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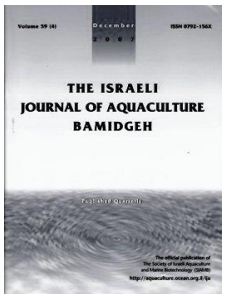

Effects of Dietary Cholesterol Levels on the Growth, Molt Performance, and Immunity of Juvenile Swimming Crab, *Portunus trituberculatus*

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Key Words: swimming crab (*Portunus trituberculatus*), cholesterol, growth performance, immunity

Abstract

The effects of dietary cholesterol levels on growth, molt performance, and immunity of juvenile swimming crab *Portunus trituberculatus,* were investigated at four cholesterol levels (0.2%-1.4%) of purified diets. Each diet was fed in triplicate to 18 crabs per replicate for 50 days. Crabs fed the diet with 1.0% cholesterol showed significantly higher (P<0.05) specific growth rate (SGR) than the other groups, who suffered from relatively lower molt death syndrome (MDS). Cholesterol content in the serum, whole body, and hepatopancreas increased in relation to dietary cholesterol. Muscle lipid content was significantly higher (P<0.05) in crabs fed the diet with 0.2% cholesterol compared to the other treatments. Crabs fed moderate dietary cholesterol levels showed higher alkaline phosphatase (AKP) or acid phosphatase (ACP) levels than those fed 0.2% or 1.4% cholesterol diets. The present study also showed that dietary cholesterol supplementation generally increased serum superoxide dismutase (SOD) activity. Overall, moderate dietary cholesterol (1.0 %) enhanced the performance of growth, survival, molting, and immunity of juvenile swimming crab *P. trituberculatus.*

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Introduction

Cholesterol is an important sterol that occurs almost exclusively in animal tissues. It is regarded as an essential dietary component for crustaceans (Sheen *et al*., 1994). It is well known that crustaceans are incapable of de novo synthesis of sterols and require an exogenous dietary source of cholesterol for growth, development, and survival. Cholesterol is the ultimate precursor for the synthesis of ecdysone, which is closely involved in the process of molting in crustaceans (Sheen, 2000; Tao *et al*., 2014). Moreover, cholesterol is a precursor of vitamin D, which enhances the immune system (Tao *et al*., 2014).

Results from studies on the quantitative requirements of cholesterol in crustacean larvae and juveniles are not conclusive, with reported values ranging from 0.4%-1.4 % (National Research Council, 2011). Several investigations have also demonstrated that moderate amounts of a cholesterol supplement enhance growth performance and survival in *Scylla serrata* (Holme *et al*., 2006), *Penaeus monodon* (Sheen *et al*. 1994), *Litopenaeus Vannamei* (Gong *et al*., 2000), *Cherax quadricarinatus* (Hernández *et al*., 2004) and *Penaeus japonicus* (Teshima *et al*., 1997). An increase in tolerance to salinity was also found in *L. vannamei* (Duerr and Walsh, 1996) and *P. monodon* (Paibulkichakul *et al*., 1998) fed diets supplemented with different cholesterol levels. However, determining appropriate dietary cholesterol levels is important as high dietary sterol levels have been found to prevent growth in crustaceans (Teshima *et al.*, 1997).

The swimming crab*, Portunus trituberculatus*, is distributed along the coasts of China, Korea, and Japan, and is well known for its taste. The industry of *P. trituberculatus* cultivation is still in the developmental stage because of low production and high mortality rates. Reports indicate that mortalities occur when crustaceans are in the process of molting, or shortly after shedding the exuvium (Bowser and Rosemark, 1981). This is referred to as molt death syndrome (MDS). There have been intensive studies on the nutritional requirements of this species however there is limited information on use of cholesterol supplements for *P. trituberculatus* feed formulations. The objective of this study was to investigate the growth performance and incidence of molt death syndrome (MDS) in *P. trituberculatus* fed various levels of cholesterol in purified casein based diets.

