Full length article

Antioxidative status, immunological responses, and heat shock protein expression in hepatopancreas of Chinese mitten crab, *Eriocheir sinensis* under the exposure of glyphosate

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ABSTRACT

As a broad-spectrum herbicide, glyphosate was extensively utilised in China for several decades. The contradiction between glyphosate spraying and crab breeding in the rice-crab co-culture system has become more obvious. In this study, the antioxidative status and immunological responses of Chinese mitten crab, *Eriocheir sinensis,* under sublethal exposure of glyphosate were investigated by detecting the antioxidative and immune-related enzyme activity, acetylcholinesterase (AChE) activity and relative mRNA expression of heat shock proteins (HSPs) in hepatopancreas. The results showed that high concentrations of glyphosate (44 and 98 mg/L) could induce significant alteration of superoxide dismutase (SOD), peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase (AKP), and phenoloxidase (PO) activities by first rising then falling during the exposure. However, AChE activity in all treatments including 4.4 mg/L was inhibited markedly after 6 h of exposure. In addition, the relative mRNA expression of HSP 60, HSP 70, and HSP 90 was significantly upregulated at both 48 h and 96 h. These results revealed that glyphosate has a prominent toxic effect on *E. sinensis* based on antioxidative and immunological response inhibition and AChE activity reduction even at the lowest concentration of 4.4 mg/L, and a protective response by upregulation of HSPs was carried out by the species to ease the environmental stress.

1. Introduction

Glyphosate, an organophosphorus herbicide with broad-spectrum activity, has been extensively used worldwide in agricultural crops including corn, soybean, cotton, rice, and trees in orchards and groves over the past 40 years [1]. In 2014, approximately 125,000 tons of glyphosate were used in the USA and 825,000 tons worldwide [2]. The main action of glyphosate is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate acid pathway. Because of the absence of the shikimate pathway in nontarget organisms, glyphosate is considered to have low toxicity to animals [3]. However, glyphosate is known to be resistant to degradation because of the inert C−P linkage in the molecule [4]. To date, several studies have demonstrated that glyphosate, or glyphosate-based herbicide, is toxic to nontarget aquatic organisms including microorganisms [5,6], aquatic plants [7], invertebrates [8], amphibians [9], and fishes [10–13]. However, there are no reports on economically important crustaceans such as crabs.

The Chinese mitten crab, *Eriocheir sinensis*, is one of the most important freshwater species widely bred in China. Rice-crab co-culture is a high-benefit ecobreeding pattern that has been extensively developed in east China in recent decades [14]. In the meantime, China has become the top producer of glyphosate with extensive use in non-agricultural areas such as aquatic ponds, for invasive plants, or for weed control. Glyphosate and its degradation products have been measured in both surface and ground water with a concentration ranging from 2 to 430 μg/L in the USA due to the rain wash and runoff from agricultural and urban land [1]. However, few data are available on glyphosate in the environment in China. Because of its relatively long half-life in water (most commonly 45–60 days) [15] and repeated application in practice, the residue of glyphosate in aquatic water could reach up to 0.765 mg/L within 1 day after spaying in a commercial pond [16]. That exceeds the maximum detection concentration in the water of the USA and Europe and the median lethal concentration of most aquatic species [17–20]. As a bottom dweller, crabs are more sensitive to the xenobiotics such as pesticides in water. These animals could be used as bioindicators for monitoring the state of pollution in an aquatic environment [21]. Therefore, the use of glyphosate may be an obstacle to

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both the crab breeding industry and ecological environment.

Herbicides are proved to cause oxidative stress in aquatic animals by the excessive production of reactive oxygen species (ROS). ROS may cause damage to DNA and biological macromolecules to induce cell injury [22]. As a consequence, the breakdown of the equilibration between antioxidant defence and generation of ROS could be the main reason for the toxic effect of herbicide to non-target organisms. In the first line of oxidative defence, superoxide dismutase (SOD) and peroxidase (POD) are the main enzymes to catalyse the conversion of the superoxide anion into hydrogen peroxide and the hydrogen peroxide into water and molecular oxygen. Thus, the activities of SOD and POD in different tissues were proposed as indicators of pollutant-mediated oxidative stress [12,23]. However, herbicides are generally considered to suppress the immune system in fishes, mussels, and crustaceans by tissue damage, enzyme inhibition, and decreased resistance to pathogens [24–26]. Therefore, two oxidative stress parameters, SOD and POD, and three immune-related enzymes, acid phosphatase (ACP), alkaline phosphatase (AKP) and phenoloxidase (PO), were detected to evaluate the potential toxic effect of glyphosate to *E. sinensis* in this study.

