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RESEARCH REPORT

Effect of cyclic serious/medium hypoxia stress on the survival, growth performance and resistance against *Vibrio parahemolyticus* of white shrimp *Litopenaeus vannamei*

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Abstract

The effect of cyclic serious/medium hypoxia stress in the presence of hypoxia environment on shrimp is not well explored. The survival, growth performance, osmoregulation gene expression, digestive enzyme activity, histology, and resistance against *Vibrio parahemolyticus* of the white shrimp *Litopenaeus vannamei* reared under conditions of cyclic serious/medium hypoxia (CSMH, 0.8 - 3.5 mg/L) versus normoxia (N, 6.4 - 7.5 mg/L) were investigated during an experimental period of 28 days. Consequently, the cumulative mortality rate of CSMH shrimp increased continuously. The weight gain percentage and length gain percentage of CSMH shrimp decreased continuously. The *Na^+/K^+-ATPase*, cytoplasmic carbonic anydrase (*CAc*), and glycosyl-phosphatidylinositol-linked carbonic anhydrase (*CAg*) transcripts in the gill of CSMH shrimp increased first and then returned to normal or decreased. The amylase, lipase, and trypsin activities in the hepatopancreas of CSMH shrimp decreased continuously. The hepatopancreas of CSMH shrimp showed histopathological lesions in a time-dependent manner. In the *V. parahaemolyticus* immersion challenge test, the mortality rate of CSMH shrimp increased continuously. Therefore, cyclic serious/medium hypoxia could reduce survival and growth performance of *L. vannamei* during long-term exposure, which was resulted from broken osmoregulation mechanism of the gill, and suppressed digestive enzyme activities of the hepatopancreas caused by growing histopathological lesions. Meanwhile, cyclic serious/medium hypoxia would probably lead to outbreak of infectious diseases in the shrimp farming.

Key Words: *Litopenaeus vannamei*; cyclic serious/medium hypoxia; mortality; growth performance; histology; *Vibrio parahaemolyticus*

Introduction

Hypoxia has been rising in marine ecosystems since the 1960s (Diaz and Rosenberg, 2008). Biological and non-biological factors, such as photosynthesis, respiration, eutrophication, tidal cycles, weather conditions, global warming, stratification of the water column, and so on, often cause the occurrence of chronic hypoxia and cyclic hypoxia/normoxia in coastal and estuarine environments (Rabalais *et al*., 2002, 2007; Chen *et al*., 2007; Stramma *et al*., 2008; McAllen *et al*., 2009; Tyler *et al*., 2009). Meanwhile, the bottom layer of pond waters, where shrimp spend most of their time, may become hypoxic or even anoxic, due to respiration of the organisms and decomposition of accumulated organic matter such as unconsumed feed and feces, especially at night (Cheng *et al*., 2003). This occurs particularly in rearing ponds that do not use aerators, in which dissolved oxygen (DO) concentrations may reach critical values, and shrimp can be exposed to hypoxia as DO levels drop from 3 mg/L to less than 1 mg/L (Chantal *et al*., 2008).

Most aquatic species can maintain an adequate oxygen uptake at DO above 5 mg/L (Gray *et al*., 2002; Vaquersunyer and Duarte, 2008; Diaz and Breitburg, 2009). Below their specific optimum DO level, aquatic species display physiological and behavioral adaptations to maintain satisfactory oxygen uptake rates (Morris and Taylor, 1985;

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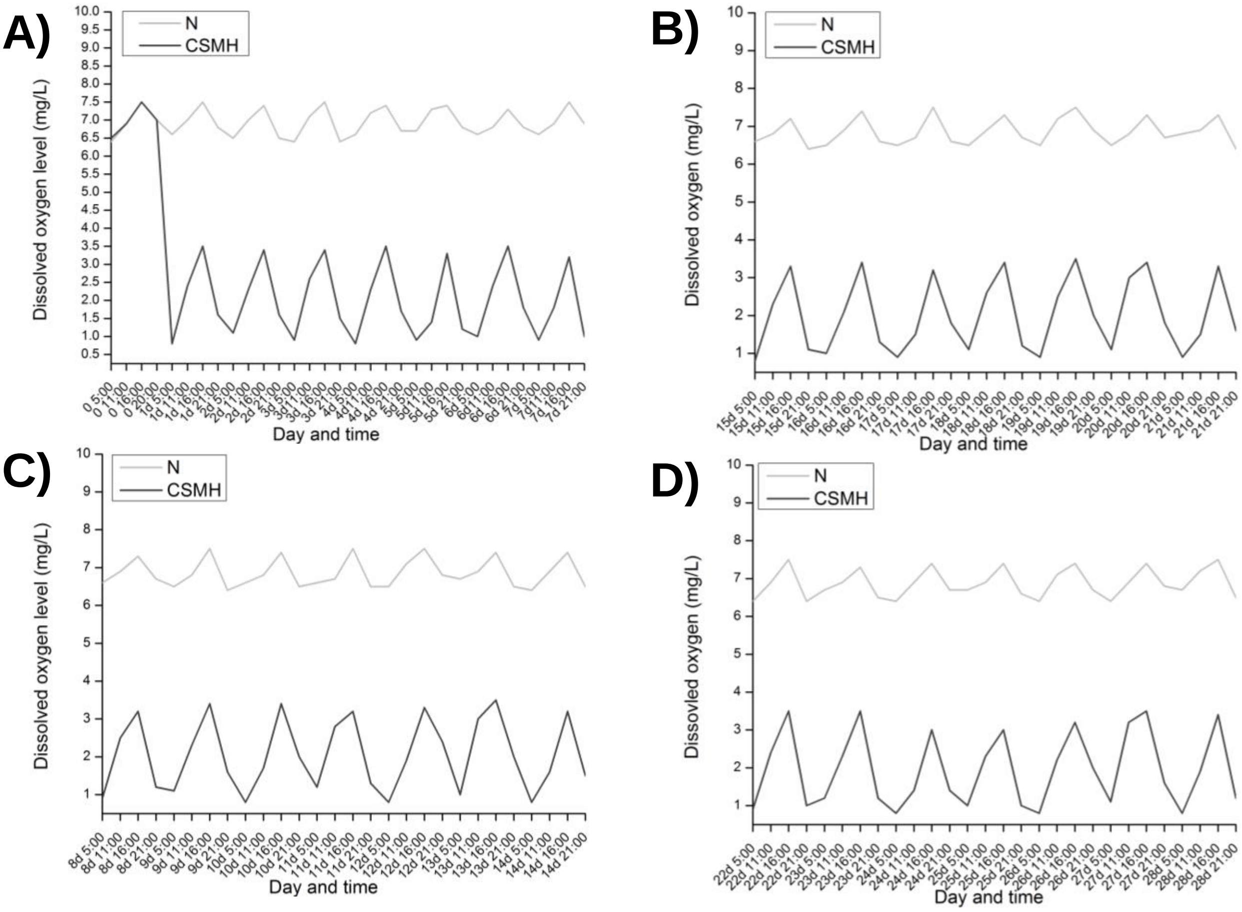

