Fish & Shellfish Immunology 43 (2015) 181–190

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Detrimental effect of CO_2-driven seawater acidification on a crustacean brine shrimp, *Artemia sinica*

Chao-qun Zheng [a], Joseph Jeswin [a], Kai-li Shen [a], Meghan Lablche [a], Ke-jian Wang [a, b], Hai-peng Liu [a, b, *]

[a] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, Fujian, PR China
[b] Fujian Engineering Laboratory of Marine Bioproducts and Technology, Xiamen 361102, Fujian, PR China

ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form
19 December 2014
Accepted 23 December 2014
Available online 30 December 2014

Keywords:
CO_2
Ocean acidification
Artemia sinica
Brine shrimp

ABSTRACT

The effects of the decline in ocean pH, termed as ocean acidification due to the elevated carbon dioxide in the atmosphere, on calcifying organisms such as marine crustacean are unclear. To understand the possible effects of ocean acidification on the physiological responses of a marine model crustacean brine shrimp, *Artemia sinica*, three groups of the cysts or animals were raised at different pH levels (8.2 as control; 7.8 and 7.6 as acidification stress according to the predictions for the end of this century and next century accordingly) for 24 h or two weeks, respectively, followed by examination of their hatching success, morphological appearance such as deformity and microstructure of animal body, growth (i.e. body length), survival rate, expression of selected genes (involved in development, immunity and cellular activity etc), and biological activity of several key enzymes (participated in antioxidant responses and physiological reactions etc). Our results clearly demonstrated that the cysts hatching rate, growth at late stage of acidification stress, and animal survival rate of brine shrimp were all reduced due to lower pH level (7.6 & 7.8) on comparison to the control group (pH 8.2), but no obvious change in deformity or microstructure of brine shrimp was present under these acidification stress by microscopy observation and section analysis. In addition, the animals subjected to a lower pH level of seawater underwent changes on their gene expressions, including *Spätzle*, *MyD88*, *Notch*, *Gram-negative bacteria binding protein*, *prophenoloxidase*, *Apoptosis inhibitor 5*, *Trachealess*, *Caveolin-1* and *Cyclin K*. Meanwhile, several key enzyme activities, including superoxide dismutase, catalase, peroxidase, alkaline phosphatase and acid phosphatase, were also affected by acidified seawater stress. Taken together, our findings supports the idea that CO_2-driven seawater acidification indeed has a detrimental effect, in case of hatching success, growth and survival, on a model crustacean brine shrimp, which will increase the risk of juvenile brine shrimp and possibly also other crustaceans, as important live feeds for aquaculture being introduced in the ecosystem especially the marine food webs.

1. Introduction

The increasing release of CO_2 into the atmosphere by human activities, such as fossil fuel combustion, cement production and land-use change, leads to global climate change. Approximately one third of the CO_2 that entered the atmosphere over the past 100 years has been absorbed by the ocean surface water through air to sea equilibration and led to a reduction of about 0.1 pH units since pre-industrial times, and a further decline by 0.3–0.5 pH units is predicted by 2100 if no more measures are taken to control the current CO_2 emission [1,2]. The increase of CO_2 emission gives rise to the decrease of the pH level of the ocean, thus resulting in ocean acidification. It has been universally acknowledged that ocean acidification may cause a suite of changes in the carbonate system of seawater as: the concentrations of dissolved CO_2, totally dissolved inorganic carbon, and the bicarbonate ion become higher; on the contrary, the pH, carbonate ion concentration, and calcium carbonate saturation state become lower. These changes may affect the shell building in marine organisms since the formation of skeletons of shells in most marine organisms is an internal process where most organisms appear to convert bicarbonate to carbonate

* Corresponding author. State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, Fujian, PR China. Tel./fax: +86 592 2183203.
E-mail address: Haipengliu@xmu.edu.cn (H.-p. Liu).

http://dx.doi.org/10.1016/j.fsi.2014.12.027
1050-4648/

to form calcium carbonate. However, as this conversion creates protons, hydrogen ions, the organisms must exert energy to expel the hydrogen ions into the seawater. Considerable research on the effects of ocean acidification has been conducted in a number of calcareous marine organisms. For example, Kaniewska et al. [3] found that ocean acidification would cause long term negative effects in coral growth, fecundity and mortality by changing the expression of genes involved in membrane cytoskeletal interactions and cytoskeletal remodeling. Meanwhile, multiple studies on sea urchin have shown a consistent result that ocean acidification has a range of negative effects on larvae of sea urchin, such as decrease of fertilization rate, cleavage rates and arm length [4–8]. Recent studies [9–11] have revealed a number of potential negative effects that ocean acidification has brought on calcareous marine organisms such as copepods, lobster, oyster and so on, including their development, morphology, growth, hatching success and survival ability during their early developmental stages.

Artemia sinica, also known as brine shrimp, is a microcrustacean found worldwide in natural salt lakes and salterns. It has been widely used as important live feeds for different aquaculture species. Juvenile and adult brine shrimp have become a popular model organism for studies of stress response [12–14]. In addition, the lethality test of brine shrimp is usually employed to study the biological effects of cyanobacteria in coastal environments such as estuarine, marine and hypersaline ecosystems [15]. Thus, the brine shrimp has been regarded as a useful animal model for the investigation of environmental stress on zooplankton community. Besides, studies on acid-base regulation and on the ability of different species to counteract pH disturbances become more and more important in predicting the future ocean acidification for marine ecosystems. So far, the effects of ocean acidification on calcifying organisms such as marine crustacean are unclear.

In the present study, to gain a better understanding of physiological effects, negative or positive, of the ocean acidification on marine crustaceans, the brine shrimp was employed as a model. The cysts or animals were hatched or cultured, respectively, with three different pH treatments of acidified seawater driven by elevated pCO_2, in which pH 8.2 was used as a control treatment, while pH 7.8 and pH 7.6 were taken as different acidification stress according to the predictions for the end of this century and next century accordingly. After seawater acidification treatment, the hatching success of cysts, the morphological appearance such as deformity and microstructure of animal body, growth (body length) and survival rate of the animals were then determined. Furthermore, the transcript expression of selected genes involved in development, immunity and cellular activity, and biological activity of several key enzymes participated in antioxidant and physiological responses were also examined.

