RESEARCH REPORT

Effects of ammonia and nitrite accumulation on the survival and growth performance of white shrimp *Litopenaeus vannamei*

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Abstract

Ammonia and nitrite levels caused by shrimp excreta and metabolic waste and organic detritus are important limiting factors in intensive aquaculture system, the purpose of this study was to determine how ammonia and nitrite accumulation caused by accumulated these compounds affected survival and growth performance of white shrimp *Litopenaeus vannamei*. Unconsumed feed, feces and seawater in treatment group were not removed or replaced over a 33-day period, while unconsumed feed and feces in control group were removed with a siphon tube and 60 % seawater was replaced once-daily. Significantly higher ammonia and nitrite concentrations were accumulated in the seawater of treatment group from day 6 to day 33 and from day 9 to day 33, compared with control group, respectively. Significantly lower survival rate, weight gain percentage, length gain percentage and specific growth rate were recorded in treatment shrimp, compared with control shrimp. Significantly higher lipase, superoxide dismutase, and catalase activities, malondialdehyde content, relative expression of 4E-binding protein 1, p70S6 kinase, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase mRNA were detected in the hepatopancreas of treatment versus control shrimp. Significantly lower protease, glutathione reductase and glutathione peroxidase activities, and glutathione content were detected in the hepatopancreas of treatment versus control shrimp. Meanwhile, hepatopancreas in treatment shrimp showed disorganized tubules, blurred boundaries, decreased or disappeared B, R and E cells, injured connective tissue between liver tubule, infiltrated hemocytes, narrowed lumen, and vacuolization compared with control shrimp. These findings might indicate that ammonia and nitrite accumulation caused by accumulated waste in aquaculture tanks could significantly reduce survival, growth performance of *L. vannamei* with hepatopancreas damage, which was resulted from accumulated reactive oxygen species. Therefore, ammonia and nitrite accumulation may significantly impact shrimp production in intensive aquaculture system.

Key Words: *Litopenaeus vannamei*; ammonia and nitrite accumulation; survival; growth performance; hepatopancreas

Introduction

Decapod crustaceans release nitrogenous waste as ammonia in aquaculture system, the end product of protein catabolism (Regnault, 1987); nitrite is an intermediate product either during bacterial denitrification of nitrate or bacterial nitrification of

ammonia (Mevel and Chamroux, 1981). Both ammonia and nitrite are the most common pollutant in intensified aquaculture or in recirculated water, they increase with culture duration which can increase to 0.81 mg/L total ammonia-N, 0.12 mg/L nitrite-N in the hatcheries and 6.497 mg/L total ammonia-N, 4.611 mg/L nitrite-N in the grow-out ponds even with frequent water exchange (Chen *et al.*, 1986, 1989). Pond water in massive cultivation may accumulate high concentrations of ammonia and nitrite as the result of excretion by the animals in the system and the mineralization of organic detritus,

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such as unconsumed food and feces (Tacon et al., 2002; Chen et al., 2012; Ren et al., 2015).

Previous studies demonstrated that ammonia stress could affect a variety of physiological functions of crustaceans, including respiration, metabolism, excretion (Racotta and Hernandez-Herrera, 2000; Cheng and Chen, 2001; Miranda-Filho et al., 2009; Barbieri 2010; Martin et al., 2011; Ren et al., 2015), the immune system (Yue et al., 2010; Peaydee et al., 2014; Ren and Pan, 2014; Chang et al., 2015; Duan et al., 2015; Wongsasak et al., 2015; Pinto et al., 2016), osmoregulation (Romano and Zeng, 2007; Romano and Zeng, 2010), and apoptosis and molting (Mugnier et al., 2008; Liang et al., 2016). Like ammonia, nitrite stress could also affect crustacean growth, respiration, metabolism (Chen and Cheng, 1995, 2000; Mallasen and Valenti, 2006; Hong et al., 2009; Xian et al., 2011), the immune system (Tseng and Chen 2004; Wang et al., 2004, 2006; Liao et al., 2012), excretion (Chen and Cheng, 1995, 2001, 2002) and apoptosis (Xian et al., 2012; Guo et al., 2013). Furthermore, ammonia and nitrite could act synergistically, resulting in toxic effects that were greater than either compound alone (Schuler et al., 2010; Cheng et al., 2013; Zhang et al., 2015).

The tropical white shrimp Litopenaeus vannamei has become an attractive cultivar for inland aquaculture in many parts of the world, including the USA, Thailand, and China. It is widely cultured in extensive, intensive, and semi-intensive systems (Frías-Espericueta et al., 1999). Due to the limitations and availability of land to construct, the culture of L. vannamei has been intensified to boom the production and economic benefit. In this intensive culture system, shrimp excreta and metabolic waste produced and organic detritus caused concern with water pollution and shrimp diseases, ammonia and nitrite levels are important limiting factors during the rapid accumulation of these compounds. It has been reported that the concentration of nitrite increases directly with culture period, and might reach as high as 20 mg/L in grow-out ponds of L. vannamei (Tacon et al., 2002). However, we know little about the toxic mechanism of ammonia and nitrite accumulation caused by accumulated those compounds in intensive aquaculture system on shrimp.