Fig. 1 DO levels in the seawater of tanks during 0-7 d (A), 8-14 d (B), 15-21 d (C), and 22-28 d (D).

Wannamaker and Rice, 2000; Bernatis *et al*., 2007). Specific adaptations vary depending on the severity and duration of hypoxia. Previous studies have investigated the observable effects of: acute hypoxia on survival, carbohydrate metabolism, immune response, apoptotic processes, comparative proteomics, and resistance against viruses (Cota-Ruiz, *et al*., 2015; Martínez-Quintana *et al*., 2016; Wei *et al*., 2016; Felix-Portillo *et al*., 2016; Cheng *et al*., 2002; Sun *et al*., 2016; Lehmann *et al*., 2016); chronic hypoxia on growth, reproductive expression, aerobic and anaerobic metabolism, antioxidant response, gene expression profile, and mitochondrial expression (Coiro *et al*., 2000; Brouwer *et al*., 2007, 2008; Li and Brouwer, 2009; Dupont-Prinet *et al*., 2013; Pillet *et al*., 2016); cyclic hypoxia/normoxia on gene expression profile, growth, and reproductive expression (Coiro *et al*., 2000; Brown-Peterson *et al*., 2011; Li and Brouwer, 2013); and hypoxia and reoxygenation on DNA damage and oxidative and antioxidant states of shrimp (Parrilla-Taylor and Zenteno-Savín, 2011; Li *et al*., 2016; García-Triana *et al*., 2016). However, we know little about the effects of cyclic serious/medium hypoxia that may occur as part of a subsistence process in shrimp in an aquaculture system.

The white shrimp, *Litopenaeus vannamei* can suffer fluctuations of oxygen levels and experience hypoxia (Parrilla-Taylor and Zenteno-Savín, 2011), as they can convert aerobic into anaerobic metabolism to produce energy under these circumstances (Soñanez-Organis *et al*., 2012). In the present study, we would evaluate the survival, growth performance, and disease outbreak's possibility of *L. vannamei* under cyclic serious/medium hypoxia and analyze related mechanism in an aquaculture system. The osmoregulatory capacity measurement was confirmed as a convenient tool to monitor the physiological condition and the effect of stress in crustaceans (Charmantier and Soyez, 1994), and digestive enzyme analysis is an excellent tool for the physiologists as the animal grows and matures (Lee *et al*., 1984). Therefore, we investigated: (1) mortality and growth performance; (2) Na$^+$/K$^+$-ATPase, cytoplasmic carbonic anhydrase (CAc), and glycosyl-phosphatidylinositol-linked carbonic anhydrase (CAg) gene expression of the gill; (3) trypsin, amylase, and lipase activity of the hepatopancreas; (4) histology of the hepatopancreas; (5) resistance against *Vibrio parahemolyticus* in *L. vannamei* reared under conditions of normoxia and cyclic serious/medium hypoxia during a 28 d experiment.

Table 1 Primers for the genes encoding *Na+/K+-ATPase*, *CAc*, *CAg*, and *β-actin* in shrimp

Gene	Nucleotide sequence (5'→3')	Amplicon
Na^+/K^+-ATPase	F- GTATCCATCCACGAGACTGAG R- AAGGTAGGCATTGTTGAAAGC	135 bp
CAc	F- CCCGTGCGACAGTAACCTAA R- GGCTCCTCGAAGACAATCCA	147 bp
CAg	F- ACGAGCAATGTGGA R- GTGGAACTGAGCGAAGATGT	126 bp
β-actin	F- GCCCATCTACGAGGGATA R-GGTGGTCGTGAAGGTGTAA	121 bp

Materials and Methods

Experimental shrimp

Nine hundred healthy juvenile *Litopenaeus vannamei* that were of similar size (mean weight 1.20 ± 0.03 g) were obtained from the Ruizi Seafood Development Co. Ltd. (Qingdao, China), where the experiment was also conducted. Only shrimp in the intermoult stage were used for this study. They were placed in six 640-L cylindrical tanks with net cover (*N* = 150 per tank), and each 640-L cylindrical tank contained 500 L of aerated seawater (DO 6.4 - 7.5 mg/L). The initial seawater was unfiltered and had the following characteristics: pH 8.0 - 8.4, salinity 30 - 31 ‰, total ammonia 0.022 - 0.038 mg/L, nitrite 0.015 - 0.032 mg/L, and nitrate 0.120 - 0.205 mg/L at 28 - 32 °C. The shrimp were acclimated for 3 weeks under a ‘photoperiod’ (12 h light:12 h dark). They 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 am, 11:00 am, and 7:00 pm, with a daily feeding rate that was 10 % of the weight of the shrimp. Unconsumed feed and feces were removed with a siphon tube and 50 % of the seawater was replaced once daily. Unfiltered seawater was prepared in two 1000-L cylindrical tanks to use for daily exchange.

