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Mating behaviour and cholesterol nutritional strategies promoted ovarian development of female swimming crab (*Portunus trituberculatus*)

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Abstract

Female crabs enter a stage of rapid ovarian development after mating, and cholesterol is a substrate for steroid hormone synthesis. Therefore, in this experiment, an 8-week feeding trial was conducted to investigate the effects of mating treatments (mated crab and unmated crab) and three dietary cholesterol levels (0·09 %, 0·79 % and 1·40 %) on ovarian development, cholesterol metabolism and steroid hormones metabolism of adult female swimming crab (*Portunus trituberculatus*). The results indicated that crabs fed the diet with 0·79 % cholesterol significantly increased gonadosomatic index (GSI) and vitellogenin (VTG) content than other treatments in the same mating status. Moreover, mated crabs had markedly increased GSI and VTG content in the ovary and hepatopancreas than unmated crabs. The histological observation found that exogenous vitellogenic oocytes appeared in the mated crabs, while previtellogenic oocytes and endogenous vitellogenic oocytes were the primary oocytes in unmated crabs. The transmission electron microscopy analysis showed that when fed diet with 0·79 % cholesterol, the unmated crabs contained more rough endoplasmic reticulum and mated crabs had higher yolk content than other treatments. Furthermore, mating treatment and dietary 0·79 % cholesterol level both promoted cholesterol deposition by up-regulation of the mRNA and protein expression levels of class B scavenger receptors 1 (Srb1), while stimulating the secretion of steroid hormones by up-regulation of the mRNA and protein expression of steroidogenic acute regulatory protein (Star). Overall, the present results indicated that mating behaviour plays a leading role in promoting ovarian development, and dietary 0·79 % cholesterol level can further promote ovarian development after mating.

Keywords: ***Portunus trituberculatus*: Mating: Cholesterol: Ovarian development: Steroid hormones**

The swimming crab (*Portunus trituberculatus*) is a widely cultivated marine economic species in China[1]. In 2021, the total catch of swimming crab reached 454 513 tons, with a cultured production of 105 283 tons and a cultivation area exceeding 21 359 hectares[2]. The life cycle of swimming crab is about 2 to 3 years, reaching sexual maturity in the first year[3]. When the crab finishes pubertal moult, it transitions from subadult to adult crab[4]. The ovary starts vitellogenesis and enters a stage of rapid development after crabs successfully mating[5]. During mating, sperm is transferred from males to females, and along with the sperm, seminal fluid proteins (such as prohormones, lectins, protease inhibitors, antioxidants and peptides) are also transferred to the female's body[6,7]. However, decapod crustaceans have a delay between fertilisation and spawning, as females retain the sperm for a particular duration[8]. This period is known as the post-mating period, which can range from hours to years[9]. The duration of this period is influenced by factors including species, the type of female spermathecae, reproductive biology and environmental conditions such as water temperature[6,9].

Vitellogenesis in crustaceans is a process of forming and accumulating various nutrients (such as proteins, lipids and carbohydrates) in oocytes, which is a necessary factor for oocyte maturation[10]. Vitellin is the primary storage protein in the

Abbreviations: EN, endogenous vitellogenic oocytes; EX, exogenous vitellogenic oocytes; GSI, gonadosomatic index; HSI, hepatopancreas index; Hsl, hormone-sensitive lipase; Ldlr, LDL receptor; PR, previtellogenic oocyte; RER, rough endoplasmic reticulum; Srb1, class B scavenger receptors 1; Star, steroidogenic acute regulatory protein; VTG, vitellogenin; 3β-hsd, 3β-hydroxysteroid dehydrogenase.

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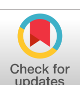

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mature ovaries of crustaceans and can undergo processing by substances such as vitellogenin (VTG), lipids, carbohydrates, proteins, pigments and other substances[11]. During the process of vitellogenesis, VTG can transport a certain amount of lipophilic compounds (such as triglycerides (TG) and phospholipids) and steroidal hormones to the ovary from the hemolymph, and it is deemed a vital biomarker related to ovarian development[12]. The VTG has proven to be synthesised in hepatopancreas and ovaries in most crustaceans, and these two tissues play essential roles in different stages of ovarian development[13,14]. Vitellogenesis can be divided into two phases based on different VTG synthesis sites: endogenous vitellogenesis and exogenous vitellogenesis[15]. VTG is synthesised primarily in the ovary during the endogenous vitellogenesis phase, while the hepatopancreas is the leading site of VTG synthesis during the exogenous vitellogenesis phase[16].

In crustaceans, ovarian development is complex and regulated by many factors, mainly manipulated by hormone regulation, including steroid hormones and neuropeptides[17]. Vertebrate steroids, including estradiol, progesterone and pregnenolone, have been found in the hepatopancreas and ovary in several crustacean species[12]. The fluctuation of these substances during gonadal maturation suggests that they regulate reproduction[18]. For example, estradiol administration to crayfish increased the *vtg* mRNA expression levels in the hepatopancreas, while progesterone administration to crayfish stimulated VTG concentration accumulation in the hemolymph[19]. Moreover, in swimming crabs, exogenous estradiol also increased the transcripts of *vtg* in both the hepatopancreas and ovary[16].

Cholesterol is a crucial component in cellular structure, serving as a precursor to anabolic steroid hormone, vitamin D and bile acid[20]. At the same time, lipoproteins, which are formed from cholesterol, are involved in the formation of the egg yolk. Thus, cholesterol is vital in vitellogenesis[21]. However, numerous studies have reported the effects of cholesterol on growth performance in crustaceans, such as Chinese mitten crab (*Eriocheir sinensis*)[22], mud crab (*Scylla paramamosain*)[23] and swimming crab[24]. There has been limited research on the impact of cholesterol on ovarian development in crustaceans. In general, for anabolic steroid hormones, cholesterol is transported to the ovaries by two primary mechanisms: (1) cholesterol-rich LDL is taken up through endocytosis, which is regulated by the LDL receptor (Ldlr). (2) Cholesterol esters from HDL are selectively absorbed regulated by the scavenger receptor-BI (Srb1)[25,26]. Cholesterol esters enter cells via Srb1 or Ldlr and are further transformed into free cholesterol by hormone-sensitive lipase (Hsl)[27]. Subsequently, free cholesterol is transported to the mitochondrial inner membrane under the control of the steroidogenic acute regulatory protein (Star) and converted into pregnenolone and estradiol through a series of enzymes, such as cholesterol side-chain cleavage enzyme (Cyp11a) and 3β-hydroxysteroid dehydrogenase (3β-hsd)[25,27].

The nutritional status of crustacean broodstock significantly affects gonad maturation, reproductive performance, egg and juvenile crab quality[28]. Most formulated diets for crustaceans consist of different types of proteins, lipids and essential amino acids, which are important for the ovarian maturation of crustacean broodstock[29]. Feeding broodstock with diets that have a higher protein content can increase fertilisation rates, while feeding them with high-fat diets can result in shorter incubation periods and hatching times[29]. To improve the nutritional status of crustacean broodstock, it is important to provide them with a balanced diet containing the proper nutrients. Up to now, studies on dietary nutrients in swimming crabs mainly focus on the nutritional requirements of protein[30], lipid[31] and carbohydrate[32] in juvenile crabs, while there are few studies on broodstock of swimming crabs. However, the effects of cholesterol nutrition and mating behaviour on ovarian development are currently unclear. Therefore, this experiment aims to investigate the effects of cholesterol and mating behaviour on ovarian development, cholesterol metabolism and steroid hormone metabolism of swimming crabs. The goal is to provide a reference for producing a high-quality crustacean broodstock fattening diet and provide basic information for further study of reproductive physiology of swimming crabs.

Materials and methods

Experimental diets

Based on a previous research[33], three isolipidic and isonitrogenous diets with 44·0 % protein and 9·5 % lipid were formulated to contain 0·09 %, 0·79 % and 1·40 % cholesterol. In order to maintain a balanced lipid content, palmitic acid was supplemented in the experimental diets, resulting in a consistent total amount of 1·5 % in each diet. The protein sources utilised in the diets included Peru fish meal, soybean meal, krill meal, soybean protein concentrate and corn gluten meal. All raw materials were ground by a hammer mill and passed through a 60-mesh percolator. Subsequently, the materials were carefully weighed and mixed step by step, and liquid ingredients (oils and deionised water) were added to the mixture to make a dough. Finally, the obtained dough was squeezed out through a double-helix grinder to make 3-mm and 5-mm diameter pellets. The pellets were steam-dried to approximately 10 % moisture and stored in vacuum plastic bags at –20°C until use in the feeding trial. The formulation and proximate composition of the diets are shown in Table 1.

The cholesterol content in the diets was determined through the GC (Shimadzu) method, as outlined in a study by Zhu *et al.*[24]. Ten millilitres chloroform was added to 500 mg experiment diets to extract lipids. Subsequently, 1 mL of the lipid solution was dried under pure nitrogen. Then, 1 mL of diethyl ether was added and analysed using GC.

Feeding trial conditions

The experimental crabs were obtained from a local commercial farm in Hengma, Ningbo, China. This feeding trial was implemented in a pilot base of the Meishan Campus of Ningbo University (Ningbo, China). Before the feeding trial, all female crabs underwent a 2-week acclimation period in 100 L of recirculation aquaculture system aquaria to acclimate to laboratory conditions. During this period, they were provided with a commercial diet containing 8 % lipid and 45 % protein.

