Original Research Article

Dietary cholesterol promotes growth and ecdysone signalling pathway by modulating cholesterol transport in swimming crabs (*Portunus trituberculatus*)

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ARTICLE INFO

ABSTRACT

Cholesterol, as an indispensable nutrient, regulates molting and growth in crustacean. As crustaceans are unable to biosynthesize cholesterol de novo, it is central to understand how dietary cholesterol affects molting in crustaceans. An 8-week feeding trial was conducted to evaluate the effects of dietary cholesterol level (0.12%, 0.43%, 0.79%, 1.00%, 1.30% and 2.50%) on growth, cholesterol metabolism and expression of genes related to lipid and ecdysone metabolism in female swimming crabs (*Portunus trituberculatus*). A total of 192 crabs (1.41 ± 0.05 g) were randomly distributed into 192 aquaria. Each treatment had 4 replicates with each replicate containing 8 crabs. Crabs fed the 1.00% cholesterol diet showed best growth performance, and thus based on percent weight gain, the optimal dietary cholesterol requirement was calculated at 1.01%. Tissue cholesterol concentrations were positively correlated with dietary cholesterol level. The contents of functional fatty acids in hepatopancreas significantly increased as dietary cholesterol increased from 0.12% to 2.50% ($P < 0.05$). The expression levels of genes related to lipogenesis pathway, lipid catabolism and fatty acid oxidation were significantly down-regulated with increased dietary cholesterol level ($P < 0.05$). The highest expression levels of cholesterol transport genes, low-density lipoprotein receptor (*ldlr*) and low-density lipoprotein receptor-related protein 2 (*lrp2*) occurred in crabs fed the 1.30% cholesterol diet. Moreover, hormones related to molting such as crustacean hyperglycemic hormone (CHH), methyl farnesoate (MF), molt-inhibiting hormone (MIH), and ecdysone in hemolymph were significantly influenced by dietary cholesterol level ($P < 0.05$). The highest expression levels of ecdysone receptor (*ecr*) and chitinase 1 (*chi1*) in eyestalk and hepatopancreas were found in crabs fed the diet containing 1.00% cholesterol ($P < 0.05$). In conclusion, the optimal dietary level was beneficial to functional fatty acid accumulation, regulated lipid metabolism, promoted the ecdysone signalling pathway by improving the cholesterol transport, and improved the molting rate and growth of swimming crabs.

1. Introduction

The molting cycle, a fundamental process of growth and development in crustaceans, generally has 4 phases including pre-molt, molt, post-molt and inter-molt (Diez and Lovrich, 2013). However, crustaceans have a lower survival than many other marine aquaculture species, with mortality attributed to 'molting death syndrome' (MDS), in which crustaceans cannot completely shed the old carapace during molting (Bowser and Rosemark, 1981; Hamasaki et al., 2002). Previous studies reported that MDS was

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

influenced by lipid nutrition and was associated particularly with cholesterol and highly unsaturated fatty acids (Hamasaki et al., 2002; Gong et al., 2004). As a precursor of several functional molecules in tissues, cholesterol in steroidogenic tissues is the precursor for the synthesis of steroids, including steroid hormones that are classic nuclear hormones having genomic effects (i.e., affecting gene transcription), but also more rapid nongenomic effects (Wehling, 1997; NRC, 2011). Thus, cholesterol can be converted through the action of a series of enzymes to molting hormones and sex hormones, steroids that regulate molting and reproduction in crustaceans (Sheen et al., 1994; Mykles, 2011). However, a characteristic of crustacean is that they are unable to biosynthesize cholesterol de novo and, therefore, it is an essential nutrient that they must obtain through the diet and so in farming it must be include in feeds (Grieneisen, 1994; Spaziani et al., 1999). A previous study demonstrated that methyl farnesoate (MF) and ecdysone are both important hormones for the induction of molting (Raghavan and Ayanath, 2019). Ecdysone, the major ecdysteroid produced is converted to the active form, 20-hydroxyecdysone (20E), that is dominant in mediating the changes of the ecdysis process, which is regulated by intricate multi-hormonal system (Chang and Mykles, 2011). Furthermore, 20E activates the ecdysone receptor (EcR)/retinoid-x receptor (RXR) complex, that promotes a series of downstream events including up-regulation of several genes related to processes involved in molting regulation, such as the biosynthesis and degradation of chitin, osmotic flux and cuticle release (Abdullah-Zawawi et al., 2021). In addition to ecdysteroids, 2 neuropeptides including molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH) are involved in molting and inhibit ecdysone synthesis in the Y-organ (Chung and Webster, 2003; Techa and Chung, 2015).

Cholesterol is a key component of all animal cell membranes with important structural roles, reducing both the fluidity and permeability of the plasma membrane to protons and sodium ions (Lange and Steck, 2008; NRC, 2011). Membrane cholesterol also has further functional roles in intracellular transport as an important component in caveola-dependent and clathrin-dependent endocytosis, cell signaling through the formation of lipid rafts, and nerve conduction as an important part of the insulating myelin sheath (Simons and Ikonen, 2000). As a component of lipoproteins, cholesterol impacts the absorption and transportation of lipids in animals (Yepiz-Plascencia et al., 2000). At least 90% of cholesterol in the blood is associated with low-density lipoprotein (LDL) and high-density lipoprotein (HDL). In mammals, LDL transports cholesterol from the liver to the peripheral tissues, while HDL does the opposite, transporting cholesterol from body tissues to the liver (Huang et al., 2019). Numerous genes and metabolic enzymes play vital roles in cholesterol uptake and transport, including low-density lipoprotein receptor (LDLR), low-density lipoprotein receptor-related protein (LRP), scavenger receptor BI (SRB1) and members of the ATP-binding cassette subfamily (Cortes et al., 2014). Furthermore, cholesterol regulates the expression of genes related to lipid metabolism including lipogenesis, lipolysis, fatty acid β-oxidation and uptake (Martin et al., 2006; Wang et al., 2010).

Swimming crabs (*Portunus trituberculatus*) is widely distributed on the coasts of Japan, Korea and China (Yuan et al., 2019). Due to its delicious meat and market demand, swimming crabs has become one of the most important crustaceans farmed in China, with production of via aquaculture of 100,895 t in 2020 (Song et al., 2019; China Fishery Statistical Yearbook, 2021). Studies on dietary cholesterol in swimming crabs have demonstrated a true requirement that has been established generally through observations of reduced growth response and comparatively higher mortality in crabs fed low cholesterol, with the dietary cholesterol requirement commonly reported in the range of 0.6% to 1.0% of diet (Han et al., 2013, 2015; Sun et al., 2017). However, there has been little focus on impacts of dietary cholesterol on lipid metabolism or the ecdysone signalling pathway. Therefore, the primary aims of the present study were to reveal the important roles of cholesterol in the regulation of crustacean molting and lipid metabolism, with specific objectives to evaluate the influence of dietary cholesterol level on growth performance and molting, and the expression of genes involved in cholesterol metabolism and the ecdysone signalling pathway. The results of the present study will provide vital insights to the feasibility of dietary cholesterol and feeding strategies to improve the growth of swimming crabs.

2. Materials and methods

2.1. Animal ethics

Animal experimentation within the present study was conducted in accordance with the Animal Research Institute Committee guidelines of Ningbo University, China and approved by the Committee of the Animal Research Institute, Ningbo University, China.