Preparation of Vibrio parahaemolyticus

The pathogenic strain *V. parahaemolyticus* ATCC 17802 was donated by the First Institute of Oceanography, State Oceanic Administration, People’s Republic of China. The strain was cultured in tryptic soy broth (supplemented with 2 % NaCl, Difco) for 24 h at 28 °C, and then centrifuged at 7155*g* for 20 min at 4 °C (Yeh and Chen, 2009). The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution at $1×10^8$ colony forming units (cfu) mL^{-1} as the stock bacterial suspension for the resistance test.

Experimental design for the DO challenge

Following acclimation, we randomly divided the six 640-L cylindrical tanks into two groups. Each group of three 640-L cylindrical tanks constituted three repetitions of either (1) normoxia (N) or (2) cyclic serious/medium hypoxia (CSMH). The experiments were conducted over 28 d, and the photoperiod, feeding conditions, water exchange, and waste disposal were handled in exactly the same way as during the acclimation period. During each day, the N group was aerated enough to generate a DO level of 6.4 - 7.5 mg/L automatically; however, the CSMH group was aerated sufficiently to generate a DO level of only 0.8 - 3.5 mg/L automatically, and was also characterized by having the lowest DO in the early morning and the highest DO in the afternoon with exposure to a DO of 0.8 - 2 mg/L for 16 h and 2 - 3.5 mg/L for 8 h during every 24-h cycle (Figs 1A-D). The DO levels were monitored four times a day using a YSI model 55 DO meter (YSI Incorporated, Ohio, USA) during the experimental period.

Measurement of mortality and growth performance and sampling procedure

The number of dead shrimp in 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. Twenty shrimp from each tank were randomly selected before the experiment (day 0) and on days 7, 14, 28 during the experimental period and replaced after their weights and lengths were measured. Mortality and growth performance was evaluated in terms of their cumulative mortality rate (CMR), weight gain percentage (WGP), and length gain percentage (LGP) based on the following standard formulae:

CMR (%) = 100 × (cumulative dead shrimp number)/(initial shrimp number);

WGP (%) = 100 × (final weight-initial weight)/initial weight;

LGP (%) = 100 × (final length-initial length)/initial length.

Three shrimp were randomly selected and removed from each tank before the experiment (day 0) and on days 7, 14, 28 during the experimental period, and the hepatopancreas and gill of each shrimp were collected using sterilized scissors and forceps. Three hepatopancreases were immediately ground in liquid nitrogen and stored at -80 °C prior to the digestive enzyme activity assay; three gills were immediately placed into RNAlater (Applied Biosystems, Austin, TX, USA) and stored at -20 °C until RNA isolation and osmoregulation gene expression analysis. Another three shrimp were randomly selected and removed from each tank before the experiment (day 0) and on days 1, 3, 7, 14, 28 during the experimental period; the hepatopancreas of each shrimp were also collected using sterilized scissors and forceps, then immediately fixed in 10 % formaldehyde to be used for histological study.

Assays of osmoregulation gene expression in the gill

RNA was extracted from the gill using an RNA fast extraction kit according to the manufacturer's protocol (Fastagen, Shanghai, China). RNA solution (6 μL) was mixed with homogenates of the three gills from each tank (2 μL RNA solution per gill). This 6-μL RNA solution was employed 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 relative expressions of osmoregulation genes encoding *Na^+/K^+-ATPase*, *CAc*, and *CAg* were evaluated by relative quantitative real-time PCR (qPCR) using TransStar Top Green qPCR Supermix according to the manufacturer's protocol (TransGen Biotech Co., Ltd). Each of the aforementioned osmoregulation genes were analyzed with three replicates of each sample. *β-Actin* was selected as a reference gene and specific primers were used for each gene (Table 1). 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 melting 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).

Assays of digestive enzyme activity in the hepatopancreas

Approximately 100 mg of hepatopancreas tissue was dissected from a single hepatopancreas. The three hepatopancreas tissues from 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 collected for the assays. Digestive enzyme activities, including trypsin activity, amylase activity, and lipase activity, were evaluated using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The activities of each of the above digestive enzymes were analyzed with three replicates of each sample. Activities were expressed as a relative unit per milligram of soluble protein (U/mg protein).

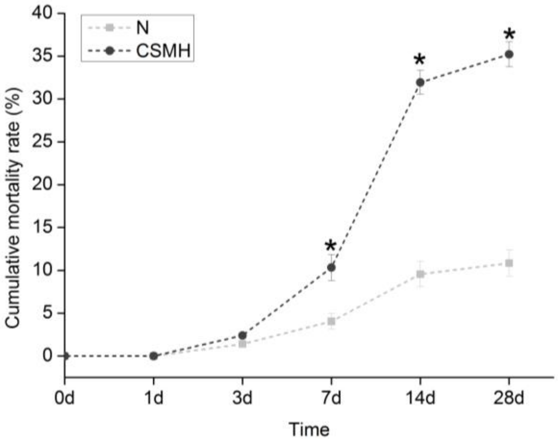

Fig. 2 Cumulative mortality rates of shrimp during the experimental period. Each bar represents the mean value from three repetitions with standard error (SE). *Indicates a significant (p <0.05) difference compared with the N group.

