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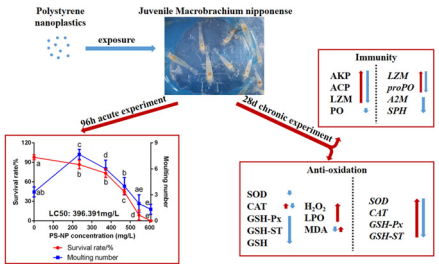
Effects of nanoplastics on antioxidant and immune enzyme activities and related gene expression in juvenile *Macrobrachium nipponense*

Yiming Li[a,1], Zhiquan Liu[a,1], Maofeng Li[a], Qichen Jiang[c], Donglei Wu[a], Youhui Huang[a], Yang Jiao[a], Meng Zhang[a], Yunlong Zhao[a,b,*]

[a] School of Life Science, East China Normal University, Shanghai 200241, China
[b] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200241, China
[c] Freshwater Fisheries Research Institute of Jiangsu Province, Nanjing 210017, China

GRAPHICAL ABSTRACT



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ABSTRACT

Nanoplastics are widely distributed in aquatic environments, and nanoplastic pollution has become a global concern. However, few studies have evaluated the toxicity of nanoplastics to freshwater crustaceans. In this study, by adding different concentrations of nanoplastics to water, we explored the effects of nanoplastics on the survival, antioxidant activity, immune enzyme activity, and related gene expression levels in juvenile *Macrobrachium nipponense*. The results showed that the 96 -h half-lethal concentration of nanoplastics to juvenile shrimp was 396.391 mg/L. As the concentration of nanoplastics increased, the activities of antioxidant enzymes generally decreased, while the contents of hydrogen peroxide and lipid peroxidation products increased. The activities of non-specific immune enzymes first increased and then decreased with increasing nanoplastic concentration. The trends in the expressions of antioxidant-related genes were generally consistent with those in the activities of antioxidant enzymes. As the nanoplastic concentration increased, the expressions of immune-related genes generally increased at first and then decreased. These results indicate that low concentrations of nanoplastics (5 mg/L) may enhance the viability of juvenile shrimp, whereas high concentrations (10,20, 40 mg/L) have inhibitory and/or toxic effects. The findings provide basic information on the toxic effects of nanoplastics in juvenile shrimp.

* Corresponding author at: School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China.
E-mail address: ylzhao426@163.com (Y. Zhao).
[1] These authors contributed equally to this work.

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1. Introduction

Plastic is widely used worldwide because of its low weight, durability, and low price (Leal Filho et al., 2019), and its demand is increasing as the world population grows. Global plastic production in 2018 was approximately 359 million tons(PlasticsEurope, 2019). While plastics bring convenience to human life, they also damage the environment due to poor management and careless disposal (Laskar and Kumar, 2019). In the environment, plastic can be degraded into plastic fragments smaller than 5 mm in diameter via ultraviolet radiation, biodegradation, and mechanical wear; these fragments are called microplastics (Thompson et al., 2004; Xie et al., 2020). However, the degradation of microplastics does not stop at the millimeter or micrometer scale; these microplastics can continue to be broken down into nanometer-scale particles(Lambert and Wagner, 2016). Nanoplastics are more toxic than larger plastics. Nanoplastics have the characteristics of small size and large specific surface area. Compared with larger plastics, it is easier to enter biological tissues through biological barriers (Mattsson et al., 2015), and studies have shown that polystyrene nanoplastics have a stronger ability to cause inflammation due to their larger surface area (Brown et al., 2001).Nanoplastics can easily spread through the water body, resulting in environmental pollution (Alimi et al., 2018; Kögel et al., 2020). Nanoplastics have been shown to have toxic effects on aquatic organisms. For example, studies have shown that nanoplastics can accumulate in aquatic organisms to affect digestion and absorption, antioxidant and immune systems, energy reserves, and reproductive capabilities (Liu et al., 2019a; Lu et al., 2016; Sökmen et al., 2019; Yu et al., 2018). Ultimately, nanoplastics may enter the human food chain through aquatic organisms, affecting human health (Lehner et al., 2019). Therefore, the effects of nanoplastics on aquatic organisms have become a hot research topic in recent years (Franzellitti et al., 2019; Xu et al., 2020). Currently, most research on nanoplastics has focused on the marine environment and marine organisms, while relatively few studies have examined freshwater organisms such as *Eriocheir sinensis* and *Daphnia pulex*(Liu et al., 2018; Yu et al., 2018). The abundance of nanoplastics was found in aquatic environment which is likely due to physical and photo-degradation of macro- and micro-plastics in environment (da Costa et al., 2016; Rios Mendoza et al., 2018). Therefore, it is necessary to study the effects of plastics on freshwater organisms.

Macrobrachium nipponense is a freshwater shrimp (phylum Arthropoda, subphylum Crustacea, and order Decapoda) that is mainly distributed in tropical and subtropical areas of the world (New, 2005). *M. nipponense* is an economically important species for aquaculture in China due to its good meat quality, fast growth rate, and strong reproductive ability (Li et al., 2018). The prawn's growth is also restricted due to its rigid exoskeleton with molting cycle recurring throughout the life periodically (Wilder et al., 2009). To date, research on *M. nipponense* has focused on germplasm resources, nutrition, heavy metal stress, and diseases (Ding et al., 2019; Gu et al., 2017; Li et al., 2019a; Sun et al., 2019). However, nanoplastics are also widespread in the freshwater waters where *M. nipponense* live (Chae and An, 2017; Mao et al., 2020). The effects of nanoplastics exposure on some freshwater aquatic organisms have been studied. For example, nanoplastics exposure can affect the growth and reproduction of *D. pulex* (Liu et al., 2018), destroy the immune defense of *E. sinensis* (Liu et al., 2019a). Therefore, it is necessary to study the effect of nanoplastics exposure on *M. nipponense*. In this study, juvenile *M. nipponense* were selected as the research object to investigate the effects of different concentrations of nanoplastics on the antioxidant activity, immune enzyme activities, and the expressions of related genes. The results provide a theoretical basis for studying the antioxidant and immune capacities of aquatic organisms.