Materials and Methods

Four isonitrogenous purified diets containing various levels of cholesterol (CH 0.2%, CH 0.6%, CH 1.0% and CH 1.4 % on dry basis) were formulated. Each diet was fed in triplicate to 18 crabs per replicate for 50 days. Ingredients, proximate composition, and gross energy of the diets are presented in Table 1. Casein and corn starch were used as the major protein and carbohydrate sources, respectively. Fish oil and soybean oil were used as lipid sources. All dry ingredients were finely ground and mixed for 15 min in a mixer (SM-201, Sinmag, Wuxi, China). Micro components were mixed by the progressive enlargement method. Blended oil was added to the diets and mixed for other 15 min. Distilled water was then added to the mix to produce homogeneous dough. The diets were sieved through a 3-mm die with a laboratory pelleting machine (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China). The diets were then dried overnight at 50^0C, sieved and stored in plastic bags at -20^0C until use.

Table 1. Composition of six experimental diets formulated for the *Portunus trituberculatus*

	CH 0.2	*CH 0.6*	*CH 1.0*	*CH 1.4*
Casein[1]	49.0	49.0	49.0	49.0
Corn starch[2]	20.8	20.4	20.0	19.6
Fish oil[3]	3.5	3.5	3.5	3.5
Soybean oil	3.0	3.0	3.0	3.0
Cholesterol[4]	0.2	0.6	1.0	1.4
Vitamin mix[5]	5.0	5.0	5.0	5.0
Choline chloride	1.0	1.0	1.0	1.0
Mineral mix[4]	3.0	3.0	3.0	3.0
Monocalcium phosphate	2.0	2.0	2.0	2.0
Cellulose	8.0	8.0	8.0	8.0
Taurine	1.5	1.5	1.5	1.5
Sodium alginate	3.0	3.0	3.0	3.0
Approximate composition (Dry matter %)				
Dry matter	92.68	93.19	93.74	90.01
Protein	44.09	43.39	45.37	43.32
Lipid	5.73	7.14	5.64	5.58
Ash	4.16	4.21	4.22	3.92
Gross Energy (kJ/g)	19.8	19.7	19.0	19.1

1. Purchased from Fonterra Co-operative Group Ltd, New Zealand.
2. Purchased from Sanzhenzhai Foodstuff Co., Ltd, China.
3. Purchased from Zhejiang Industrial Group Co., Ltd, China.
4. Purchased from Sinopharm Chemical Reagent Co., Ltd, China.
5. Purchased from Minsheng Pharmaceutical Group Co., Ltd, China. Vitamin mixture (g kg-1 mixture): thiamine B1, 5.0; riboflavin, 8.0; nicotinamide, 26.0; biotin, 1.0; calcium pantothenate, 15.0; vitamin B6, 3.0; folic acid, vitamin B, 5.0; vitamin C, 121.0; vitamin K, 2.02; p-aminobenzoic acid, 3.0; vitamin B12, 1.0; cellulose, 504.0; vitamin A, 25.0; vitamin D3, 25.0; vitamin E, 50.0; inositol, 181.0;.
6. Mineral mixture (g kg-1 mixture): calcium dihydrogen phosphate, 122.87; lactate, 474.22; sodium dihydrogen phosphate, 42.03; potassium persulfate, 163.83; ferrous sulfate, 10.78; iron citrate, 38.26; magnesium sulfate, 44.19; zinc sulfate, 4.74; manganese sulfate, 0.33; copper sulfate, 0.22; cobalt chloride, 0.43; iodate, 0.02; sodium chloride, 32.33; potassium chloride, 65.75.