Histology assays on the hepatopancreas

The fixed hepatopancreas tissues were dehydrated using 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).

Resistance of shrimp to Vibrio parahaemolyticus

Shrimp from the two groups were evaluated before the experiment (day 0) and on days 7, 14, 28 during the experimental period. At each evaluating point, there were four treatment groups (two challenged and two unchallenged treatment groups). 20 shrimp and 20 L of seawater from each 640-L cylindrical tank were transferred to two 40-L cylindrical tanks, 10 shrimp and 10 L of seawater per 40-L cylindrical tank. Challenge testing was conducted by adding 100 mL of a stock bacterial suspension to the 20 L of seawater, resulting in immersion of the shrimp at 5×10^5 cfu/mL, while unchallenged testing required no further processing. Other culture conditions during these treatments were handled in exactly the same way as the 640-L cylindrical tanks, except that no seawater was exchanged. Dead shrimp were recorded for each treatment after 2 days. The mortality rate (MR) was expressed as: MR (%) = 100 × (dead shrimp number)/(initial shrimp number).

Statistical analysis

The data are all presented as mean ± standard error (SE). Statistical analysis was performed using SPSS (version 17.0) (IBM, Armonk, NY, USA), and the *t*-test was used to analyze differences between the two experimental groups. The significance level was $p < 0.05$. All images were generated with Origin 8.6 software (OriginLab, MA, USA).

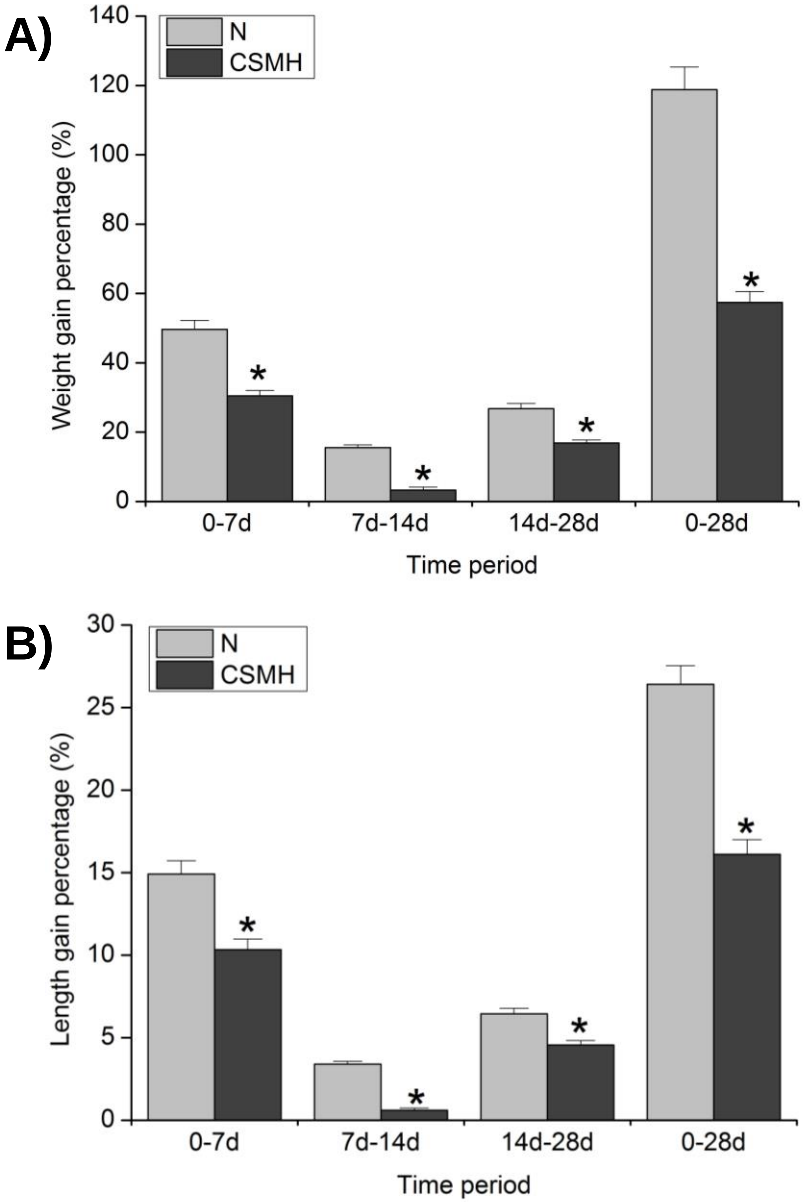

Fig. 3 Weight gain percentages (A) and length gain percentages (B) of shrimp during the experimental period. See Fig. 2 for statistical information.

Results

Mortality of shrimp under cyclic serious/medium hypoxia

Compared with N shrimp, the CMR of CSMH was not significantly ($P>0.05$) different on days 1, 3, but significantly ($p < 0.05$) higher on days 7, 14, 28. Specifically, the CMR of CSMH shrimp showed an increasing trend with time until 36.5 % on days 28 (Fig. 2).

Growth performance of shrimp under cyclic serious/medium hypoxia

The WGR and LGR of CSMH versus N shrimp significantly ($p < 0.05$) decreased during the periods 0-7d, 7-14d, 14-28d, and 0-28d (Fig. 3).