Previous research has shown that pollutants in aquatic environments can be toxic to living organisms by stimulating the production of reactive oxygen species (ROS) (Sussarellu et al., 2016), generating oxidative damage (Livingstone, 2001; Lushchak, 2011). The ROS produced in the body is mainly removed through the antioxidant defense system. The antioxidant defense systems of aquatic organisms include free radical scavengers such as reduced glutathione (GSH) and specific antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), Se-glutathione peroxidase (GSH-Px), and glutathione S-transferase (GSH-ST) (Livingstone, 2001). Some studies have suggested that the extent of oxidative stress can be characterized by detecting the ROS content and the content of lipid peroxidation products related to excessive ROS, including lipid peroxide (LPO) and malondialdehyde (MDA) (Livingstone, 2001). Therefore, oxidative stress products and antioxidants are often used as indicators of environmental pollution levels (Valavanidis et al., 2006; Zhang et al., 2020). Immunization is another important way that organisms resist pollutant stress (Huang and Ren, 2020). Crustaceans lack acquired immunity and rely mainly on non-specific immunity (Li and Xiang, 2013). Alkaline phosphatase (AKP), acid phosphatase (ACP), lysozyme (LZM), and phenoloxidase (PO) play important roles in the innate immunity of crustaceans. PO is obtained by the activation of inactive prophenoloxidase (proPO) via the ProPO-activation pathway, which requires the participation of activators such as α-2-macroglobulin (A2M), serine protease, and serine protease homolog (SPH) (Ding et al., 2014; Huang and Ren, 2020; Yu et al., 2019). These immune factors are effective indicators for assessing the toxicity of ammonia nitrogen, heavy metals, and pathogens in *M. nipponense* (Ding et al., 2019; Li et al., 2019b; Yu et al., 2019). The above indicators were selected to characterize the effects of nanoplastics on the antioxidant and immune defense systems of juvenile shrimp in this study.

In summary, polystyrene nanoplastics and juvenile *M. nipponense* were selected as the research material and object, respectively, in this study. The objectives were to elucidate: (i) the effects of 96-h exposure to nanoplastics at different concentrations on the survival of juvenile shrimp; the effects of 28-d exposure to nanoplastics at different concentrations (ii) on the antioxidant and immune enzyme activities of juvenile shrimp; and (iii) on the antioxidant- and immune-related gene expressions of juvenile shrimp. The findings provide reference data for studying the effects of nanoplastics on freshwater organisms.

2. Materials and methods

2.1. Experimental animals and test materials

The juvenile shrimp used in the experiments were obtained from Qingpu aquafarm (Qingpu District, Shanghai, China). The juvenile shrimp were temporarily kept in several 300-L aquariums for two weeks. Polyvinyl chloride pipes and nets were placed in the aquariums as shelters to prevent the shrimp from eating each other during molting. The water temperature was 25 °C ± 1 °C, the pH was 6.5–7.5, and air was continuously bubbled into the water for 24 h to maintained the dissolved oxygen content above 6 mg/L. During the holding period, commercial feed (9812, Shanghai Harmony Feed Co., Ltd.) was provided at 08:00 and 18:00 each day in an amount of 5% of the shrimp weight.

Polystyrene nanoplastics were purchased from BaseLine Chromtech Research Centre (Tianjin, China). The nanoplastics were monodisperse polystyrene microspheres 75 nm in nominal diameter and supplied in 10-mL aqueous suspensions of 2.5 %(w/v) (1.06×10^{13} particles/mL) concentrations. The sizes and shapes of the nanoplastics were measured by transmission electron microscopy (TEM). Dynamic light scattering analysis showed that the nanoplastics were stable in fresh water without obvious agglomeration or aggregation. No aggregation/agglomeration was observed during the test.TEM data showed that the average size of the 75-nm nanoplastics was 71.18 nm. According to the DLS data, the Z-average particle diameter was dav = 85.86 nm and the average relative half-width of the distribution was σ = 25.31 nm. All these results have been published by Liu et al. (2018).

2.2. Acute experiment of nanoplastic exposure for 96h

In a pre-experiment, the nanoplastic concentration was set to 0, 20, 40, 80, 160, 320, 640, or 1280 mg/L, and 96 -h breeding experiments were performed in 200-mL beakers. Ten juvenile shrimp with similar sizes and shapes were randomly placed in each beaker, and the death rate was recorded at 24, 48, 72, and 96 h to determine the half-lethal nanoplastic concentration (LC50).

The pre-experimental results indicated that the 96-h LC50 value was between 320 and 640 mg/L. According to the pre-experiment results, the nanoplastic treatment concentrations in the acute experiment were 0, 235, 372, 469, 545, and 606 mg/L. The breeding experiment was performed in 300-mL beakers of aqueous solution with three parallel groups. Referring to a previous experiment on lead stress in juvenile shrimp (Ding et al., 2019), 15 juvenile shrimp with weights of (0.12 ± 0.04) g and lengths of (24.46 ± 2.5) mm were placed in each group. At 24, 48, 72, and 96 h, the numbers of dead and moulting shrimp were counted. Half of the nanoplastic solution was replaced with the solution of the same concentration each two days, and the dead shrimp and residual bait were removed.

2.3. Chronic experiment of nanoplastic exposure for 28d

The nanoplastic concentrations in the chronic experiment were selected as 0, 5, 10, 20, and 40 mg/L. Since nanoplastics are difficult detected in the environment and can be originated by the degradation of plastics/microplastics, their concentration in the environment may be directly correlated with the concentration of plastics/microplastics (Rocha-Santos et al., 2019; Yu et al., 2020). Thus, the nanoplastic concentrations in the present study were chosen based on previous studies that detected the presence of microplastics in the environment (Dubaish and Liebezeit, 2013; Eriksen et al., 2013; Sendra et al., 2019).The solution volume in the chronic experiment was 3 L. Each group included three parallel groups of 40 juvenile shrimp with weights of (0.14 ± 0.06) g and body lengths of (22.96 ± 3.87) mm. The breeding experiment was carried out in a glass tank with dimensions of 35 cm × 27 cm × 24 cm for 28 d. Half of the original nanoplastic solution was replaced every 2 d, and dead shrimp and bait were removed. Whole shrimp samples were taken at 7, 14, 21, and 28 d and stored in a refrigerator at −80℃ for subsequent analysis of biochemical indicators and gene expression.

2.3.1. Biochemical analysis

To assess the toxic effects of juvenile *M. nipponensis* exposure to nanoplastics, the changes in the biomarkers of the sampled whole shrimp were evaluated. Tissue homogenate (10 %) was centrifuged at 2500 rpm for 10 min at 4 ˚C, and the supernatant was collected for the determination of biomarkers. The protein concentration in the supernatant was determined using the Coomassie Brilliant Blue method. The activities of SOD, CAT, GSH-Px, GSH-ST, AKP, ACP, LZM, and PO along with the contents of GSH, hydrogen peroxide (H_2O_2), LPO, and MDA were determined using commercially available kits (Nanjing Jiancheng Biotechnology Research Institute, China).

SOD activity was analyzed by xanthine oxidase (Elstner and Heupel, 1976). CAT was measured following hydrogen peroxide reduction at 405 nm (Aebi, 1984) and expressed as units per milligram protein. GSH reduction was analyzed to determine GSH-Px activity (Sedlak and Lindsay, 1968). GSH-ST activity was measured based on the method by Pradeepa et al. (2016). GSH contents were determined by the modified method of Vardi et al. (2008). The H_2O_2 concentration was determined according to Islam et al. (2008). Lipid peroxidation was measured according to Ohkawa et al. (1979). MDA content was measured using the thiobarbituric acid method and expressed as nmol/mgprot (Ohkawa et al., 1979). Acid phosphatase (ACP) activity was measured by a disodium phenyl phosphate method (Subramanian et al., 2013). Alkaline phosphatase (AKP) activity was analyzed by the method reported by Rausch and Moore (1976). LZM activity was detected by turbidimetric assay (Hultmark et al., 1980). PO enzyme activity was measured using a method described by Ashida and Söderhäll (1984).