The recognised effect of organophosphorus in both invertebrates and vertebrates is the inhibition of the enzyme acetylcholinesterase (AChE), which is responsible for terminating the transmission of the nerve impulse [22]. The detection of AChE in different tissues was applied as a sensitive biomarkers for the assessment of several herbicides exposure to fish and shrimps [27,28]. In this study, we also detected the variation of AChE in hepatopancreas of *E. sinensis*.

Heat shock proteins (HSPs) commonly exist in cells from both eukaryotic and prokaryotic organisms with highly conservative evolution. It is proved that HSPs have important biomolecular functions including molecular chaperone, antioxidation, cell apoptosis, and immune response [29]. Several studies on the response of HSPs to the exposure of heavy metal and pesticides in aquatic animals have been reported [30,31]. To our knowledge, no information about the responses of HSPs to glyphosate exposure to crustaceans has been provided from any scholar. However, the variation of HSPs may provide a better explanation about the mechanism of antioxidation and immune response during a herbicide exposure.

Hepatopancreas is an important tissue in crustacean for metabolism and detoxication. The impaired activity of hepatopancreas marker enzymes such as ACP, AKP, SOD and POD is directly related to the degree of hepatopancreas damage in *E. sinensis* induced by toxins including pesticides [32]. From our previous study, glyphosate induced evident immunosuppression by haemocyte composition alteration, immune-related enzyme inhibition, and phagocytic activity decline, as well as haemocyte DNA damage [33]. In the current work, we investigated several oxidative stress and immunological response parameters in the hepatopancreas to evaluate the toxic effect of glyphosate to the important commercial species, *E. sinensis*.

2. Methods and materials

2.1. Animals and chemicals

Adult experimental crabs, *Eriocheir sinensis* (Crustacean: Decapoda: Grapsidae), with average weight of 104.4 ± 8.7 g, were collected from a commercial farm in Jiangsu Province. Crabs were acclimated to laboratory conditions for 2 weeks before the beginning of the experiment. A recirculation system containing filtered freshwater and ultraviolet-treated PVC tubes as shelters in $150 \, \text{cm} \times 100 \, \text{cm} \times 120 \, \text{cm}$ glass aquariums was utilised in this period. The system had controlled light (12 h light:12 h dark) and regulated temperature ($20 \pm 1.0 \, ^\circ\text{C}$). The animals were fed once a day at 7:00 P.M. with commercial crab ration, and residuals and faeces were removed 2 h after feeding. The water conditions were monitored daily and showed as follows: temperature $20 \pm 1.0 \, ^\circ\text{C}$, pH 7.6 ± 0.5, dissolved oxygen 6.7 ± 0.3, ammonia

nitrogen $< 0.2 \, \text{mg/L}$, nitrite $< 0.005 \, \text{mg/L}$. Glyphosate (analytical standard) used in this test was purchased from Sinochem Crop Care Co. LTD (Shanghai, China).

2.2. Glyphosate exposure

Based on the 24-h and 48-h median lethal concentration (LC_{50}) and safe concentration of glyphosate on adult *E. sinensis* [33], four sublethal concentrations including $4.4 \, \text{mg/L}$, $9.8 \, \text{mg/L}$, $44 \, \text{mg/L}$, and $98 \, \text{mg/L}$ were carried out in this experiment. In addition, a group that received no glyphosate was regarded as control. There were three replicates for each treatment with 10 crabs and a total of 150 crabs were used. Considering the results from Vera-Candioti et al. [34] that there is no significant difference between measured glyphosate concentration and 24-h interval renewals of testing solutions, in addition, less than 15% reduction of glyphosate in water after 96-h exposure [35], no measurement of the level of glyphosate in water was carried out in this study. During the experiment, water in aquariums was half-changed daily by adding fresh water containing the same concentrations of glyphosate to ensure that the concentration in each group remained invariable. Before the renewal of water, water quality in each aquarium was measured to avoid additional stress induced by water deterioration.

2.3. Sampling

Three individuals were randomly taken from each treatment at 0, 6, 12, 24, 48, and 96 h after exposure. Crabs were anaesthetised immediately with ice bath, and hepatopancreas were collected and stored at $-80 \, ^\circ\text{C}$ for use. Tissues were divided into two portion, one for biochemical and one for gene expression analysis.

