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Research Article

The Effects of Desiccation on the Antioxidant Defense, Immune Responses, and Nitrogen Metabolism of Red Swamp Crayfish *Procambarus clarkii*

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Desiccation due to air exposure is a common environmental stress that harms the health of crustaceans. How crustaceans respond to air exposure remains largely unclear. In this research, we explored the physiological responses and gene regulation under air exposure of an invasive species *Procambarus clarkii*, which is the most commonly cultured freshwater crustacean in China. Toll3 belonging to the Toll-like receptor family and proPO in the prophenoloxidase-activating system were two important immune responses to air exposure. In addition, air exposure can also influence ammonia metabolism via regulating the expression of the key genes. *Prx6* and proPO showed quick responses and high sensitivity to air exposure, suggesting the potential to be the biomarker for desiccation stress assessment in crayfish. The results of this research can provide useful information for monitoring desiccation stress, as well as transport and sale management in red swamp crayfish.

1. Introduction

Red swamp crayfish, *Procambarus clarkii*, a freshwater crustacean native to the Mississippi and Gulf of Mexico drainages, has been introduced to every continent except Antarctica. *P. clarkii* was first introduced to China in 1929 as a typical invasive species, while it has been used as an aquaculture species since 1983. Over the last few decades, the stock of this species has increased rapidly in China, reaching 2,890,684 tons in 2022, becoming the most productive freshwater crustacean in China [1]. During the farming and production of crayfish, a variety of environmental stressors, such as ammonia concentration, hypoxia, and heat shock, can influence the growth, health, and even survival of this species. Among these environmental factors, hypoxia induced by long-time air exposure is inevitable because dry delivery without water is the main method for transport and sales of crayfish [2, 3]. Previous studies suggested that long-term desiccation can cause severe respiratory metabolic disorder, tissue damage, oxidative stress and apoptosis, and even mass mortality in crustaceans [4–8]. However, the mechanism of desiccation induced by air exposure to aquatic animals is still largely unexplored.

During air exposure, crustaceans are subjected to functional hypoxic, which can induce cellular homeostasis imbalance and accumulation of reactive oxygen species (ROS) [6, 8, 9]. Generally, free radicals are essential for several physiological activities (e.g., signaling, transcription, and catalysis); however, free radicals are highly reactive and extremely unstable and, therefore, can be also deleterious [10, 11]. Naturally, the body should equilibrate the generation and neutralization of free radicals, but environmental stress can disrupt this balance, leading to the accumulation of endogenous free radicals [12]. Excessive free radicals can oxidize lipids, proteins, and nucleic acids and then cause oxidative stress [13]. The activities of many antioxidant enzymes (e.g., superoxide dismutase, lipid peroxidase, peroxiredoxins, catalase, and glutathione peroxidase) and

several nonenzymatic antioxidants (e.g., vitamin A, thiols bilirubin, and melatonin) can neutralize free radicals to avoid oxidative stress in animal species [14–16].

In addition, certain relationships between immune response and antioxidant defense have been observed in many aquatic organisms [17, 18]. Crustaceans lack adaptive immunity and their protective system relies on the innate immune system via the reciprocal complementarity of cellular components and humoral immunity [19]. In the open circulatory system of *P. clarkii*, hormones and phagocytic cells in the hemolymph are actively involved in the recognition and elimination of nonself substances and downstream coagulation [20]. Humoral immunity includes the activation of the hemagglutinin, prophenoloxidase (proPO) system, and production of antimicrobial peptides (AMPs). Cellular immunity includes phagocytosis, encapsulation, and nodulation of blood cells [21]. Heat stress proteins (HSPs) are molecular chaperones involved in many stress-stimulated processes and they are widely used as indicators for cellular homeostasis in fishes [22]. The Toll signal pathway can regulate humoral and cellular immune responses to activate the innate immune system in invertebrates [23]. However, the effect of air exposure on the immune system and the underlying molecular mechanism in crustaceans are still unclear.

Beyond immune response and antioxidant defense, air exposure can also influence nitrogen metabolism in aquatic species by changing the end product of nitrogen metabolism—ammonia. Previous studies observed significant changes in blood ammonia concentration, urate content, and glutamate dehydrogenase (GDH) enzyme activities in decapods under air exposure and hypoxia over time [24–27]. Under air exposure, the elimination of ammonia, which is usually through ionic exchange or passive diffusion through gills, can be impaired. The accumulation of ammonia can penetrate the cell membrane and impair cell function, causing osmotic imbalance and oxidative stress [28, 29]. Glutamate dehydrogenase (GDH) and glutamine synthase (GS) are important ammonia metabolism enzymes in aquatic animals [30]. The impacts of ammonia exposure have received considerable attention, while our knowledge about how air exposure influences nitrogen metabolism in decapods is still limited.

In this study, we performed an air exposure experiment on *P. clarkii* to investigate how antioxidant defense, immunity system, and nitrogen metabolism respond to the air exposure at the molecular level over time through measuring enzyme activities and expression of key genes. These results can serve as some theoretical guidance and reference materials for standardized transport and aquaculture production management.

2. Materials and Methods

2.1. Experimental Animal Preparation and Management. The crayfish used in this experiment were purchased from the breeding base of Hefei Honghe Agricultural Technology Co., Ltd. in June and then they were transported to the aquatic animal breeding facility at Anhui Agriculture University using foam box ($485 \times 330 \times 220$ mm) with ice packs. The initial weight of the animals was 20 ± 5 g, with entire appendages. *Hydrilla varticillata* were additionally placed in the temporary holding tank ($680 \times 390 \times 400$ mm) to provide shelter for *P. clarkii*. The water was aerated for more than 24 h and the temperature was kept at 22 ± 1°C. Special compound feed for crayfish (Hefei Tongwei Jiuding Feed Co., Ltd., of which 28% crude protein and 3% crude lipid) was provided at 8:00 am and 18:00 pm each day before experiment. A total of 1/4 of the water was changed every day after the daily feeding, and the feeding was stopped 24 h before the exposure started. The siphon method was utilized to clean the leftover bait and excrement in water. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the Guide for the Care and Use of Laboratory Animals, the Animal Care Committee of Anhui Agricultural University (Hefei, China). The protocol was approved by the Animal Care Committee of Anhui Agricultural University.

