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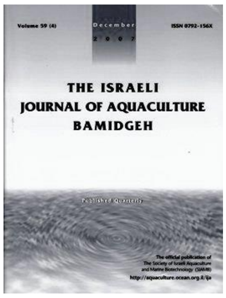

Dietary Copper Requirement of Juvenile Oriental River Prawn *Macrobrachium nipponense*, and its Effects on Growth, Antioxidant Activities, and Resistance to *Aeromonas hydrophila*

You-Qin Kong[1, 2], Zhi-Li Ding[1,2], Zhen-Yu Du[1*], Sheng-Ming Sun[1], Li-Gai Wang[1], Er-Chao Li[1], Li-Qiao Chen[1]

[1] *School of Life Sciences, East China Normal University, Shanghai 200062, PR China*
[2] *Department of Life Sciences, Huzhou Teachers College, Huzhou, Zhejiang 313000, PR China*

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Key words: *Macrobrachium nipponense*, copper requirement, growth, antioxidant activity, challenge

Abstract

The present experiment evaluated the effects of dietary copper (Cu) on growth, antioxidant activities, and susceptibility to *Aeromonas hydrophila* infection of juvenile Oriental river prawn *Macrobrachium nipponense*, as well as determining the optimal dietary copper requirement. Semi-purified diets containing seven graded levels of copper (2.8, 12.2, 20.9, 29.8, 43.1, 78.9 and 157.1 mg/kg diet) from $CuSO_4 \cdot 5H_2O$ were fed to juvenile prawn (initial weight 0.101±0.002 g). The weight gain of prawns fed with 2.8-78.9 mg/kg Cu was higher and they had lower feed conversion ratios than the group fed 157.1 mg/kg Cu. Cu concentrations in the hepatopancreas, muscle, and whole body in prawns tended to increase with increased dietary Cu concentration. Hepatopancreas Cu-Zn superoxide dismutase (Cu-Zn SOD) activity, glutathione peroxidase (GPx), and total antioxidant competence (T-AOC) were highest ($P<0.05$) in the 43.1 mg/kg Cu group. The hepatopancreas malondialdehyde (MDA) content was lower ($P<0.05$) in the prawns fed 29.8 mg/kg Cu than that in prawns fed 2.8, 12.2, 78.9 and 157.1 mg/kg Cu. After the feeding experiment, prawns were injected with *A. hydrophila*, and the cumulative mortality rate of the prawns fed with 20.9-43.1 mg/kg Cu was lower than the prawns fed with 2.8 and 157.1 mg/kg Cu. The optimum requirement of dietary Cu in juvenile prawns was estimated at 26.9-27.8 mg/kg diet based on cumulative mortality rate and whole-body Cu retention.

* Corresponding author. Li-Qiao Chen, E-mail: lqchen@bio.ecnu.edu.cn; Zhen-Yu Du, E-mail: zydu@bio.ecnu.edu.cn

Introduction

Copper (Cu) is an essential nutrient for animals, and its importance is well documented (O'Dell 1976). Physiologically, Cu is a co-factor for some enzymes including cytochrome c oxidase, lysyl oxidase, ferroxidase, tyrosinase, superoxide dismutase (SOD), and dopamine hydroxylase (Watanabe et al., 1997). Dietary Cu requirements have been determined for many animals; for crustaceans, optimal dietary copper quantitative requirements have been determined for the Chinese mitten crab *Eriocheir sinensis* (Sun et al., 2011), grass shrimp, *Penaeus monodon* (Lee and Shiau, 2002), and *Penaeus vannamei* (Davis et al., 1993). These requirements ranged from 15-41 mg/kg Cu and were much higher than that in fish with Cu requirements of 2-6 mg/kg (Gatlin and Wilson, 1986; Tan et al., 2011; Lin et al., 2010). Hemocyanin contains 40% of the entire copper load in shrimp (Depledge, 1989). Hemocyanin is a copper-containing protein that is responsible for the oxygen-carrying function in crustaceans indicating that the physiological demand for copper in crustaceans may be greater than in fish.