2. Materials and methods

2.1. Brine shrimp culture and experimental design

Before hatching incubation, brine shrimp (*A. sinica*) cysts were sterilized by being immersed in potassium permanganate solution (300 mg/L) for 5 min followed by washing sufficiently with filtered seawater prepared from sea salt (Aquarium Mangrove). As the optimal pH for hatching of the brine shrimp cysts is 8–9, the cysts were then exposed to seawater pretreated with different pCO_2 concentration (380 ppm, pH = 8.2 as normal hatching; 780 ppm, pH = 7.8; 1500 ppm, pH = 7.6) with the salinity of 28 ppt at 25 °C for hatching. CO_2 was supplied by a central automatic CO_2-mixing-facility (CE100-3 model, WuHan Ruihua Equipment Limited Company, China). For hatching rate assays, 100 cysts/50 mL seawater (see above with three pH treatment) in covered glass bottles for keeping stable pH (confirmed by pH determination at different time intervals in our preliminary test) were prepared in triplicates with gently shaking on a table concentrator. The hatching of cysts from embryo to instar I nauplii usually needs 24 h under suitable environmental conditions. After hatching for 24 h, cysts hatching rate was calculated by counting the hatched animals divided by 100 cysts in each group accordingly. This experiment was biologically repeated for three times.

In brine shrimp hatching and culture for later experiments, 0.5 g of cysts/2 L glass tank were prepared for hatching each time under three different pH treatment, respectively, in the specified incubators which were aerated with air (control group) or CO_2-enriched air as described above. After 24 h hatching, the hatched instar I nauplii were then transferred to three 30 L glass tanks bubbled with air (control group) or CO_2-enriched air, respectively, supplied with the central automatic CO_2-mixing-facility and each tank (one tank per each pH treatment) was prepared as an individual aeration system. A light–dark cycle of 10 h: 14 h was established with light intensity of 1000 lx. Food (spirulina powder) was supplied twice a day for the larvae. Approximately two thirds of the seawater was replaced with fresh seawater, unacidified for control or acidified for stress, once a week. The pH value and temperature of each seawater tank were monitored twice a day (Table 1) to ensure that any fluctuations during the experiment were noted.

For brine shrimp growth determination (i.e. body length), at least 20 animals were taken from each tank everyday to a petridish followed by transfer to a sticky glass slide for observation and calculation of the body length under a multi-function microscope (Olympus). The body length at each time point was defined as the average of 20 animals for each treatment. This experiment was biologically repeated four times. The survival rate of brine shrimp was daily recorded by calculation of the animal density. Briefly, three separate 10 mL of the seawater containing brine shrimp were taken from upper, middle and bottom levels, respectively, of each tank followed by animal counting and the average numbers were taken for calculation of the survival rate. This experiment was biologically repeated for more than three times.

To observe if there was any change of deformity or microstructure of the animal body resulted from seawater acidification stress, the sampled both of juvenile and adult brine shrimps from each pH treatment (about 10 animals/treatment) were observed directly with an Olympus multi-function microscope followed by fixation separately with 4% paraformaldehyde with gently shaking for 24 h at 4 °C. Subsequently, the fixed animals were washed with 70% ethanol for three times. The fixed animals were then dehydrated using gradient ethanol and vitrified by dimethylbenzene in embedding cassette followed by paraffin embedding individually for each section preparation. Sectioned samples were next rehydrated using gradient ethanol followed by staining with hematoxylin and eosin for observation under light microscopy.

For gene expression analysis, at least about 60, 30 and 15 animals were sampled at 1st day (nauplii), 7th day (juvenile) and 14th day (adult), respectively, from each treatment/tank at each time

Table 1
pH and temperature of the seawater during experiment.

pH		Temperature (°C)
Treatment	Measured	
8.2	8.2 ± 0.03	25.0 ± 0.2
7.8	7.8 ± 0.03	25.0 ± 0.2
7.6	7.6 ± 0.02	25.0 ± 0.2

point indicated. While for enzyme activity test, at least about 90, 60, and 30 animals according to nauplii, juvenile and adult, respectively, were sampled as described above for each test. All samples were snap-frozen in pellet accordingly in liquid nitrogen and stored at −80 °C until the extraction of total RNA or protein. Each experiment or samples taken above was biologically repeated for three times at least if not specified elsewhere.

2.2. Total RNA isolation and cDNA synthesis from brine shrimp

For total RNA isolation, about 100 mg wetweight of brine shrimp from each sample prepared above was grinded with liquid nitrogen in mortar in triplicates followed by homogenization in a 1.5 mL eppendorf tube containing 1 mL of TRIzol reagent (Invitrogen). The separated total RNA was digested with DNase I to eliminate DNA contamination and the obtained RNA was then quantified using an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Sweden) at A_{260}/A_{280} nm and its integrity was determined by 1.2% denaturing formaldehyde/agarose gel electrophoresis. One microgram of purified total RNA was used for cDNA synthesis in a final volume of 10 μL with each sample using a PrimeScript™ RT-PCR Kit (TaKaRa) according to the manufacturer's instructions.

2.3. Determination of the transcripts of selected genes by quantitative real-time PCR

Quantitative real-time PCR was performed using the fluorescent dye Power SYBR Green PCR Master Mix and ABI 7500 system. The gene-specific primers were designed based on the gene sequences obtained from NCBI as shown in Table 2. These genes included *Spätzle*, *MyD88*, *Notch*, *Gram-negative bacteria binding protein* (*GNBP*), *prophenoloxidase* (*proPO*), *Apoptosis inhibitor 5* (*API5*), *Trachealess*, *Caveolin-1* and *Cyclin K*. Beta-actin was used as an internal control. The PCR reaction was carried out by 10 uL of Power SYBR Green PCR Master Mix (Applied Biosystems, UK) according to the manufacturer's specifications in a 7500 Real-Time PCR System (ABI) with the following procedure: 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 25 s and 72 °C for 40 s plus an additional extension at 75 °C for 40 s. PCR amplifications were prepared in triplicates. DEPC water for the replacement of cDNA template was used as a negative control. The relative expression levels of the tested genes were calculated with the relative expression software (ABI) based on the $2^{-\Delta\Delta CT}$ method. The qPCR result was analyzed by 7500 system SDS software version 1.3.1.21. This assay was repeated for three times (biological replication) from the samples taken accordingly as stated above.