The aim of the present study was to evaluate the effects of ammonia and nitrite accumulation caused by accumulated waste in aquaculture tanks on L. vannamei, which would lead to develop a better understanding of the toxic mechanism of ammonia and nitrite accumulation caused by shrimp excreta and metabolic waste and organic detritus in intensive aquaculture system on shrimp. Given that the hepatopancreas is the most important digestive gland in crustacean, and is involved in digestion, nutrient absorption, storage, diseases, as well as synthesis and excretion of digestive enzymes (Bautista et al., 1994; Rosas et al., 1995; Li et al., 2008, Franceschini-Vicentini et al., 2009), and this organ in shrimp comprises branched tubules lined by different types of epithelial cell (E cells, R cells, F cells, and B cells), we investigated the effects of ammonia and nitrite accumulation caused by accumulated waste in aquaculture tank on the survival, growth performance and digestive enzyme activities, antioxidant status, metabolic gene expression, and histology in the hepatopancreas of L. vannamei.

Materials and Methods

Experimental shrimp

Fresh, healthy, juvenile Litopenaeus vannamei (mean weight 0.90 ± 0.02 g) were obtained from the Ruizi Seafood Development Co. Ltd. (Qingdao, China), where the experiment was also conducted. A total of 1200 shrimp were distributed among six 640-L cylindrical tanks with net cover ($N = 200$ per tank), every 640-L cylindrical tank contained 500-L aerated seawater (dissolved oxygen 5.4 - 6.5 mg/L). The initial seawater is unfiltered with pH 7.5 - 8.2, salinity 30 - 31 ‰, total ammonia 0.022 - 0.038 mg/L, nitrite 0.015 - 0.032 mg/L, and nitrate 0.030 - 0.065 mg/L at 28 - 32 °C. They were acclimated for 2 weeks under a natural photoperiod (12 h: 12 h light: dark). The shrimp were fed three times daily with a commercial diet (41.52 % crude protein, 7.42 % lipid, and 12.03 % crude ash, supplied by Yantai Dale feed Co. Ltd, Shandong, China) at 07:00 h, 11:00 h, and 19:00 h, at a feeding rate of 35 %, 20 %, and 45 %, respectively, representing a daily feeding rate that was 10 % of the weight of shrimp. The unfed feed and feces were removed with a siphon tube and 60 % seawater was replaced once-daily. Unfiltered seawater (28 – 32 °C, pH 7.5 - 8.2, salinity 30 - 31 ‰, total ammonia 0.022 - 0.038 mg/L, nitrite 0.015 - 0.032 mg/L, and nitrate 0.030 - 0.065 mg/L) were prepared in other three 1000-L cylindrical tanks to use for exchange daily.

Experimental design for ammonia and nitrite accumulation

Following acclimation, the experimental setup was comprised of two experimental groups, each group had three repetitions, each 640-L cylindrical tank was regarded as a repetition: (1) control group (three 640-L cylindrical tanks); and (2) treatment group (three 640-L cylindrical tanks). Shrimp were experimented for 33 days in 640-L cylindrical tanks with net cover containing 500-L aerated seawater (dissolved oxygen 5.4 - 6.5 mg/L), the photoperiod and feeding condition was handled in exactly the same way as during the acclimation period. For the control tanks, the unconsumed feed and feces were removed with a siphon tube and 60 % seawater was replaced once-daily, to maintain low ammonia and nitrite concentrations in the tanks. By contrast, for the treatment tanks, unconsumed feed, feces and seawater were not removed or replaced, to allow ammonia and nitrite accumulation in the tanks, and seawater was only added to raise the volume in the tanks to 500 L as necessary. Unfiltered seawater (28 - 32 °C, pH 7.5 - 8.2, salinity 30 - 31 ‰, total ammonia 0.022 - 0.038 mg/L, nitrite 0.015 - 0.032 mg/L, and nitrate 0.030 - 0.065 mg/L) was still prepared in other three 1000-L cylindrical tanks to use for exchange daily for control tanks, and for adding to 500 L for treatment tanks. At the end of the experiment, shrimp were deprived of feed for 24 h before any experimental treatment.

Table 1 Primers for the genes encoding p70s6k, 4ebp1, GOT, GPT, GST, and β-actin in shrimp. Primer pairs of p70s6k, 4ebp1, GOT, GPT were designed by primer5 according to transcriptome sequences in our lab, and GST, β-actin were from Zhou *et al.*(2009)

Target gene	Sequence (5'→3')
p70s6k	F-GCAAGAGGAAGACGCCATA R-CCGCCCTTGCCCAAAACCT
4ebp1	F-ATGTCTGCTTCGCCCGTCGCTCGCC R-GGTTCTTGGGTGGGCTCTT
GOT	F- CTACGACCCAAAGACCTGT R- ACCTTGCTCATTTCATCCC
GPT	F-ATGTCGTTTGTGGGTTTTCG R-TTCGCCTTGGTCACGCTGT
GST	F- AAGATAACGCAGAGCAAGG R- TCGTAGGTGACGGTAAAGA
β-actin	F- GCCCATCTACGAGGGATA R-GGTGGTCGTGAAGGTGTAA