2.4. Determination of antioxidative stress and immunological responses

Hepatopancreas was homogenised at $4 \, ^\circ\text{C}$ in 0.1 M potassium phosphate buffer with a mixer mill (MM400, Retsch, Germany) and centrifuged at 12,000 rpm for 20 min at $4 \, ^\circ\text{C}$. Then, the supernatant was analysed for the activity of SOD, POD, ACP, AKP, and PO.

Antioxidant, SOD, and POD activities in hepatopancreas were measured by a spectrophotometric method at 550 nm and 420 nm with corresponding detection kits (A001–3 and A084-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols. One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50% and was expressed as U/mg protein, which refers to unit per mg protein in tissue. One unit of POD activity was defined as the amount of enzyme that catalysed the decomposition of 1 mmol of H_2O_2 per min and was also expressed as U/mg protein.

ACP and AKP activity was determined using the corresponding detection kits (A060–2 and A059-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with a spectrophotometric method. One ACP and AKP activity unit was expressed as the production of 1 mg of phenol by reaction between 1 g of protein in tissue and the substance in 30 min. The specific PO activity was detected according to the method of Ashida [36]. Fifty μL 3 g/L levodopa, 50 μL supernatant, and 50 μL PBS (0.1 M, pH 7.0) were mixed into a 96-cell plate, incubated at room temperature for 20 min, and the optical density at 450 nm was recorded on a Bio-Rad iMark microplate reader (Bio-Rad, USA) from the beginning to 10 min. One unit of PO activity was defined as the increase of 0.001 optical density per min.

2.5. Determination of AChE activity

The AChE enzyme activity in hepatopancreas was determined by a colourimetric method according to the manufacturer's protocol from a detection kit (A024, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance was measured at 415 nm and the enzyme

Table 1
Primer sequence and gene table of *E. sinensis*.

Gene symbol	Accession no.	Primers (5′→3′)
β-actin	HM053699.1	F: TCCTGCGGCATCCACGAGAC
		R: CACGGTGTTGGCGTACAGATCC
HSP 60	KP642083.1	F: TGCTGAGGATGTGGACGGTGAG
		R: ACCTGTGGCAATGGCAATGTCC
HSP 70	KC493625.1	F: GGCAAGGCAGCGAAGGTCATC
		R: CGGCATTGGTGACAGACTGACG
HSP 90	EU809924.1	F: CTACCACACCTCCGCCTCTGG
		R: CTACCACACCTCCGCCTCTGG

activity was expressed as U/mg protein.

Protein content in samples was determined by Foline-phenol method based on a report by Lowry [37], using bovine serum albumin as standard.

2.6. Quantitative real-time polymerase chain reaction

Total RNA was extracted from tissues with RNAiso Plus Kit (Takara, Japan) according to the manufacturer's instructions. Then, RNA sediment was resuspended in RNAse-free water. RNA quality including concentration and purity was determined by a spectrophotometric method at 260/280 nm cDNA was synthesised using the PrimeScript™ RT reagent Kit with genomic DNA Eraser (Takara, Japan) from 2 μg of total RNA. The resulting cDNA was diluted five times and stored at −80 °C before use. Primers for HSP 60, HSP 70, HSP 90, and β-actin were designed by the Primer Premier Software (USA) according to the known sequence in *E. sinensis*. The primer sequences and gene bank numbers are provided in Table 1.

The qPCR was performed on a Bio-Rad CFX96 touch Real-Time PCR Detection System (Bio-Rad) in a 20-μL reaction volume for 40 cycles of 95 °C for 2 min, 95 °C for 10 s, and 60 °C for 30 s. The reaction mixture contained 10 μL of SYBR Premix EX Taq™, 2 μL of RT reaction solution (cDNA), 1 μL of each primer, and 7 μL of DNase/RNase free ddH$_2$O. All samples were run in triplicate. The changes of expression level of HSP 60, HSP 70, and HSP 90 were expressed as the fold change relative to the β-actin gene, by the 2-$\triangle\triangle$Ct method.