Studies have also shown that deficiency or excess of dietary Cu retards growth in grass shrimp (Lee and Shiau, 2002), Chinese mitten crab (Sun et al., 2011; 2013) and yellow catfish (Tan et al., 2011), and deficiency or excess dietary Cu can reduce immunity and increase infection in mammals (Omole and Onawunmi, 1979), fish (Lin et al., 2010; Shao et al., 2012), mollusks (Wang et al., 2009), and crustaceans such as grass shrimp (Lee and Shiau, 2002) and Chinese mitten crab (Sun et al., 2013) etc.

Oriental river prawn *Macrobrachium nipponense* have been widely farmed in P.R. China, Japan, and other South-East Asian countries as a traditional and popular aquaculture species. Annual production of farmed *M. nipponense* has surpassed 2 million tons since 2009 in China (Ministry of Agriculture Fisheries Bureau, 2010). The rapid development of *M. nipponense* cultivation has increased the need for formulated diets, however the nutrient requirements of *M. nipponense* have not been sufficiently researched. Some macronutrient requirements, such as proteins (Yu and Shen, 1990; Yang et al., 2004), and lipids (Yu and Shen, 1990) have been estimated, but there are few studies investigating the requirement of micronutrients, such as Cu. The purpose of the present study was to quantify the dietary Cu requirements of juvenile *M. nipponense,* and also investigate the effect of dietary copper on antioxidant activities and susceptibility to *A. hydrophila* infection in oriental river prawn.

Materials and Methods

Table 1. Ingredients & approx. compositions of experimental diets (%)

Ingredient	*% dry weight*
Casein[a]	30
Fish meal[b]	20
Corn starch	26
fish oil[c]	4
Soybean oil[d]	2
Vitamin mix[e]	2
Cu-free mineral mix[f]	3
Attractant[g]	3
Cholesterol[h]	0.5
Choline chloride[h]	0.5
lecithin[h]	0.5
cellulose[h]	6.5
sodium carboxymethylcellulose[h]	2
Proximate composition	
Crude protein	40.6
Crude lipid	7.47
Crude ash	7.02

[a]: Sigma-Aldrich Co., Shanghai, China
[b]: Tecnologica De Alimentos U.S.A.
[c]: Xiamen Xinsha Pharmaceutical Co. Ltd, Xiamen, China.
[d]: National Golden Dragon Fish Co. Ltd, Shanghai, China.
[e]: Vitamin mixture(mg/100g mixture): vitamin A 420000IU; vitamin C 6000mg; α-tocopherol acetate 2000mg; vitamin D3 120000 IU; vitamin K 1000mg; vitamin B1 1000mg; vitamin B2 1000mg; vitamin B6 1600mg; vitamin B12 2mg;niacin 5000mg; folic acid 400mg; inositol 6000mg; biotin 10mg; calcium pantothenic 3500mg;Hangzhou Minsheng Bio-Tech Co., Ltd., China.
[f]: Composition of mineral mixture (g/kg diet): KCl 0.84, $MgSO_4.7H_2O$ 3, NaH_2PO_4 6.45, KH_2PO_4 3, $Ca(H_2PO_4)_2.H_2O$ 7.95, $CaCO_3$ 3.15, $C_6H_{10}CaO_6.5H_2O$ 4.95, $FeC_6H_5O_7.5H_2O$ 0.36, $ZnSO_4.7H_2O$ 0.1428, $MnSO_4.H_2O$ 0.0321, $AlCl_3.6H_2O$ 0.0045, $CoCl_2.6H_2O$ 0.042, KI 0.0069.
[g]: alanine 0.6%, glycine 0.6%, glutamic acid 0.6%, betaine 1.2%.
[h]: China National Medicine Corporation Co., Ltd, Beijing, China.