2.2. Diet preparation and experimental design

Diets were prepared following the method described as previous study (Luo et al., 2021). Briefly, 6 iso-nitrogenous (approximately 46% protein) and iso-lipidic (approximately 8.5% lipid) diets were formulated to contain different levels of cholesterol (Shanghai Macklin Biochemical Co., Ltd., China), specifically 0.12%, 0.43%, 0.79%, 1.00%, 1.30% and 2.50% (Table 1). Palmitic acid (Shanghai Yiji Chemical Co., Ltd., China) was added to the experimental diets to balance added cholesterol, ensuring the combined amount of added cholesterol and palmitic acid was 2% in all diets. Peruvian fish meal, soybean protein concentrate, soybean meal, krill meal and corn gluten meal were used as protein sources. Fish oil and soy lecithin were used as the main lipid sources. All ingredients were purchased from Ningbo Tech-Bank Feed Co. Ltd., China. All dry ingredients were ground into fine powder and passed through a 60-mesh sieve, micro components such as minerals and vitamins premix were added followed by lipid and distilled water. The ingredients were thoroughly mixed in a Hobart type mixer, cold-extruded pellets produced (F-26, Machine Factory of South China University of Technology, Guangzhou, China) in 2 sizes via 2-mm and 4-mm dies (G-250, Machine Factory of South China University of Technology, Guangzhou, China), after which the pellets were steam-dried to approximately 5% moisture, and finally stored at −20 °C prior to use. The absolute fatty acid composition of experimental diets is presented in Supplementary Table S1.

Swimming crabs were obtained from Hengma nursery farm (Ningbo, China), and acclimatized in 100 L aquaria (length × width × height, 40 cm × 60 cm × 48 cm) with a recirculating aquaculture system (RAS) for 2 weeks during which time they were fed a commercial diet (approximately 45% protein and 8% lipid). Seawater in the RAS was continuously purified by a series of filtration treatments including physical and biofilter systems followed by ultraviolet treatment as described previously (Luo et al., 2021). Crabs were maintained under an artificial light regime of 12 h light (from 08:00 to 20:00) and 12 h dark (from 20:00 to 08:00), and water temperature was controlled at approximately 27.0 °C. A total of 192 female swimming crabs (1.41 ± 0.05 g) were randomly distributed into 192 aquaria to prevent cannibalism. Each treatment had 4 replicates (n = 4) with each replicate containing 8 crabs. During the 8-week experimental period, crabs were fed twice a day at 8:00 and 18:00, with a daily ration of 6% to 8% of wet body weight. Uneaten feed and feces were collected using a siphon

Table 1
Formulation and proximate composition of the experimental diets (dry matter basis).

Item	Dietary cholesterol level, %					
	0.12	0.43	0.79	1.00	1.30	2.50
Ingredients[1], %						
Peruvian fish meal	25.00	25.00	25.00	25.00	25.00	25.00
Soybean protein concentrate	7.00	7.00	7.00	7.00	7.00	7.00
Soybean meal	23.00	23.00	23.00	23.00	23.00	23.00
Krill meal	3.00	3.00	3.00	3.00	3.00	3.00
Corn gluten meal	5.00	5.00	5.00	5.00	5.00	5.00
Wheat flour	23.70	23.70	23.70	23.70	23.70	23.70
Fish oil	2.00	2.00	2.00	2.00	2.00	2.00
Palmitic acid[2]	2.00	1.75	1.50	1.25	1.00	0.00
Cholesterol[3]	0.00	0.25	0.50	0.75	1.00	2.00
Soy lecithin	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix	2.00	2.00	2.00	2.00	2.00	2.00
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
Sodium alginate	2.00	2.00	2.00	2.00	2.00	2.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Proximate composition, %						
Dry matter	95.46	95.49	95.03	94.85	95.24	95.45
Crude protein	46.34	46.08	46.21	46.24	46.01	46.12
Crude lipid	8.46	8.81	8.72	8.77	8.34	8.56
Ash	11.12	11.14	11.19	10.93	10.84	10.74
Cholesterol	0.12	0.43	0.79	1.00	1.30	2.50

[1] All the ingredients (except for palmitic acid and cholesterol) were obtained from Ningbo Tech-Bank Feed Co. Ltd., China.
[2] Palmitic acid: 97% of total fatty acids as palmitic acid methyl ester, Shanghai Yiji Chemical Co., Ltd., China.
[3] Cholesterol: 99%, Shanghai Macklin Biochemical Co., Ltd., China.

and spoon-net, and molts as well as mortalities were recorded daily, 50% of the seawater was renewed every three days to maintain water quality. Water quality parameters including water temperature (from 26.8 to 27.4 °C), salinity (from 23.9 to 26.1 g/L), dissolved oxygen (from 7.0 to 8.0 mg/L), pH (from 7.4 to 7.8) and ammonia nitrogen (<0.05 mg/L) were measured daily (YSI Proplus, Yellow Springs, Ohio, USA).

2.3. Sample collection

After the feeding trial, crabs were counted and weighed to determine growth performance related indices. Hemolymph was collected from the pericardial cavity from 4 crabs per replicate and centrifuged (956 × g, 10 min, 4 °C) to obtain supernatant, which was stored at −80 °C until analysis of biochemical indices and hormone concentrations ($n = 4$ per treatment). The hepatopancreas from 4 crabs per replicate were collected into 2-mL microfuge tubes, frozen in liquid nitrogen, and stored at −80 °C prior to analyzing lipid class contents, fatty acid compositions and mRNA levels ($n = 4$ per treatment). Body muscle samples were dissected from 4 crabs per replicate and placed into 4-mL microfuge tubes and stored at −80 °C until analyses of muscle cholesterol and tri-acylglycerol (TG) contents ($n = 4$ per treatment). Four eyestalk samples from each replicate were collected and stored at −80 °C for gene expression ($n = 4$ per treatment).

2.4. Lipid class and hormone analysis

The cholesterol contents of feeds were analyzed by the gas chromatography (GC) method. Briefly, after extraction of the lipids from 500 mg feed using 10 mL chloroform, 1 mL of the extracted lipid solution was dried under a stream of pure nitrogen. The obtained dry residues were resuspended in 1 mL diethyl ether, and samples analyzed using gas chromatography (Shimadzu, Japan). Lipid class parameters including cholesterol and TG in hemolymph, hepatopanceas and muscle, as well as low-density lipoprotein

cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) in hemolymph were analyzed using diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, China). The concentration of MIH and ecdysone in hemolymph were determined using assay ELISA kits (Jiangsu Meibiao Biological Technology Co., Ltd., China), and the CHH and MF in hemolymph were measured by Multiskan spectrum (Thermo Fisher Scientific, Waltham, USA) using ELISA assay kits (Shanghai Qiaodu Biological Technology Co., Ltd., China) according to the manufacturer's instructions.

2.5. Fatty acid analysis

Fatty acid compositions of feeds and hepatopancreas of swimming crabs were determined essentially as described previously (Luo et al., 2021). Briefly, around 120 mg of freeze-dried samples were placed into glass tubes and 1 mL of methyl tricosanoate (C23:0) solution (1.0 mg/mL hexane) added as an internal standard for quantification, and total lipid extracted by chloroform/methanol (2:1 by vol.). The extracted total lipids were dried under nitrogen and 3 mL methanolic sulfuric acid added and the samples heated at 80 °C in a water bath for 4 h to prepare fatty acid methyl esters (FAME). Finally, extracted FAME was analyzed by GC−MS using an Agilent Technologies GC−MS (7890B-5977A, USA). The content of each fatty acid was calculated using the following equation:

$$\text{Fatty acid content} = (\text{area of fatty acid}/\text{area of C23:0} \times \text{correction factor})/(\text{sample weight} \times \text{dry matter content}).$$

2.6. Gene expression

Total RNA extraction and qPCR were carried out as described by Luo et al. (2021). In short, total RNA was extracted from the eyestalk and hepatopancreas using Trizol Reagent (Vazyme Biotech Co., Ltd., Nanjing, China). The isolated RNA was analyzed by Nanodrop 2000

spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and gel electrophoresis (1.0% denaturing agarose) to assess quantity and quality, respectively. The cDNA was reversed transcribed using HiScript II Reverse Transcriptase Reagent Kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Specific gene primers were synthesized commercially (Tsingke Biotech Co., Ltd., Hangzhou, China), and are presented in Supplementary Table S2. Real-time qPCR was carried out in a quantitative thermal cycler (Lightcycler 96, Roche, Switzerland) in reaction volumes of 20 μL, containing 0.4 μL of each primer, 10 μL of SYBR Green I Master (Vazyme Biotech Co., Ltd., Nanjing, China), 0.8 μL of 1/5 diluted cDNA and 8.4 μL DEPC-water. The real time PCR conditions were as follows: 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s. Standard curves were generated using 6 different dilutions of the cDNA samples, and amplification efficiency determined using the $E = 10^{(-1/\text{Slope})} - 1$ equation. Amplification efficiencies of all genes were approximately equal and ranged from 90% to 110%. Results for gene mRNA expression were presented relative to the expression values in the reference group (diet containing lowest cholesterol, 0.12%). The mRNA expression levels of the candidate genes were calculated by the $2^{-\Delta\Delta Ct}$ method as described (Livak and Schmittgen, 2001).

