



The IJA is a peer-reviewed open-access, electronic journal, freely available without charge to users

Produced by the AquacultureHub non-profit Foundation  
Sale of IJA papers is strictly forbidden



## The evaluation of ammonia tolerance in introduced and local Pacific white shrimp, *Litopenaeus vannamei*, populations in China

Jing Wang, Jianyong Liu \*

Guangdong Provincial Shrimp Breeding and Culture Laboratory, Guangdong Ocean University, Zhanjiang 524088, China

(Received Nov 11, 2022; Accepted Nov 25, 2022; Published Dec 04, 2022)

Keywords: ammonia, immune, *Litopenaeus vannamei*, metabolism, relief

### Abstract

The white shrimp, *Litopenaeus vannamei*, is one of the most valuable commodities in the global seafood trade. Affected by high-density farming environments, ammonia accumulates in shrimp cultures and has a strong toxic effect, resulting in poor shrimp survival and poor immune function and metabolism. We selected six different populations of *L. vannamei* from Xing Hai No.1 (A and B), CHAI, Sy Aqua, PRIMO, and a second-generation Sy Aqua-PRIMO hybrid population (SP). The shrimps ( $3.24 \pm 0.71$  cm body length) were exposed to ammonia (24 h, 48 h), followed by recovery (R48 h, R96 h) to assess the tolerance of different populations. The survival rate (SR), immune-related enzymes (superoxide dismutase SOD, catalase CAT, and Glutathione peroxidase GSH-PX), Malondialdehyde (MDA), and metabolism (glutamate dehydrogenase *GDH*, glutamine synthetase *GS*, and aspartic acid transaminase *GOT*) and were measured at different populations under acute ammonia stress. Multiple comparisons of the ammonia resistance index from six populations showed that the expression of these indicators varied among the populations. The degree of lipid peroxidation in the Sy Aqua and PRIMO was significantly higher than in the other populations ( $P < 0.05$ ), and the ammonia metabolism index was poor. The *GDH* and *GOT* genes for the Xing Hai No.1 (A) were higher than for the other populations. Mortality and physiological indicators recovered to varying degrees for all experimental populations following 96 h of ammonia relief, whereas the Sy Aqua and PRIMO showed a noticeable lag. These results indicated that the immunity and metabolic capacity of Xing Hai No.1 (A) might be higher than those of Sy Aqua and PRIMO. These data could have value in developing future scientific breeding schemes and in the sustainability of shrimp farming.

\* Corresponding author. e-mail: [liujy70@126.com](mailto:liujy70@126.com)

## Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, distributed along the Pacific coast of western North to South America (from Mexico to Peru), supplies approximately 52 % of the global penaeid shrimp trade (Wyban 2019). In China, the annual production of *L. vannamei* is approximately 1.8 million tons and covers 70% of the total culture area and 80% of the shrimp output (FAO 2020). Because *L. vannamei* is a non-native species in China, most culture stocks are produced using imported shrimp from South American countries or closely cultured stock bred in captivity for multiple generations. Various *L. vannamei* populations have been introduced since 1988, including CHAI, Sy Aqua, and PRIMO populations. After years of selective breeding of these introduced populations, several new breeds with excellent economic traits have been successfully bred in China (Yuan et al., 2018).

High-quality shrimp stock has been the basis for selective breeding and improving economic traits for many years in China and even the world. Thus, some researchers have begun to evaluate the differences in phenotype, growth, and survival (Yuan et al., 2018) of different populations, to provide higher accuracy for evaluating resistance to the selective breeding of *L. vannamei*. Fan (2021) analyzed morphological differences among the four introduced populations, while Luo (2018) and Yuan (2015) analyzed the growth survival rates of the introduced and selective populations. In addition, researchers assessed the resistance to white spot syndrome virus (WSSV) in the underlying and selective populations in terms of survival rates and landmark physiological indicators (Huang et al., 2012). However, in addition to *L. vannamei* selected for WSSV resistance, new strains have been developed with other economically important traits in China. For instance, we successfully selected *L. vannamei* for ammonia resistance at Guo Xing Farm, Zhanjiang, China (Yuan et al., 2018). However, the differences in immune and metabolic responses of different *L. vannamei* populations to ammonia have not been reported.