2.2. Air Exposure Design and Sample Collection. The following two groups of *P. clarkii* were selected for the air exposure experiment: the control group with water and the air exposure group without water. Three plastic tanks ($580 \times 400 \times 120$ mm) were set for each group and each tank contained 50 samples of both sexes. All the tanks were put in the same room and kept in the same condition except the water environment in the control group. In the experimental group, all the samples of both sexes were randomly selected and exposed to air for 0, 2, 6, 12, 48, and 96 h. Four biological replicates from each tank were collected for the control and treatment group at each time point. Before dissection, the crayfish was frozen in −20°C for 10 minutes to induce hypothermia anesthesia. The surface water and mucus of *P. clarkii* were cleaned with gauze. The hepatopancreas was collected and cleaned with the PBS buffer to remove blood stains and mucus for each animal. All the samples were frozen in liquid nitrogen and then stored at −80°C for the following analysis.

2.3. Determination of Enzyme Activities. Antioxidant activities were assessed with the following 6 enzymes: catalase (CAT), superoxide dismutase (SOD), plasma glutathione peroxidase (*GSH-Px*), lactoperoxidase (LPO), total antioxidant capacity colorimetric (T-AOC), and malondialdehyde (MDA). Immune responses were determined by the following 3 enzymes: total nitric oxide synthase (T-NOS), polyphenol oxidase (PPO), and lysozyme (LSZ). The activity of nitrogen metabolism was assessed with two key enzymes, i.e., glutamate dehydrogenase (GDH) and glutamine synthetase (GS). All the enzyme kits used in this study were from Nanjing Jian Cheng Institute of Biological Engineering. A total of 0.1 g hepatopancreas tissues were taken, mixed with 0.9 g stroke-physiological saline solution in a homogenizer, and then centrifuged at 2500 rpm for 10 minutes. The supernatant was collected to measure the enzyme activities using the corresponding kits according to the instructions. Before the formal experiment, a standard curve was generated for each enzyme.

2.4. The Quantitative Real-Time PCR Analysis. The expression level of two antioxidant-related genes peroxiredoxin 6 (*Prx6*) and *GSH-Px* genes, five immune-related genes LSZ, astacidin (ASTA), Toll3, prophenoloxidase (proPO) and heat shock protein 70 (HSP70) genes, and one ammonia metabolism gene GS were detected to assess the effects of air exposure on gene expression. Total RNA was extracted using an adsorption column ultrapure RNA extraction kit following the instructions (Kangwei Century Biotechnology Co., Ltd.). The quality and the concentration of the RNA product were checked with 1% agarose electrophoresis and an ultramicro spectrophotometer, respectively. Reverse transcription was performed using a two-step synthesis kit (Mona Biotechnology Co., Ltd.), with the first step removing genomic DNA contamination and the second step synthesizing one-strand cDNA. The gene expression analysis in this experiment adopts the relative quantitative method. The 18S ribosomal gene was selected as the internal reference gene is 18S and the relevant gene sequences used in the experiment are obtained from NCBI GenBank and references. Primer 6.0 was used to design the primers, and the information on primers is provided in Table 1. Quantitative real-time PCR (qRT-PCR) was run in a LightCycler Roche 480. The amplification efficiency of the primers was checked using the standard curve method. The volume for the qRT-PCR was 20 μL, including 10 μL ChamQ SYBR Master Mix (2×), 0.4 μL of each primer (10 μM), 2 μL of template DNA, and 7.2 μL ddH2O. The reaction condition was 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and then annealing from 65°C to 95°C with a rising speed of 0.5°C/5 s. After the experimental process, Bio-Rad fluorescence quantitative software CFX Manager 3.1 was used to export the data. And, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes.

2.5. Statistical Analysis. Kaplan–Meier survival curves for the control and aerial exposure groups were analyzed in R v4.2.3 using survminer package. Normality of data for both groups at each time point was check by the Shapiro test implemented in R v4.2.3. Not all the groups followed normal distribution, so we used the nonparametric test for the statistical analysis. We used the Mann–Whitney test for the comparison between the control and aerial exposure groups. Also, we used the Kruskal–Wallis test followed by the multiple pairwise Wilcox test with Bonferroni adjustment for the multiple time point comparisons. All the figures were plotted in Rstudio using ggplot2 package.

3. Results

3.1. Oxidative Stress and Antioxidant Enzyme Activities. Kaplan–Meier survival curves for the control and aerial groups are shown in Figure 1. Sampling and normal death were recorded as two events for Kaplan–Meier survival analysis. At the beginning 60 hours, both groups presented 100% survival probability. First death record for the control and aerial exposure groups was presented at 60 h and 72 h, and the survival probability in the aerial group declined faster than the control group. After 96-hours experiment, the mortality in the control group and aerial exposure group in all three tanks were 5.3% and 18.7%, respectively.

The effects of air exposure on the antioxidant system were assessed according to the activities of six antioxidant enzymes (CAT, SOD, *GSH-Px*, T-AOC, LPO, and MDA) and two antioxidant genes (*GSH-Px* and *Prx6*). We compared these enzyme activities and gene expression levels between the control and aerial exposure groups, as well as changes in the control and exposure groups, respectively, across different time points. Figure 2 depicts the changes in enzyme activities in the hepatopancreas of crayfish over time in the aerial exposure and control groups. The enzyme activities of CAT, MDA, and the T-AOC content presented no difference between the control and aerial exposure groups. However, T-AOC and MDA in the aerial exposure group showed significant decline over time, while CAT in the aerial group increased significantly after 2-h exposure. In the aerial exposure group, SOD activities decreased substantially compared to the control group, and significant reductions were observed after 2-h and 12-h exposures ($P < 0.05$). By contrast, LPO contents showed higher levels in the aerial exposure group after 2-h exposure; however, the LPO activities in the aerial exposure group started to decline after 12-h exposure and showed significantly lower level at 96 h while LPO contents in the control group remain stable along time. The *GSH-Px* content in both the control and aerial exposure group showed a decline pattern after 2 hours, and the level in the aerial group after 6 h exposure was significantly higher than the control group.

The relative expression of two antioxidant-related genes *GSH-Px* and *Prx6* in the hepatopancreas were also assessed using qRT-PCR. As shown in Figure 3, at 0 h and 2 h, no significant differences were observed between the control and the aerial exposure groups for *Prx6*, while the expression increased significantly after 6-h exposure in the treatment group ($P < 0.05$). The *Prx6* expression reached the highest level at 12 h in the exposure group and then showed a slightly reduction at 48 h and 96 h ($P > 0.05$). Similarly, the aerial exposure group showed a significantly higher expression level of *GSH-Px* than that of the control group ($P < 0.05$) at 2 h, 6 h, 12 h, 48 h, and 96 h. Also, the expression of *GSH-Px* in the exposure group increased significantly after 2 h, reaching its highest level at 12 hours ($P < 0.05$) and then returning to the comparable level at 2 h.