The absorbance measurements in the above experiments were performed using a microplate spectrophotometer (BioTek Instruments, Inc, USA).

2.3.2. RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction (qRT-PCR)

Whole shrimp treated for 28 d were taken as samples to determine the expressions of antioxidant- and immune-related genes. Total mRNA was extracted from the whole shrimp using an easy-spin total RNA extraction kit (Beijing Aidlab Biotechnologies Co., Ltd., China). RNA integrity was detected by 1.0 % agarose (Transgen, Beijing, China) gel electrophoresis, and the purity and concentration of the extracted RNA were quantified based on the OD260/OD280 ratio determined using a NanoDrop 2000 spectrophotometer. Hiscript®III RT SuperMix for qRT-PCR (Vazyme Biotech Co., Ltd., Nanjing, China) was used for reverse transcription to obtain cDNA. Prior to use, the resulting cDNA was stored at −20 ˚C.

The gene sequences of *M. nipponense* were found on NCBI (https://www.ncbi.nlm.nih.gov/), and the primer sequences were designed by NCBI Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). The synthetic primer sequences are shown in Table 1. The primers were synthesized by Shanghai Platinum Biotech Co., Ltd.

The ChamQ™ Universal SYBR® qPCR Master Mix Kit (Vazyme Biotech Co., Ltd., Nanjing, China) was used to evaluate the expressions of antioxidant- and immune-related genes in juvenile shrimp. A CFX96™ Real-Time System (Bio-Rad, USA) was used for qRT-PCR. The reaction mixture (20 μL) contained 2 × ChamQ Universal SYBR qPCR Master Mix 10 μL, 1 cDNA template, and 0.4 μL (10 μM) each of the forward and reverse primers (Table 1). The reaction conditions were as follows: pre-denaturation at 95 ˚C for 30 s, denaturation at 95 ˚C for 10 s, annealing at 60 ˚C for 30 s, and 40 cycles. Three replicates were evaluated for each sample. According to the literature (Liu et al., 2019a; Yu et al., 2018), the housekeeping gene β-actin was used for correction. The specificity of the amplified product was confirmed by melting curve analysis.

2.4. Statistical analysis

The experimentally measured indicators are expressed in the form of mean ± standard error (mean ± SD). Microsoft Office Excel 2010 and SPSS Statistics 17.0 were used for data analysis. Two-way ANOVA and Duncan's multiple comparisons were used to evaluate significant

Table 1
Nucleotide sequences and sources of primers used for qRT-PCR.

Genes	Sequence(5′-3′)	Reference or Query Gene ID
MnSOD F	ACGATCAAGCATGGCTGACA	HQ852226
MnSOD R	GCAGCATTTGCGAGCTGATT	
GSH-ST F	ACATTCGAGGTCTTGCCCAG	KC485003
GSH-ST R	TGTCAAACTTTGGGGCTGGT	
GSH-Px F	CCTGATTGGCAAGGATGG	HQ651155
GSH-Px R	CAAGGATCGGGTTCGTGT	
CAT F	ACTCCTGGACGAGATGTGC	KC485002
CAT R	AACTTGACAGCGAAGCCAC	
LZM F	GACCTTGCGTCATGCCAGAT	AY257550
LZM R	CCATGGGTTTATGTGCGTCTTC	
proPO F	GGACCTCTGCGACTACTT	JN704301
proPO R	CGGTTTCCGAATGATGAC	
A2M F	GACCGACGAAAGTAACCCA	MK439847
A2M R	GGTTCGCTTCGACAATCCT	
SHP F	TGTTGCCCTGTCAGTCATG	JQ757047
SHP R	GGTCTGCCGAATCCACTT	
β-actin F	CAGGTCGTGACTTGACCGAT	KY780298
β-actin R	CGTCAGGGAGCTCGTAAGAC	

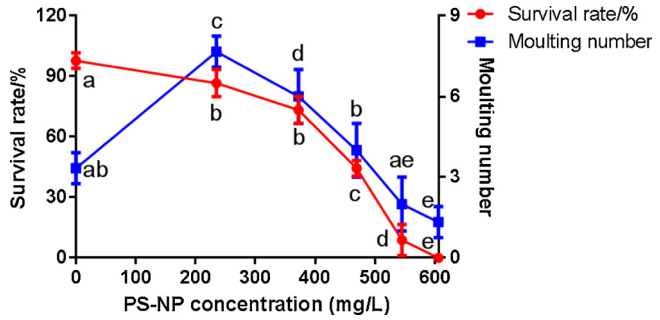

Fig. 1. Effects of different concentrations of nanoplastics on survival rate and moulting number at 96 h. Data are presented as mean ± SD. Different letters indicate significant differences between treatments ($P < 0.05$).

differences between the control and treatment groups. Before ANOVA, the Levene test was used to test for homogeneity of variance. In the case of uneven variance, the Dunn–Bonferroni method in the Kruskal–Wallis test was applied (Liu et al., 2020a).

Survival rate was calculated as follows: survival rate (%) = 100 % × number of surviving shrimp at the end of the experiment / number of shrimp at the beginning of the experiment. The 96-h LC50 of nanoplastics in juvenile shrimp was calculated using SPSS 17.0 Probit analysis along with the 95 % confidence interval (Liu et al., 2019b). The relative expressions of genes were calculated using the $2^{-\Delta\Delta Ct}$ method. ΔCt was defined as the difference between the internal reference (β-actin) *Ct* value and the target gene *Ct* value, while $\Delta\Delta Ct$ was defined as the difference between the control group ΔCt value and the treatment group ΔCt value.

3. Results

3.1. Effect of nanoplastics on the survival of juvenile M. Nipponense

No total immobilization exceeding 10 % was observed in the control at 96 h. As shown in Fig. 1, the 96-h LC50 value of nanoplastics in juvenile shrimp was calculated as 396.391 mg/L, and the 95 % confidence interval was 25.953–637.579 mg/L. The 96-h mortality of juvenile shrimp decreased with increasing nanoplastic concentration, while the number of moulting shrimp generally increased first and then decreased. The number of moulting shrimp in the group treated with nanoplastic at the concentration of 235 mg/L was significantly higher than those in the other groups ($P < 0.05$). As for Table 2, there were no significant differences in weight gain and length gain between different nanoplastics levels ($P > 0.05$).