Ammonia, a common stressor and one of the most critical water quality parameters, is a toxic aquaculture substance that can drastically degrade the function of marine ecosystems (Chatvijitkul et al., 2017). It has been reported that elevated ammonia concentrations can accumulate in aquatic animals' body fluids, leading to poor growth, tissue erosion and degeneration, immunosuppression, and high mortality (Long et al., 2021). This, in turn, affects the output of shrimp and seriously restricts the health and sustainable development of breeding shrimp populations (Wang et al., 2021). Therefore, it is necessary to evaluate stress resistance to ammonia in different populations according to their enzymes and genes to develop breeds with excellent resistance. It is essential for the accuracy and direction of actual shrimp production selective breeding efforts.

Previous reports indicated that many endogenous enzymes are involved in shrimp immune responses. These enzymes can evaluate the host's physiological state and estimate its potential resistance to ammonia (Huang et al., 2012). Ammonia can cause oxidative stress in organisms by increasing the concentration of reactive oxygen species (ROS) (Liang et al., 2016). To maintain homeostasis, organisms employ antioxidant defense systems to eliminate ROS, counter oxidative stress, and prevent oxidant damage (Wang et al., 2006). Numerous studies have shown that superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-PX) act as interrelated antioxidant enzymes in living organisms that jointly scavenge ROS and inhibit lipid peroxidation in shrimp under ammonia stress, protecting tissues from oxidative damage (Liang et al., 2016). MDA is an unsaturated fatty acid in biofilms, which produces lipid peroxide products under the action of oxygen radicals. As the ROS level rises in response to ammonia, the MDA content increases significantly and indirectly reflects the speed and extent of oxidative damage to the body's tissues (Long et al., 2021).

Ammonia metabolism is also crucial in shrimp adaptation to ammonia stress. The glutamate dehydrogenase (*GDH*), glutamine synthetase (*GS*), and aspartic acid transaminase (*GOT*) genes encode proteins with crucial roles in the ammonia metabolism (Liu et al., 2014). *GDH* catalyzes ammonia assimilation with  $\alpha$ -ketoglutaric acid ( $\alpha$ -KG) to address endogenous ammonia

poisoning in tissues; GS can catalyze the generated glutamate and excess ammonia to form non-toxic glutamine (Gomez-Jimenez et al., 2004). *GOT* catalyzes the reaction of 2-oxosuccinic acid with glutamic acid to form aspartic acid, which is required to synthesize urea in living organisms; this promotes detoxification by reducing the concentration of ammonia in the body (Li et al., 2014). Many researchers have used it to evaluate key indicators of ammonia excretion performance in aquatic organisms, such as the *L. vannamei* (Qiu et al., 2018), *Penaeus monodon* (Yang et al., 2014), *Oncorhynchus mykiss* (Wicks and Randall 2002), and *Portunus trituberculatus* (Liu et al., 2014).

Studies have found that the adaptability of *Sinonovacula constricta* to salinity was different because of the differences in salinity between the north and the south (Chen et al., 2021). The salinity and ammonia nitrogen tolerance varied between breeding families in *Penaeus monodon* (Chen et al., 2016). Therefore, we selected six *L. vannamei* populations with different origins to investigate their ammonia tolerance comprehensively. Our experimental data will provide a reference for the healthy breeding of *L. vannamei*, lay the foundation for genetic improvement of crucial economic conditions, and enable the development of new scientific breeding schemes.