Measurement of ammonia and nitrite concentrations

Ammonia and nitrite concentrations in the seawater of each tank were measured using the hypobromite oxidation method and naphthylethylenediamine photometric method (GB 17378.4 - 2007), respectively, every 3 days. Hypobromite oxidation method: 10 mL seawater was added with 1mL 168 g/L fresh sodium hypobromite solution and mixed, the mixture was mixed with 1 mL 2 g/L sulfanilamide solution and then mixed with 0.2 mL 1 g/L ethylenediamine dihydrochloride solution, absorbance (y) of this solution was measured at 543 nm wavelength, and ammonia concentration (x) was evaluated in term of drawn working curve, $y = 3.28x + 0.0155$. Naphthylethylenediamine photometric method: 10 mL seawater was added with 0.2 mL 10 g/L sulfanilamide solution and mixed, the mixture was mixed with 0.2 mL 1 g/L ethylenediamine dihydrochloride solution, absorbance (y) of this solution was measured at 543 nm wavelength, and nitrite concentration (x) was evaluated in term of drawn working curve, $y = 3.303x + 0.0126$.

Measurement of survival and growth performance and sampling procedure

The number of dead shrimp from each tank was recorded every 24 h during the experimental period. Shrimp were considered dead when they failed to move even when gently stimulated with a glass pipette. Dead shrimp were removed to prevent fouling. 20 shrimp from each tank were randomly selected at the beginning and end of the experiment, respectively, and replaced after measuring weight and length. Survival and growth performance was evaluated in terms of their survival rate (SR), weight gain percentage (WGP), length gain percentage (LGP), and specific growth rate (SGR) based on the following standard formulae:
SR (%) = 100 × (final shrimp number)/(initial shrimp number);

WGP (%) = 100 × (final weight-initial weight)/initial weight;
LGP (%) = 100 × (final length-initial length)/initial length; and
SGR (%/day) =100 × (ln final weight-ln initial weight)/days.

20 shrimp were randomly selected and removed from each tank at the end of the experiment ($N = 60$ for each group); the hepatopancreas from each shrimp was collected using sterilized scissors and forceps: five for each tank were immediately placed into RNAlater (Applied Biosystems, Austin, TX, USA) and stored at -20 °C until RNA isolation and mRNA expression analysis ($N = 15$ for each group); five for each tank were immediately stored at -80 °C to be used for the digestion and antioxidant parameter assay ($N = 15$ for each group); and ten for each tank were immersion fixed in Bouin's solution for 18-h and then transferred to 70 % (v/v) ethanol (Romano *et al.*, 2015), until processing for histological study ($N = 30$ for each group).

Hepatopancreatic digestive enzyme activity and antioxidant status

Approximately 200 mg of hepatopancreas tissue was dissected from a single hepatopancreas, five hepatopancreas tissues for each tank were mixed at a 1:5 ratio (w/v) with chilled Tris-hydrochloric acid buffer solution (pH 7.6, 10 mmol L^{-1}) and homogenized under ice-chilled conditions. The homogenates were then centrifuged at 10,000×g, 4 °C for 30 min and the supernatants were then collected for the assays. Digestive enzyme activities, including protease activity, amylase activity, cellulose activity and lipase activity, were evaluated using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. Each of the above digestive enzyme activities was analyzed
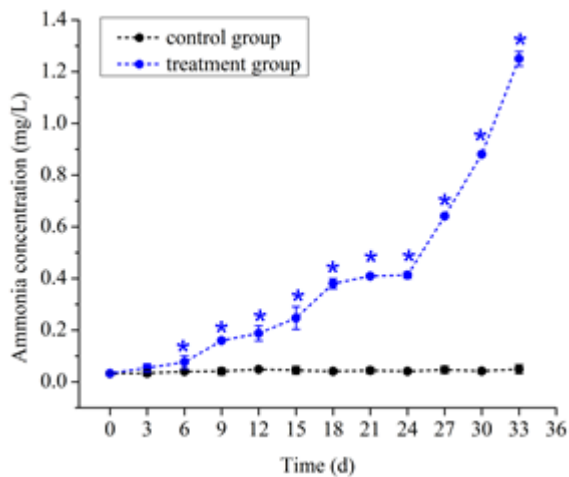
Fig. 1 Ammonia concentrations in the seawater of tanks during the experimental period; *Indicates a significant difference between the treatment (blue circles) and control (black circles) groups. Ammonia concentration could significantly increase in the treatment group versus control group.
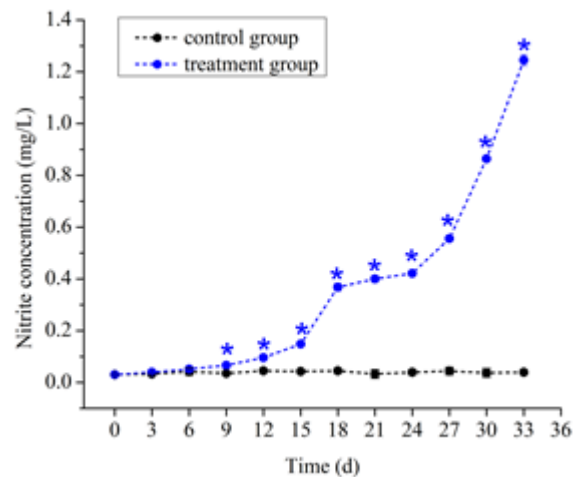