Juvenile crabs were obtained from the Zhejiang province Key Lab of Mariculture and Enhancement (Zhoushan, China) with one spawned batch spawning. Prior to the experiment, crabs were acclimatized to laboratory conditions for 7 days. A total of 216 juveniles (25.42 ± 0.21 g) were then randomly allocated into 216 plastic baskets. Crabs were fed a daily ration of 4%-7% of body weight divided into two meals per day (08:00 and 16:00h) for 50-days. Mortality and molting were checked and recorded daily. During the experiment, pH (7.4-7.8) and dissolved oxygen (DO>6 mg/L) levels were monitored every two days. Water temperature was maintained at 25.6 ± 0.74^0, salinity was 27.11±0.98 g/L. The experiment was conducted indoors under natural photoperiod.

During the experiment, molt death syndrome (MDS) was determined according to the description of Bowser and Rosemark (1981), as shown in Fig 1. At the end of the feeding trial, the crabs were starved for 24 h, and mortality in all treatments was quantified. The wet weights of the crabs were measured first, and two crabs were randomly sampled for whole body composition analysis. Hepatopancreas and muscle samples were dissected from three other crabs in each replicate. All of the samples were immediately frozen in liquid nitrogen and stored at -80^0 until needed. In each treatment, hemolymph samples were immediately collected from nine crabs using the method described by Li et al. (2013). Three samples from each replicate (nine samples for each treatment) were held at 4^0C for 4 h, followed by centrifugation (2,000 *g*, 10 min, 4^0C). The supernatant was stored at -80^0C until analysis.

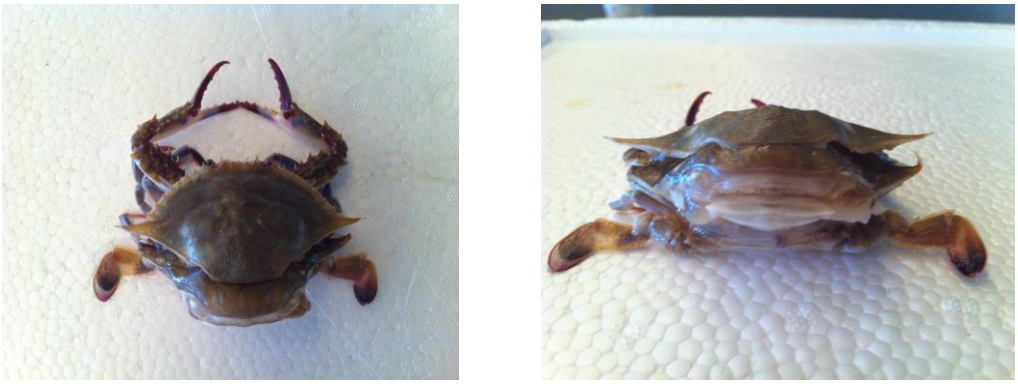

Fig.1. Typical signs of MDS, a common cause of death in *P. trituberculatus* juveniles.

All chemical composition analyses of diets, whole body, and tissues, were conducted by standard methods (AOAC, 1995). Moisture was determined by oven drying at 105^0C for 24 h. Crude protein (N×6.25) was measured using an Auto Kjeldahl System (K358/K355, BUCHI, Flawil, Switzerland). Crude lipid was determined by petroleum ether extraction using a Soxhlet extractor. Ash was determined by muffle furnace at 550^0 for 24 h. Gross energy was determined with an adiabatic bomb calorimeter (HER-15E, Shanghai Shangli, Shanghai, China). Cholesterol and triglyceride levels in serum and tissues were measured with commercial kits (ab65359/ab65336, Sigma-Aldrich, St Louis, America) according to the protocols of the manufacturer using a microplate reader (iMark, Bio-Rad, Hercules, USA) at a wavelength of 570 nm.

Alkaline phosphatase (AKP) was determined using a commercial kit (AP0100, Sigma-Aldrich, St Louis, America) and a spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan), with p-nitrophenyl phosphate (pNPP) as substrate; alkaline phosphatase hydrolyzes pNPP to *p*-nitrophenol and inorganic phosphate. During incubation of the alkaline phosphatase sample and substrate at 37^0C, the reaction was assessed by monitoring the increase in absorbance at 405 nm, and recording the increase in 405nm for 5 minutes.