Table 1. Formulation and proximate composition of the experimental diets (%, DM)

	Dietary cholesterol levels, %		
Ingredients, g/100 g	0·09	0·79	1·40
Peru fishmeal*	25·00	25·00	25·00
Soybean protein concentrate*	7·00	7·00	7·00
Soybean meal*	23·00	23·00	23·00
Krill meal*	3·00	3·00	3·00
Corn gluten meal*	5·00	5·00	5·00
Wheat flour*	24·70	24·70	24·70
Fish oil*	2·00	2·00	2·00
Palmitic acid†	1·50	0·75	0·00
Cholesterol‡	0·00	0·75	1·50
Soybean lecithin*	2·00	2·00	2·00
Vitamin premix*	0·50	0·50	0·50
Mineral premix*	1·00	1·00	1·00
$Ca(H_2PO_4)_2$*	2·00	2·00	2·00
Choline chloride*	0·30	0·30	0·30
Sodium alginate*	3·00	3·00	3·00
Proximate composition, %			
DM	89·07	88·56	88·58
Crude protein	44·61	44·69	44·86
Crude lipid	9·73	9·70	9·54
Ash	9·56	9·58	9·86
Cholesterol	0·09	0·79	1·40

* Ningbo Tech-Bank Feed Co. Ltd, China.
† Palmitic acid: 97 % of total fatty acids as palmitic acid methyl ester; Shanghai Yiji Chemical Co., Ltd, China.
‡ Cholesterol: Shanghai Macklin Biochemical Co., Ltd.

Unmated crabs were taken as crab broodstock and then put into a single aquarium for mating (the ratio of male and female was 1:1). Subsequently, a total of 144 healthy female swimming crabs (seventy-two unmated crabs and seventy-two mated crabs, with average weight of 115·5 (SEM 5·91) g) were randomly allocated to 144 individual aquaria. Three replicates of eight crabs each were randomly assigned to the three experimental diets, resulting in the six experimental treatments as follows: unmated crabs (U0·09, U0·79 and U1·40) and mated crabs (M0·09, M0·79 and M1·40). During the 8-week experimental period, the crabs were fed once a day at 18.00, with a feed amount ranging from 3 % to 5 % of their wet body weight. Uneaten diet and feces were removed with a dirt absorber. To maintain the quality of the seawater, 50 % of seawater was replaced every 3 d. Daily measurements were taken to monitor water quality parameters, including maintaining the water temperature between 25·8°C and 27·6°C, dissolved oxygen levels between 7·0 mg/L and 8·0 mg/L, salinity between 24·2 ppt and 26·7 ppt, and pH between 7·3 and 7·9 and ensuring that the ammonia nitrogen levels stayed below 0·05 mg/L.

Sample collection

At the end of the experiment, the crabs were fasted for 24 h and then anesthetised on ice before sampling. Hemolymph samples were collected with 2 mL of syringes from four crabs per replicate and stored at 4°C for 24 h. Subsequently, the hemolymph was centrifuged (956 × *g* for 10 min at 4°C) to obtain supernatant for analysing hormone concentrations and biochemical indexes. Following the hemolymph collection, the crabs were further utilised to obtain samples of the hepatopancreas and ovary to determine the hepatopancreas index (HSI) and gonadosomatic index (GSI), respectively. Then, the ovary and hepatopancreas were rapidly frozen using liquid N_2 and stored at −80°C for subsequent analyses. Before that, part of the ovary was used for histological analysis (fixed in 4 % paraformaldehyde) and transmission electron microscopy observation (fixed in 2·5 % glutaraldehyde).

Proximate composition analysis

The content of crude protein, crude lipid, ash and moisture in diets were determined by standard Association of Official Agricultural Chemists (AOAC) methods[34]. Crude protein content (N × 6·25) was determined by a protein analyser (FP-528, LECO). Total lipid was extracted with the petroleum ether extraction method. The content of ash was assayed using a muffle furnace at 550°C for 8 h. Moisture content was determined by making each sample to continuous drying until a constant weight at 105°C.

Histological analysis of ovary

Histological analysis was performed as described in detail previously[35]. For histological description, fresh ovarian tissue samples were first fixed in a 4 % paraformaldehyde solution. Following fixation for a minimum of 24 h, samples were gradient dehydration in 70 %, 80 %, 90 %, 95 % and 100 % of ethanol, cleaned and balanced with xylene. Ovary samples were embedded in paraffin and cut into 5 μm sections. The slides were deparaffinised in xylol and rehydrated in a graded alcohol series and then stained with hematoxylin and eosin (H&E). After this, the sections were again dehydrated in ethanol and xylol, mounted with the neutral resins. Finally, the images were observed under a microscope (Nikon Eclipse CIpan) to capture magnified images, and the relative areas of ovarian cells were quantified by Image J (v 1.8.0) software. For each sample, three measurements were taken.

Transmission electron microscopy observation

The transmission electron microscopy analysis was performed according to the methods described in detail previously[35]. The ovary samples were fixed in 2·5 % glutaraldehyde for 2–4 h and washed three times with sodium phosphate buffer (0·1 M, pH 7·4) for 15 min each time. Subsequently, the ovary samples were further fixed in 1 % osmium tetroxide for 1·5 h. Then, the ovary samples were washed twice in the same buffer (15 min each) and once with ultrapure water for 15 min. Then, the samples were dehydrated in a graded series of ethanol (30–50–70–90 %) followed by 100 % acetone. Different proportions of SPI-Pon 812 resin: acetone (1:3, 1:2, 1:1) were used to gradually infiltrate ovary for 1 h each time, followed by SPI-Pon 812 resin overnight, and embedded in SPI-Pon 812 resin at 70°C for more than 36 h. Lastly, the images were obtained and digitised in a Hitachi H-7650 electron microscope.

Cholesterol metabolism-related parameters analysis

The hemolymph samples were used to measure the contents of LDL-cholesterol (LDL-C) (A113–1–1) and HDL-cholesterol

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Table 2. Primer sequences used in this experiment

Gene	Nucleotide sequence (5' - 3')	Size (bp)	GenBank reference or Publication
abcg1	F: ACTCCGGCTGTCTCTTCTTC R: CACCAACAGCACATACAGCA	203	PRJNA428031
3β-hsd	F: AGGGTGACCGTTCCTTCGT R: CGGAGGCGTGTTGTCTGTT	221	PRJNA428031
β-actin	F: CGAAACCTTCAACACTCCCG R: GGATAGCGTGAGGAAGGGCATA	–	FJ641977·1
hsl	F: GTTCCCATTCTCTCCATTG R: ACCACCAGCACTGTCACCT	159	MF537408
ldlr	F: GCCTCTCTCCTCTTTGCTCAC R: GAAATAACCACCACCTTCTTGC	206	PRJNA428031
lrp2	F: GGAACTGCTGGCAAGATTTAC R: CAGTCCAATACAGCATTTCCTC	124	PRJNA432636
npc1	F: GAAGTTTGGGTGTGTGGGTC R: GTCCTCCACAGACCACTCAA	220	PRJNA428031
srb	F: CCTCTGCTTGACTGGGTACA R: TCCAGTTGTCAATCCTCCCC	208	KT899699·1
star	F: TAAATGGTCGCATGTCAGCA R: GCAGCAACCATCACAATGTC	187	PRJNA428031
vtg	F: TGCTGCCAAACTATCCTTCATCC R: CAACTTATCGGAGCCAGGCAATC	214	DQ000638·1

abcg1, ATP-binding cassette sub-family G member 1; *3β-hsd*, 3β-hydroxysteroid dehydrogenase; *hsl*, hormone-sensitive lipase; *ldlr*, LDL receptor; *lrp2*, LDL receptor-related protein 2; *npc1*, NPC intracellular cholesterol transporter 1; *srb*, class B scavenger receptors; *star*, steroidogenic acute regulatory protein; *vtg*, vitellogenin.

(HDL-C) (A112–1–1) by commercial kits from Jiancheng Bioengineering Co., Ltd following the manufacturer's procedures. The absorbance at 600 nm was recorded using a microplate reader (Spectra Max M2, Molecular Device). The analysis of very low-density lipoprotein (VLDL) content in the hemolymph was conducted utilising assay kits provided by Jiangsu Meibiao Biological Technology Co., Ltd. The absorbance of VLDL was recorded at 450 nm using a SpectraMax M2e microplate reader.

The hepatopancreas and ovary samples were weighed and homogenised in ten volumes (v/w) of the ice-cooled physiological saline (0·85 %). The homogenates were centrifuged at 1500 *g* for 10 min at 4°C to remove debris. The supernatant was used to determine the contents of TG (A110–1–1) and cholesterol (CHO) (A111–1–1) using commercial kits from Jiancheng Bioengineering Co., Ltd. The assay procedures were carried out following the instructions provided by the manufacturer. Finally, the absorbance of TG and CHO was recorded at 500 nm using a SpectraMax M2e microplate reader.

Assay of vitellogenin content and steroid hormones

The concentration of pregnenolone, progesterone, estradiol and VTG in hemolymph, ovary and hepatopancreas were assayed using ELISA commercial kits from Jiangsu Meibiao Biological Technology Co., Ltd according to the manufacturer's instructions.

Assay of quantitative real-time PCR

The quantitative real-time PCR (qPCR) assays were performed following the protocols established in our previously published study[24]. In brief, total RNA was extracted from hepatopancreas and ovary samples using Trizol Reagent, provided by the manufacturer (Vazyme Biotech Co., Ltd). The quantity and quality of isolated RNA were determined spectrophotometrically (Nanodrop 2000, Thermo Fisher Scientific) on a 1·2 % denaturing agarose gel. The cDNA was synthesised through reverse transcription using the HiScript II Reverse Transcriptase Reagent Kit (Vazyme), following the provided instructions. The qPCR program included 10 min at 95°C, 45 cycles at 95°C for 10 s, 58°C for 10 s, and, finally, 72°C for 20 s. The *β*-actin was used as the reference gene, and the primers for qPCR analysis are presented in Table 2. Standard curves were generated using six different dilutions (in triplicate) of the cDNA samples, and the amplification efficiency was analysed using the eq.$E = 10^{(-1/\text{Slope})}$-1. The amplification efficiencies of all genes were approximately equal and ranged from 87 to 109 %. The gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method[36].