2.7. Statistical analyses

The results in all figures are the average values of three replicates ± standard error. The data were processed with SPSS version 20.0 software and normality of data was tested by the Shapiro- Wilk test for all bioassays. A homogeneity test of variances was performed, followed by one-way analysis of variance, and a multiple comparison of the Duncan test was used to determine significant differences among all groups and also between each treatment and their initial value at 0 h. To compare the expression of HSPs derived from exposure time, individual *t* tests for each concentration were used. The interaction of concentration and exposure time was analysed by two-way analysis of variance and a value of P < 0.05 was considered significant.

3. Results

3.1. Effect of glyphosate exposure on antioxidative stress and immunological responses

Both concentration and time affected the SOD activity and there is interaction between glyphosate concentrations and exposure time (F = 5.728, P < 0.05), as well as other parameters. The SOD activities in hepatopancreas of *E. sinensis* were induced first at concentrations of 44 mg/L and 98 mg/L after 6 h exposure, and reached a peak at 12 h. Then, concentrations dropped dramatically at 24 h and kept decreasing until 96 h. At that time point, SOD activities in treatment of 9.8 mg/L,
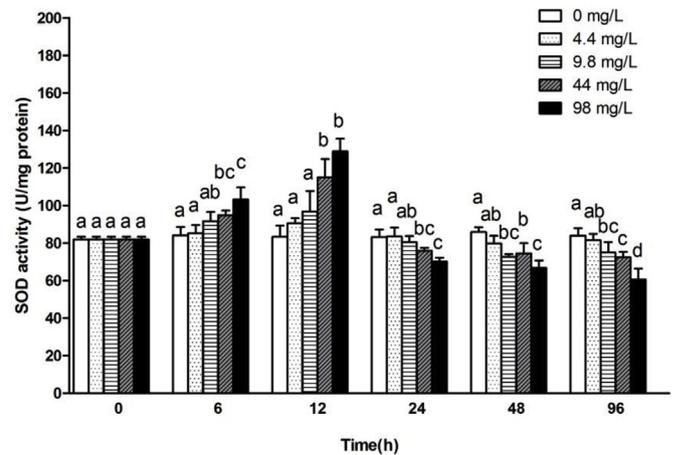

Fig. 1. The SOD activity in *E. sinensis* when exposed to glyphosate. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.
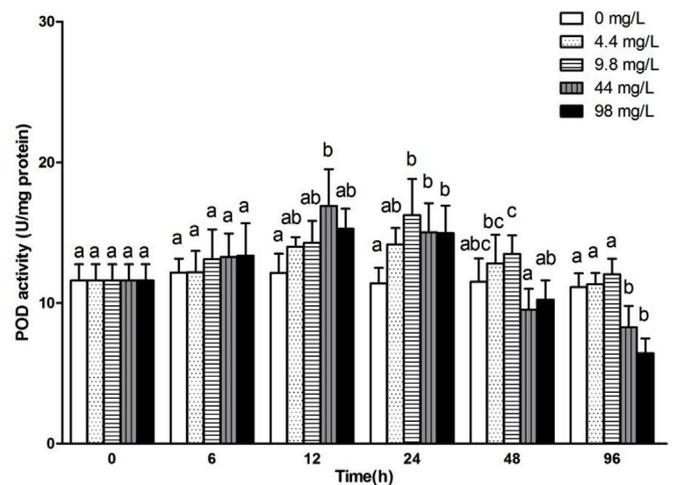

Fig. 2. The POD activity in *E. sinensis* when exposed to glyphosate. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.

44 mg/L, and 98 mg/L were significantly lower than control (P < 0.05) (Fig. 1). However, the same trend was observed in the level of POD that increased first and decreased with the progress of the exposure in the group of 44 mg/L and 98 mg/L (Fig. 2). There was no significant difference among groups of 4.4 mg/L and control in both SOD and POD activity throughout the experiment.

The ACP activity in treatments of 44 and 98 mg/L increased significantly at 12 h whereas a remarkable increase was observed at 24 h at a concentration of 9.8 mg/L. However, activity decreased in all treatments and at 96 h, it was significantly lower than that of the control group at 98 mg/L (Fig. 3). Similarly, the highest increase of AKP activity was detected at 98 mg/L at 12 h, and decreased to the lowest level at 96 h (Fig. 4). Meanwhile, the specific PO activity in the 98 mg/L group increased and reached a peak at 6 h, and then decreased gradually. After 96-h exposure, PO activities at concentrations of 44 and 98 mg/L were significantly lower than that of the control group (Fig. 5).