2.7. Calculations and statistical analysis

The following variables were calculated using the following equations:

$$\text{Percent weight gain (PWG, \%)} = 100 \times (\text{FBW} - \text{IBW})/\text{IBW},$$

$$\text{Specific growth ratio (SGR, \%/day)} = 100 \times (\ln \text{FBW} - \ln \text{IBW})/\text{T},$$

$$\text{Feed conversion rate (FCR)} = \text{FI}/(\text{FBW} - \text{IBW} + \text{DCW}),$$

$$\text{Survival (\%)} = 100 \times \text{FN}/\text{IN},$$

$$\text{Molting ratio (MR)} = 2 \times \text{the number of molting}/(\text{FN} + \text{IN}),$$

where FBW, IBW and DCW were the final body weight, initial body weight and dead crabs' weight, respectively; FI was feed intake; T was the duration of the experimental in days; FN and IN were the final and initial crabs' numbers, respectively.

Data were presented as mean ± SEM ($n = 4$), the homogeneity of variances (Levene's test) were checked prior to one-way analysis of variance (ANOVA). Tukey's multiple range test was applied, where $P < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS 19.0 (SPSS, Chicago, IL, USA). Principal component analysis (PCA) was carried out using the APT-BioCloud program, an online platform for data analysis (http://cloud.aptbiotech.com/).

3. Results

3.1. Growth and molting performance

Survival was not significantly influenced by dietary cholesterol level ($P > 0.05$) (Table 2). Final body weight, PWG, SGR and MR all increased significantly as dietary cholesterol level increased from 0.12% to 1.00%. However, FBW, PWG and SGR did not increase significantly with further increased dietary cholesterol level, with highest PWG and SGR found in crabs fed the 1.00% cholesterol diet ($P < 0.05$). Crabs fed the 0.12% cholesterol diet had the highest FCR

(lowest feed efficiency) among all treatments ($P < 0.05$). Broken-line regression analysis of PWG against dietary cholesterol level indicated that the optimal dietary level was determined to be 1.01% of dry diet (Fig. 1).

3.2. Lipid class in tissues

Lipid class in hemolymph, hepatopancreas and muscle of swimming crabs fed the experimental diets were presented in Fig. 2. Triacylglycerol contents in hemolymph, hepatopancreas and muscle were not significantly affected by dietary cholesterol level ($P > 0.05$). Cholesterol contents in hemolymph, hepatopancreas and muscle significantly increased as dietary cholesterol level increased from 0.12% to 2.50% ($P < 0.05$). HDL-C and LDL-C contents in hemolymph increased significantly as dietary cholesterol increased from 0.12% to 1.00%, and, thereafter, decreased as dietary cholesterol level increased from 1.00% to 2.50% ($P < 0.05$).

3.3. Fatty acid compositions in hepatopancreas

Selected fatty acid compositions of hepatopancreas of swimming crabs fed the experimental diets are shown in Fig. 3A and B. Full fatty acid compositions are presented in Supplementary Table S3. The contents of palmitic acid (PA, 16:0), arachidonic acid (ARA, 20:4n-6) and saturated fatty acids (SFA) in hepatopancreas were not significantly influenced by dietary cholesterol level ($P > 0.05$). However, the contents of eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), total n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA), n-3 polyunsaturated fatty acids (n-3 PUFA), n-6 polyunsaturated fatty acids (n-6 PUFA) and monounsaturated fatty acids (MUFA) increased markedly in hepatopancreas as dietary cholesterol level increased from 0.12% to 2.50% ($P < 0.05$). Crabs fed diets with 1.00%, 1.30% and 2.50% cholesterol exhibited significantly lower DHA to EPA ratio in hepatopancreas than those fed the other diets ($P < 0.05$). The PCA of full fatty acid compositions indicated that fatty acid compositions were clustered based on dietary cholesterol level, with the 2 principal components accounting for 77.5% of total variance with PC 1 of 66.8% and PC 2 of 10.7%, respectively (Fig. 3C).

3.4. Gene expression of lipid and cholesterol metabolism

The expression of genes related to lipid metabolism including lipogenesis, lipolysis and fatty acid β-oxidation as well as uptake in hepatopancreas of swimming crabs are presented in Fig. 4A, B, and C, respectively. For lipogenesis, there were no significant differences among the diets in expression levels of acetyl-CoA carboxylase (*acc*) and sterol regulatory element-binding protein-1 (*srebp1*) ($P > 0.05$), whereas the mRNA expression levels of fatty acid synthase (*fas*) and glucose 6-phosphate dehydrogenase (*g6pd*) in hepatopancreas were down-regulated with increased dietary cholesterol level ($P < 0.05$, Fig. 4A).

The expression of genes involved in lipolysis such as triacylglycerol lipase (*tgl*) and pancreatic lipase (*pl*) were significantly down-regulated as dietary cholesterol level increased from 0.43% to 0.79% or from 0.79% to 1.00%, respectively. In contrast, the expression levels of hormone-sensitive lipase (*hsl*) and intracellular lipase (*il*) were not significantly influenced by dietary cholesterol level ($P > 0.05$) (Fig. 4B).

The expression levels of genes related to fatty acid β-oxidation such as acyl-CoA oxidase 1 (*acox1*) and acyl-CoA oxidase 2 (*acox2*) were significantly down-regulated as dietary cholesterol level increased from 0.12% to 2.5% ($P < 0.05$). In contrast, the lowest expression of fatty acid binding protein 3 (*fabp3*) occurred in crabs

Table 2
Effects of dietary cholesterol level on growth, molting performance and feed utilization (%, wet weight) of juvenile swimming crabs (*Portunus trituberculatus*)[1].

Item	Dietary cholesterol level, %					
	0.12	0.43	0.79	1.00	1.30	2.50
IBW, g	1.47 ± 0.04	1.34 ± 0.05	1.41 ± 0.07	1.43 ± 0.07	1.42 ± 0.06	1.47 ± 0.08
FBW, g	27.56 ± 1.08[b]	29.58 ± 2.25[ab]	30.44 ± 1.62[ab]	35.34 ± 1.50[a]	34.46 ± 1.44[a]	34.2 ± 0.16[a]
PWG[2], %	1,790.06 ± 51.68[b]	1,867.91 ± 120.10[b]	2,185.29 ± 89.41[ab]	2,482.33 ± 123.01[a]	2,406.82 ± 85.17[a]	2,374.32 ± 101.49[a]
SGR[3], %/day	5.24 ± 0.04[c]	5.30 ± 0.08[c]	5.51 ± 0.08[bc]	5.80 ± 0.07[a]	5.76 ± 0.06[ab]	5.72 ± 0.06[ab]
FCR[4]	1.28 ± 0.06[a]	1.26 ± 0.07[ab]	1.23 ± 0.06[ab]	1.18 ± 0.05[ab]	1.06 ± 0.06[ab]	1.03 ± 0.05[b]
Survival[5], %	90.63 ± 4.57	90.63 ± 4.57	87.5 ± 4.72	89.29 ± 4.34	96.88 ± 3.12	87.5 ± 4.72
MR[6]	3.60 ± 0.05[c]	3.64 ± 0.04[c]	3.69 ± 0.03[bc]	4.00 ± 0.08[a]	3.88 ± 0.05[ab]	3.79 ± 0.03[bc]