3.2. Effects of nanoplastics on the antioxidant and immune enzyme activities of juvenile M. Nipponense

The effects of different concentrations of nanoplastics on the antioxidant enzyme activities in juvenile *M. nipponense* after 28 d of exposure are shown in Fig. 2. For SOD, the enzyme activity was only significantly higher than that in the control in the group treated with 5 mg/L nanoplastics at 14 d ($P < 0.05$). The SOD enzyme activities in the other groups were lower than that in the control group, and SOD enzyme activity showed a downward trend with increasing nanoplastic concentration (Fig. 2A). Compared to the control, the CAT enzyme activity was significantly higher in the group treated with 5 mg/L nanoplastics at 7, 14, and 21 d and in the group treated at 10 mg/L at 21 d ($P < 0.05$). In the other groups, CAT enzyme activity was lower than in the control. Overall, CAT enzyme activity first increased and then decreased with increasing nanoplastic concentration (Fig. 2B). The activities of GSH-Px and GSH-ST decreased with increasing nanoplastic concentration for all sampling days (Fig. 2C and D). With the exception of the group treated with 5 mg/L nanoplastics at 7 d, the GSH content was lower than that in the control in all groups. GSH content decreased continuously with increasing nanoplastic concentration (Fig. 2E).

At 7 and 14 d, the H_2O_2 content first decreased and then increased with increasing nanoplastic concentration. The lowest H_2O_2 contents were found in the groups treated with 10 mg/L nanoplastics at 7 d and with 5 mg/L nanoplastics at 14 d. At 21 and 28 d, the H_2O_2 content increased with both time and nanoplastic concentration (Fig. 2F). The MDA content in the group treated with 5 mg/L nanoplastics was significantly lower than that in the control group ($P < 0.05$). At 21 and 28 d, the MDA content in the group treated with 10 mg/L nanoplastics decreased as exposure time increased. In the other groups, the MDA content increased with increasing exposure time and nanoplastic concentration (Fig. 2G). The LPO content increased with increasing nanoplastic concentration at all time points (Fig. 2H). Altogether, the data indicate that antioxidant enzyme activity and the contents of oxidative stress products in juvenile shrimp depend on both exposure time and nanoplastic concentration.

The effects of nanoplastics at different concentrations on the immunoenzyme activities of juvenile *M. nipponense* are shown in Fig. 3. In the groups treated with 5 mg/L nanoplastics at 7, 14, and 21 d and 10 mg/L nanoplastics at 21 d, AKP activity was significantly higher than in the control group ($P < 0.05$). In the remaining treatment groups, the AKP activity decreased with increasing nanoplastic concentration (Fig. 3A). At 7 and 14 d, ACP activity first increased and then decreased with increasing nanoplastic concentration, with the maximum activity observed at the concentration of 5 mg/L in both cases. At 21 and 28 d, the ACP activity decreased with increasing nanoplastic concentration (Fig. 3B). The trend in LZM activity was similar to that of ACP. At 7 and 14 d, LZM activity was maximized at the nanoplastic concentration of 5 mg/L; the activity then decreased with both increasing exposure time and nanoplastic concentration (Fig. 3C). PO activity was lower than that in the control group for all treatment conditions and time points, and PO activity decreased with increasing nanoplastic concentration (Fig. 3D). In summary, the activities of AKP, ACP, LZM, and PO were dependent on the nanoplastic concentration, while the activities of AKP, ACP, and PO were also dependent on exposure time.

Table 2
Effects of different nanoplastic concentrations on the growth Macrobrachium nipponense (mean ± SE, n = 10). No significant differences were observed between conditions ($P > 0.05$).

	Nanoplastics concentration					
	0 mg/L	235 mg/L	372 mg/L	469 mg/L	545 mg/L	606 mg/L
Initial shrimp wet weight(g)	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
Initial shrimp body length(mm)	24.45 ± 1.38	24.48 ± 1.69	24.46 ± 1.13	24.47 ± 1.43	24.46 ± 1.06	24.45 ± 1.31
Final shrimp wet weight(g)	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Final shrimp body length(mm)	25.78 ± 0.99	26.15 ± 0.78	26.12 ± 0.77	26.01 ± 0.51	25.79 ± 0.70	25.83 ± 0.52
Weight gain (WG, %)	10.08 ± 7.36	12.40 ± 5.78	11.48 ± 5.73	11.67 ± 4.30	10.17 ± 5.65	10.83 ± 4.03
Length gain(LG, %)	5.45 ± 4.07	6.84 ± 3.19	6.77 ± 3.13	6.29 ± 2.10	5.45 ± 2.85	5.66 ± 2.11