Western blot analysis

Protein extraction and western blot analysis were performed according to the methods described in detail previously[37]. The ovary samples were homogenised on ice using a mixture of phenylmethylsulfonyl fluoride (1 mM, Beyotime Biotechnology) and radio immunoprecipitation assay (RIPA) lysis buffer to extract the proteins. Proteins samples were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, then transferred to a 0·45 μm polyvinylidene difluoride membrane and were incubated in 5 % bovine serum albumin blocking buffer for 30 min. The membrane was incubated overnight at 4°C with the following rabbit antibodies: *β*-actin (ABclonal, 1:5000 dilution), Srb1 (ABclonal, 1:1000 dilution) and Star (ABclonal, 1:1000 dilution). The polyvinylidene difluoride membrane was washed and incubated with a secondary antibody (ABclonal, 1:5000 dilution) for 1 h at room temperature. The western blot images were obtained using a luminescent image analyser (Tanon 5200) and quantified by Image J.

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Calculations and statistical analysis

The following variables were calculated:

Hepatosomatic index (HSI, %) = 100 × (hepatopancreas weight, g/crab weight, g)

Gonadosomatic index (GSI, %) = 100 × (ovary weight, g/crab weight, g)

Data were expressed as mean (SEM). Two-way ANOVA analysis was performed to calculate the combined effects of mating treatment (mated crabs and unmated crabs) and dietary cholesterol levels (0·09, 0·79 and 1·40 %) on all parameters. One-way ANOVA was conducted to analyse the different dietary cholesterol levels in the same mating status. Tukey's multiple range test was performed to identify significant differences ($P < 0·05$) and the significant difference between the mated and unmated crab treatments under the same dietary cholesterol level using independent-samples Student's *t*-test ($P < 0·05$ and $P < 0·01$). All statistical analyses were conducted using SPSS 19.0 (SPSS). All bar charts were carried out using GraphPad Prism software (version 9).

Results

Morphological characteristics analysis

The morphological observation and morphology index of female swimming crab are presented in Fig. 1. In unmated crab, the ovary was immature and displayed a transparent to semi-transparent coloration (Fig. 1A, B and C). After mating, the ovary displayed a yellow coloration (Fig. 1D, E and F). Besides, the two-way ANOVA analysis revealed that GSI was significantly influenced by mating and dietary cholesterol levels (Fig. 1H; $P < 0·05$). Moreover, no significant differences in HSI were detected among the treatments (Fig. 1G; $P > 0·05$). The *t*-test analysis showed that when fed with the same dietary cholesterol level, mated crabs had markedly increased GSI compared with unmated crabs in the same dietary cholesterol level ($P < 0·05$). Additionally, the one-way ANOVA indicated that the crabs fed the diet with 0·79 % cholesterol had significantly increased the GSI than other treatments in the same mating status ($P < 0·05$).

Analysis of cholesterol deposition

The two-way ANOVA showed that the contents of HDL-C, CHO, LDL-C and TG in hemolymph, as well as CHO and TG in ovary, were markedly affected by mating and dietary cholesterol levels (Fig. 2). Significant interactions between mating and dietary cholesterol level were found in LDL-C content in hemolymph and TG content in ovary (Fig. 2A and C; $P < 0·05$). Specifically, the *t*-test analysis indicated that mated crabs had significantly increased contents of CHO, LDL-C, HDL-C and TG in hemolymph as well as CHO and TG in ovary compared with those unmated crabs (Fig. 2A and C; $P < 0·05$). The VLDL has significantly increased in U0·79 and M0·79 treatments compared with the other treatments in the same mating status (Fig. 2A; $P < 0·05$). Besides, the CHO and TG content in hemolymph, hepatopancreas and ovary were increased with the increase of dietary cholesterol levels and exhibited a dose effect, to a lesser extent, in the same mating status (Fig. 2; $P < 0·05$).

Vitellogenin and steroid hormone contents

The effects of mating and dietary cholesterol level on pregnenolone, progesterone, estradiol and VTG content in hemolymph, ovary and hepatopancreas are shown in Fig. 3. The results of a two-way ANOVA showed that the content of pregnenolone in hemolymph, progesterone and VTG in the ovary were significantly influenced by mating and dietary cholesterol levels and had highly interactions between mating and dietary cholesterol level ($P < 0·05$). In detail, the results of *t*-test analysis presented that mated crabs markedly increased content of progesterone in ovary and VTG in hepatopancreas than those unmated crabs ($P < 0·05$). When fed the diet with 0·09 % and 0·79 % cholesterol, mated crabs had significantly increased contents of pregnenolone in hemolymph and VTG in ovary than those unmated crabs ($P < 0·05$). Furthermore, the one-way ANOVA indicated that crabs fed the diet with 0·79 % cholesterol led to significantly enhanced the content of pregnenolone, progesterone, estradiol and VTG in hemolymph, ovary and hepatopancreas compared with crabs fed with cholesterol-free diet in the same mating status ($P < 0·05$), except for hepatopancreatic pregnenolone and hemolymphatic progesterone content after mating treatment (Fig. 3).

Histological analysis

The two-way ANOVA indicated that ovarian histology was significantly influenced by both mating and dietary cholesterol levels in different degrees (Fig. 4). In addition, significant interactions between mating and dietary cholesterol levels were observed in the oocyte types (Fig. 4G; $P < 0·05$). Concretely, previtellogenic oocytes (PR) were the primary oocytes in unmated crabs fed the diet with 0·09 % cholesterol, and a few oocytes had developed into endogenous vitellogenic oocytes (EN) (Fig. 4A and G). In unmated treatment, crabs fed diet with 0·79 % cholesterol had significantly increased EN area than crabs fed diet with 0·09 % cholesterol (Fig. 4B and G; $P < 0·05$). However, when the dietary cholesterol levels increased further, the area of EN was dramatically decreased and PR was increased by crabs fed diet with 1·40 % cholesterol (Fig. 4C and G; $P < 0·05$). Nevertheless, exogenous vitellogenic oocytes (EX) appeared in the mated crabs but not in unmated crabs (Fig. 4D, E and F). Evidently, after mating treatment, crabs fed the diet with 0·79 % cholesterol exhibited larger diameter of EX than those fed other cholesterol (Fig. 4G; $P < 0·05$).

Transmission electron microscopy observation

As shown in Fig. 5, dietary cholesterol level and mating treatment had a significant effect on the ultrastructure of the ovary in swimming crabs. Ultrastructurally, the rough endoplasmic reticulum (RER) and mitochondria (M) in the cytoplasm of unmated crabs were abundant (Fig. 5A–F). Besides, the RER in unmated crabs fed the diet with 0·79 and 1·40 % cholesterol contained material of different electron densities compared with the U0·09 treatment (Fig. 5A–F). After mating in crabs, the ultrastructure of oocytes showed that immature yolk (IY) and many yolk (Y) granules appeared with different sizes and electron densities and were wrapped in a membrane (Fig. 5G–L).