The activity of acid phosphatase (ACP) was assayed with a commercial kit (Nanjing Jianchen Bioengineering Institute, Nanjing, China) using a microplate reader (iMark, Bio-Rad, Hercules, USA). The unit definition of ACP enzymatic activity corresponds to 1 mg of phenol liberated per 100 ml cell-free hemolymph at 37^0C for 10 min at 520 nm. The ACP activity was expressed as U/100 ml cell free hemolymph.

The activity of superoxide dismutase (SOD) was determined by the xanthine oxidase method, described by Lin et al. (2011) and Wang and Chen (2005). The optical density was measured at 550 nm. One unit of SOD activity was defined as the amount required to inhibit the rate of xanthine reduction by 50% in a 1-ml reaction system. Specific activity was expressed as SOD unit per milliliter hemolymph, which was determined with a commercial kit (Nanjing Jianchen Bioengineering Institute, Nanjing, China) using a spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan).

Data were analyzed using one-way ANOVA, and differences of means were evaluated for significance by Duncan's multiple-range tests, $P<0.05$ for homogeneous variances (Levene-test). The Kruskal-Wallis non-parametric test and Dunn's multiple comparison test were applied ($P< 0.05$), where the requirement of normality and equality of variance were not met. All statistical analyses were performed using the SPSS 18.0 (IBM, Chicago, USA) for Windows.

Results

After 50-days of the feeding trial, MDS ranged from 30%-40%, with no significant difference (P>0.05) among crabs fed different cholesterol concentrations. The occurrence of MDS was lowest in crabs fed a diet with 1.0% cholesterol (Figs. 2, 3). The total number undergoing molting (including successful molting and MDS) of each group was relatively higher in crabs fed high cholesterol levels (Fig 3). The highest specific growth rate (SGR) was found in crabs fed the diet with 1.0% cholesterol (Table 2), which was significantly higher than in the other groups (P<0.05). There were no significant differences in feed conversion ratio (FCR), protein efficiency ratio (PER), and hepatosomatic index (HIS) for all treatments (P >0.05).

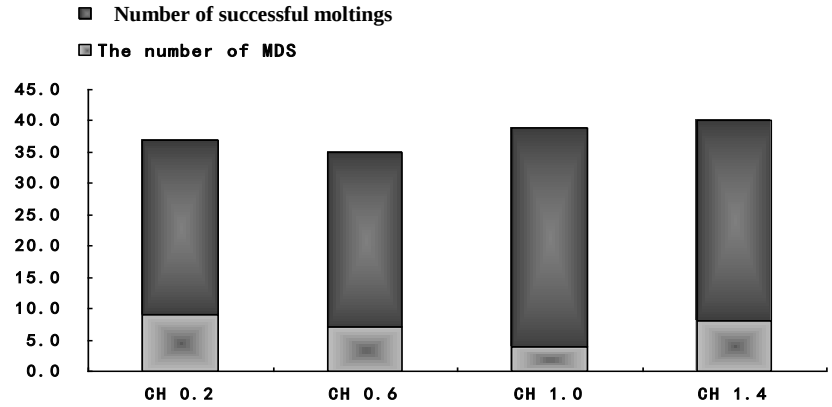

Fig. 2. The number of successful moltings, and molt death syndrome (MDS) for each treatment