Table 2
Primers used in this study.

Primer	Direction	Sequence (5′-3′)
AS-API5	F	ATCTGGTACATTAGCTCCAGAAGC
AS-API5	R	TGCTGTCCAGATACAGACTTACC
AS-Caveolin-1	F	GCTTCTCTTGGTGGATCAGAGC
AS-Caveolin-1	R	CCCACTGTAAGGTTCTCAACAACAC
AS-Cyclin K	F	GGGCTTCGATATGACACAATGGCA
AS-Cyclin K	R	GCTAGAAAGAGGCAGCAACAAGC
AS-GNBP	F	GAACCAATACTGGCGAACTGC
AS-GNBP	R	GTTCGGTCTCAGCACTCCATGT
AS-MyD88	F	CGAAAATGTTCTCTGGGCG
AS-MyD88	R	CAAGTACTCGGCATGTAGGACC
AS-Notch	F	CTTCACTTCTTGGTCATGGTGCC
AS-Notch	R	CGTTCCTGAGCGACATCACGT
AS-proPO	F	TCAGCAGACCTTGCTTGCCGT
AS-proPO	R	GGGCATCACTCGTGTTTGCAG
AS-Spätzle	F	AGGAAACTTGCGAACTCCTCG
AS-Spätzle	R	AGGGTAGATGTTCATGGCAGC
AS-Trh	F	GATGCATCTACGCCACTTGGAG
AS-Trh	R	AACGGAGCTAGGTGGTGTCAT
AS-β-actin	F	AGCGGTTGCCATTTCTTGTT
AS-β-actin	R	GGTCGTGACTTGACGGACTATCT

2.4. Preparation of crude protein extract and activity test of selected enzymes

To prepare the crude protein extract from brine shrimp, about 300 mg wetweight of each sample collected above were homogenized in an eppendorf tube containing 1 mL of normal saline (0.9% NaCl) on ice by the homogenizer in triplicates (Sigma Aldrich) as described by the commercial kits according to the manufacturer's instructions, respectively (Nanjing Jiancheng Bioengineering Institute, China). Then the mixture of each sample was centrifuged with 2500 × g for 10 min at 4 °C. The supernatant was removed as protein extract followed by the protein concentration determination using a Brandford method. The prepared crude proteins were then used for enzyme activity test of acid phosphatase, alkaline phosphatase, catalase, superoxide dismutase and peroxidase, respectively, as instructed by the different kits mentioned above accordingly. This assay was biologically repeated for three times with the samples collected as described above.

2.5. Statistical analysis

The data was analyzed by SPSS software version 16.0. Results for all determinations were presented as means ± standard deviation (M ± SD) of three separate experiments (n = 3) if not specified elsewhere. Statistical analysis was performed using one-way ANOVA test for cysts hatching rate analysis, and an overall two-way ANOVA with time and pH treatment followed by Fisher's least significant difference (Fisher's LSD) test for other assays if not specified elsewhere.

3. Results and discussion

3.1. The cysts hatching rate, morphology appearance, growth and survival rate of brine shrimp under seawater acidification driven by elevated CO_2

To elucidate the biological effect of acidification on the brine shrimp, we determined the cysts hatching rate under different pH

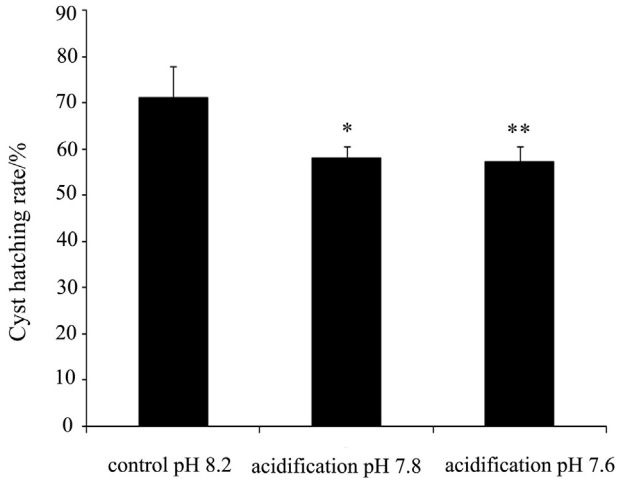

Fig. 1. The cysts hatching rate of brine shrimp under seawater acidification driven by elevated CO_2. Data were presented as means ± SD (n = 3) and analyzed by one-way ANOVA. *$p < 0.05$ and **$p < 0.01$ indicate statistical difference accordingly between the control group and the acidification groups.

values of seawater driven by CO_2. As shown in Fig. 1, the cysts hatching rate was lower in both pH 7.8 (58.1 ± 2.3%) and pH 7.6 (57.3 ± 3.3%) groups compared to that of the control group (71.1 ± 6.6%) ($p < 0.05$), indicating that the cysts hatching success was clearly negatively affected by elevated pCO_2. Other researchers have also shown that the egg hatching rate of several marine copepods was negatively affected by acidification induced under higher pCO_2 [9]. These findings together demonstrated that the accumulated detrimental effects of acidification during the exposure time of hatching did negatively affect the hatching success of the eggs of marine copepods. On the other hand, a lack of developmental deformity has been reported in sea urchin larvae at early stages under low pH conditions [6]. In the present study, whereas no morphological difference, based on deformity of the whole animal or the microstructure of the animal by section analysis, was found among the brine shrimp during their developments under these pH values when observed under light microscope (Fig. 2), suggesting that the morphological appearance of brine shrimp was lack of sensitivity to seawater acidification stress under this experimental condition. Similar phenomenon was also found in sea urchin as described previously [6].

In the case of the brine shrimp growth such as body length, no significant difference was found until the 11th day post the acidification treatment. The animal body length, however, exhibited a significant decrease at the 13th and 14th day in the acidification groups when compared to that of the control ($p < 0.05$, Fig. 3). From these observations, we speculated that the negative impacts of seawater acidification on brine shrimp might be time-dependent with the accumulation of the acidification stress. Previous studies have found that ocean acidification could inhibit growth and induce deformity toxic effect on marine organisms such as sea urchin and oyster [7,11]. Meanwhile, ocean acidification could cause less calcification process and lead to decline, loss or disorder of calcification organ function, and also result in negative impacts on the growth, development and reproduction of calcified organisms [16,17]. Here we found that the body length of brine shrimp was significantly reduced in acidification treatments from 13th day, strongly suggesting that the growth and calcification of brine shrimp might be detrimentally affected as the acidification stress accumulated by time course.