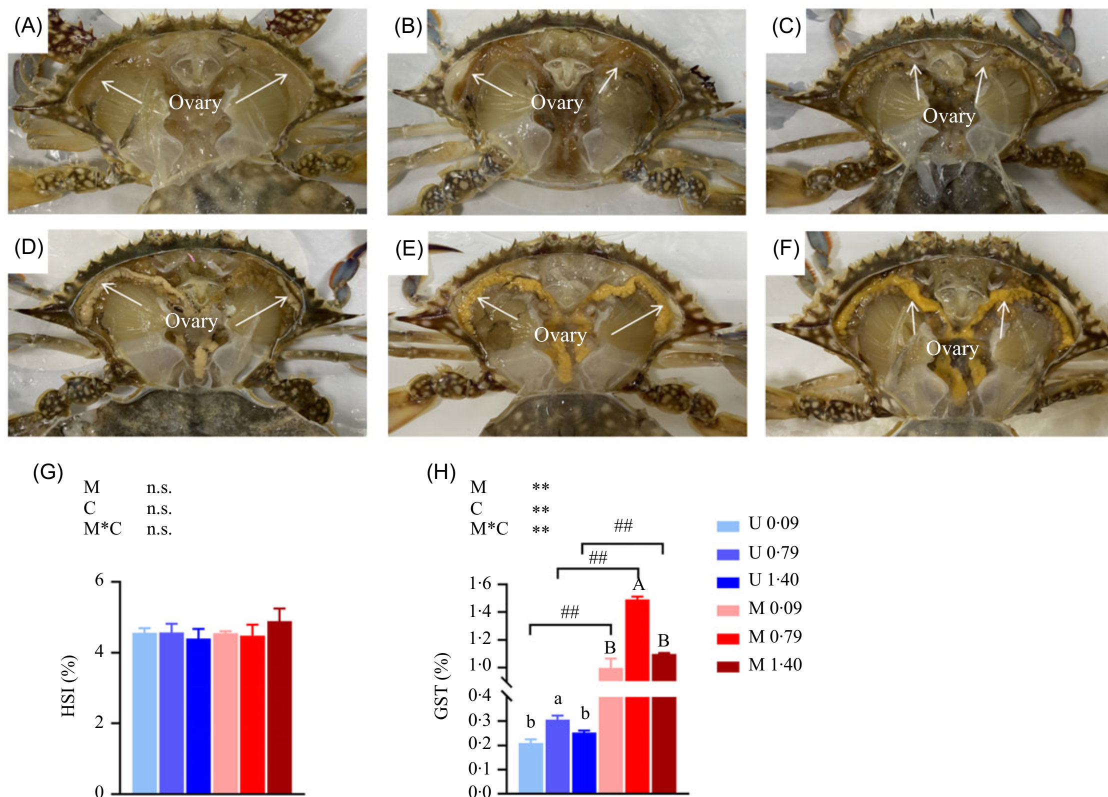

Fig. 1. Effects of mating and dietary cholesterol levels on morphological observation (A–F) and morphology index (G–H) of adult female swimming crab (*Portunus trituberculatus*). A, B, C and D, E, F represent treatment of diets 0·09 %, 0·79 % and 1·40 % cholesterol fed to unmated crabs (A, B, C) and mated crabs (D, E, F), respectively. Data were expressed as mean (SEM) (n=4). Two-way ANOVA P values are shown in each panel, with 'M' representing effects of mating, 'C' representing effects of dietary cholesterol levels and 'M * C' representing interaction between mating and dietary cholesterol levels. n.s., not significant ($P \geq 0·05$), * and ** mean significant differences ($P < 0·05$) and ($P < 0·01$). # and ## mean significant differences ($P < 0·05$) and ($P < 0·01$) between same dietary cholesterol levels with different mating treatments by performing t-test. 'a b c' means significant difference ($P < 0·05$) between dietary cholesterol levels in unmated treatment and 'A B C' means significant difference ($P < 0·05$) between dietary cholesterol levels in mated treatment by performing one-way ANOVA. HSI, hepatosomatic index; GSI, gonadosomatic index.

In mating treatment, crabs fed diet with 0·79 % cholesterol had higher yolk content compared with crabs fed diet with 0·09 % and 1·40 % cholesterol, and a small area of lipid droplets (LI) were observed near the yolk granules in the cytoplasm (Fig. 5G–L).

Related gene mRNA expression of cholesterol metabolism

The related gene mRNA expression of cholesterol metabolism in ovary and hepatopancreas is presented in Fig. 6. The two-way ANOVA showed that dietary cholesterol level markedly affected the expression level of genes involved in cholesterol metabolism in the ovary and hepatopancreas, but the effect of mating was limited ($P < 0·05$). Significant interaction between mating treatment and dietary cholesterol level were observed in the expression levels of class B scavenger receptors (*srb*) in ovary, and LDL receptor (*ldlr*), LDL receptor-related protein 2 (*lrp2*) and *srb* in hepatopancreas ($P < 0·05$). Specifically, the results of *t*-test analysis presented that when fed the diet with 0·09 % cholesterol, mated crabs significantly increased the expression levels of *ldlr* and *lrp2* in ovary than unmated crabs (Fig. 6A; $P < 0·05$). Mated crabs markedly increased the expression levels of *srb* (M0·79 and M1·40 treatments) than unmated crabs (Fig. 6A; $P < 0·05$). The same pattern was found in hepatopancreas, mated crabs dramatically up-regulated the mRNA expression levels of *ldlr* (M0·79 treatment) and *srb* (M1·40 treatment) compared with unmated crabs (Fig. 6B; $P < 0·05$). Furthermore, 0·79 % cholesterol treatment significantly up-regulated the mRNA expression of *ldlr*, *srb* and ATP-binding cassette subfamily G member 1 (*abcg1*) in the ovary than other dietary treatments in same mating status (Fig. 6A; $P < 0·05$). Similarly, compared with 0·09 % cholesterol treatment, the expression of *ldlr* and *srb* in hepatopancreas were significantly up-regulated by 0·79 % cholesterol treatment in the same mating status (Fig. 6B; $P < 0·05$). However, no statistical difference in gene expression level of NPC intracellular cholesterol transporter 1 (*npc1*) in ovary and hepatopancreas was recorded ($P > 0·05$).

Related gene mRNA expression of steroid hormone synthesis and ovary development

Two-way ANOVA showed that the mRNA expression levels of *hsl*, *3β-hsd* and *star* both in ovary and hepatopancreas were significantly influenced by mating treatment and dietary

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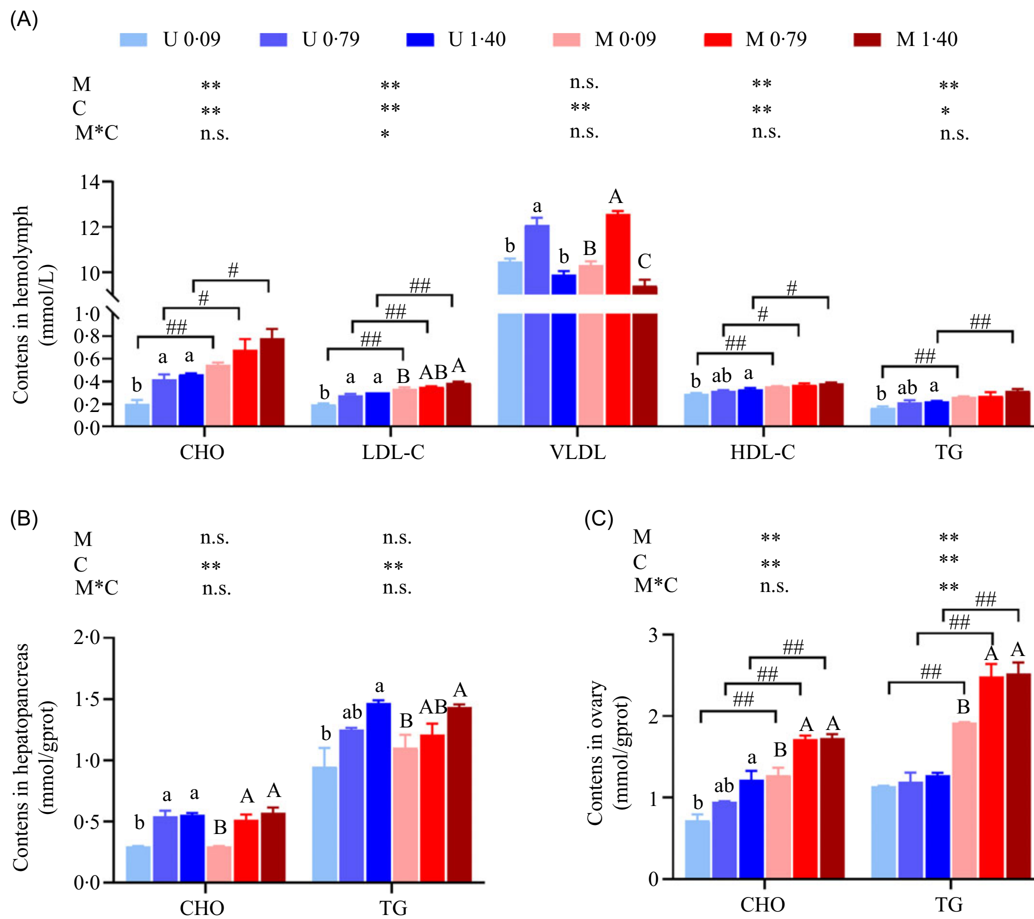

Fig. 2. Effects of mating and dietary cholesterol level on contents of lipid classes in hemolymph (A), hepatopancreas (B) and ovary (C) of adult female swimming crab (*Portunus trituberculatus*). Data were expressed as mean (SEM) (n=4). Two-way ANOVA P values are shown in each panel, with 'M' representing effects of mating, 'C' representing effects of dietary cholesterol levels and 'M * C' representing interaction between mating and dietary cholesterol levels. n.s., not significant ($P \geq 0·05$), * and ** mean significant differences ($P < 0·05$) and ($P < 0·01$). # and ## mean significant differences ($P < 0·05$) and ($P < 0·01$) between same dietary cholesterol levels with different mating treatments by performing t-test. 'a b c' means significant difference ($P < 0·05$) between dietary cholesterol levels in unmated treatment and 'A B C' means significant difference ($P < 0·05$) between dietary cholesterol levels in mated treatment by performing one-way ANOVA. CHO, cholesterol; VLDL, very low-density lipoprotein cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

cholesterol level (Fig. 7A; $P < 0·05$). However, the relative expression levels of *hsl*, *3β-hsd* and *star* only in ovary had significant interactions between mating treatment and dietary cholesterol (Fig. 7A; $P < 0·05$). Detailedly, results of t-test analysis showed that mated crabs had significantly up-regulated the relative expression levels of *hsl* (M0·09, M0·79 and M1·40 treatments) in ovary, *3β-hsd* in ovary (M0·79 treatment) and hepatopancreas (M0·79 and M1·40 treatments), and *star* (M0·79 treatment) in ovary and hepatopancreas than unmated crabs ($P < 0·05$). Moreover, one-way ANOVA indicated that crabs fed diet with 0·79 % cholesterol significantly up-regulated *hsl*, *3β-hsd* and *star* mRNA expression levels in ovary and hepatopancreas compared with other treatments in the same mating status ($P < 0·05$), except for *3β-hsd* in unmated crabs (Fig. 7A and C). Regarding the levels of mRNA expression involved in ovary development, the results showed that the mated crabs significantly up-regulated *vtg* expression levels in the ovary (M0·09 and M0·79 treatments) and hepatopancreas (M0·79 and M1·40 treatments) compared with unmated crabs (Fig. 7B and D; $P < 0·05$). Meanwhile, crabs fed diet with 0·79 % cholesterol significantly up-regulated *vtg* mRNA expression levels in ovary and hepatopancreas compared with other treatments in the same mating status ($P < 0·05$), except for the expression level of *vtg* in hepatopancreas in mated crabs ($P > 0·05$).