3.2. Immune-Related Enzyme Activities. The activities of three immune-related enzymes (PPO, LSZ, and T-NOS) and the expression of five immune-related genes (LSZ, ASTA, Toll3, proPO, and HSP70) were used to assess the aerial exposure on the immune system of crayfish. As shown in Figure 4, LSZ activities decreased significantly after 12-h exposure compared to the control group. PPO activities also showed significant increase after 6-h exposure in the control group, while the PPO level in the treatment group increased considerably after 12-h exposure and showed significantly higher level than the control group at 48 h. For T-NOS, a significantly decrease followed by a significant increase

Table 1: Primer sequences information of genes in this experiment.

Gene	Sequence (5′-3′)	Tm (°C)	GenBank accession no. or reference sequences
18S-F	CTGTGATGCCCTTAGATGTT	51.8	AF436001.1
18S-R	GCGAGGGGTAGAACATCCAA	57.23	
*Prx*6-F	CGGATCACTGGAGGGTCAAACACTT	61.29	[31]
*Prx*6-R	GCAATTTTCATCCTCGGCATCAT	55.93	
GSH-Px-F	GCTCACCGTCCCTTCATATACCCA	60.91	JN835259.1
GSH-Px-R	TAGCGGATCCCGTTCATGATCTCT	59.64	
HSP70-F	ATACCACGAAGACGACGCCAAAC	59.98	KU613184.1
HSP70-R	GAGACTGCCGACTTGATGCTGAAG	60.41	
Lyz-F	GTCAACCCACCCTCAATAAC	53.23	MG921601.1
Lyz-R	CTTGTGAATCAGGGCGTA	51.77	
proPO-F	GCACAAGTTTGTGGACGACGTC	59.47	MT720690.2
proPO-R	GTCCATCTCAGCCCAGAGGAG	60.17	
Toll3-F	CTCCGAACTTGACGACGAACTTAGG	59.55	KU680805.1
Toll3-R	GCATGACGTACCGCAGCTTCTC	61.41	
ASTA-F	GACGGCTTCCCTTCCCTCTT	60.01	GQ301199.1
ASTA-R	CCATACGCCTGACCACCTTG	58.94	
GS-F	AGAACCTCCGCTCCAAGTCCAG	61.87	JF738076.1
GS-R	CGCTGTAGAGTGCTATTGGGTGAAG	59.80	

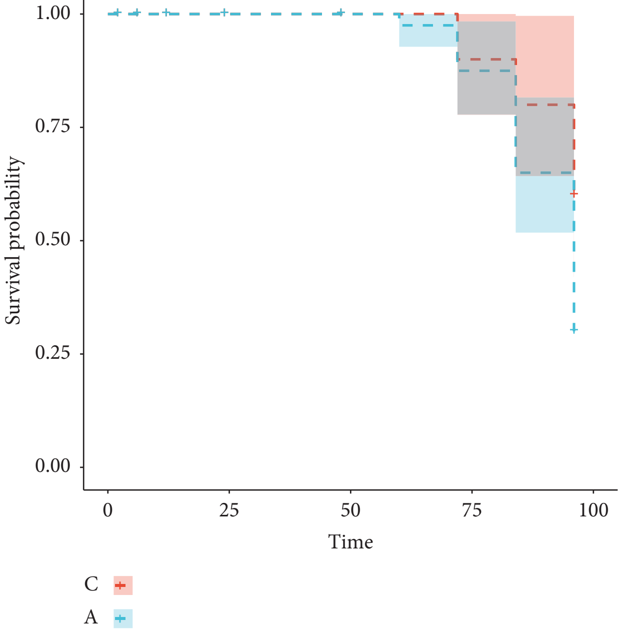
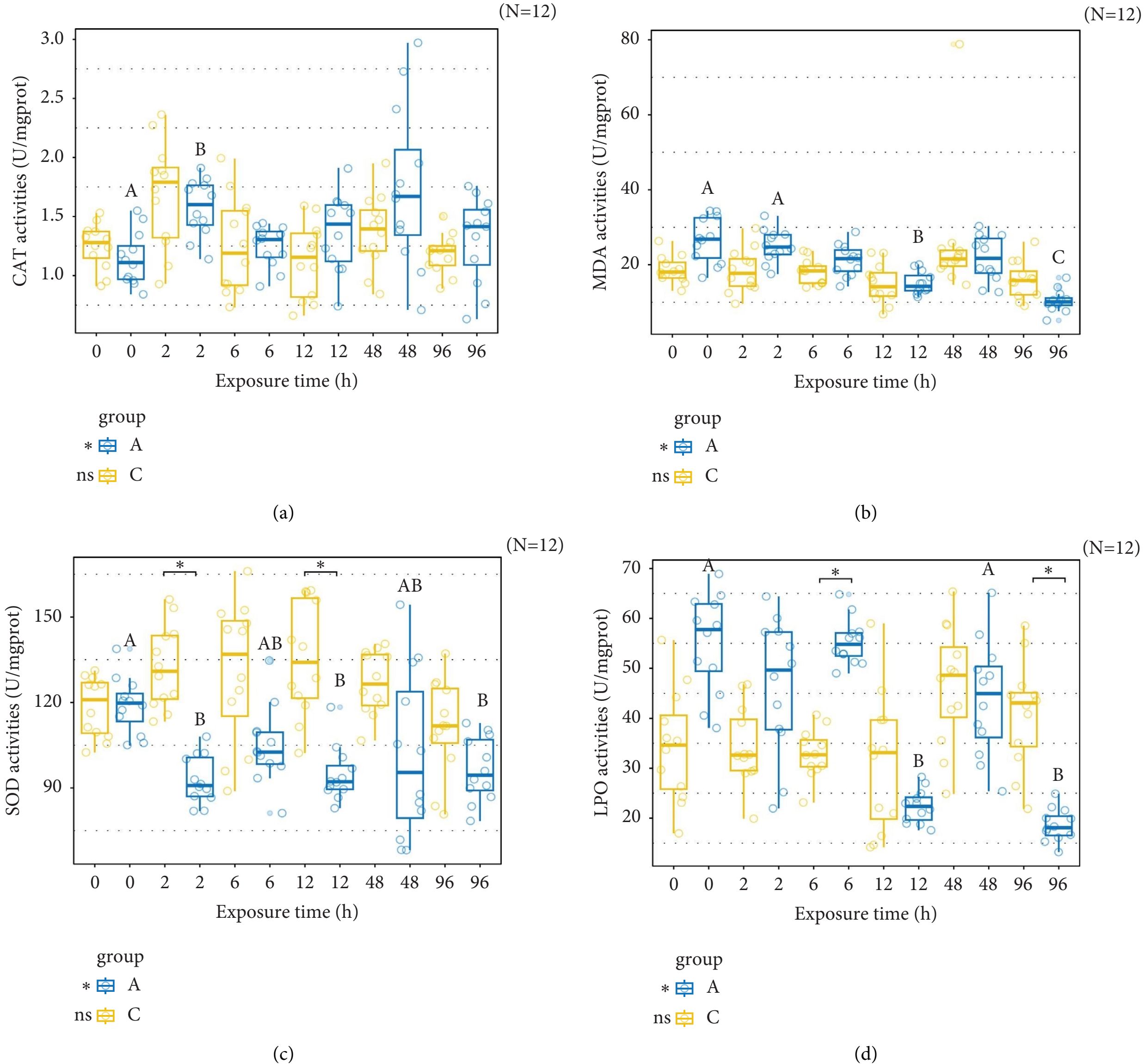