## Materials and Methods

### Shrimp sources

The experimental populations were sourced from Xing Hai No. 1 (A and B), CHAI, Sy Aqua, and PRIMO, in addition to second-generation hybrid Sy Aqua-PRIMO shrimp (SP). Six populations of *L. vannamei* from the same workshop were selected, consisting of 30 randomly selected individuals of uniform size from each population. The water from each shrimp's body was removed with a clean, dry towel, and the body weight of the experimental shrimp was measured using ImageJ-win64 software and an electronic balance (sensitivity 0.001 g).

### Ammonia exposure and sampling

The experiment was conducted at Zhanjiang Guo Xing Aquatic Technology Co., Ltd. (Zhanjiang, China). A sizeable experimental pool (6.30 x 1.40 m<sup>2</sup>) was separated by 30 x 30 x 40 network frames, and 90 young shrimp from each population were randomly divided into three groups (30 per group) and placed in the network frame for 7 days. Stock solution (1 mol/L) of high-purity NH<sub>4</sub>Cl was used as the source of the total ammonia. According to our previous experiment, *L. vannamei* exposed to 30.0 mg/L ammonia could induce significant toxic effects. Thus, 30.0 mg/L ammonia nitrogen was used in the present study to evaluate toxic reactions. Feeding was stopped the day before the experiment began. During the acute stress experiment, the total ammonia nitrogen (TA-N) levels were measured by the nesslerization method and adjusted by adding NH<sub>4</sub>Cl solution every 12 h. After exposure for 48 h, the water in ammonia stress groups was changed into normal seawater to recover for 96 h.

Samples were collected at 0 h (before stress), 24h, and 48 h after stress, R48 h and R96 h after recovery. The water temperature was kept at 28 ± 1 °C, with a pH of 7.8 ± 0.5 and a salinity of 3.24 ± 0.03‰. Nine shrimps were randomly taken out from each population for each sampling. The muscle and hepatopancreas of each shrimp in all experimental treatments were dissected, frozen in liquid nitrogen, and stored at -80 °C for further analysis.

### Detection of non-specific immune indicators

The activities of SOD, CAT, and GSH-PX in the hepatopancreatic and muscle tissues, as well as the contents of MDA in hepatopancreatic and muscle tissues, were determined using commercial kits produced by the Meilian Institute (Shanghai, China) following the manufacturer's protocols.

### Detection of metabolic detoxification indices

The expression levels of *GS*, *GDH*, and *GOT* in hepatopancreatic and muscle tissues were determined by real-time polymerase chain reaction (PCR) analysis;  $\beta$ -actin was selected as the reference gene. The primer sequences of the genes are presented in **Table 1**.

**Table 1** Oligonucleotide primers used in experiments

Genes	Forward primer	Reverse primer
<i>GS</i>	TATCTATTACCTCCTCCTTGGG	GGCTGTTGGTGAACCTTCTCA
<i>GDH</i>	ACCCACACGACCAAAACG	TGGCTGAAGAATCTCAACCAC
<i>GOT</i>	AAATGCCAAACTTGCCCC	ACCAATACTTCCTCCCCTGC
$\beta$ -Actin	CCCTCGCTCCCTCCACCATG	CTCCTGCTTGCTGATCCACATCTG

The total RNA from each tissue was extracted with Trizol Reagent (Invitrogen, Waltham, MA, USA). Reverse transcription of the first-strand cDNA was completed using a Prime Script™ RT Reagent Kit (TransGen Biotech, Beijing, China). Real-time PCR was performed using a real-time PCR amplification instrument (CFX96; Bio-Rad, Hercules, CA, USA) following the instructions provided with SYBR® Premix Ex Taq™ II. Each experiment was repeated three times.

The reaction mix had a total volume of 20  $\mu$ l, and it contained 10  $\mu$ l of 29 SYBR Green Real-time PCR Master Mix, 1  $\mu$ l of the diluted template, 0.4  $\mu$ l (10  $\mu$ m) of both the sense and antisense primers, and 7.2  $\mu$ l of double-distilled water. Three biological replicates were prepared for each time point. The reaction parameters were: denaturation at 95 °C for 10 s, denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s for a total of 40 cycles. Experimental data were obtained using the relative CT method ( $2^{-44C_T}$  method) to analyze each sample's relative expression of the immune-related genes.