Osmoregulation gene expression of shrimp under cyclic serious/medium hypoxia

The *Na*$^+$/*K*$^+$-*ATPase* and *CAg* transcripts of CSMH versus N shrimp significantly ($p < 0.05$) increased on days 7, then significantly ($p < 0.05$) decreased on days 14, 28 (Figs 4A, C); the *CAc* transcripts of CSMH versus N shrimp significantly ($p < 0.05$) increased on days 7, then returned to normal on days 14, 28 (Fig. 4B).

Digestive enzyme activity of shrimp under cyclic serious/medium hypoxia

The amylase and lipase activities of CSMH shrimp were not significantly ($p > 0.05$) different from N shrimp on days 7, 14; and 7, respectively (Figs 5A, B); but the amylase, lipase, and trypsin activities of CSMH versus

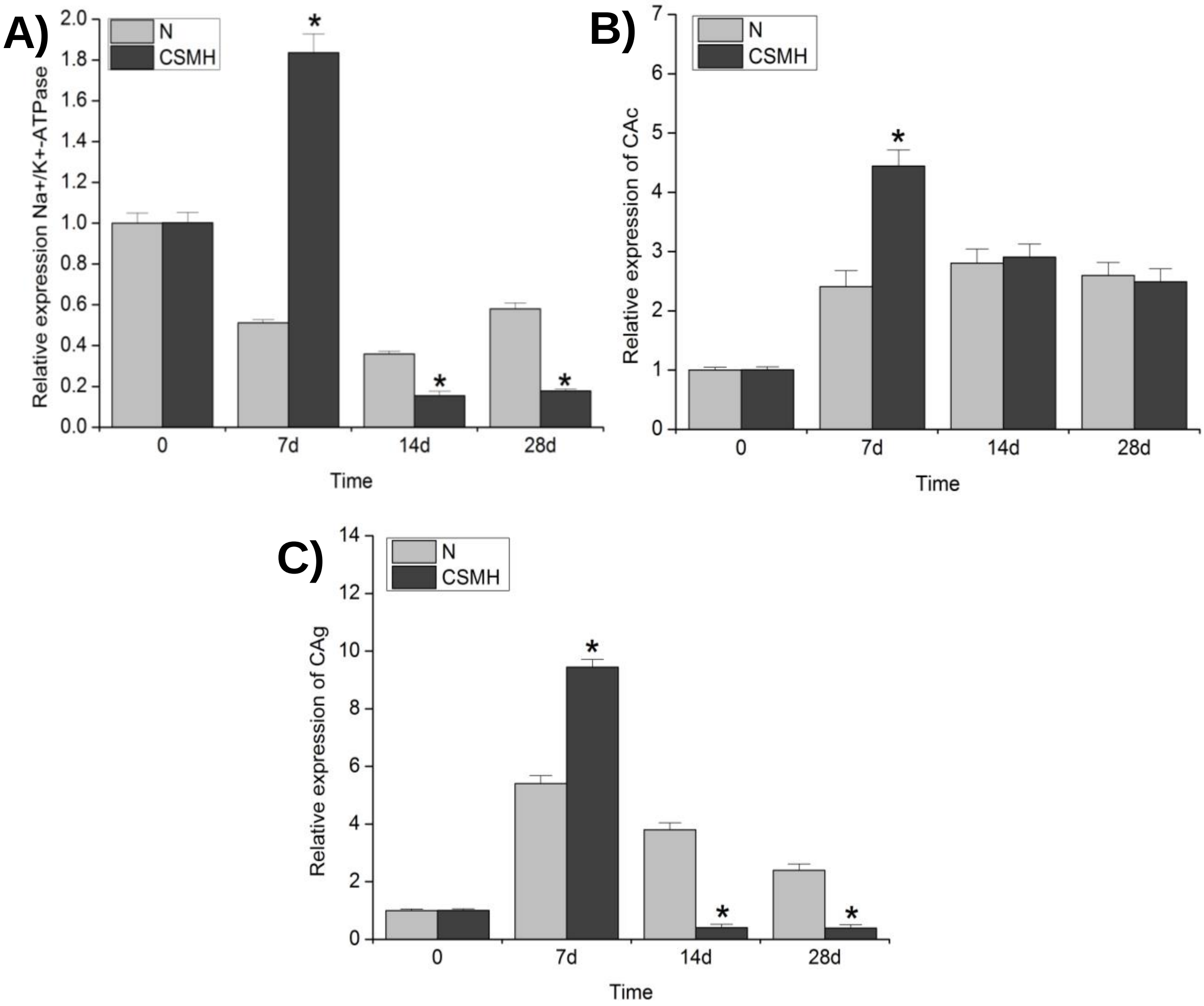

Fig. 4 *Na^+/K^+-ATPase* transcription (A), *CAc* transcription (B), and *CAg* transcription (C) of shrimp during the experimental period. See Fig. 2 for statistical information.

N shrimp significantly ($p < 0.05$) decreased on days 28; 14, 28; and 7, 14, 28, respectively (Figs 5A-C).

Histology of shrimp under cyclic serious/medium hypoxia

In the hepatopancreases of CSMH versus N shrimp, stellate tubule lumen appeared dilatation on day 1, some vacuoles appeared on days 3, full-scale vacuoles generated on days 7, these dispersive vacuoles gathered into big vacuoles on days 14, and ruptured to make epithelial cell layer thinner on days 28 (Figs 6A, B).

Resistance against Vibrio parahaemolyticus *of shrimp under cyclic serious/medium hypoxia*

All unchallenged shrimp that had been reared at different DO levels survived. In contrast, deaths occurred among challenged shrimp. The MR of CSMH versus N shrimp significantly ($p < 0.05$) increased on days 7, 14, 28 (Fig. 7).