Fig. 2 Nitrite concentrations in the seawater of tanks during the experimental period.*Indicates a significant difference between the treatment (blue circles) and control (black circles) groups. Nitrite concentration could significantly increase in treatment group versus control group.

with three replicates of each sample. The antioxidant status, including superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione peroxidase (GPx) activity, glutathione reductase (GR) activity, glutathione S-transferase (GST) activity, glutathione (GSH) content, and malondialdehyde (MDA) content, was also evaluated using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions. Each of the above antioxidant status was analyzed with three replicates of each sample. Activities were expressed as a relative unit per milligram of soluble protein (U/mg protein), and GSH and MDA contents were expressed as the relative concentration per milligram of soluble protein (nmol/mg protein).

Hepatopancreatic metabolic gene expression assays
RNA was extracted from a single hepatopancreas using a RNA fast extraction kit according to the manufacturer's protocol (Fastagen, Shanghai, China). RNA solution from five hepatopancreases for each tank were mixed into 5 µl, 1 µl RNA solution per hepatopancreas; this 5 µl RNA solution was used for cDNA synthesis using a TransScript® One-Step gDNA Removal and cDNA Synthesis Kit according to the manufacturer's protocol (TransGen Biotech Co., Ltd, Beijing, China). The expression of the metabolic genes encoding 4E binding protein 1 (*4ebp1*), p70S6 kinase (*p70s6k*), glutamic-oxaloacetic transaminase (*GOT*), glutamic-pyruvic transaminase (*GPT*), and *GST* was investigated. *β-Actin* was selected as a reference gene and specific primers were used for each gene (Table 1). The relative expression of each of the above metabolic genes was analyzed by relative quantitative real-time PCR (qPCR) using TransStar Top Green qPCR Supermix according to the manufacturer's protocol (TransGen Biotech Co., Ltd)

with three replicates of each sample. qPCR was performed with the following two steps: denaturation at 94 °C for 30 s and then 40 cycles of 94 °C for 5 s and 60 °C for 30 s. The dissociation curve analysis was performed at the end of qPCR to confirm the specificity of the PCR products, and relative expressions were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Hepatopancreatic histology assays
The fixed ten hepatopancreases for each tank were dehydrated in ascending concentrations of alcohol, cleared in toluene, embedded in paraffin, and sectioned with a rotary microtome at 5 µm. Sectioned tissues were stained with hematoxylin and eosin (H&E), and examined with a light microscope (Casado *et al.*, 2001).

Statistical analysis
The data were all presented as the mean ± standard error (SE). Statistical analysis was performed with SPSS (version 17.0) (IBM, New York, America), and t-test was used to analyze differences between two experimental groups. The significance level was $p < 0.05$. All images were generated with Origin 9.0 software (OriginLab, Massachusetts, America).

Results

Ammonia and nitrite concentrations
There were no significant changes of both ammonia and nitrite concentrations in the seawater of control group during the experimental period (Figs 1, 2). By contrast, there were a gradual increase in both ammonia and nitrite concentrations in the seawater of treatment group, such that they were significantly higher ($p < 0.05$) than the control group from day 6 to day 33 and from day 9 to day 33, respectively (Figs 1, 2).

Table 2 Growth performance of shrimp after the 33-d experiment

Group	SR (%)	WGP (%)	LGP (%)	SGR (%)
Control	86.83±3.01	547.05±6.47	62.80±1.35	5.66±0.11
Treatment	34.56±2.92*	341.10±4.85*	39.60±1.14*	4.50±0.06*

SR, WGP, LGP, and SGR represent survival rate, weight gain percentage, length gain percentage, and specific growth rate respectively. * Indicates a significant difference between the treatment and control groups.

Survival and growth performance

SR, WGP, LGP, and SGR of treatment shrimp were significantly lower ($p = 0.001$, $p = 0.024$, $p = 0.031$, and $p = 0.042$, respectively) compared with control shrimp (Table 2).

Hepatopancreatic digestive enzyme activities

Protease activity of treatment shrimp was significantly lower ($p = 0.036$) compared with control shrimp; however, lipase activity was significantly higher ($p < 0.001$) in treatment versus control shrimp (Fig. 3). No significant difference was observed in amylase activity ($p = 0.210$) and cellulose activity ($p = 0.376$) between the two groups (Fig. 3).