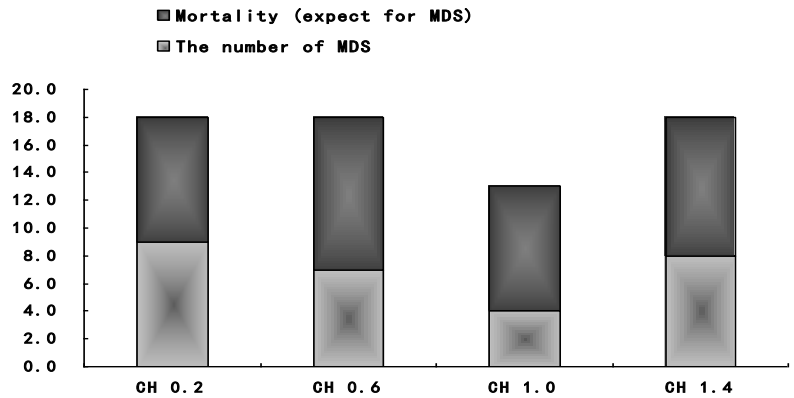

Fig. 3. The number of mortalities and molt death syndrome (MDS) for each treatment

Table 2. Growth performances of juvenile *Portunus trituberculatus* fed diets containing different cholesterol levels.

	CH 0.2	*CH 0.6*	*CH 1.0*	*CH 1.4*	*P*	*Pooled SEM**
IBW (g)[1]	25.37	25.34	25.39	25.57	AN 0.71	0.06
FBW (g) [2]	62.71[a]	61.29[a]	73.05[b]	62.84[a]	AN 0.04	1.96
SGR(%/day) [3]	1.80[a]	1.76[a]	2.11[b]	1.79[a]	AN 0.04	0.60
FCR[4]	1.17	1.34	0.91	1.24	AN 0.11	0.07
PER[5]	2.09	1.86	2.87	2.12	KW 0.28	0.20
HIS (%)[6]	5.56	5.58	5.87	6.81	KW 0.58	0.45

* Standard error of the mean (pooled). AN = one way ANOVA, KW = Kruskal Wallis and P values are given. Values in a column with different superscripts are significantly different (P<0.05).

1 Initial body wet weight (g).

2 Final body wet weight (g).

3 Special growth ratio (SGR) = 100 × (ln (final weight) – ln (initial weight))/ days.

4 Feed conversion ratio (FCR) = total feed intake/weight gain.

5 Protein efficiency ratio (PER) = weight gain/protein intake.

6 Hepatosomatic index (HSI) = 100× liver wet weight/body wet weight

Cholesterol content in serum, whole body, and hepatopancreas, displayed a general upward trend with increasing dietary cholesterol (Table 3). The cholesterol content of whole body and hepatopancreas were significantly higher (P<0.05) in crabs fed higher cholesterol (CH 1.0 and CH 1.4) than those fed lower cholesterol (CH 0.2 and CH 0.6). Crabs fed a diet with 1.0% cholesterol had significantly higher (P<0.05) triglyceride content in serum than the other treatments. In this study, there were no significant differences (P >0.05) in triglyceride content of whole body, muscle, and hepatopancreas.

Table 3. Cholesterol and triglyceride content in serum, whole body, muscle and hepatopancreas of *Portunus trituberculatus* fed the experimental diets.

	CH 0.2	*CH 0.6*	*CH 1.0*	*CH 1.4*	*P*	*Pooled SEM**
Serum						
Cholesterol (mg/dl)	1.04^{a}	1.58^{bc}	1.41^{ab}	1.89^{c}	AN 0.07	0.10
Triglyceride (nmol/ul)	0.14^{a}	0.17^{a}	0.25^{b}	0.17^{a}	AN 0.03	0.02
Whole body						
Cholesterol (ug/mg)	1.62^{a}	1.46^{a}	1.97^{b}	2.07^{b}	AN 0.03	0.84
Triglyceride (ug/mg)	3.09	2.16	2.04	3.09	KW 0.69	0.36
Muscle						
Cholesterol (ug/mg)	1.66	1.61	1.70	1.68	AN 0.85	0.04
Triglyceride (ug/mg)	0.61	0.58	0.49	0.51	AN 0.23	0.02
Hepatopancreas						
Cholesterol (ug/mg)	3.25^{a}	3.32^{a}	4.81^{b}	4.35^{b}	AN 0.19	0.26
Triglyceride (ug/mg)	12.97	11.61	13.12	15.70	AN 0.70	0.85

* Standard error of the mean (pooled). AN = one way ANOVA, KW = Kruskal Wallis and P values are given. Values in ROW with different superscripts are significantly different (P<0.05).

