to the ovary during ovary maturation [13, 33]. Steroid hormones are present in crustaceans, including 17 β -estradiol, estrone, 17 α -hydroxyprogesterone, and 17 α -hydroxypregnenolone [34–36]. The hepatopancreas and ovaries synthesize hormones promoting

ovarian development, such as estradiol [37]. CH is an important precursor of steroid hormones, and ovarian development in crustaceans can be induced by adding CH to the diet [38–40]. Gong et al. [23] reported a significant interactive effect between PL and CH on the growth of the juvenile *Litopenaeus vannamei*. However, more research has shown that no interaction effects were observed between PL and CH in the growth performance of the commercial crustaceans [17, 18, 24].

The objective of the present study was to investigate the effects of the interaction between PL and CH on the growth performance, hepatopancreas fatty acid composition, molting performance, and ovary development in female juvenile crayfish (*P. clarkii*) fed semipurified diets.

2. Materials and Methods

2.1. Experimental Diets. Nine isonitrogenous semipurified diets containing three PL levels (0, 20, and 60 g/kg) and three CH levels (0, 5, and 10 g/kg) were formulated for the present study, and the diets are defined as Diet1 (0/0), Diet2 (20/0), Diet3 (60/0), Diet4 (0/5), Diet5 (20/5), Diet6 (60/5), Diet7 (0/10), Diet8 (20/10), and Diet9 (60/10) (Table 1). PL was supplied by soybean lecithin powder at a purity of 95.6% and contained phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidate at 21%, 20%, 14%, and 8%, respectively. The purity of the dietary CH was over 90%. The levels of soybean oil were adjusted to balance the total lipid content.

TABLE 2: Primers and relative information used for quantitative real-time PCR analysis in this study.

Gene name	Forward (5'-3')	Revised (5'-3')	Product length (bp)
EcR	F: ATGGACTTCCCTCGCGAC	R: GATGTTACTGGATGTAA	162
RXR	F: AGCCCCGTTACCAT	R: CGCTCCTCCTCTACTGC	125
Chitinase	F: TTTGACTCGGTGGGTGCT	R: TGTATGGTCCAGGCTTTCC	385
TBP	F: AAGGAAATACGCTCGGATTG	R: CTGGCTGTGAGTGAGGACAA	136

EcR: ecdysteroid receptor; RXR: retinoid X receptor; TBP: TATA-binding protein.

Prior to preparing the experiment feeds, all dry ingredients were ground into a fine powder through a 250 μ m mesh. The diets were then thoroughly mixed manually with soybean oil. Water was added to form a dough that was immediately extruded through a 2 mm die orifice and air-dried at room temperature for 48 h. All diets were stored at -20°C in black plastic bags until use.

2.2. Experimental Rearing System. The indoor experiment rearing system was composed of 12 plastic tanks. Each tank (300 cm $\times$ 100 cm $\times$ 20 cm, $L \times W \times H$) contained 30 independent culture units (30 cm $\times$ 30 cm $\times$ 20 cm, $L \times W \times H$). There were several holes between the adjacent units to ensure water exchange. All units were continuously aerated, and a 14 h light and 10 h dark cycle was applied. Polyvinyl chloride tubes (diameter = 6 cm) were provided as shelters for *P. clarkii* at the bottom of each unit. The culture system was supplied with filtered freshwater, and the water depth of each unit was maintained at 10 cm. A 50% water exchange occurred each day.

2.3. Experimental Animals and Feeding Trial. The experiment was conducted at the Chongming Research Base of Shanghai Ocean University (Shanghai, China). Healthy, active, and intact *P. clarkii* juvenile females with immature ovaries containing undeveloped oocytes were obtained from a local crayfish farm in mid-June, 2019. All juvenile crayfish were kept individually in an experiment unit and fed Diet1 twice daily (07:00 and 17:00) for 7 days to acclimatize them to the feed and experimental conditions.

After the conditioning period, all crayfish were weighed (precision = 0.01 g) on an electronic balance (JA4002, Shanghai Puchun Measure Instrument Co., Ltd., Shanghai, China), and 270 *P. clarkii* (average initial weight: 5.92 ± 0.03 g) were randomly divided into nine treatments. Each dietary treatment had three replicates, with each replicate having 10 crayfish (1 crayfish unit⁻¹) that were randomly placed into 12 plastic tanks (270 units). All crayfish were fed a daily ration of 3%–5% body weight divided into two meals (07:00 and 18:30, 30% and 70% of total daily feed input, respectively). Any uneaten feed and produced feces were cleaned at 13:00 daily during the culture trial. Mortality and molting performance were monitored and recorded daily.

During the feeding phase of the experiment, the water quality was monitored daily using a water quality meter (YSI ProPlus, Visay Instruments Inc., USA). Water temperature was maintained at $25.1 \pm 2.7^\circ\text{C}$ throughout the trial, pH was 7.0 to 7.5, ammonia nitrogen was less than 0.5 mg/L,

dissolved oxygen was more than 5.0 mg/L, and nitrite was less than 0.15 mg/L.

2.4. Sample Collection. At the end of the feeding trial, the crayfish were fasted for 24 h before sampling. Crayfish weights were measured using an electronic balance (precision = 0.01 g). All crayfish were sampled. Blood samples were collected through the ventral sinus in the first abdominal segment using a 1 mL syringe and centrifuged at 3000 r/min for 10 min. The supernatants were collected and stored in 2 mL tubes at -80°C for subsequent measurements. The crayfish were dissected to obtain the hepatopancreas and gonadal tissues to calculate the gonadosomatic index (GSI) and hepatosomatic index (HSI) and were stored at -80°C for subsequent analyses of the fatty acid profile. The hepatopancreas which fed different CH levels when PL addition level was 60 g/kg was stored at -80°C for the RNA extraction. The hepatopancreas and gonads which fed different PL levels when CH addition level was 10 g/kg, with a length of 1 cm, were then sampled and immersed in Bouin's solution for histology analysis. The survival rate (SR, %), weight gain rate (WGR, %), specific growth rate (SGR, % per day), HSI, and GSI were calculated using the following formulas:

$$\begin{aligned}
 \text{Survival rate (SR, \%)} &= \frac{100 \times \text{final crayfish number}}{\text{initial crayfish number}}, \\
 \text{Weight gain rate (WGR, \%)} &= \frac{100 \times (\text{final weight} - \text{initial weight})}{\text{initial weight}}, \\
 \text{Specific growth rate (SGR, \% per day)} &= \frac{100 \times (\ln \text{final weight} - \ln \text{initial weight})}{60 \text{ days}}, \\
 \text{Molting rate (\%)} &= \frac{100 \times \text{total molting times}}{\text{final crayfish number}}, \\
 \text{Hepatosomatic index (HSI, \%)} &= \frac{100 \times \text{wet hepatopancreas weight}}{\text{wet body weight}}, \\
 \text{Gonadosomatic index (GSI, \%)} &= \frac{100 \times \text{wet gonad weight}}{\text{wet body weight}}, \\
 \text{Coefficient of variance (CV, \%)} &= 100 \times \frac{\text{standard deviation of final body weight}}{\text{mean final body weight}}.
 \end{aligned}
 \tag{1}$$

2.5. Biochemical Analysis and Fatty Acid Composition. The chemical composition of diets and tissues was measured according to standard methods with slight modifications. The moisture of the diet samples was measured by oven drying at 105°C to a constant weight, and the moisture of tissues was determined according to freeze-dried analysis. Ash was measured using a muffle furnace at 550°C for 8 h [41]. The crude protein content was determined by measuring

TABLE 3: Growth and survival performance of juvenile female *Procambarus clarkii* fed with the experiment diets with different phospholipid and cholesterol levels under experimental culture conditions.

PL (g/kg)	CH (g/kg)	IBW (g)	FBW (g)	SR (%)	WGR (%)	SGR (%/d)	CV (%)
0	0	5.91 ± 0.10	9.60 ± 0.38	93.33 ± 3.33	64.74 ± 6.42	0.80 ± 0.06	20.53 ± 1.65
0	5	5.94 ± 0.08	9.50 ± 0.37	100.00 ± 0.00	60.52 ± 5.40	0.76 ± 0.06	17.98 ± 3.38
0	10	5.94 ± 0.09	9.25 ± 0.25 ^A	93.33 ± 6.67	57.22 ± 4.66 ^A	0.73 ± 0.05 ^A	12.32 ± 2.18
20	0	5.93 ± 0.10	10.00 ± 0.38	93.33 ± 3.33	69.01 ± 6.93	0.84 ± 0.06	18.03 ± 4.02
20	5	5.90 ± 0.09	9.15 ± 0.29	96.67 ± 3.33	56.26 ± 5.77	0.71 ± 0.06	15.63 ± 1.31
20	10	5.93 ± 0.10	9.08 ± 0.35 ^A	96.67 ± 3.3	53.35 ± 5.88 ^A	0.68 ± 0.06 ^A	19.64 ± 5.15
60	0	5.93 ± 0.11	9.85 ± 0.36	100.00 ± 0.00	68.65 ± 7.24	0.83 ± 0.07	19.32 ± 1.02
60	5	5.91 ± 0.10	9.79 ± 0.31	96.67 ± 3.33	66.57 ± 5.82	0.82 ± 0.06	17.88 ± 0.25
60	10	5.90 ± 0.12	10.48 ± 0.35 ^B	100.00 ± .00	79.76 ± 7.23 ^B	0.94 ± 0.07 ^B	18.05 ± 1.60
Pooled SE		0.00	0.15	0.96	2.72	0.03	0.88
Means of main effect							
0		5.93	9.45 ^A	95.56	60.83 ^A	0.77 ^A	16.95
2		5.92	9.41 ^A	95.56	59.54 ^A	0.75 ^A	17.77
6		5.92	10.04 ^B	98.89	71.66 ^B	0.87 ^B	18.42
	0	5.92	9.82	95.56	67.47	0.83	19.29
	0.5	5.92	9.48	97.78	61.12	0.77	17.16
	1	5.92	9.60	96.67	63.44	0.79	16.67
Two-way ANOVA: <i>P</i> values							
PL		0.987	0.039	0.387	0.032	0.036	0.805
CH		0.998	0.468	0.721	0.448	0.481	0.473
PL × CH		0.997	0.186	0.522	0.258	0.241	0.382

PL: phospholipid; CH: cholesterol. Data are presented as the mean ± SE (*n* = 3). Values in the same line with different superscripts are significantly different (*P* < 0.05). ^{AB}Significant difference between phospholipid levels within the same cholesterol level (*P* < 0.05).

TABLE 4: Correlation coefficient between dietary PL levels and growth performance.

Alteration in PL levels	Growth performance		
	FBW (g)	WGR (%)	SGR (%/d)
CH 0 g/kg	0.037	0.037	0.027
CH 5 g/kg	0.092	0.102	0.103
CH 10 g/kg	0.323**	0.316**	0.299**

***P* < 0.01.

TABLE 5: Correlation coefficient between dietary CH levels and growth performance.

Alteration in CH levels	Growth performance		
	FBW (g)	WGR (%)	SGR (%/d)
PL 0 g/kg	-0.079	-0.106	-0.093
PL 20 g/kg	-0.213*	-0.193	-0.202
PL 60 g/kg	0.148	0.124	0.132

*. *P* < 0.05.

nitrogen (N × 6.25) using the Kjeldahl method. Total lipids were extracted with chloroform-methanol (2:1, *v/v*) [42].