Discussion

The course of hypoxia is known to influence the mortality and behavior of crustaceans (Llansó, 1991; Alexandra *et al*., 2010). Many researchers have studied the effects of hypoxia on the survival and growth of shrimp. Avault (1986) reported that *Macrobrachium rosenbergii* were stressed when DO fell below to 2 mg/L, and 0.5 mg/L is normally lethal. Moreover, the survival of penaeid shrimp including *Penaeus setiferus*, *Farfantepenaeus californiensis, vannamei*, and *M. rosenbergii* were not greatly affected by chronic hypoxia (1.5 mg/L< DO < 3 mg/L) (Rosas *et al*., 1998; Ocampo *et al*., 2000; Racotta *et al*., 2002; Cheng *et al*., 2003). However, Li and Brouwer (2013) pointed out that the weights and lengths of *Palaemonetes pugio* exposed to cyclic hypoxia/normoxia (1.05 - 8.87 mg/L DO) were significantly lower than for shrimp (6.34 mg/L DO) in their natural habitats. Coiro *et al*. (2000) found that

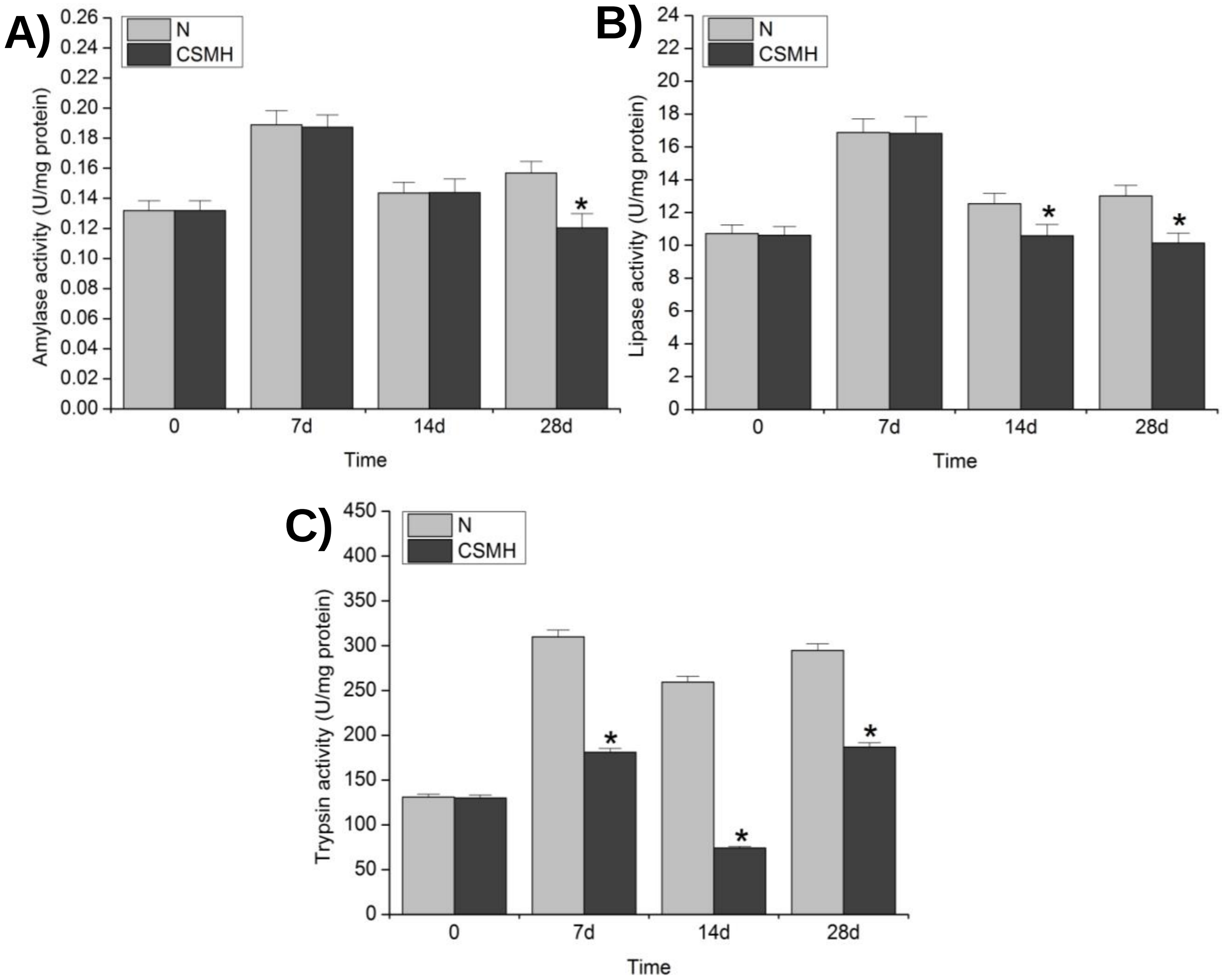

Fig. 5 Amylase activities (A), lipase activities (B), and trypsin activities (C) of shrimp during the experimental period. See Fig. 2 for statistical information.

chronic hypoxia (2 mg/L DO) exposure resulted in more serious growth impairment of larval *Palaemonetes vulgaris* than cyclic hypoxia/normoxia (2 - 8 mg/L DO) in their natural habitats. In the present study, the CMR of CSMH versus N shrimp increased continuously, and the WGP and LGP of CSMH versus N shrimp decreased continuously. These results were consistent with previous hypoxia reports on growth, but different from those on survival. It is well known that growth of an organism is often the sublethal endpoint used and has been shown to be a more sensitive indicator of low DO than survival (Morrison, 1971; Das and Stickle, 1993; Thursby *et al*., 1997), all current studies also proved that any form of hypoxia could cause growth impairment no matter whether they affected survival or not. As the ecological effects of hypoxia on the biota depend partly on their severity, duration and frequency and partly on the tolerance of the affected organisms to hypoxia (Diaz and Rosenberg, 1995; Modig and Ólafsson, 1998; Sagasti *et al*., 2001), the incessant impairment of both survival and growth performance in the present study indicated that cyclic serious/medium hypoxia might be more severe than previous reported hypoxia forms.