Diet preparation. Experimental diets compositions are given in Table 1. A semi-purified basal diet was formulated using casein and fish meal as protein sources, and fish oil and soybean oil as lipid sources. Copper sulfate (Analytical Reagent, Shanghai Chemical Co., Shanghai, China) was added to a basal diet at 0, 10, 20, 30, 40, 80, and 160 mg/kg Cu dry diet with corresponding decreasing amounts of cellulose. The process of diet preparation was similar to that described by Li et al. (2010). Copper concentrations in the diets were analyzed by flame atomic absorption photometry (AOAC, 1980) to be 2.8, 12.2, 20.9, 29.8, 43.1, 78.9 and 157.1 mg/kg, respectively. Contents of crude protein, crude lipid and ash in feed were analyzed to be 40.6%, 7.47% and 7.02%, respectively. The dry pellets were sealed in plastic bags and stored at -20°C until use.

Experimental procedure. The juvenile prawns were obtained from a local farmer. Prior to the experiment, prawns were acclimatized to the laboratory conditions for 2 weeks. At the beginning of the experiment, healthy prawns with an average initial weight of 0.101±0.002g were randomly stocked in thirty-five 300 l tanks/ 50 prawns per tank/five replicates per dietary group. Prawns were fed to apparent satiation twice a day (0800 and 1700h) for 8 weeks. One third of the tank water was exchanged daily to maintain water quality. The water temperature ranged from 27°C-30°C, dissolved oxygen was maintained above 6.5mg/l, with ammonia and nitrate below 0.1 mg/l during the feeding trial. Prawns were exposed to a natural photoperiod. The Cu concentration in rearing water was monitored regularly and ranged from 1.4-1.7μg Cu/l. At the end of the 8 week feeding period, 60 prawns from each dietary group (12 prawns from each of original tanks) were randomly chosen and challenged with *A. hydrophila* (10^7CFU/ml). Each prawn was injected 50μl bacterial suspension provided by the Shanghai Ocean University (Shanghai, China). Dead prawns were collected twice daily over a period of 1 week.

Sample collection. At the end of the feeding trial, the prawns were starved for 24h. Muscle and hepatopancreas samples were dissected and stored at -80°C. The concentration of maleic dialdehyde (MDA) and activities of total antioxidant competence (T-AOC), copper-zinc superoxide dismutase (Cu-Zn SOD), and glutathione peroxidase (GPx) of the hepatopancreas were determined. One prawn from each tank was also randomly collected and stored at -20°C.

Analysis and measurement. All prawns were counted and weighed as follows:

Survival rate = 100 × (final prawn number) / (initial prawn number)),

Weight gain rate (WGR) = 100 × (final body weight - initial body weight) / initial body weight

Specific growth rate SGR = ($\ln W_f$ - $\ln W_i$ x 100) / t ,

Feed conversion rate (FCR) = feed intake / (final body weight - initial body weight) were calculated.

Crude protein, crude lipid, and ash in diets were measured following standard methods. Crude protein was analyzed by the Kjeldahl method, crude lipid was determined by ether extraction using the Soxhlet system (2055 Soxhlet Avanti; Foss Tecator, Hoganas, Sweden), and ash content was determined by heating in a muffle furnace at 550° for 6h. The copper concentrations of whole-body, muscle, and hepatopancreas were determined by flame atomic absorption photometry (AOAC, 1980).

MDA was measured by thiobarbituric acid (TBA) method (Buege & Aust 1978). T-AOC was determined based on the Fe^{3+} reduction process (Tang et al., 2005). Cu-Zn SOD activity was analyzed according to the Xanthine oxidase method (Elstner & Heupel 1976). Reduced GSH in the enzymatic reaction was analyzed to determine GPx activity (Sedlak & Linday 1968).

Dietary Cu requirements for juvenile *M. nipponense* were estimated by linear regression analysis of the dietary Cu levels versus whole-body Cu balance values and by the broken-line regression method based on cumulative mortality rate in the challenge test.

Statistical analysis. All data are expressed as means ± SD One-way ANOVA and post hoc Duncan multiple range tests were used to determine difference between groups using SPSS 11.0. Differences were regarded as significant at P<0.05.

Results

Growth performance and copper retention. Survival rate of prawns was not affected by the dietary copper levels (Table 2).