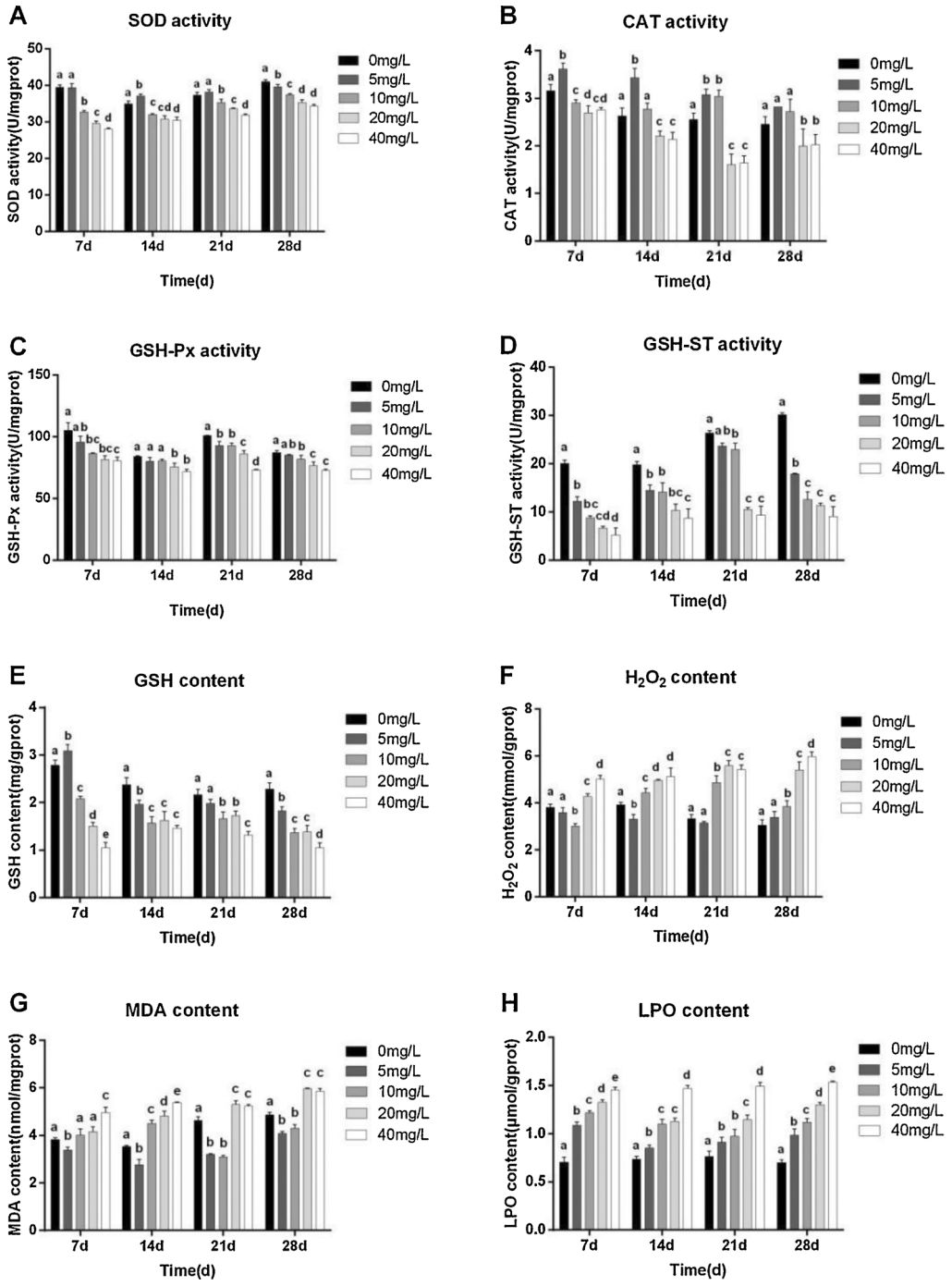

Fig. 2. Effects of different concentrations of nanoplastics on antioxidant enzyme activity. Data are presented as mean ± SD (three replicates per group). $P < 0.05$ was considered to indicate statistical significance. Different letters denote statistical differences between treatments. SOD: superoxide dismutase; CAT: catalase; GSH-Px: Se-glutathione peroxidase; GSH-ST: glutathione S-transferase; GSH: reduced glutathione; H_2O_2: hydrogen peroxide; MDA: malondialdehyde; LPO: lipid peroxide.

3.3. Effects of nanoplastics on antioxidant- and immune-related genes in juvenile M. Nipponense

As shown in Fig. 4, as the nanoplastic concentration increased, the expressions of *SOD*, *CAT*, *GSH-Px*, and *GSH-ST* genes in juvenile shrimp first increased and then decreased. The expressions of *SOD* gene were significantly higher in the groups treated with 5 and 10 mg/L nanoplastics than in the control group ($P < 0.05$). No significant difference in *SOD* gene expression was observed between the groups treated with 20 and 40 mg/L nanoplastics and the control group ($P > 0.05$; Fig. 4A). *CAT* gene expression was significantly higher in the groups treated with 5 and 10 mg/L nanoplastics compared to the control group ($P < 0.05$); meanwhile, the *CAT* gene expressions in the groups treated with 20 and 40 mg/L nanoplastics were significantly lower than in the control group ($P < 0.05$; Fig. 4B). The *GSH-Px* gene expression was significantly higher in the group treated with 5 mg/L nanoplastics compared to the control ($P < 0.05$), while no significant difference was observed between the control and the other treatment groups ($P > 0.05$; Fig. 4C). The expression of *GSH-ST* gene in the group treated with 40 mg/L nanoplastics was not significantly different from that in the control group ($P < 0.05$), while in the other treatment groups, *GSH-ST* gene expression was significantly higher compared to the control ($P < 0.05$;

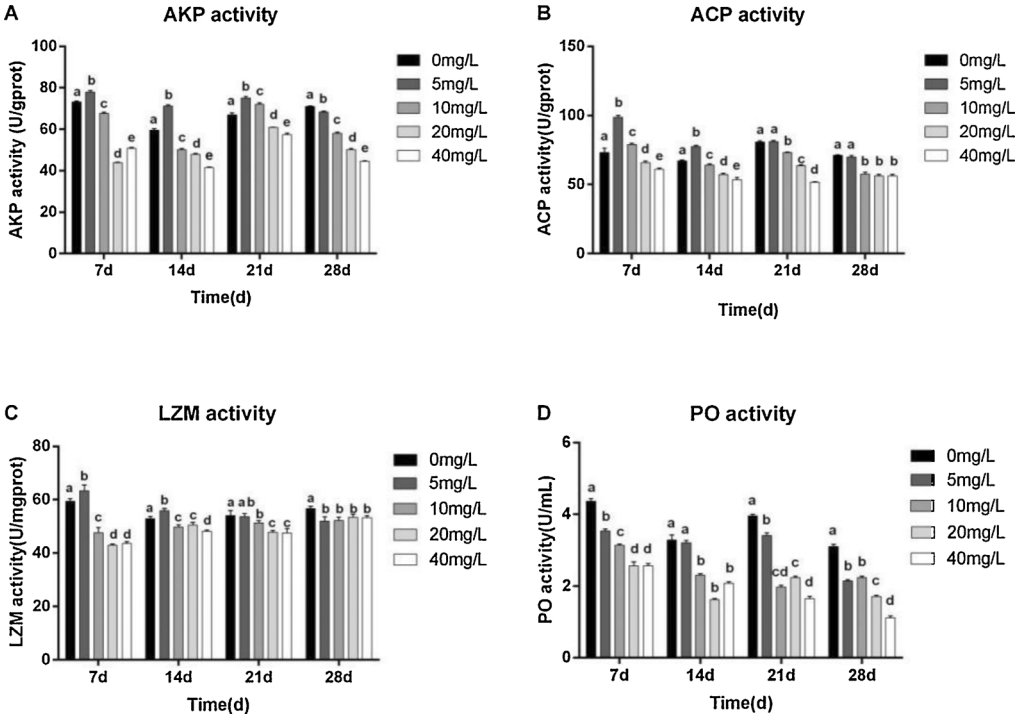

Fig. 3. Effects of different concentrations of nanoplastics on immunoenzyme activity. Data are presented as mean ± SD (three replicates per group). $P < 0.05$ was considered to indicate statistical significance. Different letters denote statistical differences between treatments. AKP: alkaline phosphatase; ACP: acid phosphatase; LZM: lysozyme; PO: phenoloxidase.

Fig. 4D).

The effects of different concentrations of nanoplastics on the expressions of immune-related genes in juvenile *M. nipponense* are shown in Fig. 5. The expressions of *LZM*, *proPO*, *A2M*, and *SPH* genes generally first increased and then decreased with increasing nanoplastic concentration. The expression levels of *LZM* and *proPO* genes were significantly higher in the groups treated with 5 and 10 mg/L nanoplastics than in the control group ($P < 0.05$), and the expressions were highest at the nanoplastic concentration of 5 mg/L; meanwhile, no significant differences in *LZM* and *proPO* gene expressions were observed between the control and the groups treated with 20 and 40 mg/L nanoplastics ($P > 0.05$; Fig. 5A, B). The expressions of *A2M* and *SPH* genes in the group treated with 5 mg/L nanoplastics were not significantly different from those in the control group ($P > 0.05$); however, the *A2M* and *SPH*