Figure 1: Kaplan–Meier survival curves for the control group (C) and the aerial exposure group (A).

were observed at the beginning of exposure in the treatment group then the level decreased again in a significant level after 48-h exposure. By contrast, T-NOS showed a decline trend in the control group. Notably, significant fluctuation over time was observed in both the control group and the aerial exposure group for several enzymes. The enzyme activities of PPO and LSZ also changed considerably over time in the control group.

Contrary to the enzyme activity, the expression level of LSZ was significantly impacted by air exposure stress (Figure 5). The expression level of LSZ peaked after 2-h air exposure and decreased slightly afterward, but it was still higher than the control group. The expression level of ASTA increased significantly at the first 12-h air exposure and declined significantly at 48 h, followed by a sharp increase at 96 h. Notably, the expression level of ASTA in the aerial exposure was significantly higher than the control group at 12 h, 48 h, and 96 h. As a common stimulation indicator, the expression of HSP70 after exposure was significantly higher than the control group and reached the peak at 12 h and then back to a level comparable to the control group. Toll3 was also sensitive to aerial exposure, and the treatment group showed consistently higher expression compared to the control group during exposure in all measured time points. Similar to Toll3, proPO expression was also significantly affected by aerial exposure. Although the expression of



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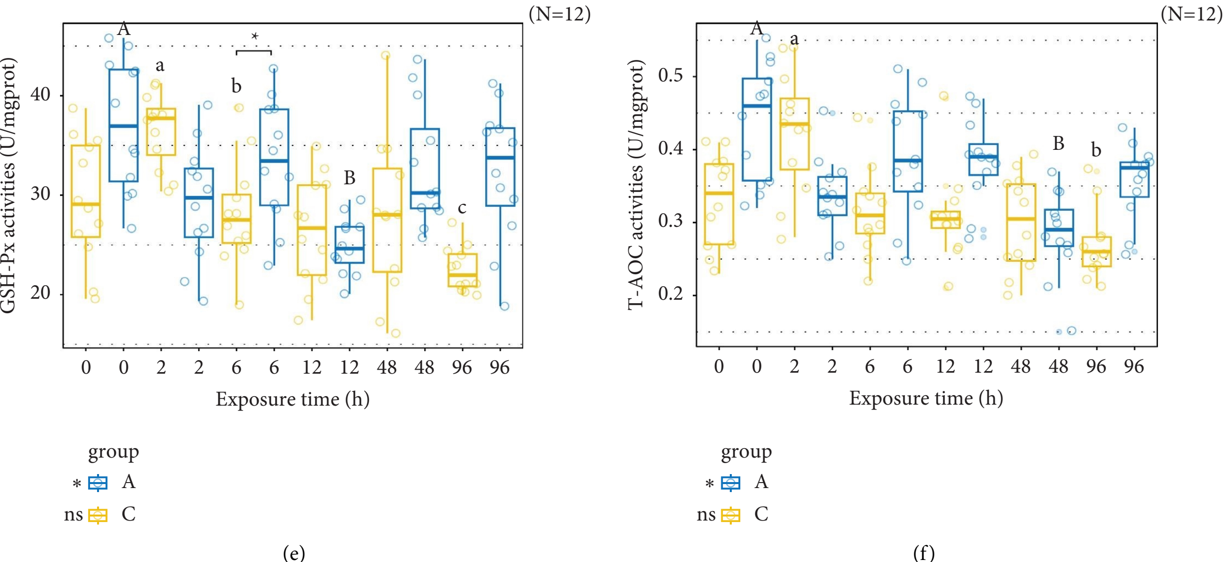


Figure 2: Effect of air exposure on the antioxidant enzyme activities. (a–f) CAT, MDA, SOD, LPO, GSH-Px, and T-AOC enzyme activities. C and A on the topright of the figures indicate the control group and the aerial exposure group, respectively. * above the boxplot in each time point indicates significant difference between the control and aerial exposure groups. * and ns (nonsignificant) on the left of the figure legend indicate the significance for the control and aerial exposure groups across time points after the Kruskal–Wallis test. Different lowercases and capital letters between pairwise time points indicate significant difference in the control group and the aerial exposure group, respectively.