Key protein expression of cholesterol transport and steroid hormone synthesis

Two-way ANOVA indicated that key protein expression of cholesterol transport (Srb1) and steroid hormone synthesis (Star) in ovary were markedly affected by dietary cholesterol levels, but mating treatment only significantly influenced the protein expression levels of Star (Fig. 8; $P < 0·05$). Significant interactions between mating treatment and dietary cholesterol were observed in protein expression levels of Star in ovary (Fig. 8A and C; $P < 0·05$). Additionally, mated crabs significantly up-regulated Srb1 (M1·40 treatment) and Star (M0·79 and M1·40 treatments) protein expression levels compared with unmated crabs ($P < 0·05$). Furthermore, the highest protein expression levels of Srb1 and Star were recorded in crabs fed diet with

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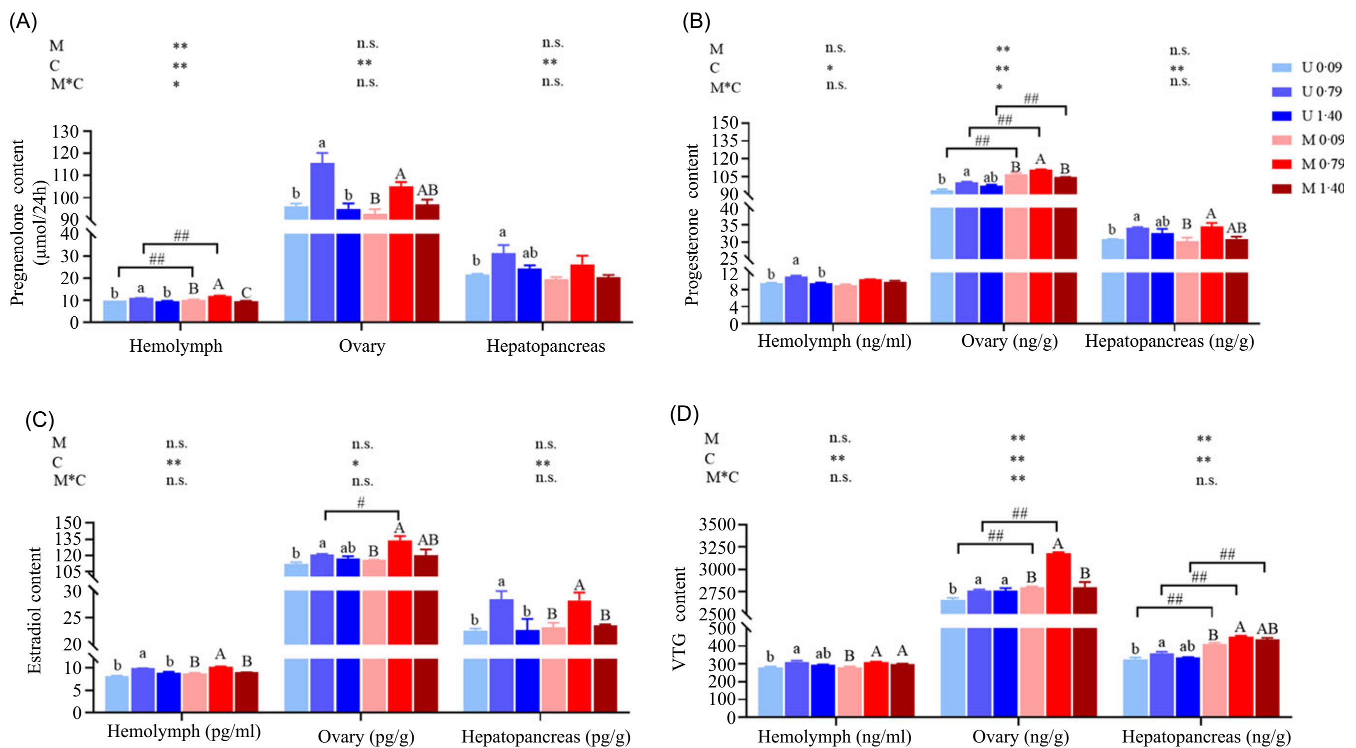

Fig. 3. Effects of mating and dietary cholesterol level on contents of pregnenolone (A), progesterone (B), estradiol (C) and VTG (D) in different tissues of adult female swimming crab (*Portunus trituberculatus*). Data were expressed as mean (SEM) (n=4). Two-way ANOVA P values are shown in each panel, with 'M' representing effects of mating, 'C' representing effects of dietary cholesterol levels and 'M * C' representing interaction between mating and dietary cholesterol levels. n.s., not significant ($P \geq 0·05$), * and ** mean significant differences ($P < 0·05$) and ($P < 0·01$). # and ## mean significant differences ($P < 0·05$) and ($P < 0·01$) between same dietary cholesterol levels with different mating treatments by performing t-test. 'abc' means significant difference ($P < 0·05$) between dietary cholesterol levels in unmated treatment and 'ABC' means significant difference ($P < 0·05$) between dietary cholesterol levels in mated treatment by performing one-way ANOVA. VTG, vitellogenin.

0·79 % cholesterol in the same mating status and significantly higher than crabs fed with 0·09 % cholesterol diet ($P < 0·05$).

Discussion

Optimal broodstock nutrition plays a crucial role in the ovary maturation of crustaceans and is central to successful reproduction[38]. Lipoproteins forming from cholesterol are a major component of egg yolks. However, crustaceans cannot synthesise cholesterol *de novo* to meet the nutritional requirements of ovarian development[21]. Hence, supplementing cholesterol in the diet is necessary to ensure ovarian development. The present study evaluated the effects of mating treatment and dietary cholesterol levels on HSI and GSI in swimming crabs. The two-way ANOVA indicated that GSI had significant interaction between mated treatment and dietary cholesterol levels. This result suggested that ovarian development is regulated by both reproductive behaviour and dietary nutrition. Mating is a complex physiological process, and a previous study has pointed out that mated swimming crabs significantly increased GSI compared with unmated crabs[4]. Moreover, Vandekerkhove *et al.*[39] also found that mated *Macrolophus caliginosus* had more eggs laid compared with unmated *Macrolophus caliginosus*. Those conclusions were also borne out in this study, when crabs fed the diet with the same cholesterol level showed a significant increase in GSI in mated crabs than unmated crabs. Regarding the effect of dietary cholesterol levels on GSI, the one-way ANOVA indicated that crabs fed diet with 0·79 % cholesterol recorded the highest GSI compared with other dietary cholesterol treatments in same mating status. Similar results were reported in a study on Chinese mitten crab[10], which indicated that suitable dietary cholesterol levels could improve ovarian development. In the ovarian development stage of crustaceans, the nutrients from hepatopancreas continuously transported to ovary results in a negative correlation between HSI and GSI[40]. However, no notable differences were observed in HSI among the treatments, which may be ascribed to suitable water temperature and adequate diet supply in this study[41]. Therefore, female crabs could feed normally, resulting in a relatively stable HSI during periods of ovary development. The findings of the present study suggested that mating behaviour had a better promoting function on ovarian development than cholesterol for swimming crab.

Vitellogenesis in crustaceans can be divided into endogenous and exogenous vitellogenic stages[42]. Generally, the ovary becomes the primary vitellogenesis site for the endogenous vitellgenesis, while the hepatopancreas has been confirmed to be a major site for exogenous vitellgenesis[11]. Moreover, there are six types of gametocytes of swimming crab, including oogonia (OG), PR, EX, EN, near-mature oocyte (NO) and mature oocyte (MO)[5,43]. Before mating, swimming crab is mainly in

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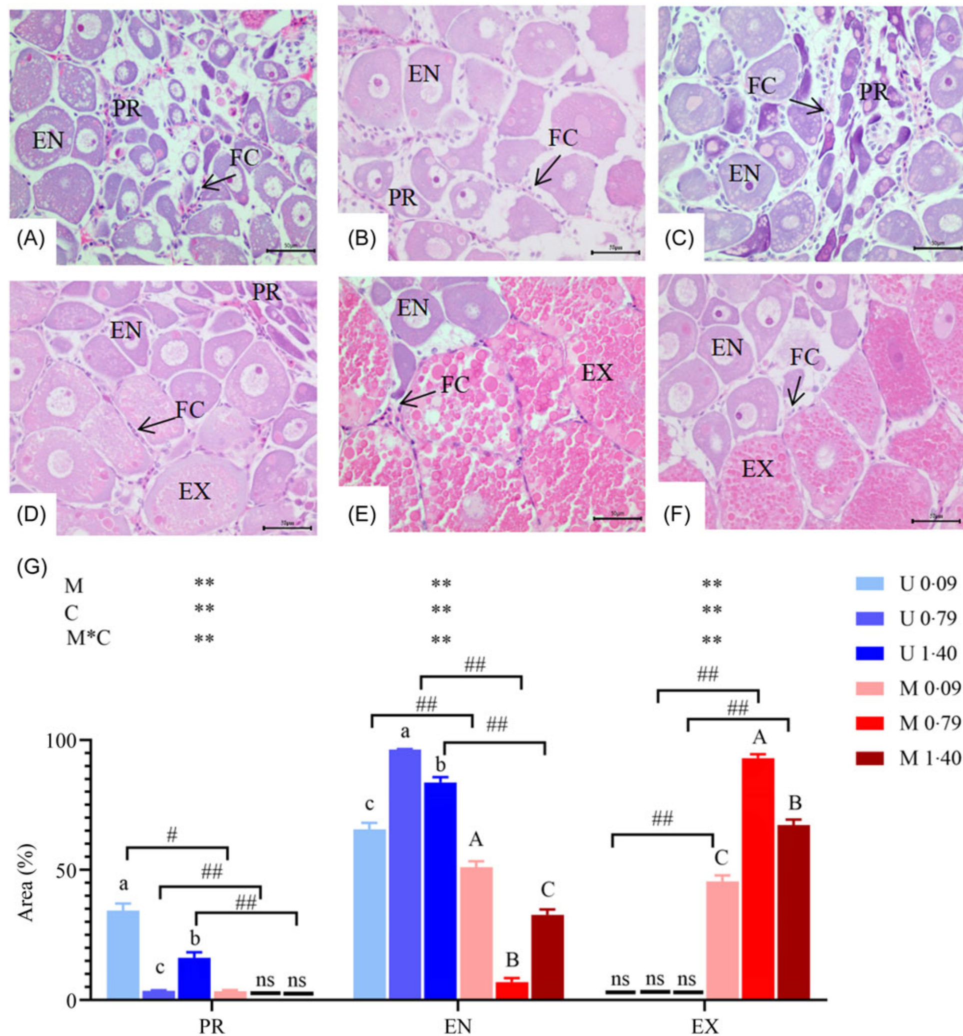