For fatty acid analysis, total lipids from the hepatopancreas and diets were esterified by boiling 14% boron trifluor-

ide/methanol (*w/w*), and fatty acid methyl esters (FAMES) were extracted using hexane [43]. FAMES were esterified analyzed using an Agilent 7890B GC/5977A gas chromatograph-mass spectrometer with an Omegawax 320-fused silica capillary column (30 m × 0.32 mm × 0.25 μm; Supelco, Bellefonte, PA, USA). The injector and detector temperatures were maintained at 260°C. The column temperature was initially set at 40°C and increased at a rate of 10°C min⁻¹ to 170°C and held for 1 min, followed by an increase at a rate of 2°C min⁻¹ to 220°C and held for 1 min. It was then further increased at a rate of 2°C min⁻¹ to a final temperature of 230°C and held for 30 min until all FAMES had been eluted. The peaks were identified by comparing retention times with a 37-component FAME standard mixture (18919-1AMP, Sigma-Aldrich Co., St. Louis, MO, USA). The fatty acid composition was expressed as the percentage of each fatty acid to the total fatty acids (% total fatty acids).

2.6. Hepatopancreas and Gonad Histology. The tissue samples which fed different PL levels when CH addition level was 10 g/kg were dehydrated in a series of alcohol solutions and embedded in paraffin. The paraffin blocks were sectioned (7 μm) in serial sagittal sections using a Lecia RM 2245 rotary microtome, and the slides were stained with haematoxylin-eosin. The morphological structure of the

FIGURE 1: Linear regression analysis of the level of dietary phospholipid (a) and cholesterol (b) supplementation with weight gain (WG), respectively.

FIGURE 2: The weight gain rate of juvenile female *P. clarkii* fed with various experimental diets. CH0: diets contain 0 g/kg cholesterol; CH5: diets contain 5 g/kg cholesterol; CH10: diets contain 10 g/kg cholesterol. Data are presented as the mean $\pm$ SE ($n = 3$). ^{AB}Significant difference between phospholipid levels within the same cholesterol level ($P < 0.05$).

FIGURE 3: The molting rate of juvenile female *P. clarkii* fed with various experimental diets. CH0: diets contain 0 g/kg cholesterol; CH5: diets contain 5 g/kg cholesterol; CH10: diets contain 10 g/kg cholesterol. Data are presented as the mean $\pm$ SE ($n = 3$). ^{ab}Significant difference between cholesterol levels within the same phospholipid level ($P < 0.05$).

tissue was observed using a light microscope (Nikon YS100), and photographs were taken.

2.7. 17β -Estradiol, Total Cholesterol, High-Density Lipoprotein Cholesterol, and Low-Density Lipoprotein Cholesterol Content Assays. The 17β -estradiol (E_2) in ovaries was measured using an enzyme immunoassay kit (ELISA) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total cholesterol (TCH), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) determinations in the hepatopancreas and serum were analyzed with a spectrophotometer and corresponding detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's guidelines.

2.8. RNA Extraction, cDNA Synthesis, and Gene Expression. Total RNA was extracted from the *P. clarkii* hepatopancreas with RNAiso Plus (TaKaRa, Japan) following the manufacturer's protocol and then dissolved in DEPC treated water. The quantity and quality of isolated RNA were evaluated using spectrophotometric (OD260/280) analysis and 1% agarose gel electrophoresis, respectively. TATA-binding protein was used as the reference gene in this study, and primers (Table 2) for molting genes were designed using Primer Premier 5.0 software based on the published mRNA sequences of *P. clarkii* [4]. cDNA synthesis via reverse transcription was performed using a PremeScript™ RT reagent kit (TaKaRa, Japan) according to the manufacturer's protocol. The quantitative real-time PCR was performed using a TaKaRa SYBR® Prime Script™ RT-PCR kit (Takara, Japan).

TABLE 6: Effects of dietary phospholipid and cholesterol levels on development performance in juvenile female *P. clarkii*.

PL (g/kg)	CH (g/kg)	Hepatopancreas		Ovary	
		HSI (%)	Lipid (g/kg)	GSI (%)	E ₂ (ng/gprot)
0	0	6.61 ± 0.41	524.1 ± 5.60 ^B	4.01 ± 0.28 ^A	14.44 ± 0.57 ^A
0	5	5.86 ± 0.20	548.5 ± 58.8 ^B	4.07 ± 0.23 ^A	19.08 ± 0.24 ^B
0	10	6.50 ± 0.28 ^B	456.5 ± 43.2	4.21 ± 0.34 ^A	15.66 ± 0.70 ^A
20	0	6.59 ± 0.29	488.8 ± 27.4 ^{AB}	4.96 ± 0.38 ^{AB}	15.38 ± 1.23 ^A
20	5	6.04 ± 0.28	481.9 ± 6.50 ^B	4.52 ± 0.33 ^A	16.61 ± 0.98 ^A
20	10	6.55 ± 0.27 ^B	412.4 ± 47.9	4.03 ± 0.36 ^A	15.00 ± 0.64 ^A
60	0	6.47 ± 0.31 ^b	417.0 ± 15.7 ^{b,A}	4.83 ± 0.36 ^{AB}	15.48 ± 0.35 ^A
60	5	5.73 ± 0.39 ^{ab}	269.5 ± 9.90 ^{a,A}	4.88 ± 0.46 ^{AB}	14.50 ± 0.56 ^A
60	10	5.48 ± 0.27 ^{a,A}	410.2 ± 15.50 ^b	5.83 ± 0.33 ^B	16.17 ± 1.44 ^A
Pooled SE		0.14	2.72	0.20	0.47
Means of main effect					
0		6.32	509.7 ^B	4.10 ^A	16.40
2		6.39	461.0 ^B	4.50 ^A	15.66
6		5.89	372.3 ^A	5.18 ^B	15.38
0		6.56 ^b	483.3	4.60	15.10 ^a
0.5		5.87 ^a	433.3	4.49	16.73 ^b
1		6.18 ^{ab}	426.4	4.69	15.61 ^{ab}
Two-way ANOVA: P-values					
PL		0.095	≤0.001	0.001	0.317
CH		0.023	0.081	0.771	0.060
PL × CH		0.427	0.010	0.075	0.011

PL: phospholipid; CH: cholesterol; HSI: hepatosomatic index; GSI: gonadosomatic index; lipid: total content of the lipid; E₂: estradiol. Data are presented as the mean ± SE ($n = 3$). Values in the same line with different superscripts are significantly different ($P < 0.05$). ^{AB}Significant difference between phospholipid levels within the same cholesterol level ($P < 0.05$). ^{ab}Significant difference between cholesterol levels within the same phospholipid level ($P < 0.05$).