IBW = initial body weight; FBW = final body weight; PWG = percent weight gain; SGR = specific growth ratio; FCR = feed conversion ration; MR = molting ratio.
[a,b,c] Different letters in the same line show a significant difference between treatments ($P < 0.05$).
[1] Data are expressed as mean and SEM ($n = 4$).
[2] PWG (%) = 100 × (FBW - IBW)/IBW.
[3] SGR (%/day) = 100 × (ln FBW - ln IBW)/culturing days.
[4] FCR = feed intake/(FBW - IBW + dead crabs' weight).
[5] Survival (%) = 100 × final crabs' number/initial crabs' number.
[6] Molting ratio (MR) = 2 × the number of molting/(final crabs' number + initial crabs' number).
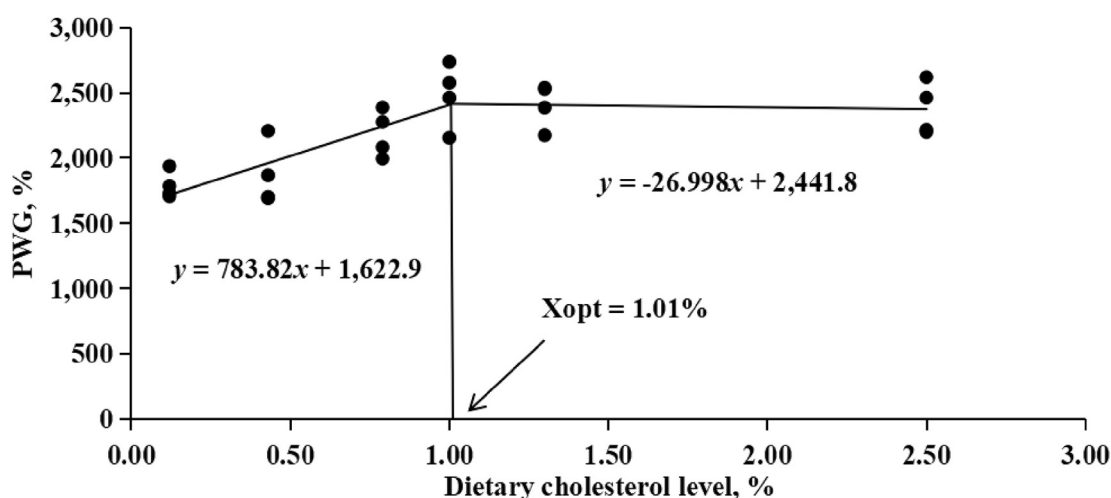

Fig. 1. The linear broken-line model for the relationship between percent weight gain (PWG) and dietary cholesterol level in juvenile swimming crabs. The horizontal axis represents the measured dietary cholesterol level. The Xopt represents the optimal dietary cholesterol level for maximum PWG of swimming crabs.

fed the 0.12% cholesterol diet ($P < 0.05$) and crabs fed the 1.00% cholesterol diet exhibited the highest expression of genes related to fatty acid uptake such as *fabp3* and fatty acid transport protein 4 (*fatp4*) (Fig. 4C).

The expression levels of class B scavenger receptors (*srb*) and NPC intracellular cholesterol transporter 1 (*npc1*) were not significantly affected by dietary cholesterol level ($P > 0.05$). However, the expression levels of low-density lipoprotein receptor (*ldlr*) and low-density lipoprotein receptor-related protein 2 (*lrp2*) were significantly up-regulated as dietary cholesterol level increased from 0.12% to 1.30% ($P < 0.05$). Moreover, the highest mRNA expression level of *ldlr* and *lrp2* were observed in crabs fed the diet containing 1.30% cholesterol ($P < 0.05$) (Fig. 4D).

3.5. Impacts on the ecdysone signalling pathway

The impacts of dietary cholesterol on the ecdysone signalling pathway including hormones related to molting in hemolymph, and expression levels of genes related to molting in eyestalk and hepatopancreas, are presented in Fig. 5A, B, and C, respectively. The concentration of MIH and CHH in hemolymph significantly decreased as dietary cholesterol level increased from 0.12% to 1.00% ($P < 0.05$), and then increased with further increased dietary

cholesterol level. However, crabs fed the 1.00% cholesterol diet had the highest ecdysone concentration in hemolymph among all treatments ($P < 0.05$) (Fig. 5A). The concentration of MF in hemolymph significantly increased with increased dietary cholesterol level, and the lowest level of MF observed in crabs fed the 0.12% cholesterol diet ($P < 0.05$) (Fig. 5A).

In eyestalk, the expression levels of retinoid-x receptor (*rxr*) and nuclear hormone receptor E75 (*e75*) were not significantly influenced by dietary cholesterol level ($P > 0.05$, Fig. 5B). However, crabs fed the diet containing 1.00% cholesterol showed the highest expression of ecdysone receptor (*ecr*), broad-complex (*br-c*) and chitinase 1 (*chi1*) among all treatments. The expression level of molt-inhibiting hormone (*mih*) in crabs fed the 0.12% cholesterol diet was significantly higher than levels in crabs fed the other diets ($P < 0.05$) (Fig. 5B).

The expression levels of *ecr*, *e75* and *chi1* in hepatopancreas were significantly up-regulated in crabs fed the 1.00% cholesterol diet ($P < 0.05$), although the expression level of *rxr* and *br-c* were not significantly affected by dietary cholesterol level ($P > 0.05$) (Fig. 5C). Crabs fed the 1.00% and 1.30% cholesterol diets had higher expression levels of methoprene-tolerant protein (*met*) than those fed the 0.12% cholesterol diet, and crabs fed the 1.00%, 1.30% and 2.50% cholesterol diets exhibited higher expression of farnesoic
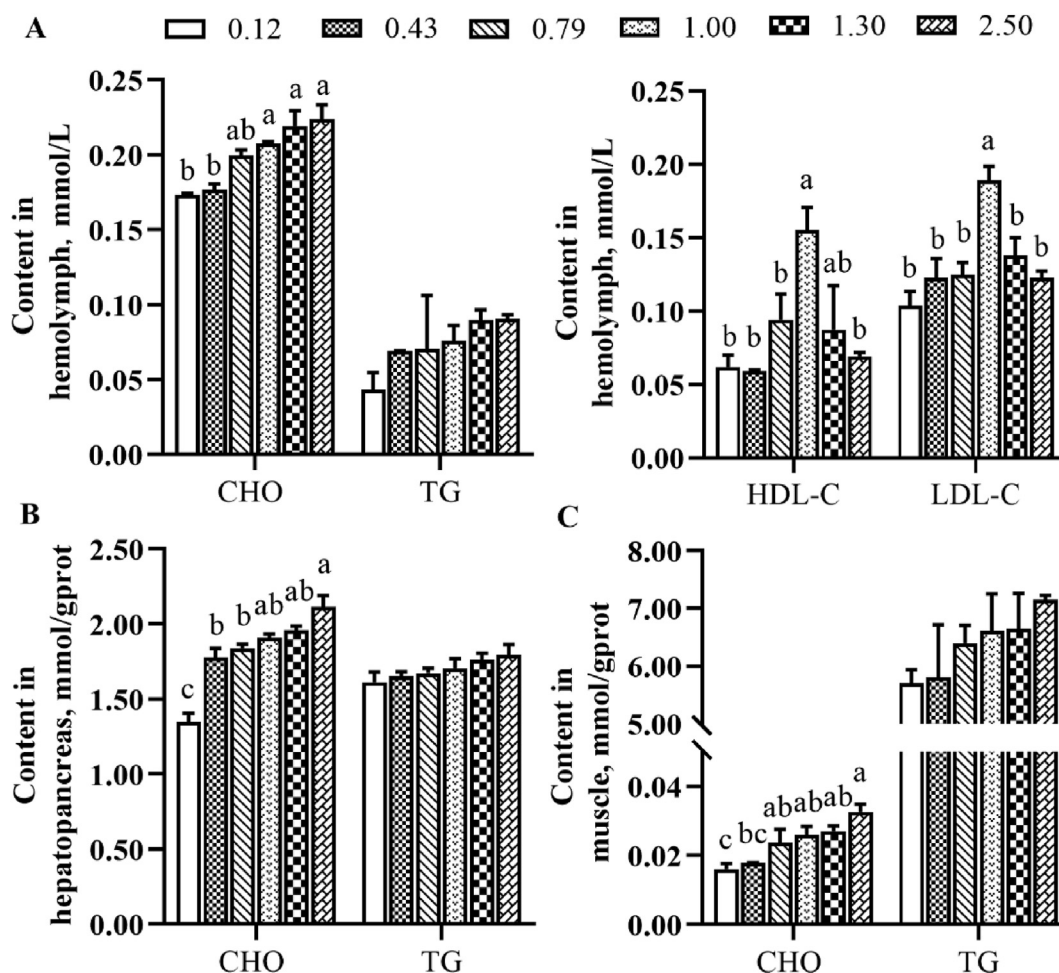
Fig. 2. Effects of dietary cholesterol level on lipid class contents in (A) hemolymph, (B) hepatopancreas and (C) muscle of swimming crabs (*Portunus trituberculatus*). [a, b, c]Mean values of bars for the same parameter with different superscript letters are significantly different (*P* < 0.05). Data are reported as the mean and SEM of 4 replicates. CHO = cholesterol; TG = triglyceride; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol.