Hepatopancreatic antioxidant status

MDA content, SOD activity, and CAT activity in the hepatopancreas of treatment shrimp were significantly higher ($p = 0.006$, $p < 0.001$ and $p = 0.035$, respectively) compared with control shrimp; however, GSH content, GPx activity, and GR activity in treatment shrimp were significantly lower ($p < 0.001$, $p = 0.015$, and $p = 0.001$, respectively) than control shrimp (Figs 4, 5). Meanwhile, no significant difference was observed in GST activity ($p = 0.087$) between the two groups (Fig. 5).

Hepatopancreatic metabolic gene expression

Relative expression of *p70s6k*, *4ebp1*, *GOT*, and *GPT* mRNA of treatment shrimp were significantly higher ($p = 0.003$, $p < 0.001$, $p = 0.035$, and $p = 0.005$, respectively) compared with control shrimp (Fig. 6). However, no significant difference was observed in the relative expression of *GST* mRNA ($p = 0.104$) between the two groups (Fig. 6).

Hepatopancreatic histology structure

Histology structure of 30 hepatopancreases in control shrimp appeared consistent look, and a representative figure (Fig. 7a) was chosen. Meanwhile, histology structure of 30 hepatopancreases in treatment shrimp also appeared consistent look, and a representative figure (Fig. 7b) was chosen. Compared with the control shrimp (Fig. 7a), histology structure of hepatopancreas in treatment shrimp showed remarkable change, including disorganized tubules, blurred boundaries, decreased or disappeared B, R and E cells, injured connective tissue between liver tubule, infiltrated hemocytes, narrowed lumen, and vacuolization (Fig. 7b).

Discussion

Previous studies (Schuler *et al.*, 2010; Cheng *et al.*, 2013; Zhang *et al.*, 2015) revealed the physiological effects of isolated and combined ammonia and nitrite on crustacean, the experimental nitrite and ammonia concentrations in aquaculture water were all achieved by adding dissolved $NaNO_2$ and NH_4Cl, resulting in animals being exposed to steady-state concentrations of ammonia and nitrite. However, the present study adopted a new approach, which showed that ammonia and nitrite were accumulated constantly in the treatment tanks, and that concentrations of both were significantly higher in the seawater of treatment group than control group after a certain period of time, presumably the result of the accumulation and breakdown of unconsumed food, faeces and excretion by the treatment shrimp (Tacon *et al.*, 2002; Chen *et al.*, 2012; Ren *et al.*, 2015). Therefore, this approach was a more accurate representation of
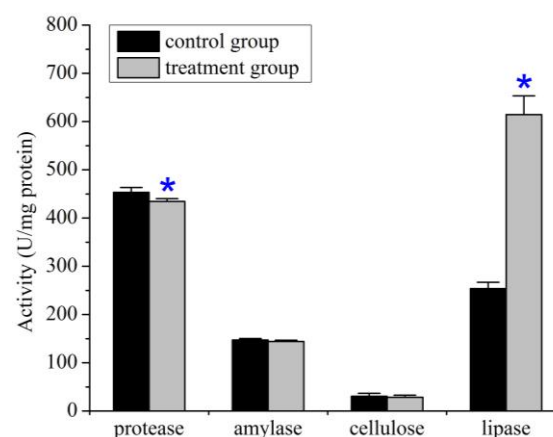

Fig. 3 Protease, amylase, cellulose, and lipase activities in the hepatopancreas of shrimp after the 33-d experiment. *Indicates a significant difference between the treatment (blue circles) and control (black circles) groups. Lipase activity increased significantly in the treatment group versus control group; protease activity decreased significantly in the treatment group versus control group.
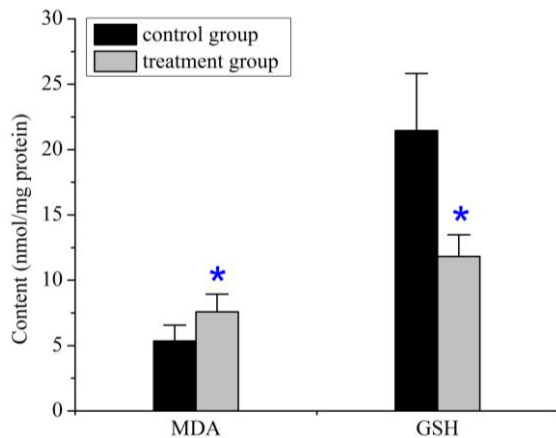
Fig. 4 MDA and GSH contents of the hepatopancreas of shrimp after the 33-d experiment. *Indicates a significant difference between the treatment (blue circles) and control (black circles) groups. MDA content increased significantly in the treatment group versus control group; GSH content decreased significantly in the treatment group versus control group.
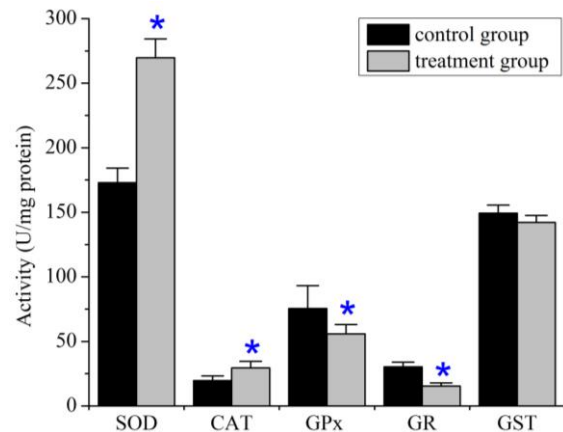