FIGURE 4: The GSI (a) and E₂ (b) of juvenile female *P. clarkii* fed with various experimental diets. CH0: diets contain 0 g/kg cholesterol; CH5: diets contain 5 g/kg cholesterol; CH10: diets contain 10 g/kg cholesterol. Data are presented as the mean ± SE ($n = 3$). ^{AB}Significant difference between phospholipid levels within the same cholesterol level ($P < 0.05$). ^{ab}Significant difference between cholesterol levels within the same phospholipid level ($P < 0.05$).

Amplification was performed in a 96-well plate with a 20 μ L reaction volume containing 10 μ L 2 × T5 Fast qPCR Mix, 0.8 μ L of each primer (10 μ M), 2 μ L cDNA template, and

6.4 μ L PCR-grade water. The amplification procedure was per denaturation (95°C, 1 min), denaturation (95°C, 10 s, 40 cycles), annealing (60°C, 5 s, 40 cycles), extension (72°C,

FIGURE 5: Histological transverse section of hepatopancreas of the crayfish (*P. clarkii*) fed with (1, 2) 0 g/kg, (3, 4) 20 g/kg, and (5, 6) 60 g/kg PL diets using different magnification (1, 3, and 5: 10x; 2, 4, and 6: 20x). R: R cells; B: B cells.

FIGURE 6: Histological transverse section of ovary of the red swamp crayfish (*P. clarkii*) fed with (1, 2) 0 g/kg, (3, 4) 20 g/kg, and (5, 6) 60 g/kg PL diets using different magnification (1, 3, and 5: 2x; 2, 4, and 6: 4x). Yg: yolk granules.

TABLE 7: Prevalence (number of cells in each tubule) of B-cells and R-cells in the hepatopancreatic tubules of the crayfish fed diets with increasing levels of phospholipid.

	Experiment diets		
	0 g/kg PL	20 g/kg PL	60 g/kg PL
R-cells	28.50 ± 2.23 ^c	19.50 ± 1.09 ^b	12.50 ± 1.87 ^a
B-cells	5.83 ± 0.54 ^a	10.50 ± 1.18 ^b	16.83 ± 2.79 ^c

Values in the same row with different superscripts are significantly different ($P < 0.05$).

15 s, 40 cycles), and melting curve at 95°C after 30 min. The $2^{-\Delta\Delta CT}$ comparative CT method was used to analyze the expression level of these genes [44].

2.9. Statistical Analysis. The data are presented as the mean ± standard error of the mean. Statistical analysis for homo-

geneity was undertaken with Levene's test, and when necessary, arcsine, square root, or logarithmic transformations were performed prior to analysis. One-way analysis of variance (ANOVA) analyses were performed and differences determined using Duncan's multiple range test. The relationship between CH levels and growth performance and PL levels regarding fatty acid composition was associated using Pearson's correlation coefficients. Two-way ANOVA analysis was used to separate the effects of PL, CH, and their interaction. A P value of <0.05 was considered statistically significant. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ equation [44].

3. Results

3.1. Growth and Molting Performance. The growth performances of *P. clarkii* fed diets with different PL and CH

supplementations are presented in Table 3. Growth performance was significantly affected by PL level ($P < 0.05$) based on the two-way ANOVA. The WGR and SGR were highest in crayfish fed a diet of 60 g/kg PL and 10 g/kg CH supplementation (Tables 4 and 5; Figures 1 and 2). No significant difference was detected for the SR of crayfish among the different dietary treatments ($P > 0.05$). With an increasing CH level up to 10%, the optimum molting rate was obtained when the crayfish were fed the 60 g/kg PL diet (Figure 3).

3.2. Hepatopancreas and Ovary Development. As shown in Table 6 and Figure 4, the PL and CH interaction significantly affected the total lipid content in the hepatopancreas and E_2 in the ovaries ($P < 0.05$). The total lipid levels decreased significantly with increasing PL levels up to 60 g/kg in the diet ($P < 0.05$). Additionally, the HSI was significantly affected by the CH level ($P < 0.05$), and the GSI was significantly affected by the PL level ($P < 0.05$) in the feed. The highest HSI was obtained in the juvenile crayfish fed the 0 g/kg CH, and the poorest GSI was obtained when juvenile crayfish were fed the 0 g/kg PL diet ($P < 0.05$).

Figures 5 and 6 show the hepatopancreas and ovary morphological structure of *P. clarkii* fed different diets. B-cells in the 60 g/kg PL level group were more abundant than the crayfish fed the other feed groups. A noticeable increase in the number of vacuoles in R-cells was observed in the group without PL supplementation compared to crayfish fed the other diets ($P < 0.05$) (Table 7). The eggs were more compactly arranged and signified advanced developmental stages in the 60 g/kg PL diet.

3.3. Muscle Proximate and Hepatopancreas Fatty Acid Composition. The proximate composition of crayfish muscle is summarized in Table 8. Based on the two-way ANOVA analysis, the muscle total lipid, ash, and moisture showed no significant difference among the different treatments ($P > 0.05$). The muscle protein level showed significant differences among the different PL and CH levels ($P < 0.05$), and the highest content occurred in the 5 g/kg CH group.

Table 9 shows the fatty acid composition (% total detectable fatty acids) in the diets of juvenile female *P. clarkii*, with a higher C18:2n-6 content found in diets containing increasing dietary PL levels (Table 10). The results for hepatopancreas fatty acid composition are shown in Tables 11 and 12. The C18:2n-6 content showed a decreasing trend, whereas C20:4n-6, C20:5n-3, and C22:6n-3 contents showed an increasing trend with an increase in dietary PL supplementation (Table 10 and Figure 7).