acid O-methyltransferase (*famet*) than those fed the other diets (*P* < 0.05) (Fig. 5C).

4. Discussion

Cholesterol is of great importance for survival and growth in crustaceans (Sheen et al., 1994; Teshima et al., 1997). NRC (2011) reported dietary cholesterol requirements of crustaceans ranged from 0.4% to 2.0% dependent upon species, age and culture conditions. The optimal dietary cholesterol requirement was reported at 0.51% for mud crabs (*Scylla serrata*) (Sheen, 2000), 0.41% for white shrimps (*Litopenaeus vannamei*) (Gong et al., 2000), and 1.0% for oriental river prawns (*Macrobrachium nipponense*) (Gu et al., 2017) as well as larvae and postlarvae black tiger prawns (*Penaeus monodon*) (Paibulkichakul et al., 1998). In the present study, swimming crabs fed with 1.00% cholesterol showed highest FBW, PWG, SGR and MR compared to crabs fed the other treatments. The optimal dietary cholesterol level based on PWG was determined to be 1.01% of dry diet according to broken-line regression analysis. The results were consistent with previous studies, on swimming crabs that reported crabs fed 1.00% cholesterol had higher growth performance and number of successful molting than other treatments as well as better FCR (Han et al., 2015). Meanwhile, some studies have reported that excess dietary cholesterol was a disadvantage for growth performance in crustaceans, such as mud crabs and white

shrimps (Sheen, 2000; Niu et al., 2012). Consistent with this, the present study confirmed that crabs fed high dietary cholesterol levels (1.30% and 2.50% cholesterol) had numerically lower PWG and SGR compared to crabs fed 1.00% cholesterol, albeit the differences were not statistically significant. This pattern might be attributed to the "nutrient-response characteristic", which classified the physiological response of nutrients generates a characteristic curve of nutritional-response, increasing to a certain point and then tending to stabilize (Mercer, 1982).