Whole body and hepatopancreas composition were not affected by the different dietary cholesterol levels (Table 4). However, the muscle lipid content was significantly higher (P<0.05) in crab fed with CH 0.2 diet than other treatments.

Table 4. Whole body, muscle and hepatopancreas composition of juvenile *P. trituberculatus* fed the experimental diets.

	CH 0.2	*CH 0.6*	*CH 1.0*	*CH 1.4*	*P*	*Pooled Sem**
Whole body %						
Moisture	74.43	75.99	74.81	74.41	AN 0.87	1.00
Protein	11.20	10.39	10.21	11.00	AN 0.78	0.49
Lipid	2.02	1.65	1.34	2.05	AN 0.56	0.24
Ash	18.33	16.84	16.61	17.58	AN 0.87	1.02
Muscle %						
Moisture	80.16	80.43	79.98	80.29	AN 0.93	0.38
Protein	14.99	14.84	15.19	14.90	AN 0.89	0.24
Lipid	2.40^{b}	1.92^{a}	1.66^{a}	1.62^{a}	AN 0.31	0.11
Hepatopancreas %						
Moisture	70.34	72.62	66.60	65.18	AN 0.74	1.04
Protein	9.32	9.39	11.03	10.87	AN 0.56	0.38
Lipid	15.85	18.66	18.73	19.27	AN 0.03	0.35

* Standard error of the mean (pooled). AN = one way ANOVA, KW = Kruskal Wallis and P values are given. Values in a row with different superscripts are significantly different (P<0.05).

The serum AKP activity was highest in crabs fed a diet with 1.0% cholesterol and significant higher (P<0.05) than other groups (Fig 4). The serum ACP activity was highest in crabs fed a diet with 0.6% cholesterol and significantly higher (P<0.05) than other groups (Fig 5). The serum SOD activity decreased as dietary cholesterol increased (Fig 6). Crabs fed diets with 1.4% cholesterol levels showed significantly higher (P<0.05) SOD activity than those fed with low cholesterol levels (CH 0.2 and CH 0.6).

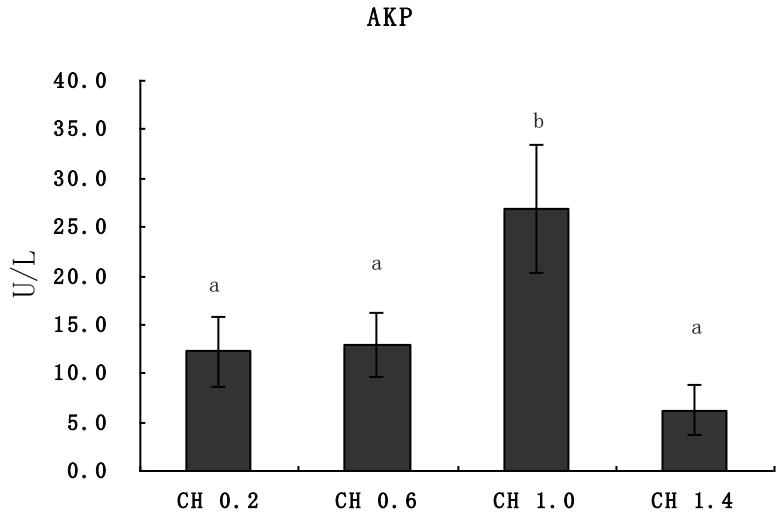

Fig. 4. Serum alkaline phosphatase (AKP) activity of *P. trituberculatus* fed experimental diets. Data with different letters are significantly different (p<0.05) between treatments.