3.4. Hepatopancreas and Serum CH Composition. Table 13 shows the hepatopancreas and serum CH composition of juvenile *P. clarkii* fed the nine diets. The two-way ANOVA showed that the serum TCH content, HDL-C, and LDL-C, and the hepatopancreas TCH content were significantly affected by dietary PL and CH levels ($P < 0.05$). With an increase in the PL level, the serum HDL-C/LDL-C showed a decreasing trend (Figure 8). In contrast, the TCH of the hepatopancreas showed an increasing trend ($P > 0.05$). Like-

TABLE 8: Muscle composition of juvenile female *P. clarkii* fed various experimental diets.

PL (g/kg)	CH (g/kg)	Protein (g/kg)	Lipid (g/kg)	Ash (g/kg)	Moisture (g/kg)
0	0	870.0 ± 14.7 _{ab}	34.1 ± 2.3	42.5 ± 0.8	789.3 ± 2.6
0	5	893.7 ± 25.4 _b	31.0 ± 1.8	43.3 ± 2.6	782.5 ± 2.4
0	10	817.6 ± 12.0 _{a,A}	34.7 ± 0.9	42.0 ± 1.3	795.6 ± 6.3
20	0	862.3 ± 45.1 _{ab}	36.3 ± 2.3	34.9 ± 3.1	788.7 ± 1.7
20	5	876.4 ± 43.2 _b	30.3 ± 3.1	42.9 ± 3.8	790.5 ± 3.6
20	10	849.7 ± 42.1 _{a,B}	37.8 ± 2.3	39.4 ± 5.3	794.3 ± 6.1
60	0	852.2 ± 54.0 _a	32.7 ± 2.3	36.2 ± 9.5	783.2 ± 5.3
60	5	884.3 ± 29.0 _b	33.6 ± 2.0	49.2 ± 1.8	797.1 ± 4.4
60	10	864.8 ± 51.2 _{a,B}	32.1 ± 2.5	32.3 ± 8.2	785.7 ± 1.2
Pooled SE		0.74	0.08	0.17	0.18
Means of main effect					
0		860.4	33.2	42.6	789.2
2		862.8	34.8	39.1	791.1
6		867.1	32.8	39.2	788.7
0		861.5 ^b	34.3	37.9	787.1
0.5		883.7 ^c	31.6	45.1	790.0
1		844.0 ^a	34.9	37.9	791.9
Two-way ANOVA: <i>P</i> values					
PL		0.679	0.524	0.669	0.747
CH		≤0.001	0.168	0.172	0.382
PL × CH		0.016	0.366	0.551	0.073

PL: phospholipids; CH: cholesterol. Data are presented as the mean ± SE ($n = 3$). Values in the same line with different superscripts are significantly different ($P < 0.05$).^{AB} means significant difference between phospholipid levels within the same cholesterol level ($P < 0.05$). ^{ab} indicates significant difference between cholesterol levels within the same phospholipid level ($P < 0.05$).

wise, the serum TG content increased with an increase in dietary CH supplementation ($P < 0.05$).

3.5. mRNA Expression of Genes Related to Molting. Gene expression related to molting is shown in Figure 9. Diet9 had the highest levels of ecdysone receptor- (EcR-) mRNA in the hepatopancreas among the three treatments ($P < 0.05$). When the addition amount of PL was 60 g/kg, *P. clarkii* fed with 10 g/kg CH supplementation had significantly higher EcR expression level than the *P. clarkii* fed with 0 and 5 g/kg CH supplementation ($P < 0.05$), which showed a positive trend with increasing CH levels in the diet.

TABLE 9: Fatty acid composition (% total fatty acids) of the experimental diets.

Fatty acid	Diets (supplement level%, PL/CH)								
	Diet1 (0/0)	Diet2 (20/0)	Diet3 (60/0)	Diet4 (0/5)	Diet5 (20/5)	Diet6 (60/5)	Diet7 (0/10)	Diet8 (20/10)	Diet9 (60/10)
C14:0	0.13	0.14	0.12	0.11	0.12	0.11	0.11	0.12	0.09
C15:0	0.03	0.03	0.06	0.03	0.04	0.07	0.07	0.07	0.08
C16:0	13.00	14.43	18.78	13.24	14.61	19.74	14.11	15.42	20.75
C17:0	0.14	0.16	0.17	0.14	0.15	0.19	0.15	0.16	0.19
C18:0	5.33	5.38	5.34	5.48	5.50	5.50	5.26	5.51	5.66
C20:0	0.53	0.50	0.26	0.55	0.52	0.26	0.48	0.48	0.24
C21:0	0.04	0.06	0.05	0.04	0.04	0.05	0.04	0.04	0.06
C22:0	0.80	0.76	0.74	0.72	0.78	0.79	0.72	0.68	0.86
C24:0	0.34	0.46	0.58	0.46	0.36	0.73	0.34	0.45	0.71
Σ SFA ^a	20.34	21.92	26.1	20.77	22.12	27.44	21.28	22.93	28.64
C16:1n-5	0.02	0.01	0.05	0.02	0.01	0.04	0.00	0.01	0.04
C16:1n-7	0.13	0.16	0.15	0.16	0.14	0.17	0.23	0.21	0.22
C17:1n-7	0.07	0.07	0.07	0.07	0.07	0.08	0.07	0.08	0.07
C18:1n-5	0.06	0.06	0.07	0.06	0.06	0.06	0.06	0.06	0.06
C18:1n-9	21.71	19.98	14.28	22.06	20.12	13.15	19.88	19.67	12.07
C20:1n-9	0.29	0.24	0.15	0.27	0.25	0.15	0.24	0.24	0.15
Σ MUFA ^b	22.28	20.52	14.77	22.64	20.65	13.65	20.48	20.27	12.61
C17:2n-5	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.03
C18:2n-6	47.09	47.59	49.55	47.76	48.28	49.14	46.34	47.52	49.60
C18:2n-7	0.15	0.14	0.13	0.17	0.16	0.13	0.16	0.18	0.14
C20:2n-6	0.04	0.06	0.02	0.04	0.06	0	0.06	0.04	0.11
C18:3n-3	7.28	7.14	6.66	7.49	6.63	6.56	6.96	6.84	6.19
Σ PUFA ^c	54.6	54.97	56.4	55.5	55.17	55.87	53.56	54.62	56.07
n-3 PUFA ^d	7.28	7.14	6.66	7.49	6.63	6.56	6.96	6.84	6.19
n-6 PUFA ^e	47.13	47.65	49.57	47.80	48.34	49.14	46.40	47.56	49.71
n-6/n-3	6.47	6.67	7.44	6.38	7.29	7.49	6.67	6.95	8.03