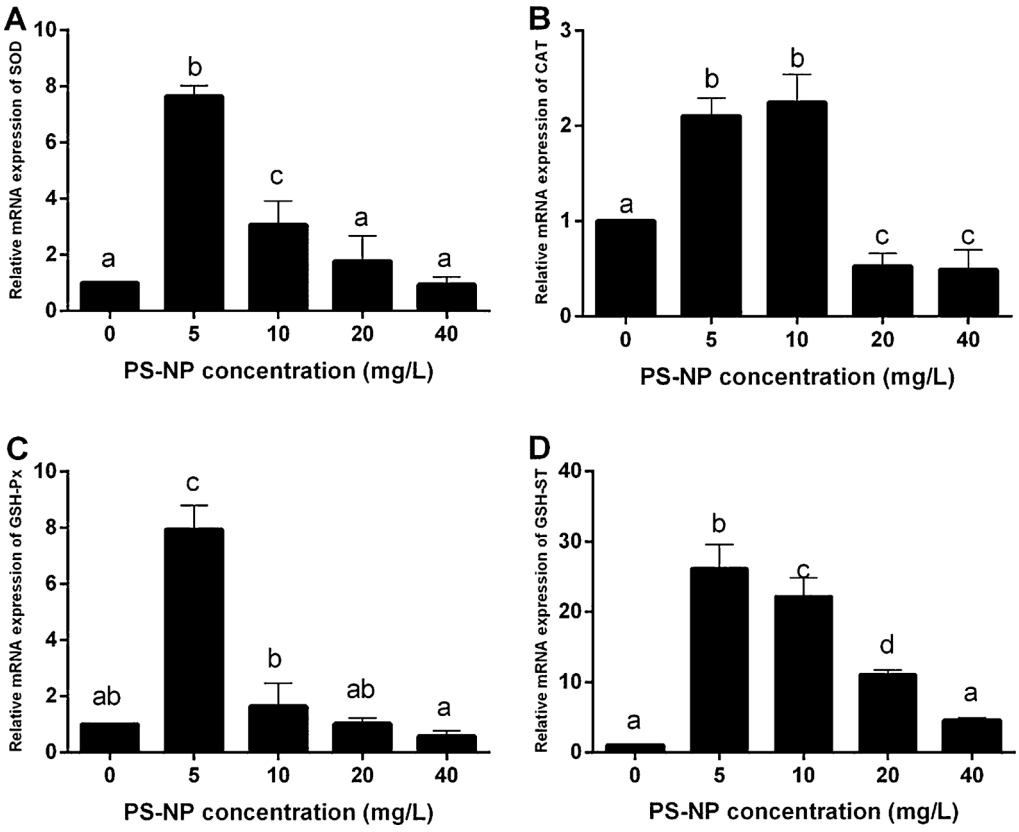

Fig. 4. Effects of different concentrations of nanoplastics on the expressions of antioxidant-related genes. Data are presented as mean ± SD (three replicates per group). $P < 0.05$ was considered to indicate statistical significance. Different letters denote statistical differences between treatments. SOD: superoxide dismutase; CAT: catalase; GSH-Px: Se-glutathione peroxidase; GSH-ST: glutathione S-transferase.

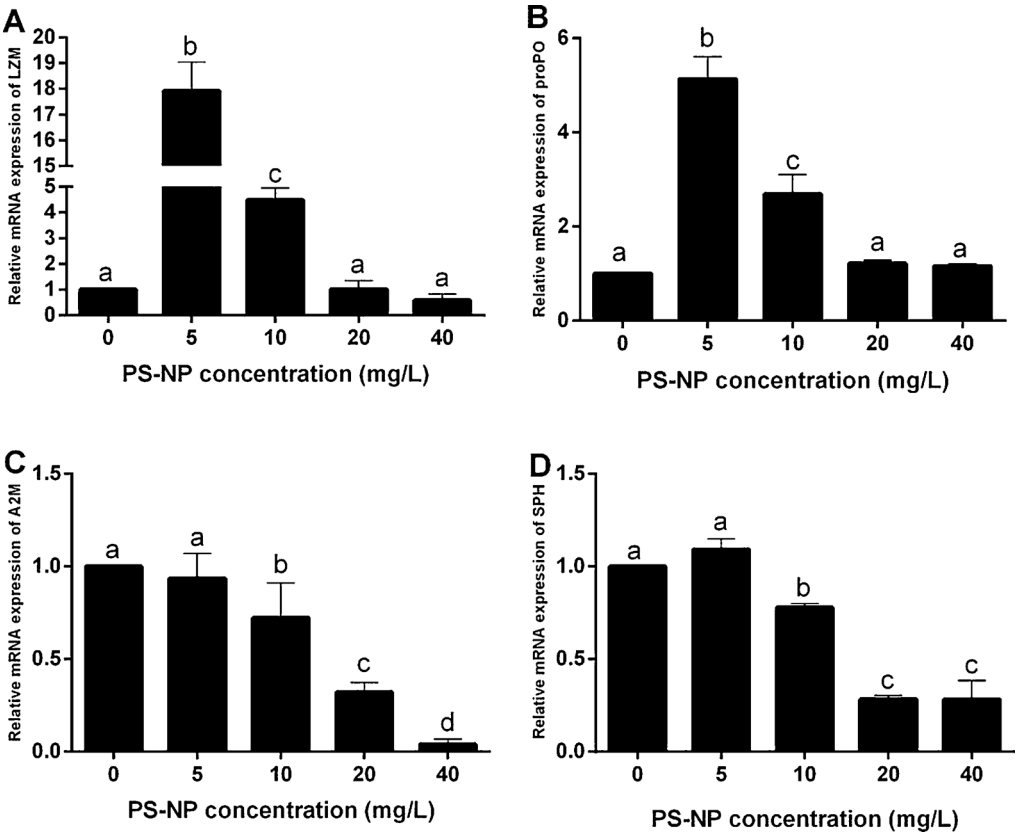

Fig. 5. Effects of different concentrations of nanoplastics on the expressions of immune-related genes. Data are presented as mean ± SD (three replicates per group). $P < 0.05$ was considered to indicate statistical significance. Different letters denote statistical differences between treatments. LZM: lysozyme; proPO: prophenoloxidase; A2M: α-2-macroglobulin; SPH: serine protease homolog.

gene expressions were significantly lower in the groups treated with 10, 20, and 40 mg/L nanoplastics compared to the control ($P < 0.05$; Fig. 5C and D).

4. Discussion

4.1. Effects of nanoplastics on the survival of juvenile M. Nipponense

In this study, the survival rate of juvenile *M. nipponense* decreased with increasing nanoplastic concentration. Similar results have also been reported in *Daphnia magna* and *D. pulex* treated with nanoplastics (Liu et al., 2019b; Nasser and Lynch, 2016). The decreased survival rate may be attributed to the adsorption of nanoplastics by young shrimp during feeding, resulting in obstruction of the digestive tract, loss of appetite, and death (Wright et al., 2013). The findings show that nanoplastics have a negative effect on the survival of juvenile shrimp.