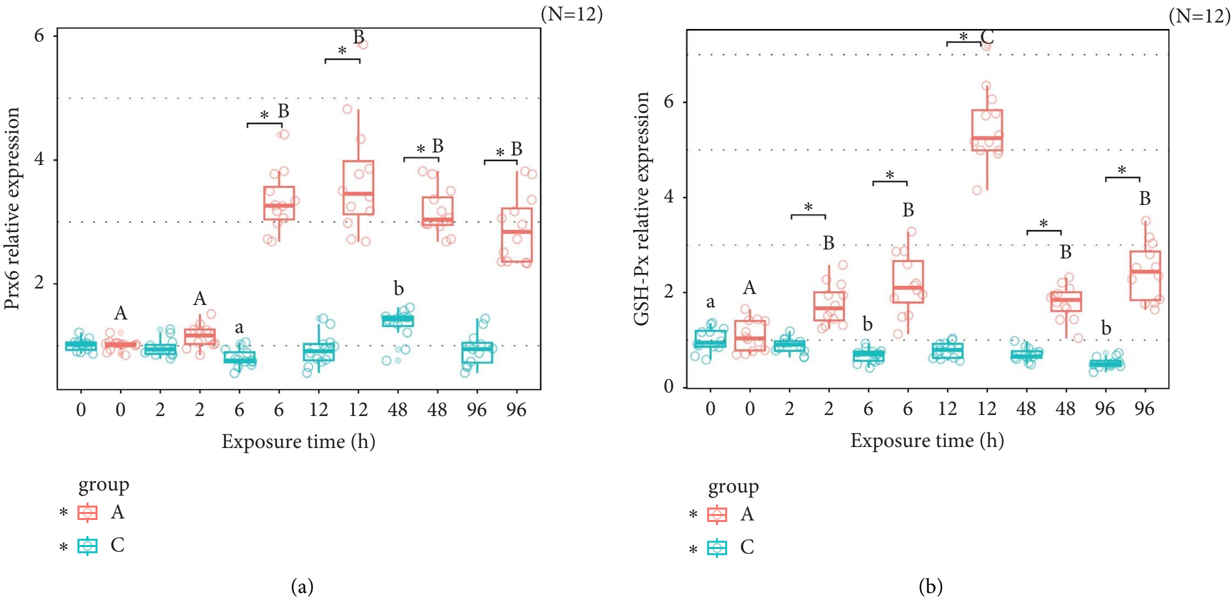


Figure 3: Effect of air exposure on the antioxidant-related genes. (a, b) Prx6 and GSH-Px expression levels. C and A on the topright of the figures indicate the control group and the aerial exposure group, respectively. * above the boxplot in each time point indicates significant difference between the control and aerial exposure groups. * and ns (nonsignificant) on the left of the figure legend indicate the significance for the control and aerial exposure groups across time points after the Kruskal–Wallis test. Different lowercases and capital letters between pairwise time points indicate significant difference in the control group and the aerial exposure group, respectively.

proPO also fluctuated over time under air exposure, the relative expression of proPO was 4–10 times higher than the control group.

3.3. Desiccation Stress on the Ammonia Metabolism. The changes in enzyme activities (GDH and GS) and the gene expression (GS) related to the ammonia metabolism under

air stress are shown in Figure 6. We did not detect a significant difference in GDH and GS enzyme activities between the control group and the aerial exposure group nor the differences over time for both the control and the exposure group. By contrast, the gene expression level of GS increased significantly and peaked at 6 h under exposure, followed by a decrease but still significant level between the control group and the exposure group after 12 h. GS expression in the control group showed significant decrease after 48 h.

4. Discussion

Many environmental factors such as air exposure, salinity, and density stress may lead to oxidative stress and an excess of free radicals or ROS, detrimental to the health of aquatic animals [13]. Elevated intracellular levels of ROS may cause damage to lipids, proteins, DNA, and other biological macromolecules, leading to oxidative stress and invoking immune defense. Due to the lack of the adaptive immune system, hepatopancreas is a key organ for both metabolism and immune defense. To combat the adverse effects caused by oxidative stress, enzymes, and antioxidant systems (i.e., SOD, CAT, and *GSH-Px*) can eliminate ROS and protect tissue from oxidative damage, playing a key role in maintaining the balance of ROS production [6]. SOD is the essential baseline for ROS elimination since it can effectively convert $O_2^{\bullet -}$ into H_2O_2, which is then decomposed by cooperating with CAT or *GSH-Px* [14]. The content of LPO is an important indicator of lipid peroxidation and protein carbonylation induced by ROS [32]. Likewise, the MDA content is one of the end products of polyunsaturated fatty acids peroxidation; therefore, it is a common lipid peroxidation marker for assessing oxidative stress [33]. Previous studies revealed that aerial exposure can trigger changes in antioxidant enzyme activities in a time-dependent pattern, causing oxidative damage and apoptosis in crustaceans [6, 9]. In the present study, we also observed a significant reduction of SOD activity and accumulation of LPO and MDA contents in the exposure group, indicating a quick antioxidant response to the air exposure. However, the reduction of LPO and MDA content over time under air exposure may attribute to the enhanced antioxidant defenses against oxidative stress in the hepatopancreas of crayfish. Similar results were also observed in other crustaceans [6]. Although the GSH-Px enzyme showed no significant differences between the control group and the aerial exposure group in most of the time, the expression of *GSH-Px* increased significantly after 2-h exposure, and a relatively high expression level in the exposure group lasted for a long time. Similar results were also detected for *Prx6*, a gene encoding thiol-specific antioxidant protein that can activate the Toll signal pathway to protect the host against oxidative stress and DNA damage in crayfish [31]. Elevated high expression levels of *GSH-Px* and *Prx6* were consistent with the antioxidant responses under aerial exposure, which also indicates that these two genes appear to be more sensitive to the oxidative stress caused by air exposure.

Several previous studies revealed that air exposure in crustaceans can also invoke immune-related pathways through transcriptome and physiological analysis [9, 34–36]. However, the mechanisms of how air exposure impacts the immune system are still unclear. We selected 3 commonly used immune-related enzymes and 5 genes on the immune pathways to understand the effects of air exposure on the immune defense. In this study, significant differences were observed between the control and exposure groups for all three enzymes at different exposure time point, indicating variant signature of immune responses. By contrast, the expression of all five immune-related genes used in this study showed a significant increase under air exposure. These results suggested that immune-related genes and enzymes have different sensitivities to hypoxic stress. ASTA is an antibacterial peptide that has a crucial function in antibacterial innate immune response [37]. LSZ usually involves a series of immune activities including stress responses and pathogen infection, playing important roles in physiological metabolic activities and defense against exogenous pathogens [38]. Toll3 belongs to Toll-like recognition receptors, which are key substances in the signal recognition pathway and they can be triggered by pathogens via multistep cascade reactions [39]. In crustaceans, the Toll pathway has been demonstrated to be involved in immune responses induced by many environmental factors such as pathogen inflection, oxidative stress, metal stress, and sulfide stress [40–43]. In this study, we found that air exposure can also trigger immune responses by regulating the expression of Toll3. In invertebrate humoral immunity, the prophenoloxidase-activating system (proPO-AS) is a sophisticated and important immune defense system. Besides the common function of antimicrobial infection, the proPO cascade can generate many intermediates that can enhance phagocytosis and host defense in crayfish [44]. In addition, the proPO system is sensitive to a variety of stimulations, such as lipopolysaccharide and ammonia stress [44, 45]. In this study, we found that the proPO system can be quickly activated by air exposure in *P. clarkii* and the responses remained at a high level for a relatively long time, indicating that proPO is a promising marker for monitoring desiccation stress in red claw crayfish.