3.2. Effect of glyphosate exposure on AChE activity

The AChE activity in hepatopancreas was significantly diminished (P < 0.05) in all treatments after 6-h exposure. Then, it continued to decrease and there was a dose-dependent response from 6 to 24 h. Although it recovered slightly from 48 h, the levels of AChE in all
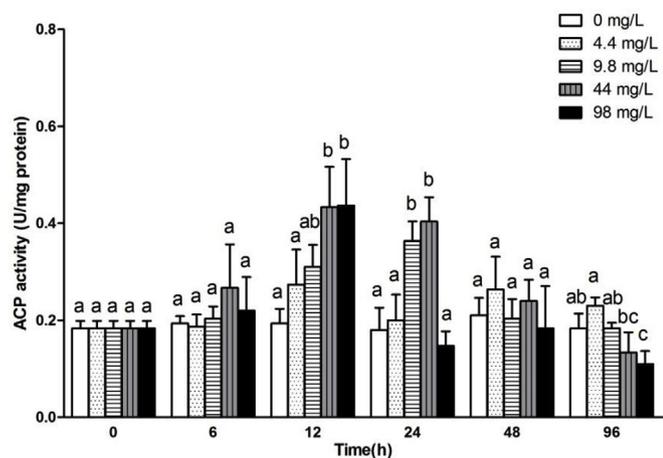
Fig. 3. The ACP activity in *E. sinensis* when exposed to glyphosate. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.
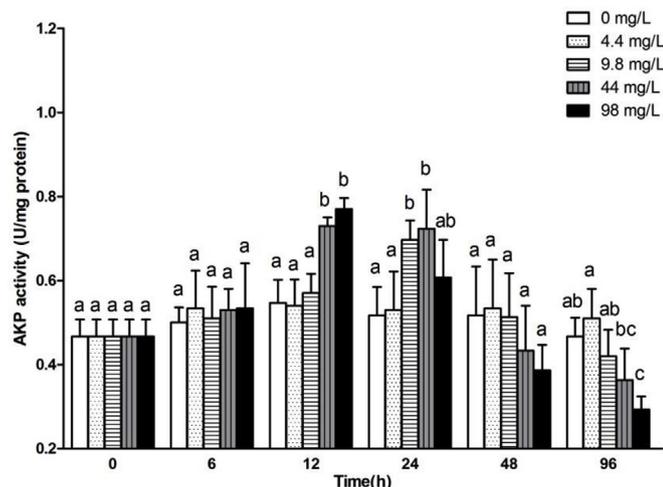

Fig. 4. The AKP activity in *E. sinensis* when exposed to glyphosate. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.
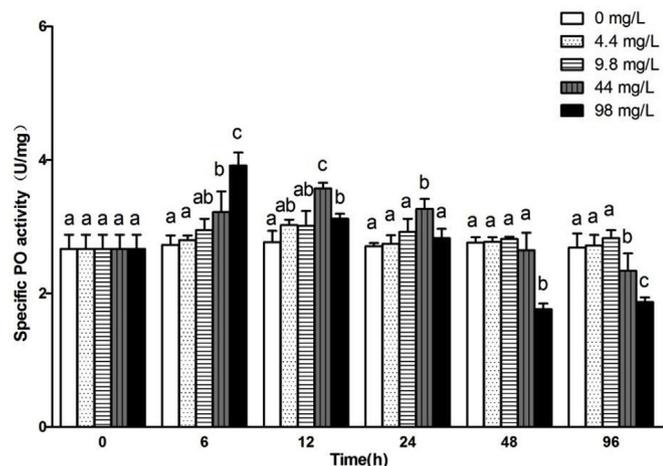

Fig. 5. The specific PO activity in *E. sinensis* when exposed to glyphosate. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.
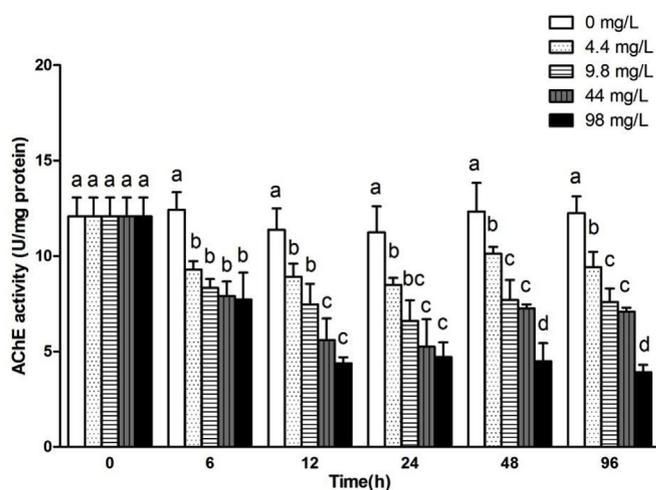

Fig. 6. The AChE activity in *E. sinensis* when exposed to glyphosate. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.

treatments were still significantly lower than control at 96 h (Fig. 6).