The hemolymph osmotic balance of invertebrates can be altered by environment changes (Cameron and Mangum, 1983; Truchot, 1983). Regulation of hemolymph osmotic pressure in crustacean mainly depends on inorganic ion and the concentrations of free amino acid, and the concentrations of inorganic ion are the most important contributors (Pan *et al*., 2007). Ion-regulation in crustacean is mostly accomplished by Na$^+$/K$^+$-ATPase, CA and other ion-transport enzymes in gill epithelium membrane (Morris, 2001). It has been reported that short-term (≤3d) ammonia exposure of *Penaeus chinensis*, *Macrobrachium nipponense*, *Portunus pelagicus*, and *Macrobrachium amazonicum* increased Na$^+$/K$^+$-ATPase activity (Lin *et al*.,1993; Wang *et al*., 2003; Romano and Zeng, 2010; Pinto *et al*., 2016), thus maintaining cell function and body fluid ammonia levels within a tolerable range (Weihrauch *et al*., 2004), while down-regulated of *Na$^+$/K$^+$-ATPase* transcription of *Metacarcinus magister* exposed to ammonia after 14 d was not a protective measure taken by the gill epithelium to prevent unwanted ammonia influxes, but may rather occur due to a toxic

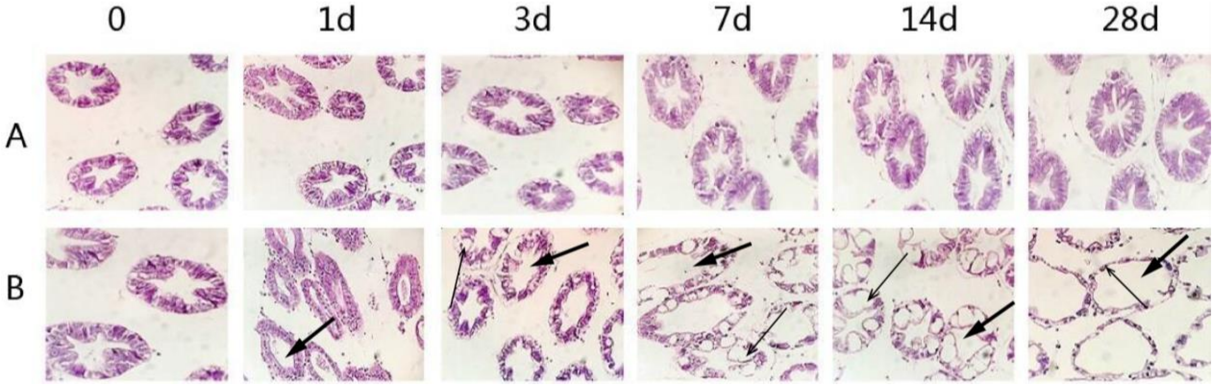

Fig. 6 Photomicrographs of the hepatopancreases of N shrimp (A) and CSMH shrimp (B). H&E stain (×400), scale bar = 100 μm. Thick arrow Indicates dilatated stellate tubule lumen; thin arrow Indicates vacuole.

effect of ammonia (Martin *et al*., 2011). Pan *et al*. (2016) also found that *CAc* and *CAg* transcription increased under short-term (2 d) low and high pH conditions in *Portunus trituberculatus*, to maintain the acid-base balance, while Wang *et al*. (2002) pointed out that a decrease in Na^+/K^+-ATPase activity resulted from impairment of the active transport mechanism for sodium ions was the primary cause of the deaths of *F. chinensis* after 14 d in acid and alkaline water. Henry *et al*. (2006) indicated that *CA* transcription in *carcinus maenas* under short-term (4d) low salinity conditions remained elevated as an adaptation mechanism, and began to decline by 7 d because of a breakdown in the mechanism of transport-related protein induction, resulting in more difficult low salinity adaptation. In the present study, the *Na^+/K^+-ATPase*, *CAc*, and *CAg* transcripts of CSMH versus N shrimp increased in the short term (7 d) , then returned to normal or decreased afterwards, which were similar to previous reports related to ammonia, pH, salinity stress. Therefore, cyclic serious/medium hypoxia might break osmoregulation mechanism in shrimp after long-term exposure, leading to incessant death.