Fig. 5 SOD, CAT, GPx, GR, and GST activities in the hepatopancreas of shrimp after the 33-d experiment. *Indicates a significant difference between the treatment (blue circles) and control (black circles) groups. SOD and CAT activities increased significantly in the treatment group versus control group; GPx and GR activities decreased significantly in the treatment group versus control group.

the toxic mechanism of ammonia and nitrite accumulation caused by shrimp excreta and metabolic waste and organic detritus in intensive aquaculture system on shrimp. Though changes in water quality within the outdoor zero-water exchange culture system included accumulated total nitrogen and total phosphorus, decreased pH, and increased microorganism over the course of the 56-day experimental test period (Tacon *et al.,* 2002), we would like to focus more on ammonia and nitrite accumulation. On the one hand, ammonia and nitrate are the main toxic product of protein catabolism in the aquatic system, which are generated mainly in the mineralization process of organic wastes such as unconsumed feed and feces (Chen *et al.*, 1989; Cheng *et al.*, 2013; Ren *et al.*, 2015); on the one hand, ammonia and nitrate can increase the concentration of hydrogen ions in aquatic ecosystem, resulting in acidification (Camargo and Alonso, 2006), and are essential, life-sustaining, nitrogen-containing compounds used by many aquatic microorganisms (Pinto *et al.*, 2016). Thus, ammonia and nitrite accumulation might be the primary pollution in intensive aquaculture system.

Previous studies on the chronic physiological effects of isolated and combined ammonia and nitrite on crustaceans were limited (Miranda-Filho *et al.*, 2009); it was shown that survival of juvenile pink-shrimp *Farfantepenaeus paulensis* was ≥ 90 % under ammonia concentrations (0.016 - 0.287 mg/L) after 75 days, whereas growth (carapace length and wet body mass) was reduced at ammonia concentration as low as 0.033 mg/L. By contrast, the present study indicated that ammonia and nitrite accumulation had significantly higher lethality but

similar inhibitory effects on physiological processes involved in body mass increase in treatment shrimp; this could be because exposure to high levels of ambient ammonia induces increased energy expenditure that regulates osmotic and ionic stress (Young-Lai *et al.*, 1991; Spaargaren 1982; Chen and Cheng 1993b, c), resulting in reduced growth; meanwhile, there might be a resulting synergistic effect of ammonia and nitrite accumulation in that energy expenditure for osmoregulation in treatment shrimp according to previous report (Cheng *et al.*, 2013), which led to higher lethality in the present study.

The activities of digestive enzymes in crustacea have a central role in their nutritional physiology, which can supply nutrition requirements and metabolic energy for body, and directly or indirectly regulate growth (Van Wormhoudt 1973; Lee *et al.*, 1984; Lovett and Felder 1990). As a cheaper, primary, and immediate source of energy for organisms under stress conditions (Tseng and Hwang 2008; Wang *et al.*, 2012), carbohydrate nutrition might have an essential role in the osmoregulation of shrimp because carbohydrates are often included in their diet (Cruz-Suarez *et al.*, 1994; Cuzon *et al.*, 2004). However, the results of the present study showed that ammonia and nitrite accumulation had no effects on amylase and cellulose activity in the hepatopancreas of treatment shrimp, which was similar to previous findings for the activity of digestive carbohydrate enzymes under salinity stress (Li *et al.*, 2008; Wang *et al.*, 2014). Thus, we speculate that digestive carbohydrate enzymes were already saturated with ammonia and nitrite accumulation, and that more dietary carbohydrate content could contribute to providing

energy for increased osmoregulation. The observed changes in the present study were characterized by a marked reduction in protease activity paralleled by a significant increase in lipase activity in the hepatopancreas of treatment shrimp, which might suggest the increased use of lipid as an energy source to respond to the increased osmotic and ionic stress, but the reduced use of protein to limit the internal accumulation of nitrogenous waste products induced by ammonia and nitrite accumulation (Miranda-Filho *et al.*, 2009; Pinto *et al.*, 2016). Meanwhile, the fact that the change of protease activity in the hepatopancreas was consistent with weight gain in shrimp supported previous review that lower protease activity contributes to reduced growth (Xu *et al.*, 1987, 1995). Therefore, ammonia and nitrite accumulation might disrupt the synthesis and excretion of digestive enzymes in the hepatopancreas, resulting in reduced metabolic energy for survival and growth performance.