Most studies have indicated that body cholesterol content increased with increased dietary cholesterol level in crustaceans (Niu et al., 2012; Han et al., 2015). Similar results were also observed in the present study, where cholesterol contents on hemolymph, hepatopancreas and muscle were significantly increased by increasing dietary cholesterol level. Cholesterol is transported via hemolymph after digestion and absorption, largely in LDL and HDL, the 2 main lipoproteins involved in cholesterol transport in fishes and crustaceans (Huang et al., 2019; Tian et al., 2020), and can be stored in different tissues or metabolized (NRC, 2011). The results of this study observed that the lipoprotein cholesterol contents, HDL-C and LDL-C, in hemolymph increased as dietary cholesterol increased from 0.12% to 1.00%, and thereafter decreased as dietary cholesterol increased to 2.50%. The LDL-C data were in agreement with a previous study in crayfishes (*Procambarus clarkii*), HDL-C data were different (Tian et al., 2020), which may due to
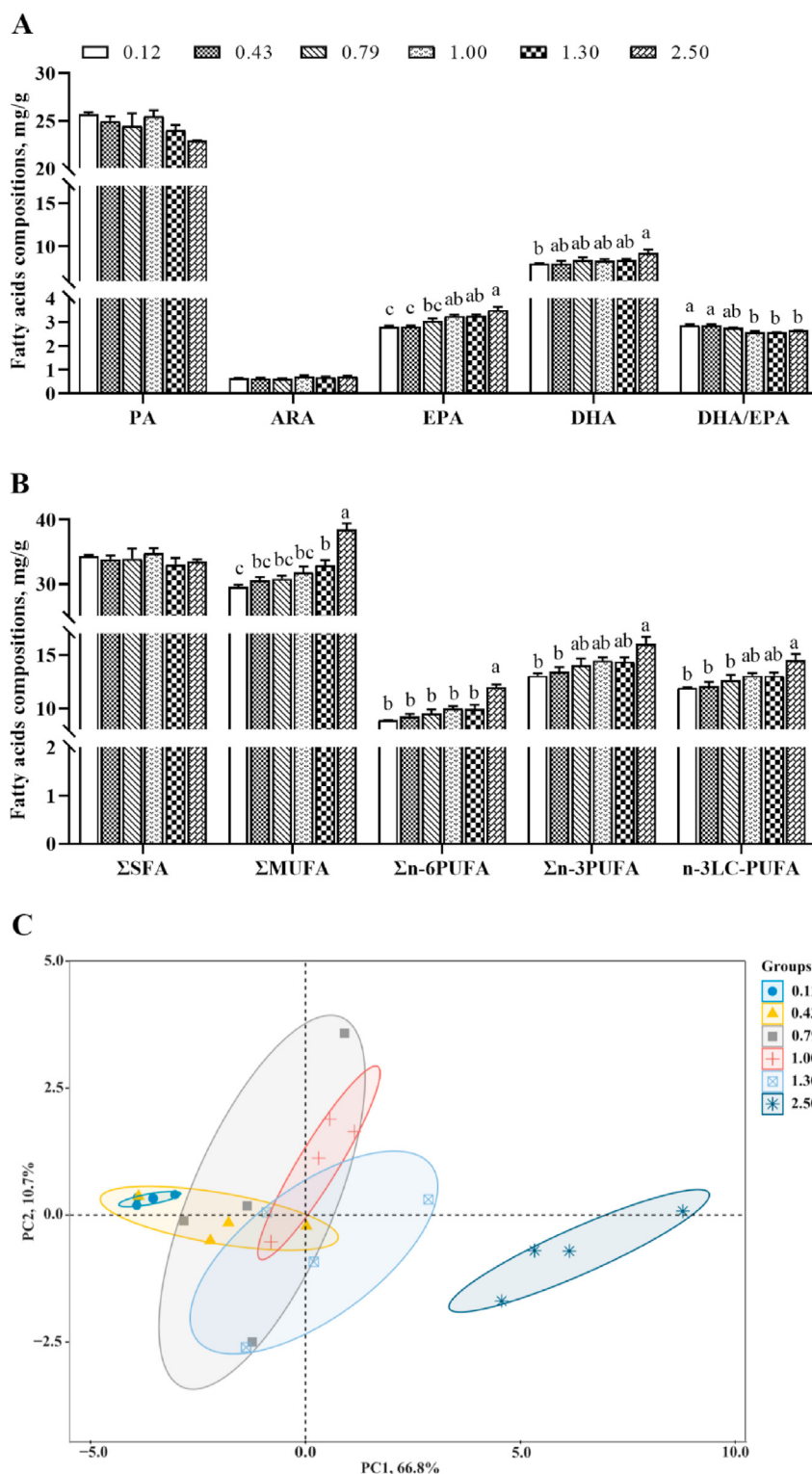
Fig. 3. Effects of dietary cholesterol level on fatty acid compositions (dry matter basis) in hepatopancreas of swimming crabs (*Portunus trituberculatus*). (A and B) selected fatty acid compositions in hepatopancreas. (C) Principal component analysis of full fatty acid compositions in hepatopancreas. [a, b, c] Mean values of bars for the same parameter with different superscript letters are significantly different ($P < 0.05$). Data are reported as the means and SEM of 4 replicates. PA = palmitic acid (16:0); ARA = arachidonic acid (20:4n-6); EPA = eicosapentaenoic acid (20:5n-3); DHA = docosahexaenoic acid (22:6n-3); DHA/EPA = 22:6n-3/20:5n-3; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; n-6 PUFA = n-6 polyunsaturated fatty acids; n-3 PUFA = n-3 polyunsaturated fatty acids; n-3 LC-PUFA = n-3 long chain polyunsaturated fatty acids.
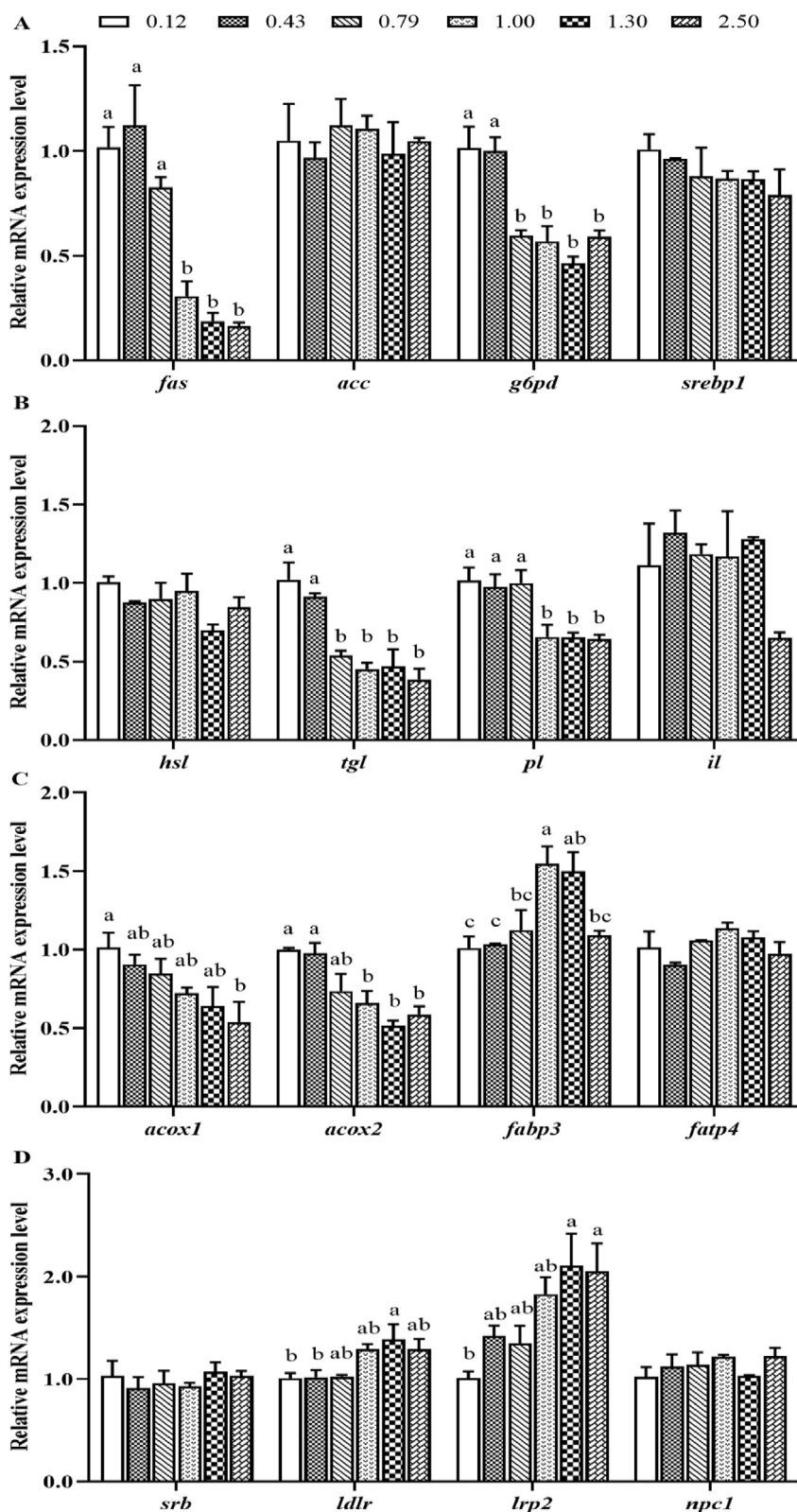
Fig. 4. Effects of dietary cholesterol level on relative expression of genes involved in (A) lipid anabolism, (B) lipid catabolism, (C) fatty acid β-oxidation and uptake, and (D) cholesterol metabolism in hepatopancreas of swimming crabs (*Portunus trituberculatus*). [a, b, c]Mean values of bars for the same parameter with different superscript letters are significantly different (*P* < 0.05). Data are reported as the means and SEM of 4 replicates. The gene expression of the control diet group (diet containing lowest cholesterol, 0.12%) was set at 1. *fas* = fatty acid synthase; *acc* = acetyl-CoA carboxylase; *g6pd* = glucose 6-phosphate dehydrogenase; *srebp1* = sterol regulatory element-binding protein-1; *hsl* = hormone-sensitive lipase; *tgl* = triacylglycerol lipase; *pl* = pancreatic lipase; *il* = intracellular lipase; *acox1* = acyl-CoA oxidase 1; *acox2* = acyl-CoA oxidase 2; *fabp3* = fatty acid binding protein 3; *fatp4* = fatty acid transport protein 4; *srb* = class B scavenger receptors; *ldlr* = low-density lipoprotein receptor; *lrp2* = low-density lipoprotein receptor-related protein 2; *npc1* = NPC intracellular cholesterol transporter 1.
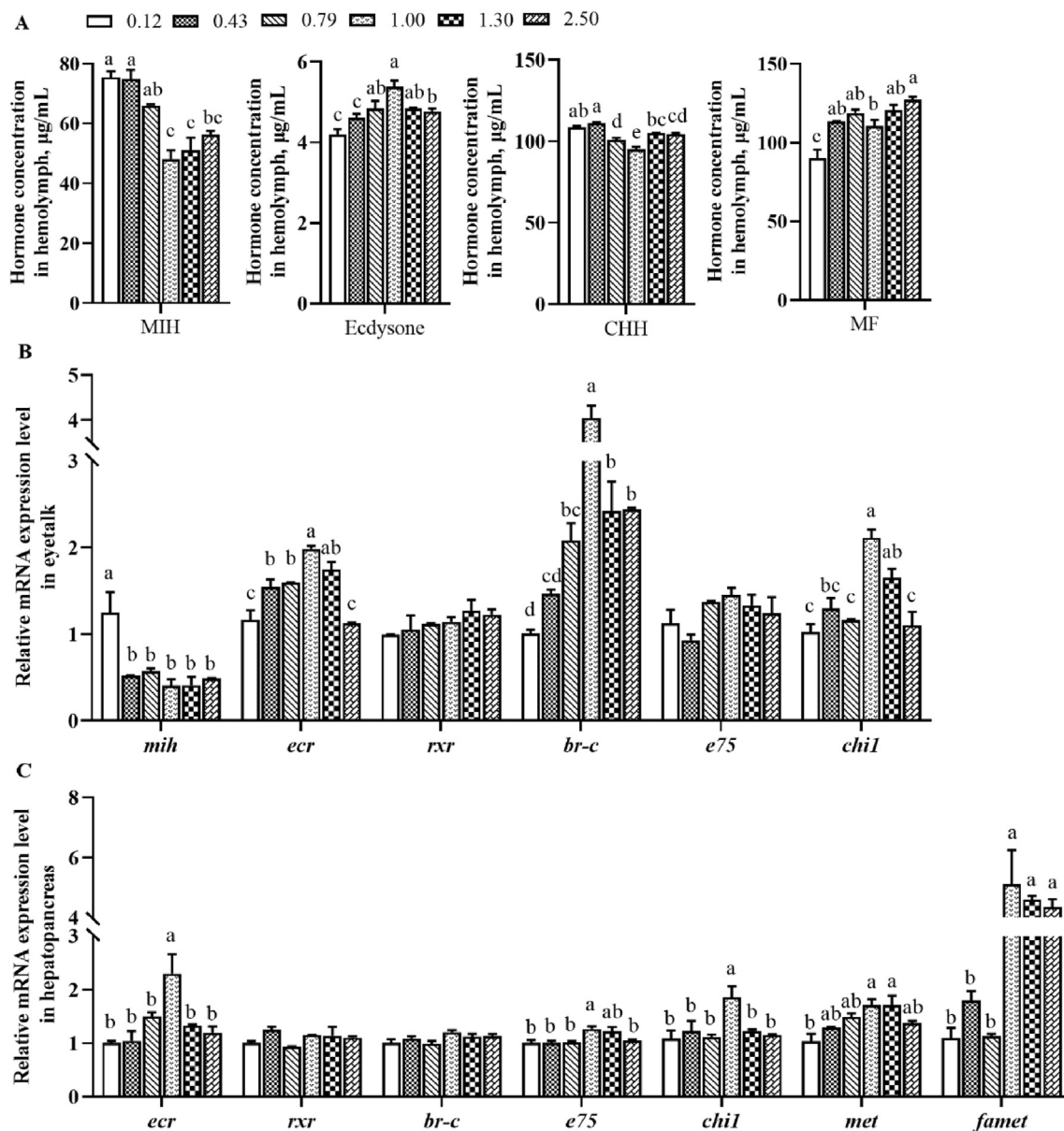
Fig. 5. Effects of dietary cholesterol level on (A) hormones related to molting in hemolymph, relative expression of genes involved in molting in (B) eyestalk and (C) hepatopancreas of swimming crabs (*Portunus trituberculatus*). [a, b, c]Mean values of bars for the same parameter with different superscript letters are significantly different ($P < 0.05$). Data are reported as the means and SEM of 4 replicates. The gene expression of the control diet group (diet containing lowest cholesterol, 0.12%) was set at 1. MIH = molt-inhibiting hormone; CHH = crustacean hyperglycemic hormone; MF = methyl farnesoate; *mih* = molt-inhibiting hormone; *ecr* = ecdysone receptor; *rxr* = retinoid-x receptor; *br-c* = broad-complex; *e75* = nuclear hormone receptor E75; *chi1* = chitinase 1; *met* = methoprene-tolerant protein; *famet* = farnesoic acid O-methyltransferase.