After the brine shrimps were cultured under acidification, the survival rate of the animals was also monitored. Obviously, the animals exhibited high death rate in the acidification group with pH of 7.6 ($p < 0.05$), but not with pH of 7.8 compared to that of non-acidification group. With the acidification stress accumulating, the survival rate of the pH 7.6 group gave a significant decrease in contrast to that of the other two groups (pH 7.8 and pH 8.2) from the 11th day post the acidification treatment ($p < 0.01$, Fig. 4), implying that mortality of organisms would be found and increased with the level of pCO_2 and the duration of exposure to acidified seawater. Reduced survival and fitness of calcareous marine organisms was likely due to the physiological compensation of maintaining normal processes such as growth, shell formation and metamorphosis in low pH marine environment [18]. Taken together, our findings here are in consistent with other studies on the impacts of acidification on marine organism [18], in which we found that acidified seawater does have negative effects on brine shrimp, to some extent, in physiological aspect such as hatching success, growth and survival rate.

3.2. Transcripts expression of selected genes from brine shrimp in response to seawater acidification driven by elevated CO_2

Given the fact that no significant difference does not mean a lack of effect [4,19], we speculated that there were still some unknown changes in homeostasis concerning the physiology such as gene expressions of the brine shrimp under acidification stress. To reveal whether the seawater acidification had impacts on genes expression of the brine shrimp, we determined the transcript level of selected genes involved in development, immunity, and cellular activity, including *Spätzle*, *MyD88*, *Notch*, *Gram-negative bacteria binding protein, prophenoloxidase*, *Apoptosis inhibitor 5*, *Trachealess*, *Caveolin-1* and *Cyclin K* under acidification stress with different developing stages by real-time PCR. Obviously, we found that the

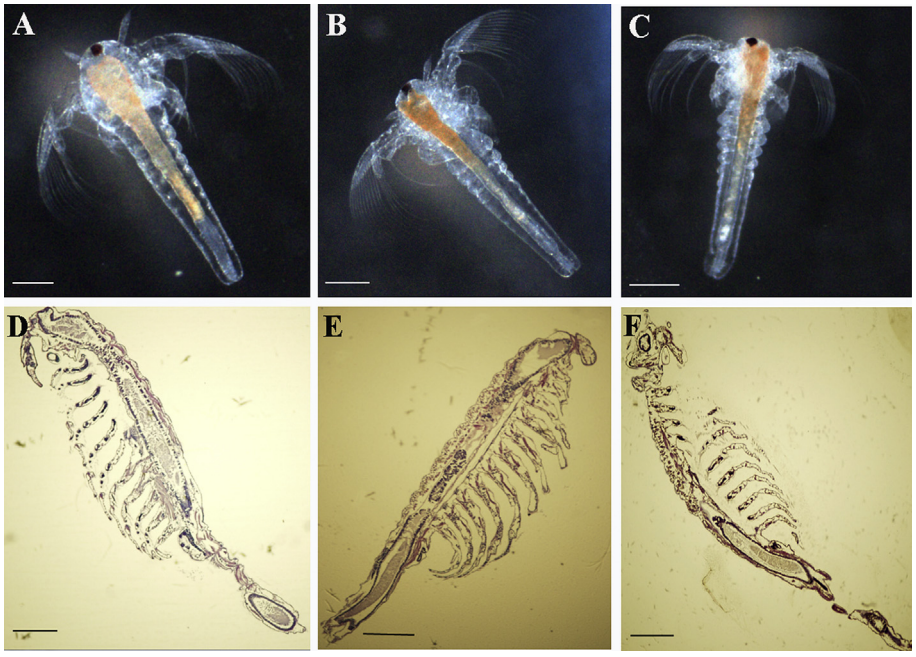

Fig. 2. The morphological observation of brine shrimp under seawater acidification driven by CO_2. The external morphology of brine shrimp with different pH of 8.2 (A), 7.8 (B) and 7.6 (C), respectively. The paraffin vertical section of brine shrimp with different pH of 8.2 (D), 7.8 (E) and 7.6 (F), respectively. Bars, 100 μm.

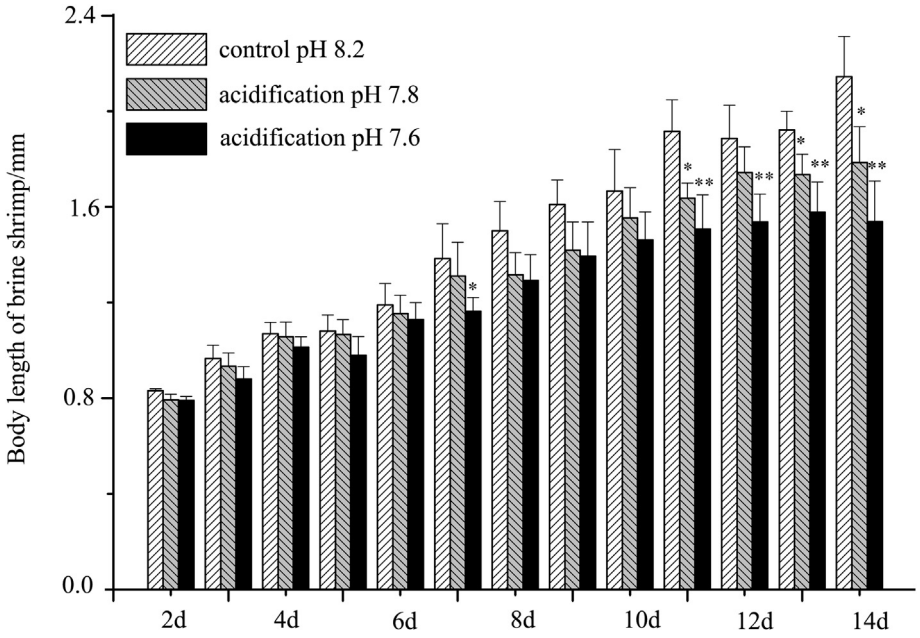

Fig. 3. The body length of brine shrimp under seawater acidification driven by elevated CO_2. Data were presented as means ± SD (n = 4). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical difference accordingly between the control group and the acidification groups at each time point.

gene expressions of all the selected genes were responsive to the seawater acidification driven by elevated CO_2 as discussed below. This finding strengthened the idea that seawater acidification elevated by CO_2 clearly impacted on gene expression of the brine shrimp.