In terms of the antioxidant status, assaying antioxidant enzymes can indicate the antioxidant status of organisms and serve as a biomarker of oxidative stress (Kohen and Nyska, 2002). SOD and CAT provide a first line of defense against reactive oxygen species (ROS) because SOD catalyzes the conversion of $O_2^{\cdot -}$ to H_2O and H_2O_2, and the latter is further degraded into H_2O and O_2 by CAT, GPx, GSH, GR, GST, and multiple different peroxidases (Dandapat *et al.*, 2003; Cheng *et al.*, 2006; Yeh *et al.*, 2009; Sinha *et al.*, 2015). Generally, higher SOD and CAT activities indicate that there are more ROS present that need to be metabolized (Chien *et al.*, 2003; Ross *et al.*, 2001). Therefore, significantly higher SOD and CAT activities in the hepatopancreas of treatment shrimp might indicate that ammonia and nitrite accumulation resulted in the increased accumulation of ROS. Our results also found that the lower GPx activity was in contrast to the increase in CAT activity in the hepatopancreas of treatment shrimp, which was consistent with previous reports, given that GPx and CAT function differently under stress conditions (Dandapat *et al.*, 2003; Zhang *et al.*, 2015). GPX catalyzes the reduction of H_2O_2 and a variety of lipid peroxides by using GSH, which is further oxidized to GSSH. GSSH is re-reduced to GSH via GR in a reduced form of nicotinamide-adenine dinucleotide phosphate-dependent reaction. The parallel augmentation of GR with GPX activity in liver tissue requires the proficient renewal of GSH (Sinha *et al.*, 2015). Therefore, significantly lower GR and GPx activities and GSH concentrations in the hepatopancreas of treatment shrimp might indicate that the hepatopancreas had lost its function to renew its GSH capacity as a result of toxicity effect of ammonia and nitrite accumulation. In addition, MDA is the final product of lipid peroxidation, providing direct evidence of the toxic processes resulting from ROS metabolism (Doyotte *et al.*, 1997; Lushchak, 2011). Thus, the significantly higher MDA concentration in the hepatopancreas of treatment shrimp might further reveal that accumulated ROS might be the substance, which caused abnormal hepatopancreas function. GST activity remained unaltered in the hepatopancreas of treatment shrimp, suggesting its limited role in ammonia and nitrite
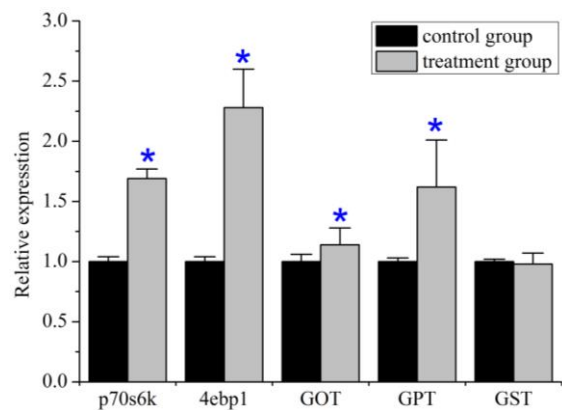

Fig. 6 Relative expressions of *p70s6k*, *4ebp1*, *GOT*, *GPT*, and *GST* mRNA in the hepatopancreas of shrimp after the 33-d experiment. * Indicates a significant difference between the treatment (blue circles) and control (black circles) groups. Relative expressions of *p70s6k*, *4ebp1*, *GOT* and *GPT* mRNA increased significantly in the treatment group versus control group.

detoxification. Therefore, higher SOD and CAT activities were not sufficient to scavenge the accumulated ROS resulted from ammonia and nitrite accumulation. In contrast, previous studies reported that SOD and CAT activities significantly decreased in response to either ammonia or nitrite, although the MDA concentration significantly increased (Liu *et al.*, 2004; Wang *et al.*, 2004; Liang *et al.*, 2016). We speculate the main factor that might have caused this discrepancy in results might be the exposure to incessant ammonia and nitrite accumulation process rather than steady-state concentrations of ammonia and nitrite immediately in the present study.