Fig. 4. Effects of mating and dietary cholesterol level on ovary histological structure of adult female swimming crab (*Portunus trituberculatus*). A, B, C and D, E, F represent treatment of diets 0·09 %, 0·79 % and 1·40 % cholesterol fed to unmated crabs (A, B, C) and mated crabs (D, E, F), respectively. G, Quantification of the oocyte types in different treatment (*n*=3). PR, previtellogenic oocyte; FC, follicle cells; EN, endogenous vitellogenic oocytes; EX, exogenous vitellogenic oocytes.

stage I-II of ovarian development, the dominant gametocyte type was OG, PR, and small amounts of EN in stage I, and the dominant gametocyte type was EN in stage II. However, swimming crab enters a phase of ovarian rapid development after mating (stage III–V). The dominant gametocyte type was EX in stage III, NO in stage IV and MO in stage V, respectively[4,43]. Our results further confirmed that the ovary develops rapidly after mating, with EX appearing in the mated crabs, while PR and EN were the main oocytes in unmated crabs. In addition, the U0·79 treatment increased the area of EN and M0·79 treatment increased the area of EX than other treatments. To some extent, these results were consistent with a previous study in Chinese mitten crab, where 0·4 % cholesterol treatment could enlarge the volume of oocytes and yolk granules[10]. In primary vitellogenesis (endogenous phase), a large number of mitochondria, free polyribosomes and RER were observed in *Callinectes ornatus* and *Arenaeus cribrarius*[44]. Nevertheless, materials with different electron densities can be seen in some dilated RER[44]. In secondary vitellogenesis (exogenous phase), VTG precursors are transported from the hepatopancreas to the ovaries, where they are endocytosed to form mature vitellin granules or vitellin[45,46]. In the present study, the unmated crabs showed numerous mitochondria and RER, while the mated crabs observed many numbers of yolk. Besides, U0·79 cholesterol treatment had more RER, while M0·79 cholesterol treatment had higher yolk content. Overall, the present results indicated that mating treatment significantly promoted oocyte maturation, and appropriate dietary cholesterol (0·79 % cholesterol) level could increase the area of gametocytes (EX or EN) in the same mating status, which also had a trend of promoting oocyte maturation.

Cholesterol, a fat-soluble substance, is primarily conveyed to different tissues and cells as lipoprotein cholesterol. Over 90 % of cholesterol is generally associated with HDL and LDL[25]. Mainly, HDL is responsible for transporting cholesterol from the peripheral tissues to the ovary, where it forms yolk in the oocyte and is thought to be necessary for vitellogenesis in crustaceans[21,47]. Besides, TG is important in oocyte maturation and embryonic development[48]. The two-way ANOVA indicated that mated treatment and dietary cholesterol levels did not interact with cholesterol metabolism, expect for LDL-C content in

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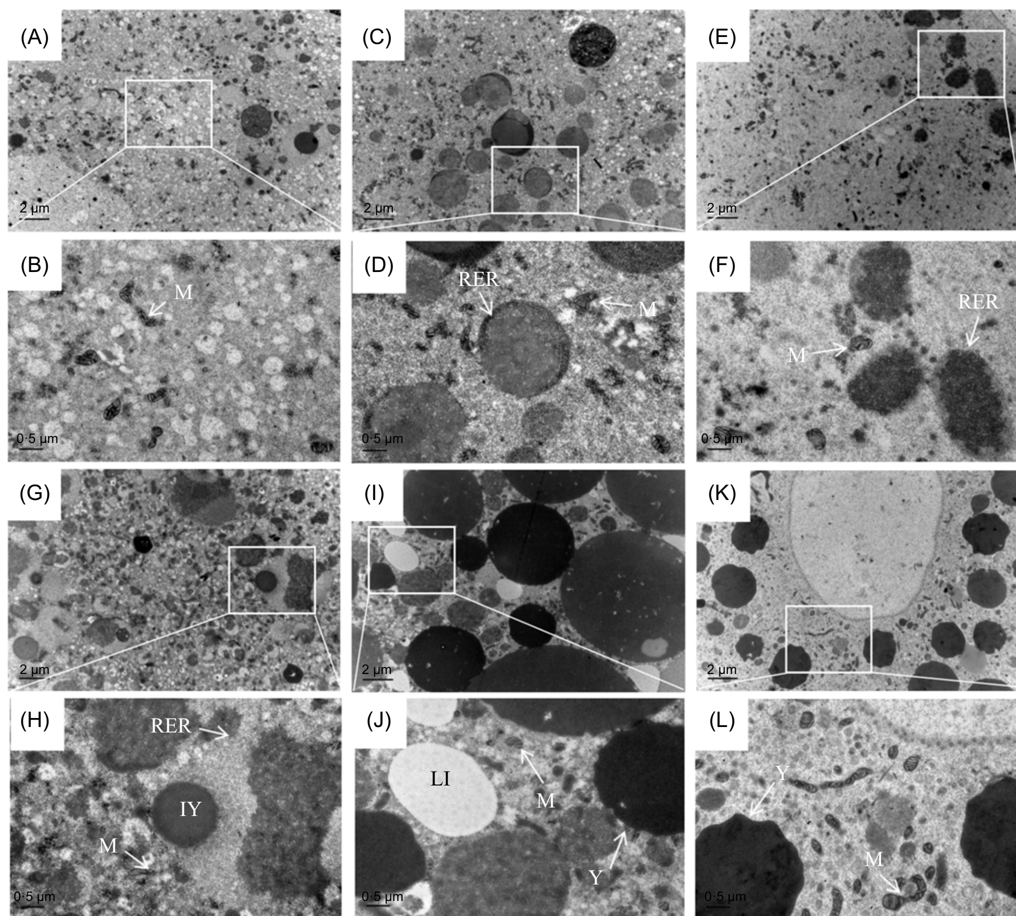

Fig. 5. Effects of mating and dietary cholesterol level on ultrastructure of oocytes in adult female swimming crab (*Portunus trituberculatus*). A-B, C-D, E-F and G-H, I-J, K-L represent treatment of diets 0·09 %, 0·79 % and 1·40 % cholesterol fed to unmated crabs (A-B, C-D, E-F) and mated crabs (G-H, I-J, K-L), respectively. M, mitochondria; RER, rough endoplasmic reticulum; IY, immature yolk granule; LI, lipid droplets; Y, yolk.

hemolymph and TG content in ovary, suggesting that cholesterol metabolism was mainly independently influenced by dietary cholesterol and mating behaviour. Moreover, in the present study, mated crabs had significantly higher LDL-C, CHO, HDL-C and TG in hemolymph, as well as CHO and TG in ovary, compared with those unmated crabs. These results were consistent with a previous study in Chinese mitten crab[48], where adult female crabs after their pubertal molt increased TG and CHO contents in ovary during the fattening period. These results potentially indicated that female crabs have a higher demand for cholesterol during periods of rapid ovarian development; thus, part of CHO in hepatopancreas may be transferred to the ovary to meet the ovarian demand for CHO[49], resulting in no significant difference in hepatopancreatic CHO and TG contents under mating treatment. Furthermore, previous studies observed that dietary cholesterol levels significantly increased CHO and HDL-C contents in crayfish (*Procambarus clarkii*)[50] and Chinese mitten crab[51], and their results were consistent with our study.