As shown in Fig. 5A, *Spätzle* gene was highly expressed in the pH 7.6 group at 1st day post acidification compared with that of the control group and the pH 7.8 group ($p < 0.05$). However, the transcript of this gene was down-regulated in both the pH 7.6 and pH 7.8 groups at 7th day post acidification ($p < 0.05$). In the course of dorsal ventral axis differentiation, *Spätzle* is activated by a protease coded by an easter gene and then binds to the Toll receptor in ventral axis of oocyte which triggers the development of ventral axis [20]. Therefore, the enhanced gene expression of *Spätzle* in pH 7.6 group indicated that acidification stress may lead to an increased ability to stimulate a type of positive regulation in brine shrimp, suggesting that seawater acidification might also affect brine shrimp development although developmental deformities was not observed in the present study. Since *Spätzle* is also a well addressed factor employed in antimicrobial peptide production [21,22], its possible immune response via Toll pathway under acidification stress needs further investigation. The *MyD88* gene of

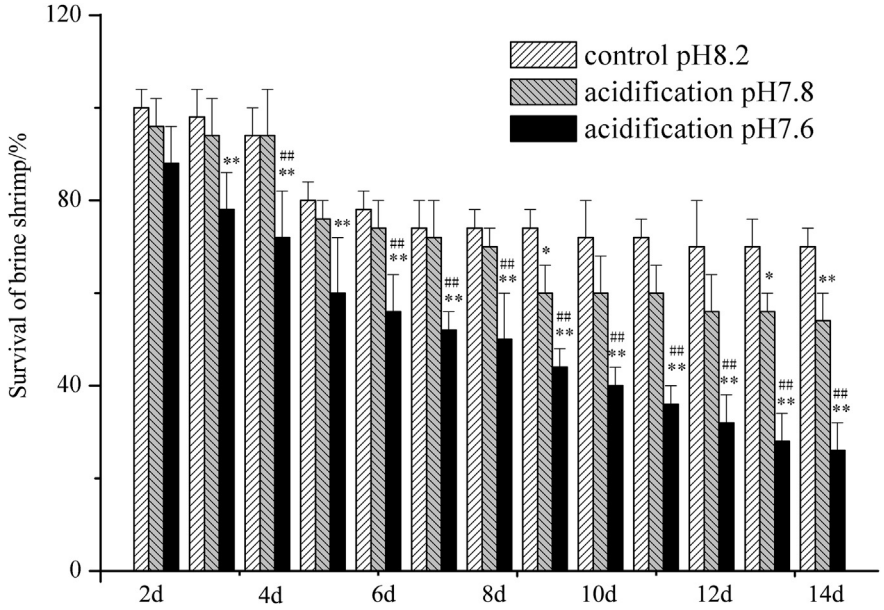

Fig. 4. The survival rate of brine shrimp under seawater acidification driven by elevated CO_2. Data were presented as means ± SD (n = 3). Data were presented as means ± SD (n = 3) and analyzed by two-way ANOVA followed by Fisher's LSD test. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical difference accordingly between the control group and the acidification groups at each time point; $\#p < 0.05$ and $\#\#p < 0.01$ indicate statistical difference accordingly between acidification groups.