The surface epidermis of *M. nipponense* is hard, which protects the organism but also restrains its growth; thus, the shrimp must molt multiple times during its lifespan (Li et al., 2019a). The molt stages of the prawns were identified based on the criteria described by de Oliveira Cesar et al. (2006). Individual prawns were divided into three molt stages: intermolt, premolt and postmolt stages. In this study, the number of moulting shrimp first increased and then decreased as the nanoplastic concentration increased, and the number of moulting shrimp was significantly lower in the group treated with 40 mg/L nanoplastics than in the control group. Juvenile shrimp gain weight and body length after moulting. Therefore, the effect of nanoplastic exposure on the moulting of juvenile shrimp suggests that nanoplastics affect the growth of juvenile *M. nipponense* in terms of weight and body length. Studies on aquatic organisms such as *E. sinensis*, *Crassostrea gigas*, adult *Danio rerio*, and *Litopenaeus vannamei* found that microplastic exposure affects growth (Chae et al., 2019; Sussarellu et al., 2016; Yu et al., 2018; Zhao et al., 2020). Therefore, the effects of nanoplastics on the growth of juvenile *M. nipponense* should be further studied.

4.2. Effects of nanoplastics on antioxidant and immune enzyme activities of juvenile M. Nipponense

Studies on the toxicity of nanoplastics to *E. sinensis*, *D. pulex*, *D. rerio*, and other organisms have shown that the toxicity of nanoplastics may arise from the stimulation of excessive ROS production in living organisms (Liu et al., 2020b; Lu et al., 2016; Yu et al., 2018). Immune defense plays an important role in defending aquatic organisms from stress. Pollutants in water have toxic effects on *M. nipponense*(Ding et al., 2014; Li et al., 2019b). To further study how nanoplastics affect the survival of juvenile shrimp, the enzyme activities and contents of substances related to antioxidant and immune systems were evaluated in this study.

ROS include metabolically produced radical and non-radical species such as O_2^- and H_2O_2. Appropriate amounts of ROS can function as signaling molecules and stimulate normal apoptosis in cells (Zucker et al., 1997). However, excessive ROS levels will disrupt the redox balance in the cells, resulting in oxidative stress (Sies, 1985). To maintain the balance, aquatic organisms have antioxidant defense systems. SOD, CAT, GSH-Px, and GSH-ST are the main antioxidant enzymes employed by aquatic organisms to reduce the harmful effects of excessive ROS production (Liu et al., 2020c; Wu et al., 2019). SOD in organisms converts O_2^- into less toxic H_2O_2. The H_2O_2 is then converted into harmless substances (O_2 and H_2O) by CAT, GSH-ST, GSH-Px, and other enzymes (Liu et al., 2020b). In this study, the activities of SOD and CAT increased upon exposure to low concentrations of nanoplastics (5 mg/L) and a short exposure time, indicating minor damage to the juvenile shrimp and activation of the oxidative defense system. Similar results were seen in *D. rerio* and *E. sinensis* (Lu et al., 2016; Yu et al., 2018). However, with the increase of nanoplastics concentration or exposure time, the activities of SOD and CAT were significantly lower than those in the control group. This may be due to

damage to the immune system. In addition, treatment with nanoplastics significantly inhibited the activities of GSH-Px and GSH-ST. The activities of GSH-Px and GSH-ST were significantly lower in the treatment groups than in the control group, and the activities decreased continuously with increasing nanoplastic concentration and increasing exposure time. This is consistent with the results reported for *E. sinensis* and *Oryzias melastigma* (Wang et al., 2019; Yu et al., 2018). The changes in the activities of these antioxidant enzymes may be related to the energy expenditure caused by oxidative stress (Yu et al., 2018). In addition to antioxidant enzymes, GSH can also eliminate excess ROS in the body by combining with GSH-Px and GSH-ST or directly reacting with some reactive oxygen molecules. GSH is also an important ROS scavenger (Regoli and Giuliani, 2014). In this study, the GSH content decreased with increasing nanoplastic concentration and increasing exposure time, indicating that GSH was oxidized due to the excessive ROS level, causing its content to decrease. This is consistent with the results reported on *Tigriopus japonicus* (Choi et al., 2020). The content of H_2O_2 in this study was found to increase with increasing nanoplastic concentration and increasing exposure time. It is possible that exposure to nanoplastics inhibited the antioxidant enzyme activity in juvenile shrimp, resulting in an excess H_2O_2 that could not be removed in time, causing the content to increase (Wang et al., 2019). Similar results were found in *D. pulex* (Liu et al., 2020b). Excessive ROS levels can induce lipid peroxidation, the production of LPO and MDA, and a variety of damages to cells and the cell membrane structure. Therefore, LPO and MDA are often used to reflect the degree of oxidative damage in the body (Livingstone, 2001; Lv et al., 2014). In this study, the activity of LPO increased with increasing nanoplastic concentrationand increasing exposure time, while the MDA content first decreased and then increased. The increases in the contents of LPO and MDA suggest that exposure to a high concentration of nanoplastics (10, 20, 40 mg/L) or exposure after a long time causes the ROS levels in juvenile shrimp to increase, eventually leading to lipid peroxidation. This is consistent with studies on *O. melastigma* and *E. sinensis* (Wang et al., 2019; Yu et al., 2018). Overall, exposure to nanoplastics causes elevated ROS levels. Exposure to low concentrations nanoplastics (5 mg/L) or a short time increases the antioxidant enzyme activity in juvenile shrimp, helping remove excess ROS and maintain redox balance. However, exposure to high concentrations of nanoplastics (10, 20, 40 mg/L) or a long time causes severe oxidative damage, thereby reducing the antioxidant enzyme activity and damaging the oxidative defense system.

Crustacean immunity is mainly non-specific immunity, which relies on PO, LZM, AKP, ACP, and other immune-related enzymes and immune factors (Huang and Ren, 2020; Li and Xiang, 2013). AKP and ACP are phosphohydrolases; AKP can enhance the recognition and phagocytosis of pathogens by changing the surface structures of pathogens (Liu et al., 2019a). ACP is one of the important marker enzymes of lysozyme to break down invading organisms (Zhang et al., 2004). LZM is a lysosomal enzyme capable of hydrolyzing the cell walls of bacteria (Ellis et al., 2011), while PO is a product of the ProPO-activation pathway, which is closely related to the activation of cell defense and the appearance of phagocytic factors (Ellis et al., 2011). These immune enzymes have important effects on immune defense in crustaceans. Thus, they were selected to study the effects of nanoplastics on the immune responses of juvenile *M. nipponense*. In this study, the activities of AKP, ACP, and LZM increased slightly upon exposure to a low concentration of nanoplastics (5 mg/L) or a short exposure time. This indicates that exposure to a low concentration of nanoplastics or a short time can activate the immune system of juvenile shrimp, stimulate the release of LZM from lysosomes, and promote the release of hydrolytic enzymes AKP and ACP to resist immune stress. When *Mytilus* and *Symphysodon aequifasciatus* were exposed to low-concentration nanoplastics, similar changes in enzyme activity were observed (Canesi et al., 2015; Wen et al., 2018). In this study, in the group treated with a high concentration of nanoplastics (10, 20, 40 mg/L), LZM activity decreased over time. This suggests that a high concentration of nanoplastics or a long exposure time impairs lysosomal degradation capacity and hinders the phagocytosis of pathogens, similar to the findings in *Tegillarca granosa* (Tang et al., 2020). The activities of ACP and AKP also decreased over time in the group treated with a high concentration of nanoplastics (10, 20, 40 mg/L), indicating that exposure to a high nanoplastic concentration inhibits the release of ACP and AKP from lysosomes and affects immune defense. Time- and dose-dependent responses could be seen in the activities of AKP, ACP and LZM. In this study, the activity of PO gradually decreased with increasing nanoplastic concentration. The activities of ACP, AKP, and PO showed similar trends when *E. sinensis* was exposed to nanoplastics (Liu et al., 2019a). Based on the above changes in immune enzyme activity, as the concentration of nanoplastics or the exposure time increased, the degree of external stress exceeded the capacity of the immune defense system of juvenile shrimp, the activities of immune enzymes decreased, and the immune system was damaged.