Srb1, the first identified HDL receptor, is a key substance for maintaining the homoeostasis of cholesterol metabolism[51]. Additionally, the expression of *srb1* gene was significantly correlated with increased cholesteryl ester (CE) uptake from HDL in rats[52]. Knocking out the *srb1* gene in mice causes disturbances in cholesterol metabolism and leads to sterility in females[53]. Therefore, to further explore the regulatory mechanism of cholesterol transport, the expression levels of *srb* were analysed. In this experiment, 0·79 % cholesterol treatment had significantly up-regulated gene expression of *srb* in the ovary and hepatopancreas, as well as protein expression of Srb1 in ovary than other treatments in same mating status. In addition to Srb1, animals could obtain LDL through endocytosis mediated by Ldlr[26]. When Ldlr was silenced, intracellular cholesterol concentration decreased by about 30 % in mice[26]. Interestingly, the mRNA expression level of *ldlr* in the present study showed the same pattern, where when crabs under the same mating status, crabs fed diet with 0·79 % cholesterol showed higher expression levels of *ldlr* than crabs fed diet with other cholesterol levels, consistent with the results of *srb*, indicating that both *srb* and *ldlr* play important roles in cholesterol transport. Moreover, mated crabs significantly increased the expression levels of *ldlr* and *srb* in ovary than unmated crabs in the same dietary cholesterol level. Overall, the findings of this study strongly supported the speculation that mated crabs mainly transport

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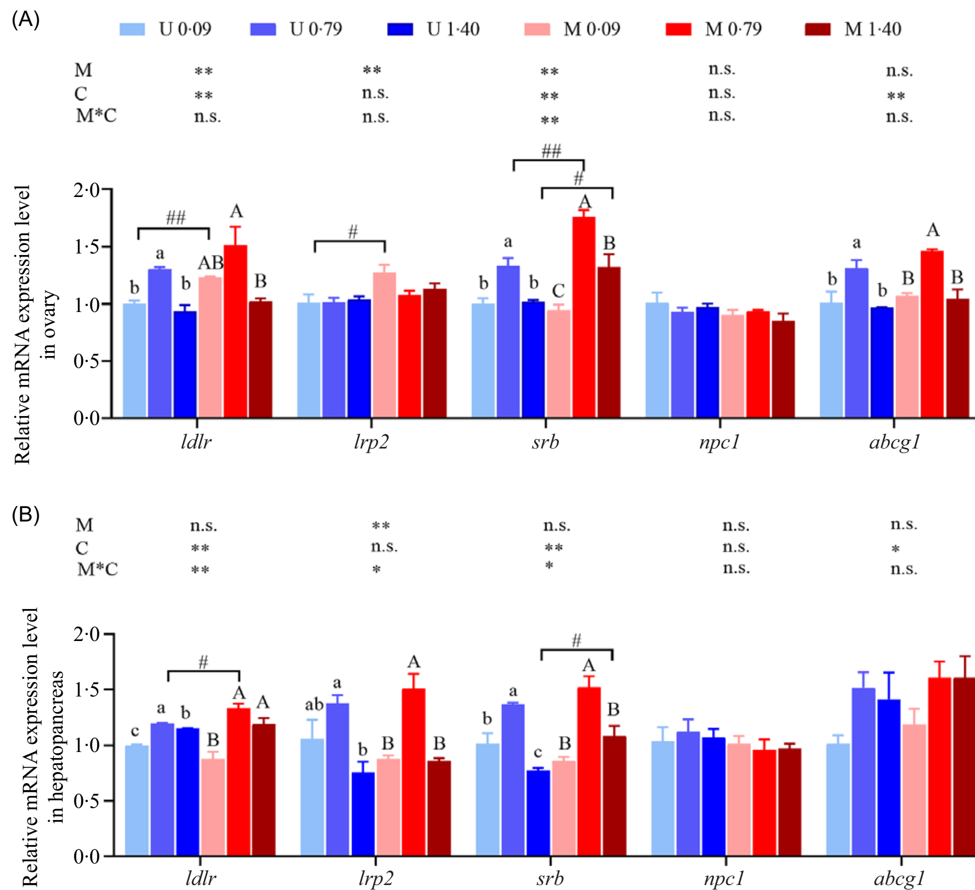

Fig. 6. Effects of mating and dietary cholesterol level on expression level of genes involved in cholesterol metabolism in ovary (A) and hepatopancreas (B) of adult female swimming crab (*Portunus trituberculatus*). Data were expressed as mean (SEM) (n=4). Two-way ANOVA P values are shown in each panel, with 'M' representing effects of mating, 'C' representing effects of dietary cholesterol levels and 'M * C' representing interaction between mating and dietary cholesterol levels. n.s., not significant ($P \geq 0{\cdot}05$), * and ** mean significant differences ($P < 0{\cdot}05$) and ($P < 0{\cdot}01$). # and ## mean significant differences ($P < 0{\cdot}05$) and ($P < 0{\cdot}01$) between same dietary cholesterol levels with different mating treatments by performing t-test. 'a b c' means significant difference ($P < 0{\cdot}05$) between dietary cholesterol levels in unmated treatment and 'A B C' means significant difference ($P < 0{\cdot}05$) between dietary cholesterol levels in mated treatment by performing one-way ANOVA. *srb*, class B scavenger receptors; *ldlr*, LDL receptor; *lrp2*, LDL receptor-related protein 2; *npc1*, NPC intracellular cholesterol transporter 1; *abcg1*, ATP-binding cassette sub-family G member 1.

LDL-C and HDL-C to the ovary by *ldlr* and *srb* in order to satisfy the requirement of more cholesterol and synthesise steroid hormones for ovarian development in mated crabs.

Previous studies have demonstrated that steroid hormones can regulate reproductive endocrine regulation[54,55]. Steroid hormones, such as pregnenolone, progesterone, estradiol, estrone and testosterone, have been reported in ovary, hepatopancreas and hemolymph in several crustaceans[56]. As an essential sex steroid hormone in vertebrates, estradiol exerts critical functions in extensive target tissues, including vitellogenesis, oocyte development and ovary maturation[56–58]. Moreover, estradiol content increased continually and peaked in the IV stage in mud crab[59]. The results of a two-way ANOVA showed that the content of pregnenolone in hemolymph and progesterone in ovary had highly interactions between mating and dietary cholesterol levels. The reason for those results may be that cholesterol can synthesise steroid hormones, and after mating, the hormones in the crab's body undergo large fluctuations, resulting in interaction with these hormones. In addition, the present study revealed that mated crabs significantly increased the contents of pregnenolone in hemolymph, progesterone and estradiol in ovary, suggesting that swimming crabs need to produce more steroid hormones to support rapid ovarian development after mating. Furthermore, the present data also showed that the highest content of pregnenolone, progesterone and estradiol was observed in crab fed diet with 0·79 % cholesterol under the same mating status, which was further confirmed that cholesterol can rapidly mobilise the production of steroids[60,61].

Due to the synthesis of steroid hormones, cholesterol esters in oocytes are first transformed into free cholesterol by *hsl*, and then cholesterol is transported to the mitochondrial inner membrane under the action of *star*, which is a speed limit step of steroid hormone synthesis[25,27]. In addition, Stocco[62] reported the estradiol synthesis pathway is blocked when *star* damaged, resulting in a dramatic drop in estradiol content. Moreover, goldfish exposed to 200 μg/g β-sitosterol significantly decreased the expression level of *star* and thus the CHO content in mitochondrial intima was reduced[63]. Furthermore, *3β-hsd* could process pregnenolone into progesterone[25]. In the

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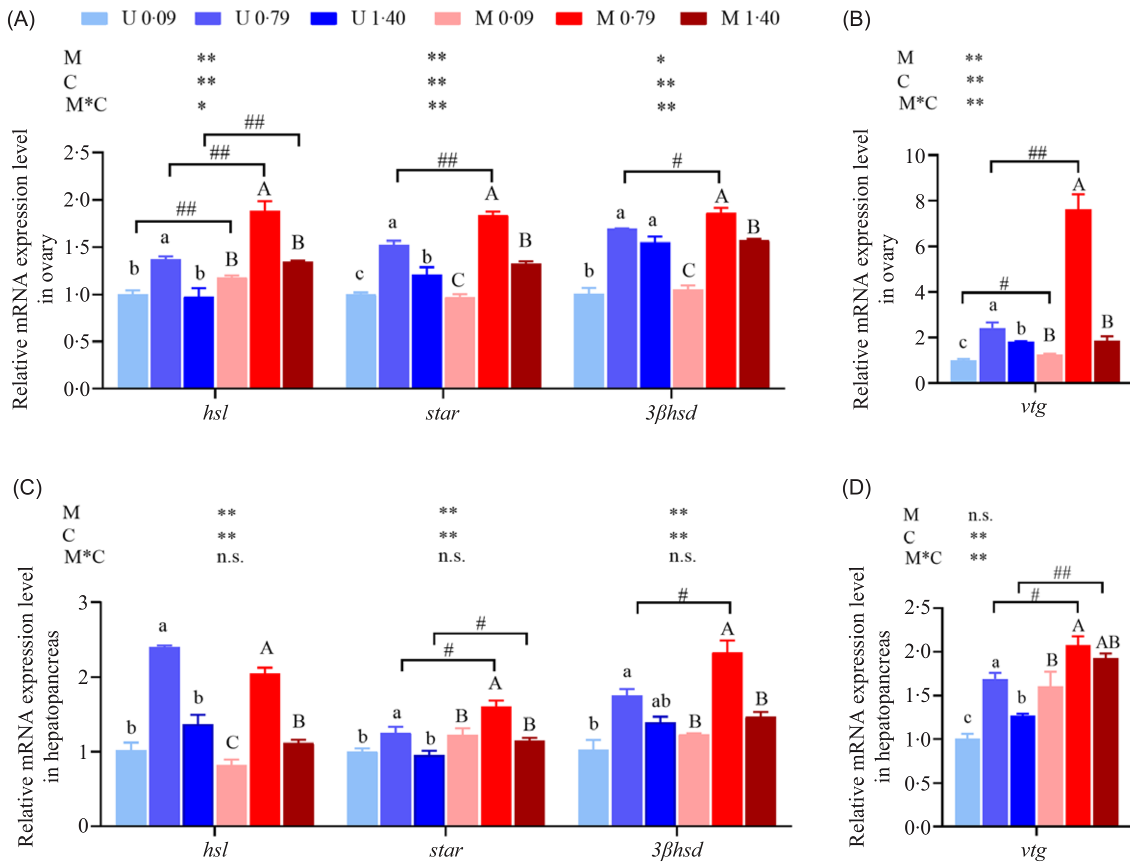

Fig. 7. Effects of mating and dietary cholesterol level on expression level of genes involved in steroid hormone synthesis and ovary development in ovary and hepatopancreas of adult female swimming crab (*Portunus trituberculatus*). Data were expressed as mean (SEM) (n=4). Two-way ANOVA P values are shown in each panel, with 'M' representing effects of mating, 'C' representing effects of dietary cholesterol levels and 'M * C' representing interaction between mating and dietary cholesterol levels. n.s., not significant ($P \geq 0·05$), * and ** mean significant differences ($P < 0·05$) and ($P < 0·01$). # and ## mean significant differences ($P < 0·05$) and ($P < 0·01$) between same dietary cholesterol levels with different mating treatments by performing t-test. 'a b c' means significant difference ($P < 0·05$) between dietary cholesterol levels in unmated treatment and 'A B C' means significant difference ($P < 0·05$) between dietary cholesterol levels in mated treatment by performing one-way ANOVA. *hsl*, hormone-sensitive lipase; *3β-hsd*, 3β-hydroxysteroid dehydrogenase; *star*, steroidogenic acute regulatory protein; *vtg*, vitellogenin.