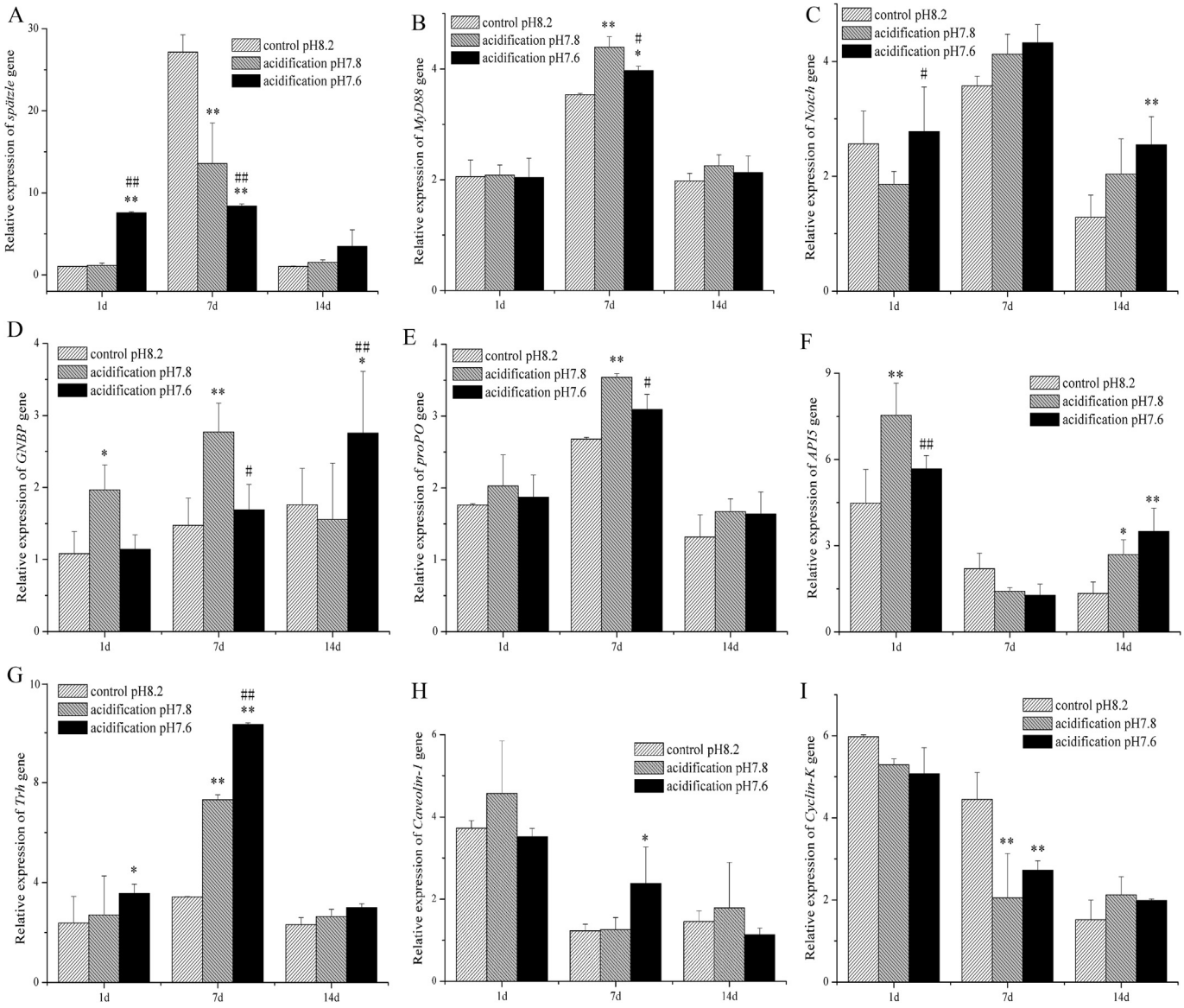

Fig. 5. Quantitative real-time PCR analysis of different genes expression at different time points in brine shrimp under seawater acidification driven by elevated CO_2. Genes determined: A, *Spätzle*; B, *MyD88*; C, *Notch*; D, *Gram–negative bacteria binding protein* (*GNBP*); E, prophenoloxidase (*proPO*); F, *Apoptosis inhibitor 5* (*API5*); G, *Trachealess* (*Trh*); H, *Caveolin-1*; I, *Cyclin-K*. Data were presented as means ± SD of triplicates and analyzed by two-way ANOVA followed by Fisher's LSD test. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical difference accordingly between the control group and the acidification groups at each time point; $\#p < 0.05$ and $\#\#p < 0.01$ indicate statistical difference accordingly between acidification groups.

brine shrimp exhibited a relatively high expression at 7th day post acidification in all three groups (Fig. 5B). Meanwhile, the expression of *MyD88* gene was clearly higher in both pH 7.8 and pH 7.6 groups compared with that of the control animals ($p < 0.05$). However, no significant difference in *MyD88* expression was observed among all experimental groups both at the 1st day and 14th day post the acidification treatment. Given that *MyD88* was a critical adapter protein involved in Toll signaling pathway which also had been found to affect the dorsal–ventral axis formation during the embryonic development in *Drosophila* [23]. The clear up-regulation of brine shrimp *MyD88* at the 7th day under the acidification stress implied that seawater acidification stress might impact on the *MyD88* mediating signal pathway down to antimicrobial peptide release via activation of NF-κB. Due to the lack of gene information of antimicrobial peptides in brine shrimp at present, this hypothesis still needs further confirmation by functional study of *MyD88* in brine shrimp under seawater acidification once the antimicrobial peptides information is available. The Notch signaling pathway functions in the developmental process of both vertebrates and invertebrates, including cell fate decision, nervous system development and the formation of organ and somite [24]. Here we observed that the gene expression of *Notch* increased significantly in the pH 7.6 group at the 14th day post acidification stress compared to the control group ($p < 0.01$, Fig. 5C), so we expected that the acidification might stimulate the expression of *Notch* gene which in turn impacted on the homeostasis of brine shrimp. We also found that the variation of *Notch* gene was similar to *MyD88* gene, as both of which are developmental genes. Furthermore, the expression of both genes were increased at 7th day followed by a decrease at 14th day, implying that similar stress responses of different pathways may occur in brine shrimp post the stress of seawater acidification.

As important pattern recognition receptors in invertebrates, *GNBPs* are well-known to be involved in innate immune response

which specifically identify and bind to features on the surface of microorganisms. This binding then triggers a variety of defensive reactions through the activation of protease cascades and intracellular immune signaling pathways [25]. The gene expression of *GNBP* was clearly enhanced in the pH 7.8 group at both of the 1st day and 7th day post acidification compared with that of the control group and the pH 7.6 group ($p < 0.05$, Fig. 5D), indicating that *GNBP* gene expression of brine shrimp was biologically responsive to the acidification stress, and the immune recognition upon bacterial invasion followed by the antimicrobial peptide release or melanization activation could also be affected by this stress. This speculation is necessary for further investigations when genomic information or wide scale of transcriptome is available in brine shrimp. proPO is well-known as a key enzyme involved in melanization in protection of invading pathogenic microorganisms. In the present study, *proPO* gene exhibited a relatively high expression at the 7th day post acidification stress in all three groups. A significant increase of *proPO* gene expression was clearly observed in pH 7.8 group compared with that of the control group at 7th day post acidification ($p < 0.01$, Fig. 5E). However, no significant difference in the expression of the *proPO* gene was found among the acidification groups and the control groups at the 1st day and 14th day post acidification treatment. This result implied that proPO-system, the important immune defense system mediating melanization against microbial infection, of brine shrimp is actively responsive to acidification stress, indicating that the melanization activity might be also affected, possibly also to the animal immune defense ability which needs further investigations such as immune protection against infection of microbials post the loss-of-function.