Ammonia is the main product of nitrogen metabolism in crustaceans [46]. Usually, aquatic animals can excrete ammonia into water through gills, whereas during air exposure ammonia excretion rate of crustaceans decreases dramatically and blood ammonia accumulates rapidly [24, 35]. To avoid toxicity due to the concentration of blood ammonia, crustaceans can transform ammonia into urea, uric, and glutamine [47]. Glutamate dehydrogenase (GDH) and glutamine synthase (GS) are important ammonia metabolism enzymes in aquatic animals. In this study, we measured the enzyme activities and gene expression related to the ammonia conversion process to check if air exposure has effects on nitrogen metabolism. Although enzyme activities showed no significant changes under air exposure, we indeed found a significant increase in GS expression after 2-h exposure. The upregulation of GS genes is consistent with the ammonia detoxification hypothesis.

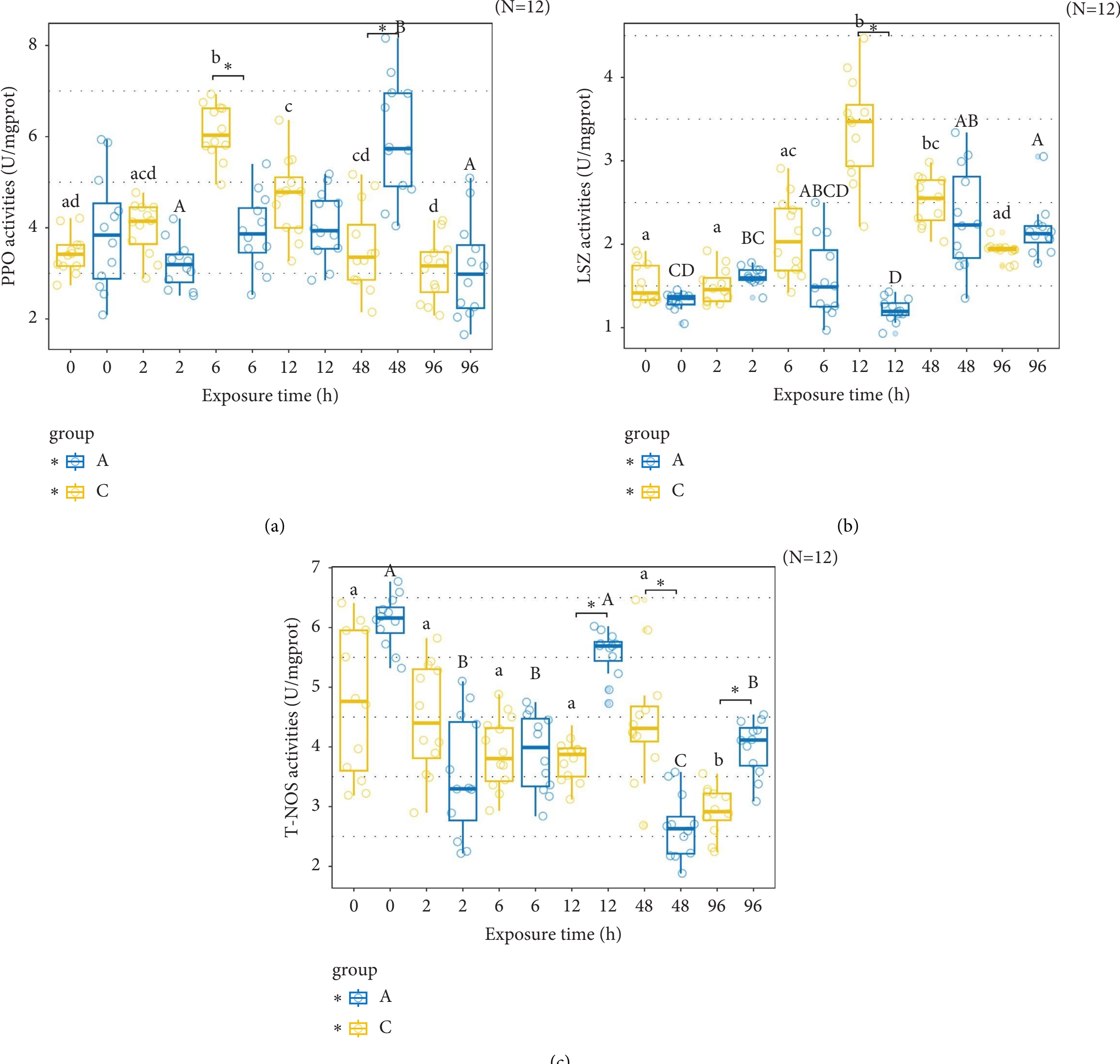
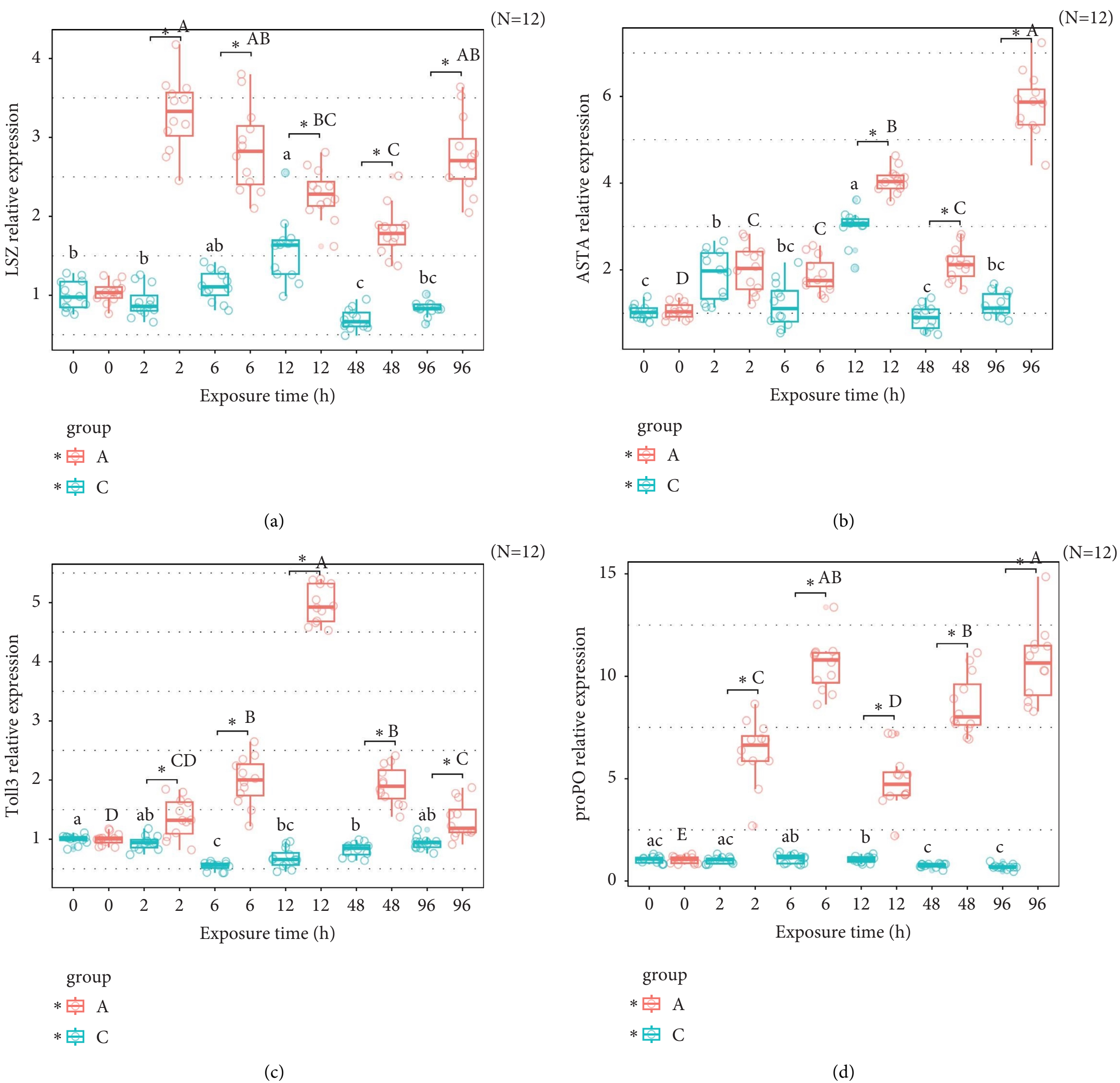