The above results are consistent with the hormesis effect. That is, exposure to low concentrations of nanoplastics (5 mg/L) or a short time has a stimulating effect on antioxidant and immune enzyme activities in juvenile shrimp; however, high concentrations of nanoplastics (10, 20, 40 mg/L) or a long exposure time produce inhibitory or toxic effects (Mattson, 2007).

4.3. Effects of nanoplastics on antioxidant- and immune-related genes in juvenile M. Nipponense

To further evaluate whether nanoplastics can regulate the antioxidant defense system, the expressions of genes related to oxidative stress (*SOD*, *CAT*, *GSH-Px*, and *GSH-ST*) were evaluated. As the nanoplastic treatment concentration increased, the expression levels of *SOD*, *CAT*, *GSH-Px*, and *GSH-ST* genes first increased and then decreased, similar to the results obtained for the SOD, CAT, GSH-Px, and GSH-ST enzyme activities. Similar experimental results have been reported for *E. sinensis* and *D. pulex* (Liu et al., 2019b; Yu et al., 2018). Combined with the changes observed in enzyme activity, the changes in gene expression levels show that treatment with low concentrations of nanoplastics (5 mg/L) increase the ROS levels in juvenile shrimp, stimulate the expressions of antioxidant-related genes, and help eliminate excessive ROS. However, as the nanoplastic concentration increased, the expressions of antioxidant-related genes (*SOD*, *CAT*, *GSH-Px*, and *GSH-ST*) decreased. This may be because the high concentration of nanoplastics stimulated the excessive production of ROS in juvenile shrimp (Liu et al., 2020b; Yang et al., 2020). When aquatic organisms are damaged or under stress, it will result in enhanced levels of ROS. High ROS levels may cause lipid peroxidation and membrane damage, thereby affecting organism (Lushchak, 2011). Previous studies have shown that nanoplastics can promote the generation of ROS in *D. pulex* (Liu et al., 2020b), microalgae (Yang et al., 2020), and carp (Xia et al., 2020), which was similar to our results. Exposure to high concentrations of nanoplastics (10, 20, 40 mg/L) also caused lipid peroxidation, inhibited the expressions of antioxidant-related genes, and destroyed the antioxidant defense system.

The immune defense system is another important system that helps organisms resist external environmental stress. In the non-specific immunity of crustaceans, LZM acts on the peptidoglycan layer of the bacterial cell wall, leading to the lysis of the bacterial cell wall (Ellis et al., 2011). The ProPO-activation pathway is also an integral part of the shrimp immune system. The main enzyme produced during system activation is PO, which is the active form of proPO. PO can activate cellular defense and affect the secretion of phagocytic factors (Ellis et al., 2011; Li and Xiang, 2013). SPH and A2M are involved in the ProPO-activation pathway and play an important role in the non-specific immune defense of *M. nipponense* (Ding et al., 2014; Yu et al., 2019). In this study, the expressions of *LZM*, *proPO*, *A2M*, and *SPH* genes were evaluated to further study the effects of nanoplastics on the immune defense of juvenile *M. nipponense*. The expressions of immune-

related genes (*LZM* and *proPO*) were significantly higher in the groups exposed to nanoplastics at low concentration (5 and 10 mg/L) compared to in the control group. When *M. nipponense* was infected by non-O1 *Vibrio cholerae*, the expressions of *LZM* and *ProPO* genes also increased (Li et al., 2019b). This may indicate that stress induces the expression of *LZM* and *proPO* genes in juvenile shrimp, strengthening the immune defense against nanoplastic stress. However, as the nanoplastic concentration increased, the *LZM*, *proPO*, *A2M*, and *SPH* gene expressions became lower than those in the control group. The decreased expressions of *proPO*, *A2M*, and *SPH* genes inhibited the activation of the phenol oxidase system, consistent with the previously reported reduction in PO activity. Based on the analysis of enzyme activities, exposure to nanoplastics a high concentration inhibits the expressions of immune-related genes and damages the immune system.

The changes in the antioxidant- and immune-related genes further confirmed the earlier speculation that low-dose nanoplastic (5 mg/L) exposure has a stimulating effect on juvenile shrimp, whereas high-dose (10, 20, 40 mg/L) nanoplastic exposure has an inhibitory or toxic effect. This conclusion is consistent with the findings in *E. sinensis* (Liu et al., 2019a).

5. Conclusion

The effects of nanoplastics on juvenile *M. nipponense* were discussed from the perspectives of survival, enzyme activity, and gene expression. Exposure to low concentrations of nanoplastics (5 mg/L) induced oxidative stress and immune response, promoted the expressions of related genes, and enhanced the antioxidant and immune enzyme activities in juvenile shrimp to resist nanoplastic stress. However, exposure to high concentrations of nanoplastics (10, 20, 40 mg/L) negatively affected the antioxidant and immune defenses of juvenile shrimp, inhibited the expressions of antioxidant- and immune-related genes, and reduced the activities of related enzymes. In total, the results show that the toxic effects of nanoplastics on juvenile shrimp may be related to the effects of nanoplastics on antioxidant defense and immune defense. This study provides reference data for further evaluating the effects of nanoplastics on aquatic organisms and studying the potential mechanisms of nanoplastic toxicity.

CRediT authorship contribution statement

Yiming Li: Conceptualization, Methodology, Resources, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Zhiquan Liu:** Conceptualization, Methodology, Resources, Visualization, Writing - original draft, Writing - review & editing. **Maofeng Li:** Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Qichen Jiang:** Conceptualization, Methodology, Resources, Visualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Donglei Wu:** Conceptualization, Methodology, Writing - review & editing. **Youhui Huang:** Investigation, Data curation, Writing - review & editing. **Yang Jiao:** Conceptualization, Methodology, Writing - review & editing. **Meng Zhang:** Conceptualization, Methodology, Resources, Writing - review & editing. **Yunlong Zhao:** Conceptualization, Methodology, Resources, Visualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Effects of Nanoplastics on Antioxidant and Immune Enzyme Activities and Related Gene Expression in Juvenile *Macrobrachium nipponense*”.

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