GOT catalyzes an important reaction in the molecular rearrangement involving amino acids linked to the citric acid cycle at two points (oxaloacetic and ketoglutaric acids); this is the most important mechanism for introducing reduction equivalents into mitochondria (Urich, 1994). GPT predominates in organs where intensive glycogenesis occurs, such as the liver (Urich, 1994; De la Torre *et al.*, 2000). High levels of GOT and GPT in the liver can result in excess liberation of these enzymes into the blood, which suggests that liver cells are damaged (Vaglio and Landriscina, 1999; Kim and Kang, 2004; Wu *et al.*, 2008). The significantly higher relative expression of *GOT* and *GPT* mRNA in the hepatopancreas of treatment shrimp was similar to results reported in a previous study (Jiang *et al.*, 2014). Therefore, this result might indicate that the hepatopancreas in treatment shrimp was damaged, which might be caused by accumulated ROS, resulting in abnormal hepatopancreas function. Translation initiation is a limiting step in protein synthesis (Holz *et al.*, 2005), and major effector of cell growth and proliferation (Hay and Sonenberg, 2004), key steps in processes involved in the growth response (Anthony *et al.*, 2001).
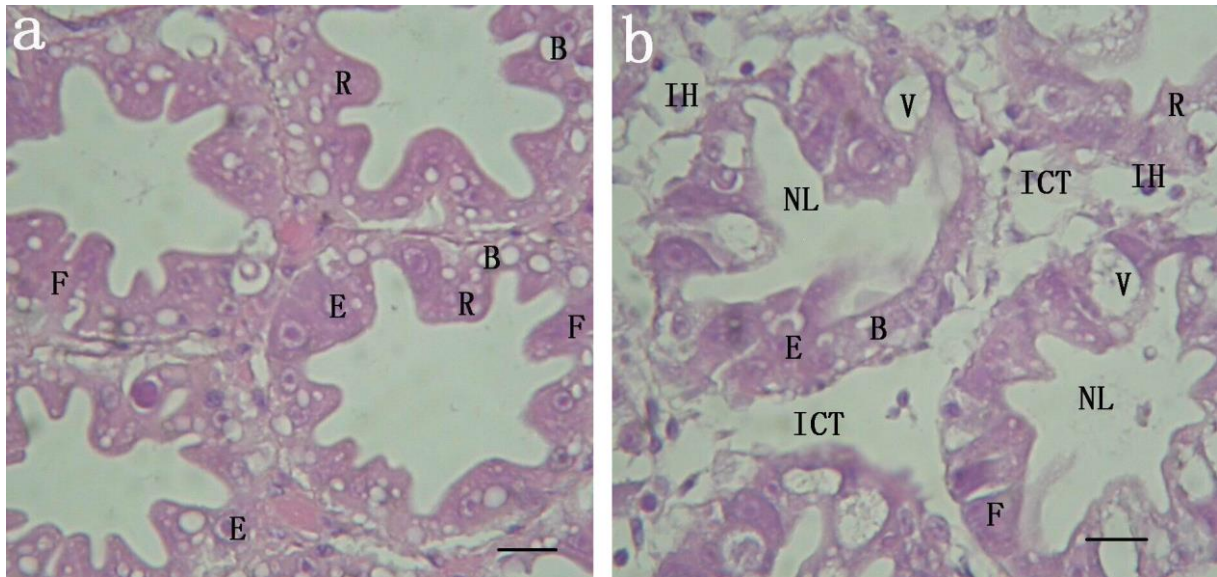
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Fig. 7 Photomicrographs of the hepatopancreas of shrimp from control group (a) and treatment group (b) after the 33-d experiment. Epithelial cell: E cell; R cell; F cell; B cell. The pathologies observation: injured connective tissue between liver tubule (ICT); infiltrated hemocytes (IH); narrowed lumen(NL); vacuolization(V) . H&E stain (×400), scale bar =100 μm. Histology structure of hepatopancreas in treatment shrimp showed remarkable change versus control group.

Target of rapamycin (TOR) promotes cap dependent translation initiation through inactivation of its downstream effector 4ebp1 or phosphorylation of p70s6k (Wullschleger *et al.*, 2006). Thus, significantly higher relative expression of *4ebp1* mRNA in the hepatopancreas of treatment shrimp induced by abnormal hepatopancreas function would inhibit translation initiation, which might result in reduced growth performance. Though significantly higher relative expression of *p70s6k* mRNA appeared in the hepatopancreas of treatment shrimp, p70s6k might not be phosphorylated correspondingly due to abnormal hepatopancreas function, and further investigation is needed in this area. Evidence suggests that the expression level of *GST* mRNA is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals (Mearns *et al.*, 2006; Zhou 2009); however, the relative expression of *GST* mRNA remained unaltered in the hepatopancreas of treatment shrimp, which is consistent with the above test result that GST activity was unaltered; thus, this is not a useful indicator of the response of shrimp to ammonia and nitrite accumulation.

All the remarkable change in the hepatopancreas of treatment shrimp corroborated that this organ was damaged by accumulated ROS, which would lead to detrimental effects on any related physiological functions. B and R cells are the main sites for the synthesis of digestive enzymes and nutrient reserves, respectively (Al-Mohanna and Nott 1986, 1987, 1989; Caceci *et al.*, 1988). E cells, as undifferentiated embryogenic cell, can supplement other types of cell by dividing and differentiating into other liver cells (Hong *et al.*, 2007). Therefore, reduced or disappeared B, R and E cells might be the essential reason, which disrupted the synthesis and excretion of digestive enzymes in the hepatopancreas of treatment shrimp, resulting in reduced metabolic energy for survival and growth performance.

In conclusion, ammonia and nitrite accumulation caused by accumulated waste in aquaculture tank could damage hepatopancreas in the *L. vannamei*, resulting from accumulated ROS; thus, the damaged hepatopancreas would disrupt synthesis and excretion of digestive enzymes and metabolic gene expression, resulting in reduced survival and growth performance. Therefore, ammonia and nitrite accumulation may significantly impact shrimp production in intensive aquaculture system, farmers would be aware of how better to manage the aquaculture system in terms of ammonia and nitrite disposal.

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