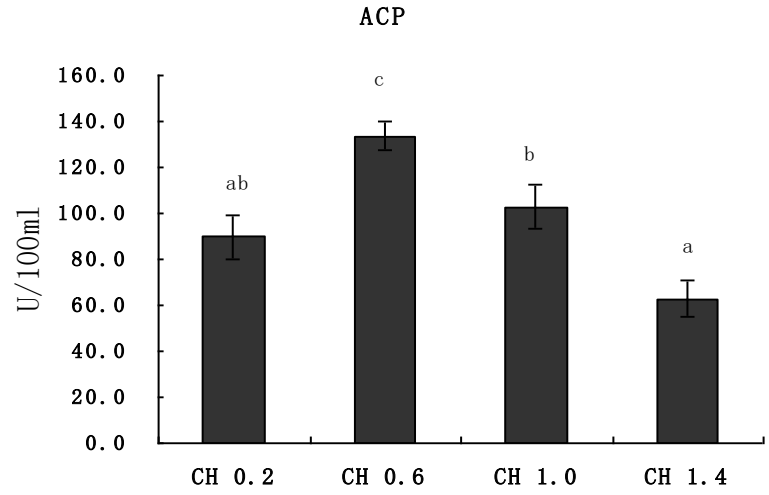

Fig. 5. The serum acid phosphatase (ACP) activity of *P. trituberculatus* fed the experimental diets. Columns with different letters are significantly different (p < 0.05).

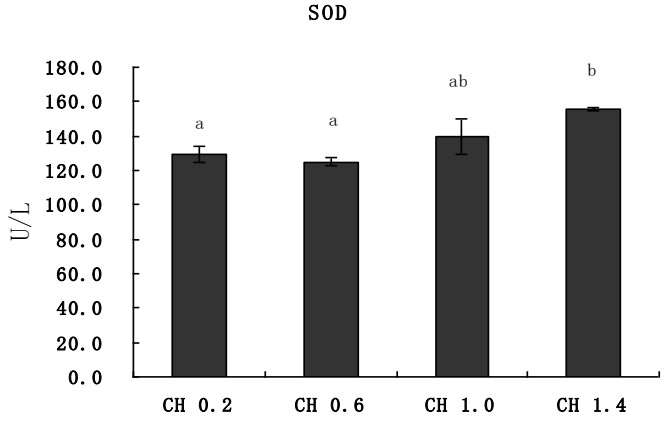

Fig. 6. The serum superoxide dismutase (SOD) activity of *P. trituberculatus* fed the experimental diets. Columns with different letters are significantly different (p < 0.05).

Discussion

Cholesterol is an essential component in the diet of crustaceans. This has been further corroborated by this study. The cholesterol requirement of crustaceans is different under a variety of culture conditions, age, and species. In this study, crabs fed a diet with 1% cholesterol showed maximum SGR, which was significantly higher ($P<0.05$) than those fed a diet with CH 0.2%. This requirement value is somewhat higher than those reported for some other aquatic crustaceans, such as 0.51% for *Scylla serrata* (Sheen, 2000) and *C. quadricarinatus* (Hernández *et al*., 2004), 0.26-0.6% for *P. japonicus* (Teshima *et al*., 1997), and 0.23-0.42% for *L. vannarnei* (Boone) (Duerr and Walsh, 1996). Our previous study suggested that the optimal dietary cholesterol requirement by juvenile swimming crabs was approximately 0.6% with defatted fish meal diets (Han *et al*., 2013). The different cholesterol requirements in the two studies are most probably due to the different dietary protein sources. Dietary protein sources modify the growth-stimulating action of cholesterol in Japanese flounder *Paralichthys olivaceus* (Deng *et al*. 2010). Juvenile *Panulirus ornatus* needed supplementary cholesterol to provide at least 0.4% dietary cholesterol with diets containing high levels of plant proteins, while no supplementary cholesterol requirements were needed in practical marine protein diets (Irvin *et al*., 2010). Several studies have shown that diets containing excess cholesterol had an adverse effect on growth performance in animals such as *S. serrata* (Sheen, 2000), *L. vannarnei* (Duerr and Walsh, 1996), *C. quadricarinatus* (Hernández *et al*., 2004) and *Scophthalmus maximus* L. (Yun *et al*., 2011). The present study showed that growth of the juvenile swimming crabs fed a CH 1.4 diet was lower than that of crabs fed a CH 1.0 diet.