Table 2. Growth performances in juvenile *M.nipponense* fed experimental diets for 8 weeks (mean±SD, n=5)

Dietary Cu (mg/kg)	*Initial weight(g)*	*Final weight(g)*	*WGR (%)*	*SGR (%)*	*Survival (%)*	*FCR*
2.8	0.101±0.002	0.798±0.019[b]	690.57±18.89[b]	3.69±0.05[b]	70.10±4.77	1.77±0.17[a]
12.2	0.101±0.001	0.817±0.032[b]	709.32±31.48[b]	3.73±0.07[b]	80.70±6.56	1.59±0.11[a]
20.9	0.101±0.003	0.867±0.043[b]	758.56±42.55[b]	3.84±0.09[b]	73.95±13.13	1.60±0.28[a]
29.8	0.100±0.001	0.823±0.074[b]	714.47±72.99[b]	3.74±0.16[b]	75.92±4.37	1.69±0.14[a]
43.1	0.102±0.004	0.822±0.113[b]	713.68±112.27[b]	3.73±0.24[b]	80.34±2.52	1.57±0.23[a]
78.9	0.101±0.003	0.825±0.082[b]	716.62±81.54[b]	3.74±0.18[b]	76.99±8.96	1.61±0.18[a]
157.1	0.101±0.002	0.690±0.027[a]	582.95±27.12[a]	3.43±0.07[a]	71.36±5.82	2.24±0.30[b]

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

Prawns in the 20.9 mg/kg Cu group exhibited the largest weight gain rate and SGR, and were significantly larger than those receiving 157.1 mg/kg Cu. Similarly, prawns in the 157.1 mg/kg Cu group had significantly lower FCR than other groups ($p< 0.05$).

Cu contents in whole-body, hepatopancreas, and muscle, generally increased as the dietary Cu level increased ($P<0.05$) (Table 3).

Table 3. Copper contents (mg/kg, dry weight) in tissues of juvenile *Macrobrachium nipponense* fed experimental diets for 8 weeks (mean±SD, n=3)

Dietary Cu (mg/kg)	2.8	12.2	20.9	29.8	43.1	78.9	157.1
Muscle(mg/kg)	11.1±0.6[a]	12.0±0.3[a]	17.9±0.9[b]	18.9±0.1[b]	21.4±1.8[c]	25.3±1.1[d]	28.6±0.1[e]
Hepatopancreas (mg/kg)	11.4±0.2[a]	14.7±0.4[a]	29.7±0.3[b]	36.9±0.5[c]	253.6±3.0[d]	429.3±3.1[e]	742.7±2.2[f]
Whole body (mg/kg)	28.3±1.4[a]	32.8±0.8[b]	40.1±1.4[c]	46.8±0.6[d]	51.0±1.1[e]	65.5±0.7[f]	100.4±1.4[g]

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

Whole-body Cu retention for each group of prawns was calculated (Table 4).

Table 4. Whole-body copper balance of *Macrobrachium nipponense* fed experimental diets for 8 weeks (mean±SD, n=3)

Analyzed dietary Cu level (mg/kg diet)	*Initial whole body Cu content (μg)*	*Total Cu Fed (μg)*	*Final whole-body Cu content (μg)*	*Final-initial whole-body Cu content (μg)*	*Whole-body Cu retention (μg)*
2.8	2.32	3.59	22.58	20.26	16.67
12.2	2.32	13.61	26.80	24.48	10.87
20.9	2.32	25.44	34.77	32.45	7.01
29.8	2.32	35.33	38.52	36.20	0.87
43.1	2.32	48.28	41.92	39.60	-8.68
78.9	2.32	92.23	54.04	51.72	-40.52
157.1	2.32	198.14	69.28	66.96	-131.18