^aΣ SFA: saturated fatty acid—C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, and C24:0. ^bΣ MUFA: monounsaturated fatty acid—C16:1n-5, C16:1n-7, C17:1n-7, C18:1n-5, C18:1n-9, and C20:1n-9. ^cΣ PUFA: polyunsaturated fatty acids—C17:2n-5, C18:2n-6, C18:2n-7, C20:2n-6, C18:3n-3. ^dn-3 PUFA—C18:3n-3. ^en-6 PUFA—C18:2n-6, C20:2n-6. Data are presented as the average values for three replicates per treatment ($n = 3$).

TABLE 10: Correlation coefficient between dietary PL levels and main fatty acid composition (% total fatty acids).

Alteration in PL levels	Experiment diets	Hepatopancreas			
	C18:2n-6	C18:2n-6	C20:4n-6	C20:5n-3	C22:6n-3
CH 0 g/kg	0.945**	-0.807**	0.896**	0.469	0.401
CH 5 g/kg	0.882**	-0.858**	0.916**	0.547	0.349
CH 10 g/kg	0.728*	-0.864**	0.411	-0.450	0.125

* $P < 0.05$; ** $P < 0.01$.

4. Discussion

The present study showed no interaction between dietary PL and CH levels on the growth performance of juvenile female *P. clarkii* under controlled environmental conditions. This result is similar to other studies that examined the swimming crab *Po. trituberculatus* [30], black tiger prawn

Penaeus monodon [17], marine shrimp *Penaeus penicillatus* [18], and freshwater prawn *Macrobrachium rosenbergii* [14]. One possible reason for observing normal growth without PL and CH supplementation was that the red swamp crayfish can synthesize PL and steroids to fulfill its growth needs. Similar results have been obtained in other studies [14, 24, 26, 45]. The second possible reason could be due to choline chloride in feed, which could serve as the material for phosphatidylcholine synthesis [46]. Crustaceans can synthesize phosphatidylcholine [45]. In the present study, 5 g/kg choline chloride was added in different experiment feeds, which could meet the normal growth requirement of juvenile female *P. clarkii*.

As a source of energy, cell membrane component, and emulsifier, PL was added to the diet of crustaceans. Increasing growth is one of the beneficial effects of supplementing PL in the diet [33, 47, 48]. The growth performance indicated that the crayfish fed a semipurified diet containing up to 6% of PL (soybean lecithin) significantly enhanced growth compared to the diet lacking supplemental PL.

TABLE 11: Fatty acid composition (% , total detectable fatty acids) in hepatopancreas of juvenile female *P. clarkii*.

Fatty acid	Diet1 (0/0)	Diet2 (20/0)	Diet3 (60/0)	Diet4 (0/5)	Diet5 (20/5)	Diet6 (60/5)	Diet7 (0/10)	Diet8 (20/10)	Diet9 (60/10)
C14:0	0.28	0.37	0.42	0.34	0.39	0.44	0.41	0.42	0.46
C15:0	0.53	0.51	0.57	0.51	0.63	0.73	0.63	0.63	0.73
C16:0	15.11	15.95	17.59	14.89	16.14	16.99	14.75	15.12	18.19
C17:0	0.23	0.22	0.25	0.24	0.23	0.32	0.25	0.31	0.24
C18:0	4.07	3.89	4.21	3.96	4.15	4.21	4.52	4.35	3.88
C20:0	0.27	0.23	0.22	0.23	0.24	0.20	0.27	0.27	0.20
C22:0	0.29	0.30	0.32	0.25	0.32	0.28	0.25	0.25	0.34
Σ SFA ^a	20.76	21.46	23.60	20.42	22.09	23.15	21.07	21.35	24.03
C16:1n-7	4.41	6.48	8.76	5.11	6.59	8.85	5.68	6.77	11.10
C17:1n-7	0.25	0.35	0.43	0.33	0.39	0.54	0.36	0.47	0.48
C18:1n-5	0.09	0.04	0.10	0.06	0.07	0.17	0.13	0.14	0.13
C18:1n-7	2.26	2.27	2.40	2.25	2.33	2.61	2.00	2.10	2.86
C18:1n-9	26.57	27.09	24.83	27.02	25.90	26.40	27.42	24.83	24.01
C20:1n-9	0.57	0.62	0.60	0.6	0.54	0.60	0.53	0.44	0.61
Σ MUFA ^b	34.15	36.84	37.12	35.37	35.82	39.17	36.13	34.75	39.22
C18:2n-6	34.83	30.90	27.61	32.83	31.42	24.65	30.66	30.24	25.28
C20:2n-6	0.96	1.02	1.08	1.14	1.03	1.48	1.26	1.26	1.00
C18:3n-3	3.77	3.04	2.72	3.49	3.22	2.05	3.25	3.33	2.37
C18:3n-6	0.07	0.03	0.11	0.08	0.09	0.14	0.11	0.11	0.11
C20:4n-6	0.79	0.99	1.27	0.83	0.94	1.68	1.17	1.35	1.45
C20:5n-3	0.87	1.06	1.15	1.19	0.86	1.78	1.08	1.03	0.98
C22:6n-3	0.39	0.43	0.46	0.44	0.37	0.52	0.43	0.53	0.47
PUFA ^c	41.68	37.47	34.4	40	37.93	32.3	37.96	37.85	31.66
HUFA ^d	2.05	2.48	2.88	2.46	2.17	3.98	2.68	2.91	2.9
n-3 PUFA ^e	5.03	4.53	4.33	5.12	4.45	4.35	4.76	4.89	3.82
n-6 PUFA ^f	36.65	32.94	30.07	34.88	33.48	27.95	33.2	32.96	27.84
n-6/n-3	7.23	7.27	6.94	6.81	7.52	6.43	6.97	6.74	7.29