Figure 4: Effects of air exposure on the immune-related enzyme activities. (a–c) PPO, LSZ, and T-NOS enzyme activities. C and A on the topright of the figures indicate the control group and the aerial exposure group, respectively. * above the boxplot in each time point indicates significant difference between the control and aerial exposure groups. * and ns (nonsignificant) on the left of the figure legend indicate the significance for the control and aerial exposure groups across time points after the Kruskal–Wallis test. Different lowercases and capital letters between pairwise time points indicate significant difference in the control group and the aerial exposure group, respectively.

In summary, air exposure can induce antioxidant defense, immune responses, and changes in nitrogen metabolism. These results can provide important information for crayfish transport and sales. One thing worth mentioning is that many commonly used enzymes showed no significant difference between the control group and the air exposure group at different exposure time point, whereas many key genes showed stable and significant changes, meaning that suitable markers would be necessary for assessing the desiccation stress. *Prx6* and proPO showed quick and stable responses with relatively high expression levels to the aerial exposure, presenting high sensitivity to the desiccation stress and, therefore, can be potential biomarkers for desiccation monitor. In addition, we observed fluctuations in several enzyme level and gene expression in the control group over time. The fluctuation of biomarkers in the control group is commonly observed in aquatic animals but less understood in many research studies under environmental stress [48–50]. One possible reason is that these enzymes or genes are highly sensitive and less stable to environmental stress, and even small differences in the control group may also trigger strong effects. Therefore, strict control during the experiment duration and a large sample size for each group would be necessary in future research to figure out this issue.



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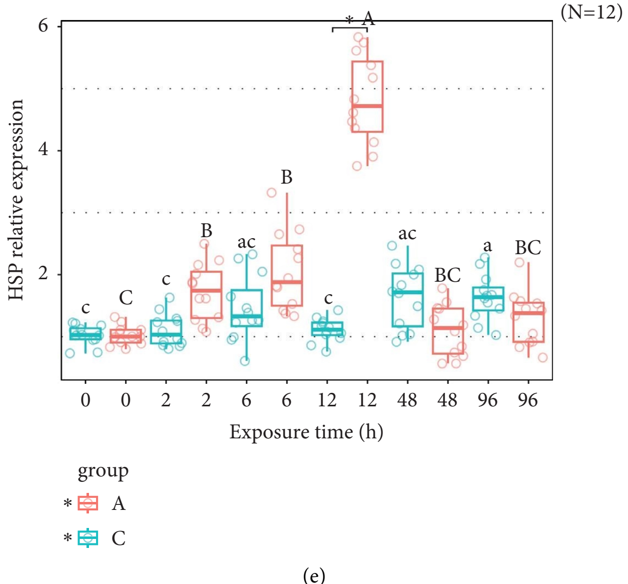
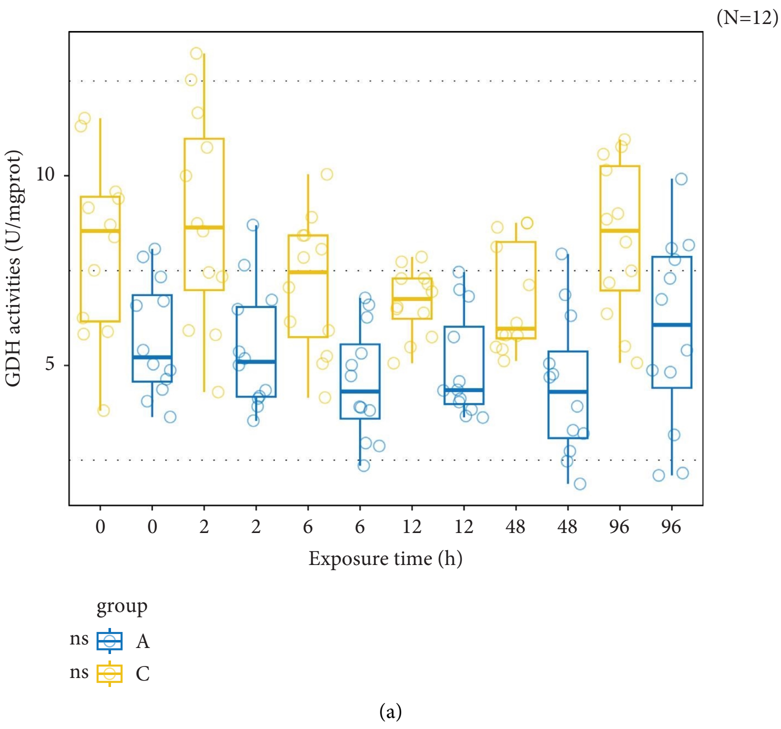


(e)

Figure 5: Immune-related gene expression patterns under air exposure. (a–e) LSZ, ASTA, Toll3, proPO, and HSP expression levels. C and A on the topright of the figures indicate the control group and the aerial exposure group, respectively. * above the boxplot in each time point indicates significant difference between the control and aerial exposure groups. * and ns (nonsignificant) on the left of the figure legend indicate the significance for the control and aerial exposure groups across time points after the Kruskal–Wallis test. Different lowercases and capital letters between pairwise time points indicate significant difference in the control group and the aerial exposure group, respectively.