Our result here indicated that the gene expression of *Apoptosis inhibitor 5* was decreased in all tested groups at the 7th day in comparison to that of the 1st day. However, it was clearly increased in all groups at the 14th day when compared to that of the 7th day (Fig. 5F). In both vertebrates and invertebrates, *Apoptosis inhibitor 5* is an apoptosis inhibitor-related protein regulating apoptosis process [26,27]. Meanwhile, the *Apoptosis inhibitor 5* gene was reported as an anti-apoptotic factor [28]. As the toxic effects of the acidified environment might be accumulated, we speculated that the brine shrimp may significantly increase the gene expression of *Apoptosis inhibitor 5* to cope with the toxic effects, possibly against cell apoptosis caused by this effect, at the 14th day post acidification but this speculation needs further confirmation. The brine shrimp survives in the high salinity water, so they have strong ability upon osmotic regulation and ion adjustment. The gene expression of *Trachealess*, an important factor of the osmo regulation in *A. sinica*, was obviously increased in the pH 7.6 group at 1st day post acidification compared to the control group ($p < 0.05$, Fig. 5G). In contrast to the control animals, the gene expression of *Trachealess* was significantly elevated in both pH 7.6 and pH 7.8 groups at 7th day post acidification ($p < 0.01$). However, no significant difference concerning the *Trachealess* gene expression was shown among the acidification groups and the control groups at the 14th day post acidification. Previous studies have shown that the adjustment organs to osmotic pressure in the different developmental period of brine shrimp were various. In the period of nauplius, they mainly relied on salt gland. However, thoracic appendages will take place of the salt gland to regulate osmotic pressure during their maturation [29,30]. We found that the relative gene expression of *Trachealess* was much higher at the 7th day if compared with that of the 1st day, suggesting that this gene was sensitively responsive to acidification stress and it might act a role in the ion balance and osmotic pressure balance in the brine shrimp under this acidification stress. As a principal structural component of caveolae [31], *caveolin-1* participates in the embryonic development, nervous regulation, osmotic adjustment and congenital immune response. In the present study, *caveolin-1* gene exhibited a relatively high expression at the 1st day post acidification in all three groups. The expression was enhanced in the pH 7.6 group compared to the control at the 7th day post acidification ($p < 0.05$, Fig. 5H). No significant difference in the expression of the *caveolin-1* gene, however, was found among the acidification groups and the control groups at the 14th day. Further biological studies with proteins are necessary to elucidate the role of *caveolin-1* along with its important cellular process in physiology of brine shrimp under the acidification stress. *Cyclin-K* serves as a crucial factor in regulating RNA polymeraseII (RNAPII) which is a key enzyme involved in the synthesis of mRNA [32,33]. *Cyclin-K* has been reported as a regulatory subunit of the positive transcription elongation factor b in the diapause embryo developmental pathways of brine shrimp [34]. In the present study, *Cyclin-K* gene was most abundantly expressed at the 1st day, suggesting that it might be involved in cell development. As shown in Fig. 5I, the expression of *Cyclin-K* gene was decreased during the different developmental stages under acidification treatment, indicating that the RNAPII enzyme activity could be negatively affected during the early developmental stages. We also found that the expression of *Cyclin-K* gene significantly decreased in the acidification groups in comparison to the control group at the 1st day and 7th day post acidification, suggesting its responsive role to an environmental stress.

3.3. Determination of the selected enzyme activities of brine shrimp in response to seawater acidification driven by elevated CO_2

Most of the cellular activities are catalyzed by cellular enzymes. To elucidate whether seawater acidification affects activity of key enzymes, including superoxide dismutase, catalase, peroxidase, alkaline phosphatase and acid phosphatase involved in cell biological process, their activities were determined accordingly at different developmental stages of brine shrimp under seawater acidification stress.

As a specific antioxidase widely present in various tissues, superoxide dismutase protects cells against oxidative damage by removing free radical O_2^- produced during the metabolic process [35]. Given that the activity of superoxide dismutase was significantly increased in the pH 7.6 group at different developmental stages of the brine shrimp under acidification ($p < 0.05$, Fig. 6A), we speculated that the brine shrimp might generate more antioxidase in response to the acidification stress resulting in the increase of free radical O_2^-. As a highly conserved molecule from invertebrates to vertebrates, catalase is a tetrameric oxidoreductase catalyzing the conversion of two molecules of hydrogen peroxide to two molecules of water and one molecule of oxygen [36]. By doing this reaction, catalase functions importantly in reducing active oxygen free radicals and maintaining cellular homeostasis in organisms. In the present study, higher catalase activity was found increasingly with both the growing developmental stages and the decrease of seawater acidification in brine shrimp, but no significant difference was observed (Fig. 6B), indicating that catalase might be biologically responsive to the stress under seawater acidification and play a protection role. Peroxidase, a class of enzyme containing ferrous metals, is considered to be one kind of enzyme related to immune responses. Peroxidase is capable of catalyzing the harmful cellular metabolites such as H_2O_2 and O_2^- into non-toxic small molecules, then reduces the intracellular toxicity and protects cell membrane [37]. In the pH 7.6 group, peroxidase activity exhibited a significant increase compared to that of the control group at different developmental stages (Fig. 6C, $p < 0.01$). This result revealed that, when brine shrimp suffered from the acidification treatment, peroxidase enzyme was responsive to the unfavorable environmental condition. We hence speculated that acidified seawater was likely to