Some investigations have been available on the effects of DO changes on the digestive enzymes in crustaceans, as they showed acclimation to low oxygen environment. For instance, Brown-Peterson *et al*. (2008) and Li and Brouwer (2013) found the transcriptional expression of papain-like cysteine proteinase in the hepatopancreas of *Palaemonetes pugio* were significantly up-regulated after exposure to cyclic hypoxia/normoxia, both in the natural habitat and 7 d in the laboratory. These conclusions suggested increased amino acids by protein degradation, which may maintain blood glucose levels as energy source through the gluconeogenic metabolic pathway from non-carbohydrate carbon substrates (Li and Brouwer, 2009, 2013; Brown-Peterson *et al*., 2011). Zeis *et al*. (2009) also considered that high-expressed glycolytic and proteolytic enzymes in the *Daphnia pulex* were remained as a process to improve carbohydrate provision for the maintenance of ATP production under hypoxia in their natural habitats. Paschke *et al*. (2010) found that total proteases, trypsin and chymotrypsin activity in the hepatopancreas of *Lithodes santolla* were not affected after 10 d chronic hypoxia. In the present study, amylase, lipase, and trypsin activities of CSMH versus N shrimp decreased continuously, especially the most sensitive trypsin. Obviously, cyclic serious/medium hypoxia as more severe hypoxia form had worse effect on digestive enzyme than previous hypoxia reports. Many studies have indicated that low DO levels reduce food consumption by crustaceans due to weakened digestive process (Rosas *et al*., 1998; Mcgaw, 2005). Moreover, crustaceans showed a preference for proteins as a metabolic substrate under hypoxia, due to an increase of free amino acids in the hemolymph, which have the double function of helping to maintain osmotic pressure and providing metabolic energy as a consequence of anaerobic mechanism (Taylor and Spicer, 1987; Hagerman and Szaniawska, 1994; Rosas *et al*., 1999). All things considered, sustained low trypsin activity in the present study was likely to decrease metabolic energy supply and disrupt osmotic balance, resulting in reduced survival and growth performance of shrimp.

Histological analysis of the hepatopancreas has been used as a practical means for assessing environmental stress in shrimp culture (Saravana and Geraldine, 2000; Li *et al*., 2007; Kuhn *et al*., 2010; Qiu *et al*., 2016). Limited study has described the effects of DO changes on hepatopancreas histology in shrimp. Sun *et al*. (2015) pointed out that the hepatopancreas of *M. nipponense* exposed to chronic hypoxia were structurally altered after 7 d, which could affect the vital physiological functions of prawns. Similarly, we found the histopathological lesions in the hepatopancreas of CSMH shrimp in a time-dependent manner. Thus, the growing lesions in the hepatopancreas of shrimp might induce the problems related to digestive enzyme in our mentioned above study (Rosas *et al*., 1995; Franceschini-Vicentini *et al*., 2009).

The resistance of shrimp against pathogens is also affected by environmental DO changes. Moullac *et al*. (1998) found that hypoxia reduced the

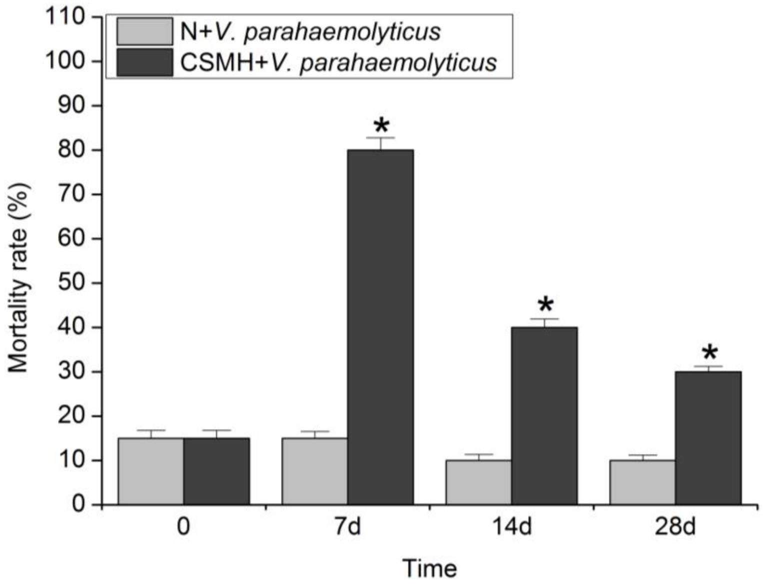

Fig. 7 Mortality rates of shrimp after + *V. parahaemolyticus* challenge during the experimental period. See Fig. 2 for statistical information.

resistance to *Vibrio alginolyticus* injection in *Penaeus stylirostris* over 24 h in consistent with variations of immunological parameters. Mikulski *et al*. (2000) pointed out that hypercapnic hypoxia reduced the resistance to *V. parahaemolyticus* injection in *L. vannamei* and *P. pugio* over 48 h due to decreased parameter of the immune response. Cheng *et al*. (2002) indicated that hypoxia reduced the resistance to *Enterococcus* injection in *M. rosenbergii* over 96 h by reducing immune ability. Brouwer *et al*. (2007) suggested that *PmAV*, a novel gene involved in virus resistance, and immunity-related genes were down-regulated in *P. pugio* after 14 d chronic hypoxia*.* Similarly, CSMH shrimp exhibited increased MR with *V. parahaemolyticus* immersion continuously. In contrast, Li and Brouwer (2013) considered that both *PmAV* and immunity-related genes were up-regulated in *P. pugio* in response to 10 d cyclic hypoxia/normoxia. Therefore, effect of hypoxia on resistance against pathogens of shrimp might mainly depend on their severity and frequency, and cyclic serious/medium hypoxia as severe hypoxia form might also decrease resistance against pathogens due to reduced immunity in the shrimp. In sum, cyclic serious/medium hypoxia as variation in the abiotic environment increased biological vulnerability to pathogens, which probably would lead to outbreaks of infectious diseases in the shrimp farming (Bachère, 2000; Lightner *et al*., 2005).

Conclusions

Cyclic serious/medium hypoxia induced incessant impairment of survival and growth performance of *L. vannamei* during long-term exposure, which might be resulted from broken osmoregulation mechanism of the gill, and suppressed digestive enzyme activities of the hepatopancreas caused by growing histopathological lesions. Meanwhile, the decreased resistance against *V. parahemolyticus* probably would lead to outbreak of infectious diseases in the shrimp farming under cyclic serious/medium hypoxia.

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