(a)

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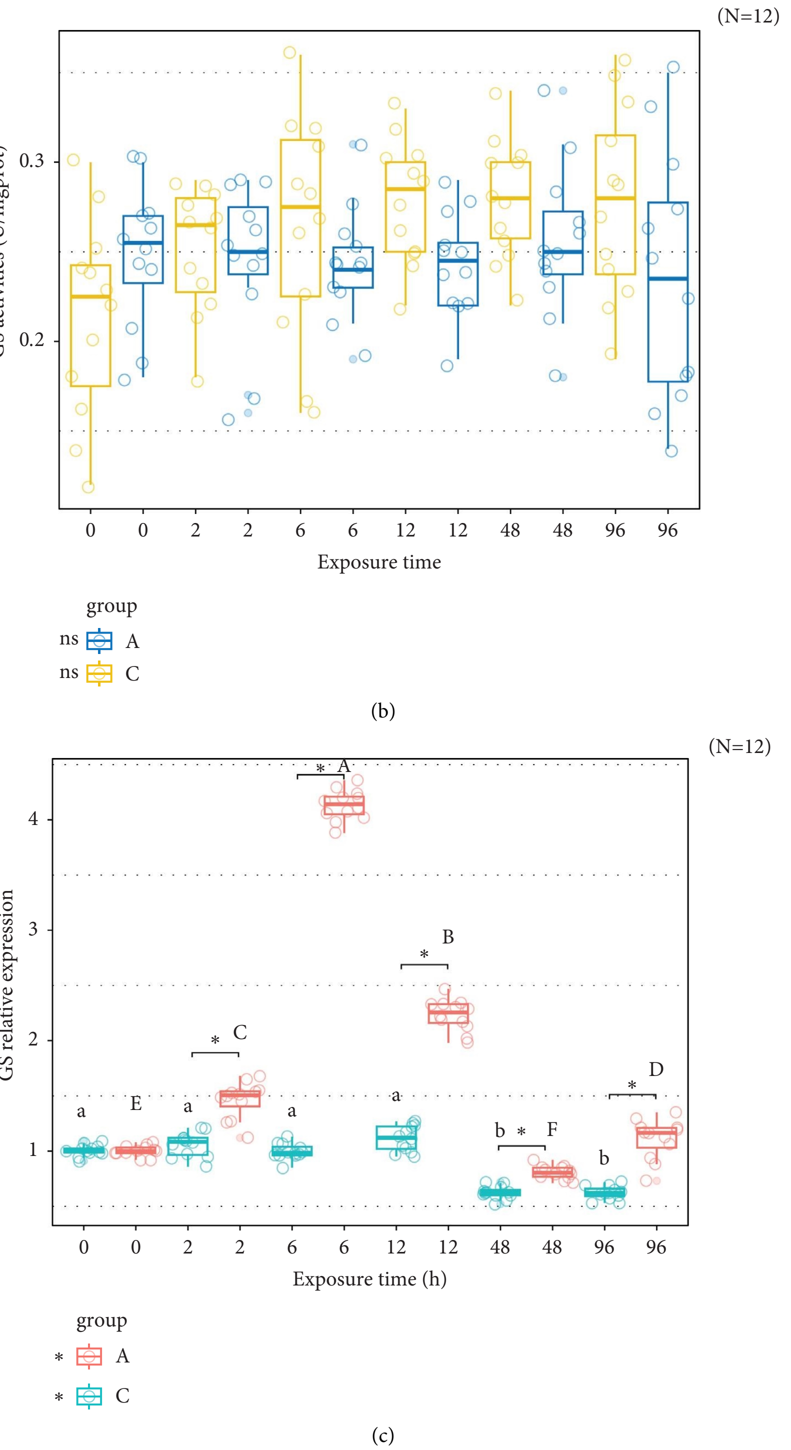

Figure 6: The effects of air exposure on ammonia metabolism. (a, b) GDH and GS enzyme activities and (c) GS gene expression level. C and A on the topright of the figures indicate the control group and the aerial exposure group, respectively. * above the boxplot in each time point indicates significant difference between the control and aerial exposure groups. * and ns (nonsignificant) on the left of the figure legend indicate the significance for the control and aerial exposure groups across time points after the Kruskal–Wallis test. Different lowercases and capital letters between pairwise time points indicate significant difference in the control group and the aerial exposure group, respectively.

5. Conclusions

In conclusion, our study indicated that air exposure can induce oxidative stress and antioxidant defense and long-term exposure may cause severe oxidative damage. In addition, we found that air exposure can also activate the immune system by regulating the expression of immune-related genes in a time-dependent manner. A key gene in ammonia homeostasis, GS, is significantly upregulated under air exposure, indicating that air exposure may also influence ammonia metabolism. In this study, we found that the activities of many enzymes are fluctuant in both the control and aerial exposure groups, suggesting that more stable and sensitive indicators for desiccation stress would be necessary. On the other hand, we found that two genes *Prx6* and proPO presented high sensitivity and high expression under air exposure, suggesting the potential for monitoring desiccation stress. In addition, to avoid random effects and individual variation in the studies investigating the effects of environmental stress, a larger sample size would be necessary.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Y.L.Z. and S.Q.D. conceptualized and supervised the study and performed project administration and funding acquisition, H.M.C., K.Y., M.H., M.X.W., L.J.Z., and X.Y.C. performed the experiment, K.Y., M.H., and R.X. performed data analysis, K.Y. and R.X. prepared the original draft, H.M.C. and K.Y. visualized the study, and R.X., Y.L.Z., and S.Q.D. reviewed and edited the study.

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