Special attention should be paid to the interaction between the growth performance and the protein source in crustaceans (Coutteau *et al*., 1997). Juvenile lobsters fed diets based on casein showed higher mortality and MDS levels than on diets with crab protein (Conklin *et al*., 1980). In this study, crabs fed diets based on casein also showed higher levels of mortality than in our previous studies (fish meal basal diets) (Han *et al*., 2013). In this study, crabs fed a CH 1.0 diet showed the lowest mortality and MDS levels indicating that moderate cholesterol supplementation could reduce occurrence of MDS in swimming crabs. Incorporating cholesterol in excess of the dietary requirement has been suggested as a potential means of improving osmoregulatory capacity and reducing occurrence of MDS in *L. vannamei,* which also leads to better survival and growth (Gong *et al*., 2004).

Previous studies have suggested that cholesterol supplementation increases the body cholesterol contents of *P. japonicus* (Teshima *et al*., 1997) and *P. olivaceus* (Deng *et al*., 2010). Similarly, cholesterol content in the serum, whole body, and hepatopancreas of juvenile *P. trituberculatus* increased with increasing levels of cholesterol. The cholesterol of whole body and hepatopancreas were significantly higher in crab groups fed diets with 1.0% and 1.4% cholesterol than those fed with 0.2% and 0.6% cholesterol supplementation. These results also suggested that cholesterol tended to accumulate in the tissues when the dietary cholesterol was equal to, or more than 1.0%. Excess cholesterol accumulation could be detrimental to health and dietary cholesterol should be kept within an appropriate range for aquatic animals (Deng *et al*. 2010).

Cholesterol is an integral component for cell membrane maintenance and is involved in the preservation of fluidity within the membrane, which is necessary for immune system function. Some studies have indicated that blood cholesterol plays an important role in the immune system in mammals (Claxton *et al*., 1998; Ravnskov, 2003). AKP and ACP have been considered to be important non-specific immune indicators for

crustaceans, and play an important role in the self defense system of the body (Lin *et al*., 2011). High levels of AKP and ACP reflect a state of good health in studies of aquatic animal immunology. In the present study, crabs fed moderate dietary cholesterol levels (0.6% and 1.0%) showed higher AKP or ACP levels than those fed diets with 0.2% or 1.4% cholesterol levels. This suggests that cholesterol supplementation improves the immune response of swimming crabs. Similar results were also observed in *Eriocheir sinensis* (Tao *et al*., 2014) and *Oncorhynchus mykiss* (Deng *et al*., 2013). However, the ACP and AKP levels of swimming crabs fed diet with 1.4% cholesterol were lower than those fed diets with 1.0% cholesterol. These results also suggested that inadequate or excessive cholesterol might limit the normal immune response of swimming crabs, which is also consistent with the poor growth performance in CH 1.4 group. Excessive dietary cholesterol may depress the normal immune response of rainbow trout (Deng *et al*. 2013). Moreover, SOD acts as one of the antioxidants needed to maintain the complex immune system of crustaceans. Several studies have suggested that cholesterol could serve as an antioxidant, protecting the body from free radicals and strengthening the immune system (Mahfouz and Kummerow, 2000). Similarly, this study showed that dietary cholesterol supplementation generally increased serum SOD activity.

In summary, the cholesterol requirement of *P. trituberculatus* was determined to be 1.0%. Dietary cholesterol supplementation could also affect the non-specific immune system capability of juvenile *P. trituberculatus* fed casein based purified diets. Moderate cholesterol was found to be helpful in improving growth and molting performance.

Acknowledgements

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