3.3. Effect of glyphosate exposure on mRNA expression of HSPs

Fig. 7 showed the variation of mRNA expression of HSP 60, HSP 70, and HSP 90 under different concentrations of glyphosate exposure. All treatments induced significant upregulated expression of all HSP genes following 48-h exposure. Subsequently, the expression of all HSP genes was downregulated to different degrees at 96 h. However, the expression of HSP 70 mRNA was still significantly higher in all concentrations compared to control (P < 0.01). Notably, the exposure of 98 mg/L of glyphosate caused the highest upregulated expression in all HSP genes at 48 h, with 2.35-, 8.13-, and 3.81-fold increases, respectively.

4. Discussion

Despite the numerous reports on the presence of glyphosate in the aquatic ecosystem and its toxicology to aquatic species, information about the toxic effects to macrocrustaceans is scarce, particularly in a co-culture system where glyphosate is commonly used. In the current study, several parameters associated with oxidative stress and immune response were measured to investigate the potential toxic effect of *E. sinensis* induced by hepatopancreas injury for the first time.

A novel research from Jia et al. showed that ROS could function as an important modulator in the hematopoiesis and promote the production of haemocytes from hematopoietic tissue of *E. sinensis* [38]. However, even under a non-lethal concentration exposure of glyphosate could lead significant decrease of total haemocyte counts by oxidation damage in a previous study [33]. As primary defenders of antioxidation, SOD and POD are proved to participate in several physiological and metabolic reactions, especially in the clearance of free radicals and prevention of biological molecular injury [39]. In this work, SOD activity in high concentration groups (44 mg/L and 98 mg/L) increased first and then decreased at 24 h with a dose-dependent response. These results are in accordance with the study from Modesto and Martinez that hepatic activity of SOD increased slightly at 6 h but significantly decreased at 24 h under the exposure of glyphosate at a concentration of 10 mg/L [27]. Regarding the POD activity, it also increased at first and declined from 48 h in high concentration groups. Interestingly, for both SOD and POD activity, there were no significant differences between group of 4.4 mg/L and control. It demonstrated that *E. sinensis* may not be sensitive to glyphosate lower than 4.4 mg/L during an acute exposure.
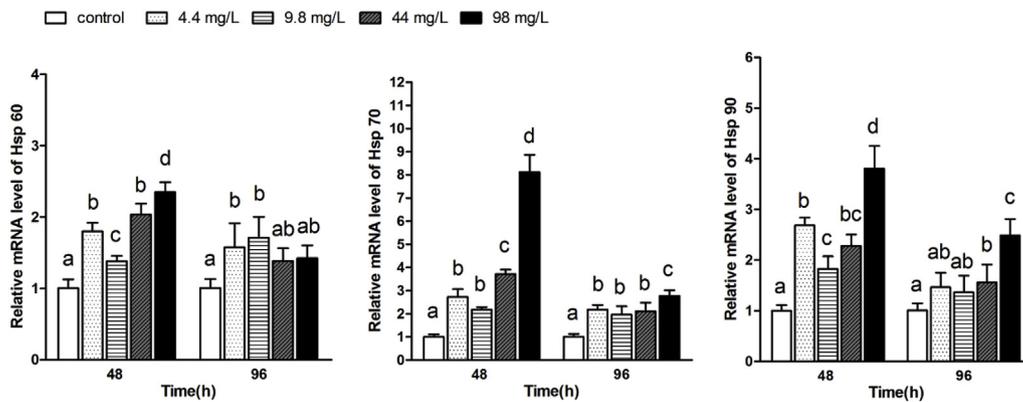
Fig. 7. The effect of glyphosate on the relative mRNA expression of HSP 60, HSP 70, and HSP 90 in hepatopancreas of *E. sinensis*. A: HSP 60; B: HSP 70; C: HSP 90. Note: different letters over the column represent the significant difference (P < 0.05) among each group, N = 3.