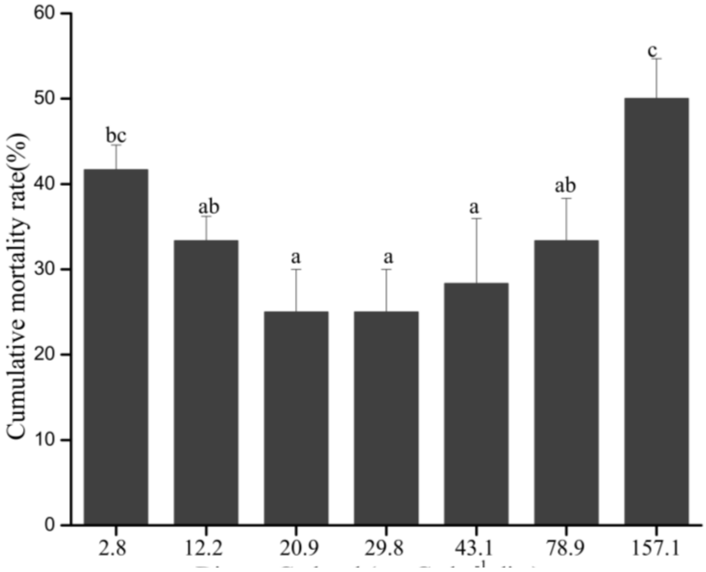
Hepatopancreas antioxidant activities. Hepatopancreas MDA in the prawns fed with 29.8 mg/kg Cu was lowest and significantly lower than those in the prawns fed 2.8, 12.2, 43.1, 78.9 and 157.1 mg/kg Cu ($p<0.05$). T-AOC activity was the highest in the prawns fed diets containing 29.8 and 43.1 mg/kg Cu, followed by the groups with 20.9, 78.9 and 157.1 mg/kg Cu, whereas, the lowest activities of T-AOC were observed in the prawns fed with 2.8 and 12.2 mg/kg Cu. Cu-Zn SOD activity in the prawns fed 43.1 mg/kg Cu was the highest, and the lowest value was seen in the dietary groups with 2.9, 12.2, 78.9 and 157.1 mg/kg Cu ($P<0.05$). The prawns fed 43.1 mg/kg Cu had significantly higher GPx activity, compared to prawns fed 2.8 mg/kg Cu diets (Table 5).

Table 5. Cu-Zn SOD, GPx, and T-AOC activitivies and MDA content of juvenile *Macrobrachium nipponense* fed experimental diets for 8 weeks (mean ± SD, n =3)

Dietary Cu (mg/kg)	*MDA(nmol/ mg prot)*	*T-AOC(U/mg prot)*	*Cu-Zn SOD (U/mg prot)*	*GPx (U/mg prot)*
2.8	14.18±1.67[c]	18.71±0.64[a]	10.28±0.61[a]	85.80±2.39[a]
12.2	16.54±1.64[c]	17.64±0.77[a]	9.91±0.35[a]	95.36±7.49[ab]
20.9	10.41±1.91[ab]	27.66±1.07[b]	15.10±1.28[b]	103.09±4.03[ab]
29.8	9.37±0.78[a]	33.77±0.42[c]	13.54±1.48[b]	105.04±12.34[b]
43.1	13.52±0.98[bc]	36.15±1.93[c]	17.25±1.89[c]	161.71±0.58[d]
78.9	15.33±1.08[c]	29.80±1.95[b]	10.42±1.31[a]	104.42±11.59[ab]
157.1	16.95±1.09[c]	28.26±0.73[b]	10.05±0.31[a]	123.64±4.76[c]

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

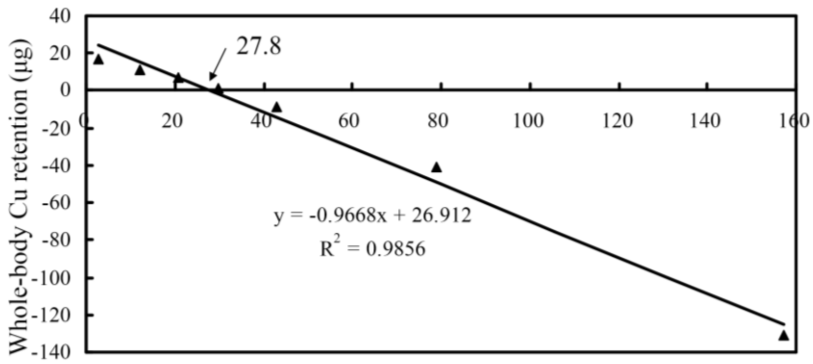
Resistance to A. hydrophila challenge. The prawns fed 2.8, 12.2, 20.9, 43.1, 78.9 and 157.1 mg/kg Cu started to die 6h after being challenged with *A. hydrophila,* whereas the prawns from the 29.8 mg/kg Cu group started to die 48h after challenge. Cumulative mortality rate in the prawns fed 20.9-43.1 mg/kg Cu were significantly lower than the prawns fed 2.8 and 157.1 mg/kg Cu ($P<0.05$) (Fig. 1).