^a#x03A3; SFA: saturated fatty acid—C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, and C22:0. ^b Σ MUFA: monounsaturated fatty acid—C16:1n-7, C17:1n-7, C18:1n-5, C18:1n-7, C18:1n-9, and C20:1n-9. ^c Σ PUFA: polyunsaturated fatty acids—C18:2n-6, C20:2n-6, C18:3n-3, C18:3n-6, C20:4n-6, C20:5n-3, and C22:6n-3. ^d Σ HUFA: highly unsaturated fatty acids—C20:4n-6, C20:5n-3, and C22:6n-3. ^en-3 PUFA—C18:3n-3, C20:5n-3, and C22:6n-3. ^fn-6 PUFA—C18:2n-6, C20:2n-6, C18:3n-6, and C20:4n-6. Data are presented as the average values for three replicates per treatment ($n = 3$).

TABLE 12: Results of two-way ANOVA for phospholipid-cholesterol interactions for several fatty acids in hepatopancreas of the juvenile *P. clarkii*.

Fatty acid	PL	<i>P</i> value CH	<i>PL</i> $\times$ <i>CH</i>
C18:2n-6	<0.01	0.077	0.460
C20:4n-6	<0.01	0.019	0.100
C20:5n-3	0.093	0.169	0.085
C22:6n-3	0.409	0.565	0.447

Many crustaceans are incapable of de novo synthesis of sterols [49]. However, high dietary sterol levels in diets retard growth in crustaceans [25, 32, 50]. For the crayfish fed diets supplemented with 0–20 g/kg PL, the final weight, WGR, and SGR showed a negative growth response with an increase in dietary CH supplementation. This result was in accordance with results for juvenile *Penaeus monodon* [51]. The best growth performance of crayfish was when

60 g/kg PL and 10 g/kg CH were supplemented in the diet. This might be caused by CH playing a crucial role in the metabolism of nutrients, such as amino acids, fatty acids, and vitamins [52]. In addition, the Y-organs of crustaceans synthesize and secrete ecdysteroid (molting hormones), which control the molting performance [53]. The ecdysone is bound to the EcR and retinoid X receptor (RXR) in the nucleus, which activates the *EcR-RXR* complex, and the complex can regulate the transcription of target genes, such as chitinase [54–57]. Moreover, CH serves as a precursor of ecdysteroid biosynthesis in crustaceans [27, 52]. Several studies have shown that the recommended CH level in the diet enhanced the molting performance in crustaceans [27, 31]. In the present study, for all PL 60 g/kg groups, the crayfish fed with a CH supplemental level of 10 g/kg had a significantly higher molting rate, and the relative EcR expression level in this group was also the highest. Previous studies have shown that dietary CH supplemental level affected ecdysteroid signal transduction [4] and molting rate determined the growth performance of crustaceans [27]. Therefore, the

FIGURE 7: Linear regression analysis between the contents of several fatty acids in hepatopancreas and dietary phospholipid levels.

increased molting rate caused by the higher PL and CH levels might be the main reason for the better growth performance of the Diet9 group.

The hepatopancreas is the crucial organ for lipid absorption, storage, and transport in crustaceans [58, 59]. Its histology can be used to monitor the nutritional value of diets in aquaculture [60]. The hepatopancreas comprises four main types of cells within the hepatopancreatic tubules: F-cells that synthesize digestive enzymes and absorption, E-cells that are undifferentiated, and R-cells and B-cells that are responsible for nutrition storage and digestion, respectively [61–64]. In the present study, the decreased numbers of R-cells were due to the decreased accumulation of lipids in the hepatopancreas of the crayfish fed higher PL content diets (60 g/kg). In contrast, the numbers of B-cells showed an increasing trend, implying an increased transport of lipids in the hepatopancreas of crayfish fed high PL level diets. In previous studies, R-cells and B-cells had a similar trend, caused by the transport of nutrients such as lipids [64, 65].

The fatty acid composition of the hepatopancreas reflects the dietary fatty acid composition [11, 66] and is a lipid storage organ that is more affected by feed than other organs [43]. At the same CH supplementation in feed, the linoleic acid (C18:2n-6) content in the diet gradually increased with an increase in PL levels. This might be attributed to the soy-

bean lecithin, which contained higher levels of C18:2n-6 [30, 67]. The content of polyunsaturated fatty acids (PUFA) and long-chain unsaturated fatty acids (lcPUFA) in the hepatopancreas presented an increasing trend with increasing PL content in the feed. Previous studies have demonstrated that the appropriate PL in feed could promote fatty acid synthase activity in the hepatopancreas. Similar speculations were observed in previous studies examining the freshwater crustacean [68, 69]. Thereby, we speculate that *P. clarkii* was capable of synthesizing PUFA and lcPUFA, which utilizes exogenous linolenic acid, although the crayfish could not synthesize de novo PUFA and lcPUFA. Thus, *P. clarkii* could be an excellent model organism for lcPUFA synthesis in crustaceans.

HDL and LDL can reflect the CH transport in the serum of crustaceans [5], and an increased or decreased HDL/LDL proportion indicates whether CH in the serum enters the hepatopancreas to participate in metabolic activities or moves from the hepatopancreas to tissues [66, 70]. In the present study, the proportion of HDL/LDL decreased with an increase in PL content. This is because PL promotes the transport of lipids [13, 71], which carry more lipids into the ovaries from the hepatopancreas during ovarian development. The serum TG content was not significantly affected by the PL addition levels. Similarly, Lin et al. [12] reported no significant difference in serum TG content

TABLE 13: Effects of various experimental diets on serum and hepatopancreas cholesterol levels in juvenile female *P. clarkii*.