species differences. However, the increasing LDL-C and HDL-C suggested that 1.00% dietary cholesterol enhanced capacity for transporting cholesterol in swimming crabs.

The ability to synthesize n-3 LC-PUFA such as DHA and EPA is relatively low in marine aquatic animals, so they generally require to acquire these essential fatty acids (EFA) from their diet (Zhu et al., 2019). Generally, the fatty acid compositions of farmed aquatic animals usually mirror the diet (Xu et al., 2020). Moreover, a previous study revealed that rats fed the diet containing 0.5% cholesterol and 5% corn oil enhanced the hepatic fatty acid synthesis rate (Fungwe et al., 1994). In the present study, the levels of EPA, DHA, n-3 LC-PUFA, n-3 and n-6 PUFA all increased in hepatopancreas as dietary cholesterol level increased. These results indicated that the efficiency of n-3 LC-PUFA accumulation in hepatopancreas was improved by dietary cholesterol. This was in contrast to earlier

studies in swimming crabs that reported n-3 LC-PUFA concentrations were not influenced by dietary cholesterol level (Han et al., 2015; Sun et al., 2017). The inconsistent results might be due to the size of crabs and/or experimental conditions. Crabs in previous studies were larger, 3.70 ± 0.03 g or 3.48 ± 0.02 g and kept in plastic baskets, whereas crabs in the present study were smaller 1.41 ± 0.05 g and kept in individual aquaria. Overall, the effects of cholesterol on LC-PUFA levels are inconclusive and, therefore, need further investigation to better understand the impact of dietary cholesterol level on LC-PUFA metabolism.

Previous studies revealed that cholesterol level affected expression of involved in lipid metabolism (Recinos et al., 2004; Wang et al., 2010). In mammal, dietary cholesterol increased expression of genes or activity of enzymes related to lipogenesis and decreased fatty acid β-oxidation, which led to TG accumulation

and unbalanced hepatic lipid homeostasis (Fungwe et al., 1993; Xu et al., 2004). However, the present study demonstrated that the expression levels of *fas* and *g6pd* related to lipogenesis were down-regulated in hepatopancreas as dietary cholesterol level increased. These results are somewhat in agreement with a previous study in rats fed a 1.0% cholesterol diet for 4 weeks (Wang et al., 2010). In addition, in the current study, the lipolysis genes (*tgl* and *pl*) were down-regulated in hepatopancreas by dietary cholesterol level. These results indicated that high dietary cholesterol leading to cholesterol accumulation could suppress lipid catabolism, which may account for higher TG level in hepatopancreas, albeit was not significant.

Fatty acid β-oxidation is another factor to affect the lipid homeostasis that is regulated by dietary cholesterol (Fungwe et al., 1993). Acyl-CoA oxidase (ACOX) is one of the vital enzymes of fatty acid β-oxidation (Sun et al., 2020). In the present study, when dietary cholesterol level increased from 0.12% to 2.50%, the expression levels of *acox1* and *acox2* in hepatopancreas were down-regulated. This result suggested inhibition of fatty acid β-oxidation by dietary cholesterol, which might in turn increase the accumulation of fatty acids in TG and cholesterol ester (CE) (Wang et al., 2010). Fatty acid-binding proteins gene transport fatty acids within tissues (Erol et al., 2004) and, in the present study, the mRNA expression level of *fabp3* was up-regulated as dietary cholesterol level increased from 0.12% to 1.00%, and then decreased when dietary cholesterol further increased to 2.50%. The up-regulated expression of *fabp3* might reflect or be associated with the increased PUFA levels in hepatopancreas, meanwhile, higher dietary cholesterol level (≥1.30% cholesterol) may prevent lipid deposition through negative feedback regulated by accumulation of LC-PUFA to suppress transport of *fabp3*.

The *ldlr* and *srb* are specific receptors for LDL and HDL, respectively, facilitating uptake of lipoprotein cholesterol (Huang et al., 2019). A previous study observed that the expression of SRB1 was up-regulated when mice were fed a combined high fat and high cholesterol diet (Zou et al., 2009). However, the present study recorded that the expression of *srb* in swimming crabs was not significantly influenced by dietary cholesterol level alone. Singh et al. (2014) reported that excess dietary cholesterol inhibited *ldlr*-mediated cholesterol uptake to maintain cholesterol homeostasis in cells. In the present study, the expression of *ldlr* and *lrp2* were up-regulated as dietary cholesterol level increased from 0.12% to 1.30%, which was further evidence to suggest that adequate dietary cholesterol levels promote cholesterol transport through increased expression of *ldlr* and *lrp2*.