Dietary Cu level (mg/kg Cu diet)

Figure 1 Cumulative mortality rate in *M. nipponense* fed experimental diets 8 weeks following a challenge with *A. hydrophila.* Values are means ± SD (n=3). Bars sharing different letters in each index are significantly different (P<0.05).

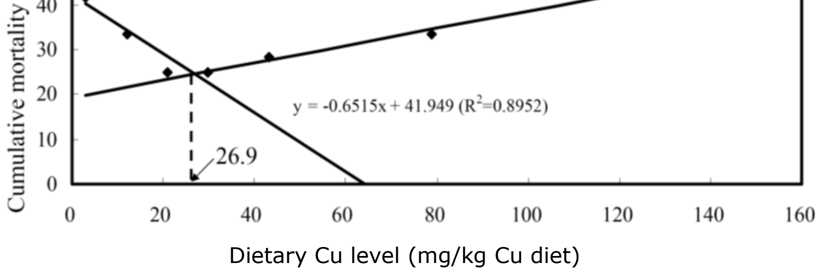
Estimation of dietary Cu requirement. A linear regression analysis of whole-body Cu retention values versus the dietary Cu level determined that the Cu requirement of oriental river prawn is 27.8 mg/kg Cu diet (Fig. 2).



Dietary Cu level (mg/kg Cu diet)

Fig. 2 Relationship between dietary copper concentration and whole-body copper retention in juvenile *M. nipponense* fed experimental diets for 8 weeks. Each point represents the mean of three replicates within a treatment prawns. Requirement derived with the linear regression method is 27.8 mg/kg Cu diet.

A broken-line regression analysis of cumulative mortality rate values versus the dietary Cu level indicated that the optimal requirement of dietary copper is 26.9 mg/kg Cu (Fig. 3).



Dietary Cu level (mg/kg Cu diet)

Fig. 3 Broken-line analysis of the relationship between dietary copper levels and the cumulative mortality rate indicates that the optimal dietary copper levels for juvenile *M. nipponense* is about 26.9 mg/kg Cu diet. Each point represents the mean of three replicates within treatment prawns.

Discussion

The present study showed that high levels of dietary Cu (157.1 mg/kg Cu) significantly decreased growth, but 2.8-78.9 mg/kg Cu had no significant effect on growth of juvenile *M. nipponense*. *Marsupenaeus japonicus* growth was not affected by dietary Cu (Kanazawa et al., 1984). The weight gain of grass shrimp (Lee and Shiau, 2002) and Chinese mitten crabs (Sun et al., 2011) responded to dietary copper content. This suggests that the effect of dietary Cu level on growth is species specific. The lowest feed conversion rate was found in the prawns fed 157.1 mg/kg Cu, suggesting that excess dietary Cu decreases growth and impairs feed conversion. This is in accordance with a previous study on grass shrimp (Lee and Shiau, 2002). Based on these growth data, we conjecture that *M. nipponense* growth is not sensitive to dietary copper levels ranging from 2.8 to 78.9 mg/kg Cu, but excess Cu may decreases growth.