PL (g/kg)	CH (g/kg)	TG (mmol/L)	TCH (mmol/L)	Serum		HDL-C/LDL-C	Hepatopancreas TCH (mmol/L)
				HDL-C (mmol/L)	LDL-C (mmol/L)		
0	0	0.17 ± 0.03	0.49 ± 0.08 ^{b,B}	0.16 ± 0.01 ^b	0.043 ± 0.00 ^b	3.82 ± 0.36 ^a	0.54 ± 0.01 ^{c,B}
0	5	0.24 ± 0.01	0.37 ± 0.04 ^{ab,B}	0.12 ± 0.01 ^a	0.036 ± 0.01 ^b	3.57 ± 0.42 ^a	0.43 ± 0.01 ^b
0	10	0.20 ± 0.07	0.21 ± 0.03 ^{a,A}	0.18 ± 0.01 ^{b,B}	0.023 ± 0.00 ^{a,A}	8.39 ± 1.02 ^{b,B}	0.34 ± 0.04 ^{a,A}
20	0	0.11 ± 0.02 ^a	0.35 ± 0.03 ^{AB}	0.14 ± 0.01	0.045 ± 0.01	3.62 ± 0.68	0.45 ± 0.05 ^{AB}
20	5	0.19 ± 0.02 ^b	0.29 ± 0.05 ^{AB}	0.15 ± 0.02	0.033 ± 0.00	5.18 ± 1.01	0.43 ± 0.03
20	10	0.23 ± 0.06 ^b	0.25 ± 0.04 ^A	0.13 ± 0.01 ^A	0.033 ± 0.00 ^B	3.87 ± 0.13 ^A	0.48 ± 0.04 ^B
60	0	0.11 ± 0.00 ^a	0.29 ± 0.02 ^{a,A}	0.14 ± 0.01 ^a	0.041 ± 0.01	3.48 ± 0.32	0.40 ± 0.04 ^{a,A}
60	5	0.17 ± 0.05 ^{ab}	0.23 ± 0.03 ^{a,A}	0.13 ± 0.00 ^a	0.034 ± 0.01	4.42 ± 0.85	0.45 ± 0.04 ^a
60	10	0.24 ± 0.02 ^b	0.56 ± 0.03 ^{b,B}	0.16 ± 0.01 ^{b,B}	0.045 ± 0.00 ^C	3.66 ± 0.30 ^A	0.62 ± 0.02 ^{b,C}
Pooled SE		0.01668	0.040	0.0063	0.0024	0.4791	0.027
Means of main effect							
0		0.20	0.36	0.15	0.034	5.26 ^B	0.44
2		0.18	0.30	0.14	0.037	4.22 ^{AB}	0.45
6		0.17	0.36	0.14	0.040	3.85 ^A	0.49
	0	0.13 ^a	0.38 ^b	0.15 ^{ab}	0.043 ^b	3.64 ^a	0.46
	0.5	0.20 ^b	0.30 ^a	0.13 ^a	0.034 ^a	4.39 ^{ab}	0.44
	1	0.22 ^b	0.34 ^{ab}	0.16 ^b	0.033 ^a	5.30 ^b	0.48
Two-way ANOVA: <i>P</i> values							
PL		0.489	0.104	0.280	0.232	0.030	0.241
CH		0.011	0.049	0.046	0.021	0.011	0.348
PL × CH		0.501	≤0.001	0.001	0.056	≤0.001	≤0.001

PL: phospholipid; CH: cholesterol; HDL-C: high-density lipoprotein; LDL-C: low-density lipoprotein. Data are presented as the mean ± SE ($n = 3$). Values in the same line with different superscripts are significantly different ($P < 0.05$). ^{AB}Significant difference between phospholipid levels within the same cholesterol level ($P < 0.05$). ^{ab}Significant difference between cholesterol levels within the same phospholipid level ($P < 0.05$).

FIGURE 8: Linear regression analysis between the HDL-C/LDL-C and dietary phospholipid levels.

between the soybean lecithin group and the control. A positive correlation between the serum TG content and dietary CH level was observed in the present study, which was in agreement with the results of previous studies [30, 70].

Lipids are crucial for the development of ovaries [72]. Appropriate dietary PL and CH supplementation are conducive to ovarian development in crustaceans [13, 39, 59, 73].

In the present study, almost all eggs were in stage IV, with staging confirmed according to the methodology described by Kulkarni et al. [74]. Crayfish fed with diets containing high PL (60 g/kg) content had higher GSI, and the eggs were more compactly arranged in tissue sections. Many researchers have shown that the hepatopancreas can synthesize steroid estrogens, such as E_2 , which are derivatives of CH, and are then transported through the hemolymph to the ovary [36, 49, 75]. E_2 can activate the vitellogenin, accelerating ovarian tissue development [76–79]. Therefore, PL facilitates CH absorption and metabolism in the hepatopancreas and increases vitellogenin activity in the ovaries, improving ovarian development. An inverse correlation between HSI and GSI was observed in the present study, confirming this inference. E_2 content shows an increasing trend followed by a decreasing trend with increasing ovarian development in crustaceans [80–82]. When *Emerita asiatica* eggs developed into stage V, the E_2 levels gradually stabilized to a low level [36]. In the present study, the significant decrease in the E_2 levels in the PL 60 g/kg and CH 5 g/kg group was similar to this situation. The comparison of ovarian development among dietary PL and CH levels showed that the addition of CH effectively improved ovarian development only when there was a sufficient PL level.

FIGURE 9: EcR (a), RXR (b), and chitinase (c) mRNA expression in the hepatopancreas of female *P. clarkii* fed with Diet3 (60/0), Diet6 (60/5), and Diet9 (60/10). * indicates significant difference ($P < 0.05$, $n = 6$).

5. Conclusion

Optimal dietary PL and CH levels improved the growth performance and promoted ovarian development in the crayfish *P. clarkii* when the CH addition level was 10 g/kg and the PL level was at least 60 g/kg. The serum CH level showed that higher dietary PL levels facilitated the transport of CH from the hepatopancreas, leading to changes in the molting rate and ovarian development.

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 no conflict of interest.

Authors' Contributions

Shouquan Hou designed and performed the whole experiment under the help of Jiayao Li. Shouquan Hou drafted the manuscript. Jinghao Li, Shaicheng Zhu, and Jin Huang revised the manuscript. Yongxu Cheng provided the experimental facilities.

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