Molting, a fundamental process for growth in crustaceans, is positively and negatively regulated by various type of hormones. Molting begins with multiple steps converting cholesterol to the steroid hormone, ecdysone in the Y-organ (Abdullah-Zawawi et al., 2021). The molt-promoting function of ecdysone is achieved via the ecdysone signalling pathway to increase expression of downstream genes. Specifically, ecdysone binds with the heterodimer complex of EcR/RXR, which activates the transcription of downstream molting response genes, such as *e75*, *br-c* and *chi* (Zhang et al., 2019). In the present study, the concentration of ecdysone increased as dietary cholesterol level increased from 0.12% to 1.00%, then decreased as dietary cholesterol increased further. Similar results were observed previously in Chinese mitten crabs (*Enricher*
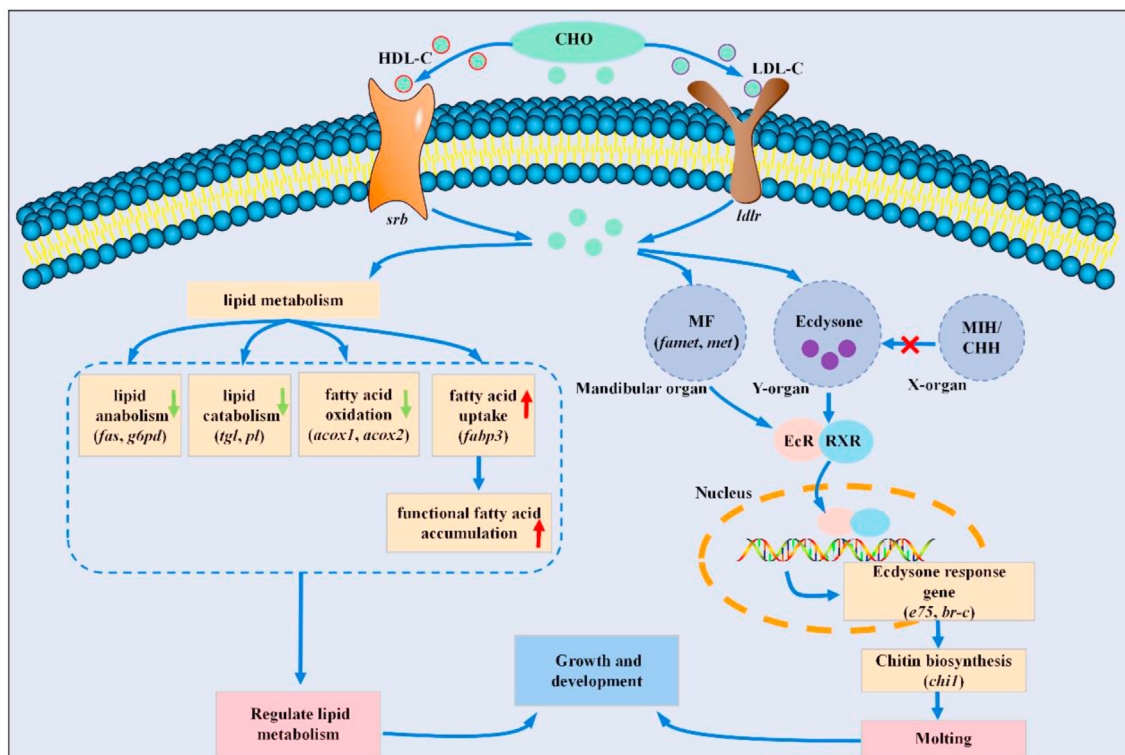

Fig. 6. Proposed pathway of how dietary cholesterol regulates lipid metabolism and molting events of the putative ecdysone signalling pathway in swimming crabs (*Portunus trituberculatus*). Red arrows represent increase; green arrows represent decrease; blue arrow indicates the path direction. CHO = cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; *srb* = class B scavenger receptors; *ldlr* = low-density lipoprotein receptor; *fas* = fatty acid synthase; *g6pd* = glucose 6-phosphate dehydrogenase; *tgl* = triacylglycerol lipase; *pl* = pancreatic lipase; *acox1* = acyl-CoA oxidase 1; *acox2* = acyl-CoA oxidase 2; *fabp3* = fatty acid binding protein 3; MF = methyl farnesoate; *famet* = farnesoic acid O-methyltransferase; *met* = methoprene-tolerant protein; MIH = molt-inhibiting hormone; CHH = crustacean hyperglycemic hormone; EcR = ecdysone receptor; RXR = retinoid-x receptor; *e75*, nuclear hormone receptor E75; *br-c* = broad-complex; *chi1* = chitinase 1.

sinensis) (Tao et al., 2014). Moreover, the expression level of the corresponding downstream genes, *ecr* and *chi1*, were significantly up-regulated in eyestalk and hepatopancreas of crabs fed the diet containing 1.00% cholesterol. These results were somewhat in agreement with some previous studies in crayfishes (Tian et al., 2020) and mud crabs (Zheng et al., 2018). MIH and CHH, both an endocrine factor from X-organ/sinus gland complex in eyestalks, inhibit ecdysone (Chung and Webster, 2003). The present study demonstrated that the concentrations of MIH and CHH in hemolymph were decreased and then increased by dietary cholesterol, with the lowest levels were recorded in crabs fed 1.00% cholesterol. Similarly, the expression level of *mih* in crabs fed the 0.12% cholesterol diet was significantly higher than those fed the other diets. These results were largely in agreement with a previous study in crayfishes (Tian et al., 2020), and another study reported that the level of ecdysone during molting showed a similar trend to body cholesterol level, however, the expression pattern of *mih* showed the opposite trend (Zhang et al., 2019), which was in accordance with the present study.

In addition to ecdysone, MF in mandibular organ, is another hormone regulating molting in crustaceans also modulated by cholesterol level (Kumar et al., 2018; Abdullah-Zawawi et al., 2021). The mechanism by which MF regulates molting is still unclear and one study reported that ecdysone and MF interact to coordinate the molt cycle through regulation of EcR/RXR (Hyde et al., 2019). Farnesoic acid O-methyltransferase (Famet) is the key last step enzyme in the biosynthetic pathway for MF and methoprene-tolerant (Met) is the primary candidate receptor for crustacean MF (Liu et al., 2016; Kumar et al., 2018). Met can also interact with EcR/RXR and bind to ecdysone response elements to drive molting (Sin et al., 2015). In the present study, the concentration of MF was lowest in crabs fed the lowest level of cholesterol (0.12%) and the expression of *met* was up-regulated as cholesterol level increased from 0.12% to 1.00%. However, crabs fed with 1.00%, 1.30% and 2.50% cholesterol showed up-regulated expression of *famet*. Therefore, the present results indicated that dietary cholesterol promoted production of MF possibly through up-regulation of *famet* expression.

5. Conclusion

In summary, based on broken-line regression analysis of PWG and dietary cholesterol, the optimal dietary cholesterol requirement of juvenile swimming crabs was estimated to be 1.01%. Moreover, dietary cholesterol regulated lipid metabolism by up-regulating the expression levels of genes related to fatty acid uptake, and down-regulating expression levels of genes related to lipid anabolism and catabolism as well as fatty acid β-oxidation. Furthermore, dietary cholesterol is transported into tissues through *srb* and *ldlr*, and then tissue cholesterol could enhance ecdysone and MF, activating EcR/RXR, subsequently increasing transcription of early genes (*e75* and *br-c*) and thus controls the transcription of late genes (*chi1*) to promote molting. MIH and CHH also impacted the molt cycle by inhibiting the pathway (Fig. 6). Overall, optimal dietary cholesterol improved growth and development of swimming crabs through regulation of lipid metabolism and ecdysone signalling pathway in swimming crabs.

Author contributions

Tingting Zhu: Formal analysis, Investigation, Writing-original draft, Writing-review and editing. **Qicun Zhou**: Conceptualization, Methodology, Supervision, Writing-review & editing, Project administration, Funding acquisition. **Zheng Yang**: Software, Formal analysis. **Yingying Zhang**: Software, Formal analysis. **Jiaxiang Luo**: Software, Formal analysis. **Xiangsheng Zhang**: Software, Formal analysis. **Yuedong Shen**: Software, Formal analysis. **Lefei Jiao**: Software, Formal analysis. **Douglas R. Tocher**: Writing-review & editing. **Min Jin**: Conceptualization, Methodology, Supervision, Writing-review & editing, Project administration, Funding acquisition.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Appendix. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.05.001.

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