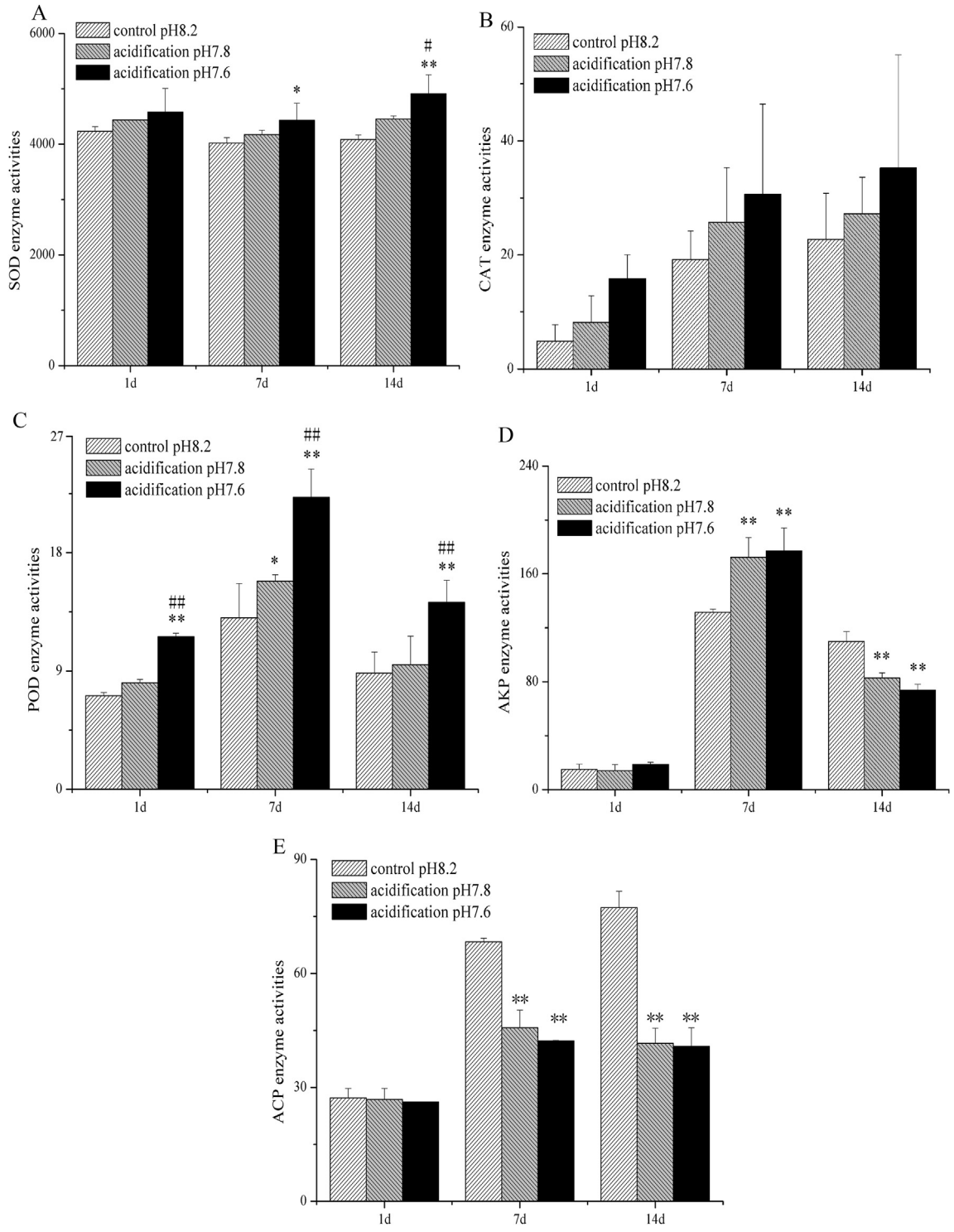

Fig. 6. Enzyme activity at different time points in brine shrimp under seawater acidification driven by elevated CO_2. Enzyme activities determined: A, superoxide dismutase (SOD); B, catalase (CAT); C, peroxidase (POD); D, alkaline phosphatase (AKP); E, acid phosphatase (ACP). The enzyme activity was presented as U/g Protein. Data were presented as means ± SD of triplicates and analyzed by two-way ANOVA followed by Fisher's LSD test. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistical difference accordingly between the control group and the acidification groups at each time point; $\#p < 0.05$ and $\#\#p < 0.01$ indicate statistical difference accordingly between acidification groups.

induce the generation of reactive oxygen species in the brine shrimp, in which the clearance of the reactive oxygen species could be subsequently enhanced with the help of the increased antioxidant enzymes like perxoidase and thus reduce oxidative damage. Taken these data together, it is obviously that the antioxidant enzymes function importantly in physiological response to the seawater acidification stress in brine shrimp. But how this response is regulated by acidification stress needs further investigations.

As we know that phosphatase plays an important role in dephosphorylation reaction, particularly in signal transduction,

physiological metabolism and environmental adaptation. We thus determined the phosphatase activity such as alkaline phosphatase and acid phosphatase in the brine shrimp under acidification stress. The alkaline phosphatase displayed a relative lower enzyme activity at 1st day during the developmental stages. After that, a significant increase of alkaline phosphatase activity showed up both in pH 7.8 and pH 7.6 groups at 7th day compared with that of the control group ($p < 0.01$, Fig. 6D). However, a significant decrease of alkaline phosphatase activity was found at the 14th day post acidification stress in both pH 7.8 and pH 7.6 groups in contrast to the control animals. Meanwhile, the enzyme activity of acid phosphatase exhibited a significant decrease in the acidification groups compared to the control group at both the 7th day and 14th day post the acidification stress ($p < 0.01$, Fig. 6E). As described above, the acidification stress resulted in significant increase of peroxidase activity. Meanwhile given that the majority of acid phosphatase is situated in cell lysosomes, the enhanced peroxidation of lysosomal membranes can lead to membrane lysis and the ensuing acid phosphatase release followed by the increase of acid phosphatase activity [38]. Similarly, Karan et al. [39] found that alkaline phosphatase activity was increased in the blood serum and gills of *Cyprinus carpio* exposed to copper, which was attributed to the damage of cell membrane resulting from the elevated membrane permeability, with higher alkaline phosphatase synthesized in cells to meet the requirements of metabolism. On the other hand, activities of acid phosphatase and alkaline phosphatase were found to be opposite at the 7th day in acidification treatments in the present study, suggesting that there might be other unknown metabolisms or pathways which were stimulated during the developmental stages of brine shrimp under acidification stress. Our findings point to the fact that phosphatase is clearly responsive to environmental stress such as acidification in brine shrimp. Hence, the cellular reactions like signal transduction and physiological metabolism might be certainly affected by ocean acidification which needs further study in the field.

4. Conclusion

The present study indicated that chronic exposure to low pH level seawater driven by elevated CO_2 significantly affected some, but not all, aspects of the discrete life phases of a crustacean model animal brine shrimp. For instance, the morphology, body length, expression of certain genes and activity of certain enzymes, were not significantly affected by seawater acidification stress from early life phases such as larvae to juvenile and adult. On the other hand, our results also demonstrated that seawater acidification did have a significant negative impact on cysts hatching rate as well as animal survival ability of brine shrimp. To date, few literatures have been concerning on the effects of ocean acidification upon gene expression and enzyme activity in crustaceans. Here, we found that some selected genes expression and enzyme activity indeed were affected by seawater acidification stress. However, the molecular mechanisms are yet unclear. Thus, a better understanding of the mechanisms behind CO_2's impact on marine organisms in case of processes of biological adaptation and evolution is very important for any attempt to accurately forecast how marine organisms and the ecosystem will respond to ocean acidification. Furthermore, the synergistic effects of seawater acidification and climate change like temperature or other pollutant stresses on organisms should be given more attention. In order to gain a better understanding of organisms' acclimatization in response to ocean acidification, further studies across natural environmental gradients, chemical and physical transformation also need to be undertaken in near future.

Acknowledgments

This work was supported by 863 (2012AA092205), MELRI1403, IRT0941, NSFC (31222056, 41176114, 41476117), NCET-10-0711, Fok Ying-Tong Education Foundation (131077), SFFPC (2013J06010) and the FRFCU (2010121030).

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