ACP and AKP are typical hydrolases with the main action of extermination of extracellular invaders, and are considered sensitive parameters in the immune response of crustaceans [40,41]. In this test, the variation of ACP and AKP activities in groups of 44 mg/L and 98 mg/L is similar to that of SOD and POD; in addition, no significant change could be seen in treatment of 4.4 mg/L for both parameters during the experiment. The results were consistent with research about the glyphosate exposure to two types of teleostean fishes in which AKP activity increased in several main tissues such as liver, intestine, and kidney [42]. Regarding the PO activity, it increased early at 6 h and decreased remarkably in high concentration groups. A previous study from Guardiola et al. showed that leukocytic PO activity of gilt-head (sea) bream *Sparus aurata* increased significantly at day 1 and day 3 but recovered at day 7 after deltamethrin exposure [43]. A study from Wei and Yang also showed that PO activity in haemolymph of crayfish *Procambarus clarkii* increased first at 24 h, but constantly decreased from 48 h to 96 h under the exposure of copper. In addition, a time- and dose-dependent response could be seen [44]. These results indicated that under environmental stress, specimens try to adapt to this adverse situation by antioxidation and immune response, although the continuous presence of the toxin can impede the hypothetic recovery of homoeostasis, especially during exposure to high concentrations of a toxic substance. Our results may provide evidence for this suggestion.

The level of AChE in tissue is commonly used as a sensitive parameter in assessment of neurotoxicity of pesticides to nontarget organisms [22]. However, this is the first report of AChE in hepatopancreas of *E. sinensis* exposed to glyphosate. In the current study, obvious inhibition of AChE activity was detected in all treatments including 4.4 mg/L throughout the experiment and a dose-dependent response was evident at each time point. The results are in agreement with the results from Glusczak et al. [45] and Modesto and Martinez [27] when *Leporinus obtusidens* and *Prochilodus lineatus* were exposed to the same herbicide. It indicated that AChE is more sensitive to the exposure of glyphosate, and suffers oxidative stress at a lower exposure concentration compared to other oxidative and immune-related parameters.

In the current study, the upregulation of the mRNA expression for HSP 60, HSP 70, and HSP 90 in hepatopancreas was observed in all treatments after 48-h exposure to glyphosate and the HSP 70 exhibited the highest upregulation at concentration of 98 mg/L. Our results are in accordance with those from Jiang that the mRNA of HSP 70 and HSP 90 was more upregulated than HSP 60 after 96 h exposure of heavy metal [17]. HSP 60 can only repair a small part of allosteric proteins and act slower than HSP 70. This may explain the difference in the expression of HSPs. HSP 90 is an abundant molecular chaperone and important component of the body's anti-stress response. To cope with the damage to the body, *E. sinensis* mobilized many immune-related proteins including HSP 90, to enhance the antioxidant ability [46]. The viewpoint is consistent with our results. However, recovery of the upregulation

was found at 96 h in all concentration groups in this experiment but there was no recovery in Jiang's research [17]. A study on the effect of avermectin on the mRNA expression of HSPs in brain tissue of King pigeons showed that the level of HSP 60, HSP 70, and HSP 90 expression significantly increased after 30-d exposure to avermectin [47], but also decreased at 60 d. It suggested the molecular regulation of *E. sinensis* when exposed to xenobiotics such as herbicide, and these changes could be important for protection from the oxidative damage caused by glyphosate. In addition, previous reports on both terrestrial and aquatic animals also indicated that a wide range of exposures trigger protective mechanisms that are mediated by HSPs [48,49]; therefore, the mRNA expression of HSPs including HSP 60, HSP 70, and HSP 90 could be used as sensitive parameters in the evaluation of acute glyphosate exposure to freshwater crabs, in particular HSP 70.

5. Conclusion

In this study, a prominent toxic effect of glyphosate on the Chinese mitten crab *E. sinensis* was detected for the antioxidative and immunological enzyme inhibition and AChE reduction in hepatopancreas, even at concentrations of 4.4 mg/L and 9.8 mg/L. Meanwhile, the protective response depending on the promotion of mRNA expression of HSP 60, HSP 70, and HSP 90 in hepatopancreas was induced to mitigate the oxidative stress. Additionally, parameters of HSP expression, especially in HSP 70, were considered sensitive for the toxicity assessment of glyphosate exposure.

Acknowledgments

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