Malondialdehyde (MDA) is a final product of lipid peroxidation. It is commonly used as an indicator of endogenous oxidative damage. The hepatopancreas MDA in the prawns fed with 29.8 mg/kg Cu was significantly lower than other prawns. Although no comparable result was reported in other crustaceans, a similar result was found in blunt snout bream, in which the diets containing moderate dietary Cu significantly reduced the accumulation of MDA in fish liver, compared to deficient or excess dietary Cu groups (Shao et al., 2012) suggesting a common mechanism that dietary copper affects the oxidative system in aquatic animals. To reduce oxidative stress and maintain the balanced state of free radicals, organisms have developed various antioxidant defenses, i.e. specialized antioxidant enzymes SOD (Nozik-Grayck et al., 2005). Cu-Zn SOD, as a Cu-containing enzyme, has been an excellent indicator in evaluating copper nutrition in animals (Gatlin and Wilson, 1986). The present study showed that dietary Cu affected the activity of Cu-Zn SOD in *M. nipponense*, and 20.9-43.1 mg/kg Cu groups displayed significantly higher Cu-Zn SOD activity. Similar results were found in channel catfish (Gatlin and Wilson, 1986). T-AOC and GPx activities respectively showed trends similar to Cu-Zn SOD activity. This could be the reason that dietary Cu deficiency may reduce antioxidant enzymes activities, leading increased free radicals and high oxidative stress (Lin et al., 2010) and dietary excess of Cu increased Cu accumulation in prawn hepatopancreas, promoting the production of hydroxyl radicals from superoxide and hydrogen peroxides, thereby leading the development of oxidative stress (Roméo et al., 2000; Winston and Di Giulio, 1991). These results indicated that the dietary Cu affects antioxidant ability of *M. nipponense*, and 20.9-43.1 mg/kg Cu could significantly promote *M. nipponense* antioxidant activity.

A deficiency or excess of dietary Cu could induce oxidative stress. One study found that oxidative stress can increase free radicals, destroy the structure of immune cells, and inhibit immune response (Lin et al., 2010). A diet which includes 10-30 mg/kg Cu could significantly increase the immune response in grass shrimp (Lee and Shiau, 2002). *A. hydrophila* is a common species of *Aeromonas* found in water and water habitants (Longshaw, 2011). *A. hydrophila* infects fish and prawn including *M. nipponense* and is one of the major diseases affecting the *M. nipponense* cultivation industry in China and elsewhere (Vivekanandhan et al., 2005; Shen et al., 2000). After the challenge experiment, cumulative mortality rate was lowest in prawns fed 20.9-43.1 mg/kg Cu ($P<0.05$). Similarly, the survival rate after infection of *A. hydrophila,* of Chinese mitten crab fed with 20.78-40.34 mg/kg Cu diet, significantly increased, compared to deficient or excess dietary Cu groups (Sun et al., 2013). Therefore, our study confirms that dietary Cu affects prawn immune response and proper dietary Cu concentrations can reduce prawn susceptibility to *A. hydrophila* infection.

Traditionally, growth is commonly used to evaluate the nutrient requirements in aquatic animals. However, in evaluating mineral requirements, whole-body retention is also widely used, especially where growth parameters are comparable between treatments. Whole-body retention of an individual mineral has been used to estimate its requirement in grouper (Lin et al., 2010), grass shrimp (Shiau and Jiang, 2006), and yellow catfish (Tan et al., 2011). In the present study, the dietary Cu requirement was estimated at 27.8 mg/kg Cu diet based on the whole-body Cu retention. Cumulative rate of mortality in a challenge test is commonly used as an indicator of aquatic animal immunity (Ai et al., 2006). When the cumulative mortality rate was used to estimate Cu requirements in prawn, the result (26.9 mg/kg Cu diet) was in line with that obtained from the whole-body Cu retention of the prawn. Therefore, the Cu requirement of prawn in the present study was set as 26.9-27.8 mg/kg Cu diet. These values are similar to those reported on crustaceans, i.e. 10-30 mg/kg Cu diet for grass shrimp (Lee and Shiau, 2002), 20.78-40.34 mg/kg Cu for Chinese mitten crab (Sun et al., 2013) and 25.27 mg/kg Cu for *Penaeus Chinensis* (Wang et al., 1997).

In conclusion, *M. nipponense* growth was not affected by dietary Cu concentrations between 2.8-78.9 mg/kg Cu. Deficient or excess dietary Cu could induce oxidative stress, affect prawn antioxidant ability, and increase prawn susceptibility to *A. hydrophila* infection. A 26.9-27.8 mg/kg Cu diet was estimated as the optimal dietary Cu requirement of juvenile *M. nipponense* . This was based on the cumulative mortality rate and whole-body Cu retention.

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