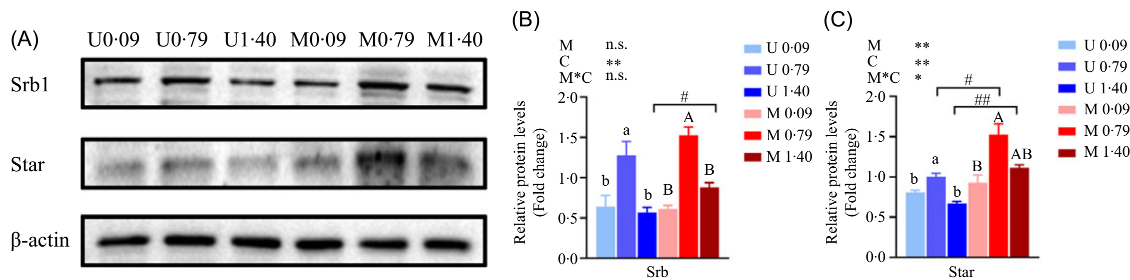

Fig. 8. Effects of mating and dietary cholesterol level on protein expressions of Srb1 and Star in ovary of adult female swimming crab (*Portunus trituberculatus*). Data were expressed as mean (SEM) (n=3). Two-way ANOVA P values are shown in each panel, with 'M' representing effects of mating, 'C' representing effects of dietary cholesterol levels and 'M * C' representing interaction between mating and dietary cholesterol levels. n.s., not significant ($P \geq 0·05$), * and ** mean significant differences ($P < 0·05$) and ($P < 0·01$). # and ## mean significant differences ($P < 0·05$) and ($P < 0·01$) between same dietary cholesterol levels with different mating treatments by performing t-test. 'a b c' means significant difference ($P < 0·05$) between dietary cholesterol levels in unmated treatment and 'A B C' means significant difference ($P < 0·05$) between dietary cholesterol levels in mated treatment by performing one-way ANOVA. Srb1, class B scavenger receptors 1; Star, steroidogenic acute regulatory protein.

present study, when crabs in same mating status, 0·79 % cholesterol treatment up-regulated the mRNA expression level of *star*, *hsl* and *3β-hsd*, while 1·40 % cholesterol treatment down-regulated transcription of those genes. Besides, the protein expression levels of Star exhibit the same pattern. The result of Star is in accordance with a previous study in Chinese mitten crab[10]. In this experiment, mated crabs can significantly up-regulated the mRNA expression level of *star*, *hsl* and *3β-hsd* than

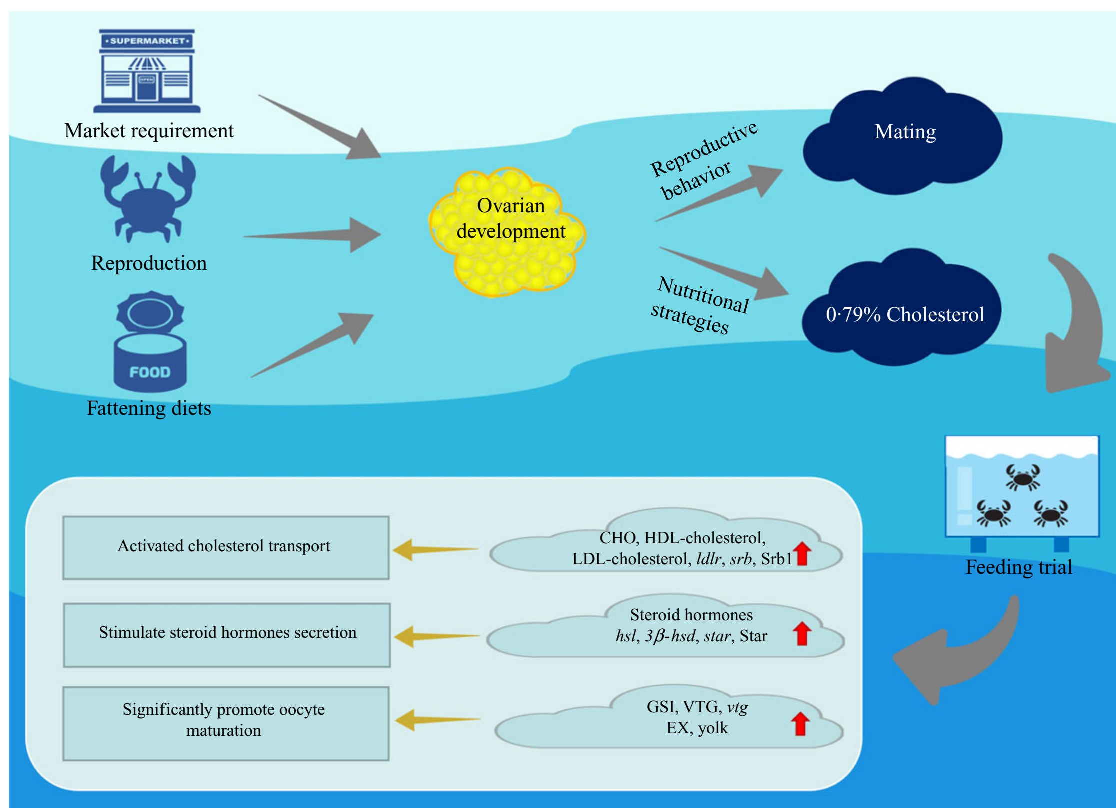

Fig. 9. A working model shows the possible mechanism. Red arrows represent promotion/up-regulation.

unmated crabs, suggesting that mating treatment could activate the expression levels of *star*, *hsl* and *3β-hsd* to promote the synthesis ability of steroid hormone.

In fact, ovarian development in crustaceans is a process of nutrient accumulation, and the VTG is the main nutrient[64]. A common concept is that VTG transports nutrients (such as lipids, carbohydrates and amino acids) to the ovary, so vitellogenesis is highly related to nutrient metabolism, especially lipid processes[65]. The results of two-way ANOVA showed that VTG in the ovary was significantly influenced by mating and dietary cholesterol levels and had highly interactions between mating and dietary cholesterol levels. This result is similar to the GSI trend and further confirms that ovarian development is a complex process that is regulated by cholesterol nutrition and mating behaviour. Moreover, in this study, mated crabs significantly increased the VTG content and *vtg* mRNA expression level than unmated crabs. To some extent, this result was consistent with a previous study in female blue crab (*Callinectes sapidus*), where the VTG content in the unmated crabs decreased and returned to the basal level after 9·5 weeks of pubertal moult, while in the mated crabs, the VTG content decreased but remained at a high level[66]. When the swimming crab finished mating, male spermatophores transferred into the female spermatheca[5]. Kubli[67] has reported that peptides in semen could promote vitellogenesis in *Drosophila*. Therefore, the variation of VTG in the mating treatment is reasonable. Most female oviparous animals synthesise VTG stimulated by liver estradiol, release VTG into the blood circulation and transport them to the ovaries to nourish oocytes[68]. Interestingly, the highest content of VTG and gene expression level of *vtg* were both recorded in crabs fed diet with 0·79 % cholesterol under the same mating status, which is in accordance with Chinese mitten crab[10]. Overall, the present results indicated that mating behaviour and cholesterol nutrition both can promote the accumulation of ovarian nutrients by increasing VTG content, but the effect of mating behaviour is significantly superior to cholesterol nutrition.

Conclusions

In conclusion, the results of present study indicated that cholesterol nutrition and mating behaviour actively regulate GSI, and there is a significant interaction. Mating treatment and dietary cholesterol supplementation both increased the contents of CHO and TG to promote the ovarian lipid accumulation. Moreover, the histological analysis showed that mating treatment significantly promoted oocyte maturation, and appropriate dietary cholesterol (0·79 % cholesterol) level could increase the area of gametocytes (EX or EN). Ultrastructurally, the unmated crabs had numerous mitochondria and RER, while the mated crabs observed many numbers of yolk. Furthermore, mating treatment enhanced the ovarian cholesterol transport and steroid hormone synthesis by activating Srb1 and Star, while dietary cholesterol supplementation could strengthen this process, which may be the main reason for promoting ovarian development. Overall, mating behaviour plays a leading role in

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promoting ovarian development, and cholesterol nutrition can further promote ovarian development after mating (Fig. 9).

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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.

T. Z.: Formal analysis, Investigation, Writing – original draft, and Writing – review and editing. M. J.: Conceptualisation, Methodology, Supervision, Writing – review and editing, Project administration, and Funding acquisition. J. L.: Software and Formal analysis. Y. Y.: Software and Formal analysis. X. L.: Software and Formal analysis. H. P.: Software and Formal analysis. Y. S.: Software and Formal analysis. Q. Z.: Conceptualisation, Methodology, Supervision, Writing – review and editing, Project administration, and Funding acquisition.

The authors declared that they had no conflicts of interest with the contents of this article.

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