### Histological assay of the hepatopancreas

In the ammonia stress (48 h), after recovery (R96h), and normal groups, the hepatopancreas from a shrimp in each population was dissected from the cephalothorax and fixed with 4% paraformaldehyde for tissue fixation, then stored in 4°C for paraffin sections. Cross-sections (5 mm) were prepared by Service bio (Wuhan, China) and observed under light microscopy.

### Data processing

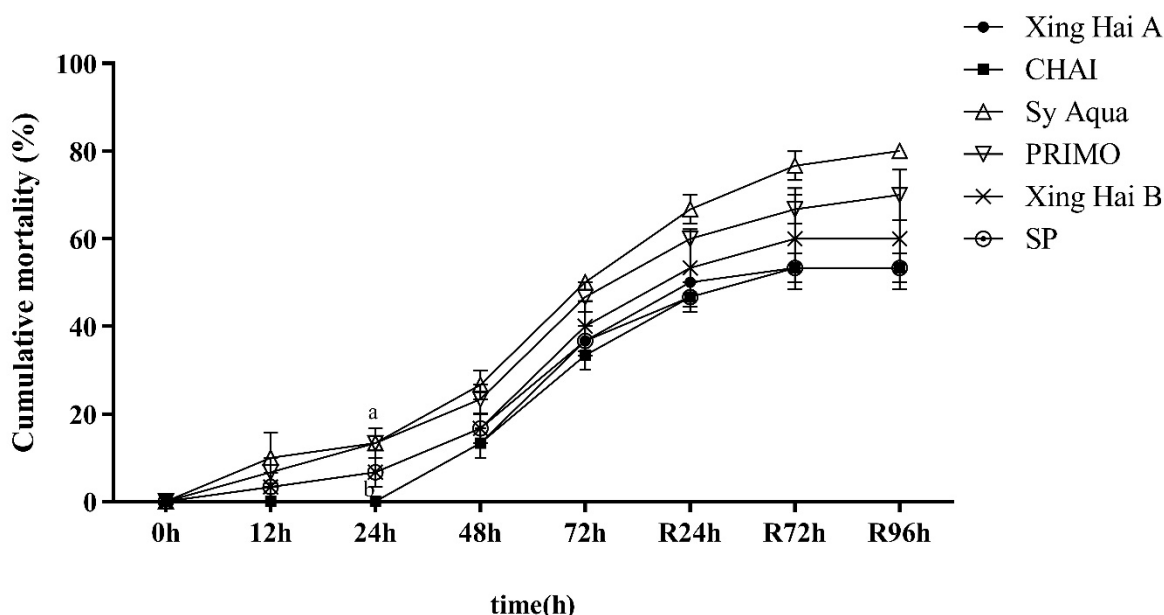
All experimental data are expressed as the mean value of three parallel populations  $\pm$  the standard deviation. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used for factor analysis of variance. Duncan's multiple comparison method and one-way analysis of variance were used to assess differences between populations. The alpha level was set to  $P < 0.05$ .

## Results

### Cumulative shrimp mortality

The cumulative mortality of *L. vannamei* exposed to ammonia stress is presented in **Figure 1**. As the experiment progressed, the mortality rate of each population increased gradually. The Sy Aqua and PRIMO began to die after 12 h of ammonia stress; the cumulative mortality rates were 26.6% and 23.3% after 48 h, which is significantly higher than for Xing Hai No. 1 (A) ( $P < 0.05$ ). The mortality rate of shrimp in the various populations began to decrease after 96 h of treatment; the death rate of the Xing Hai No. 1 (A, B) and CHAI ultimately decreased to 0.



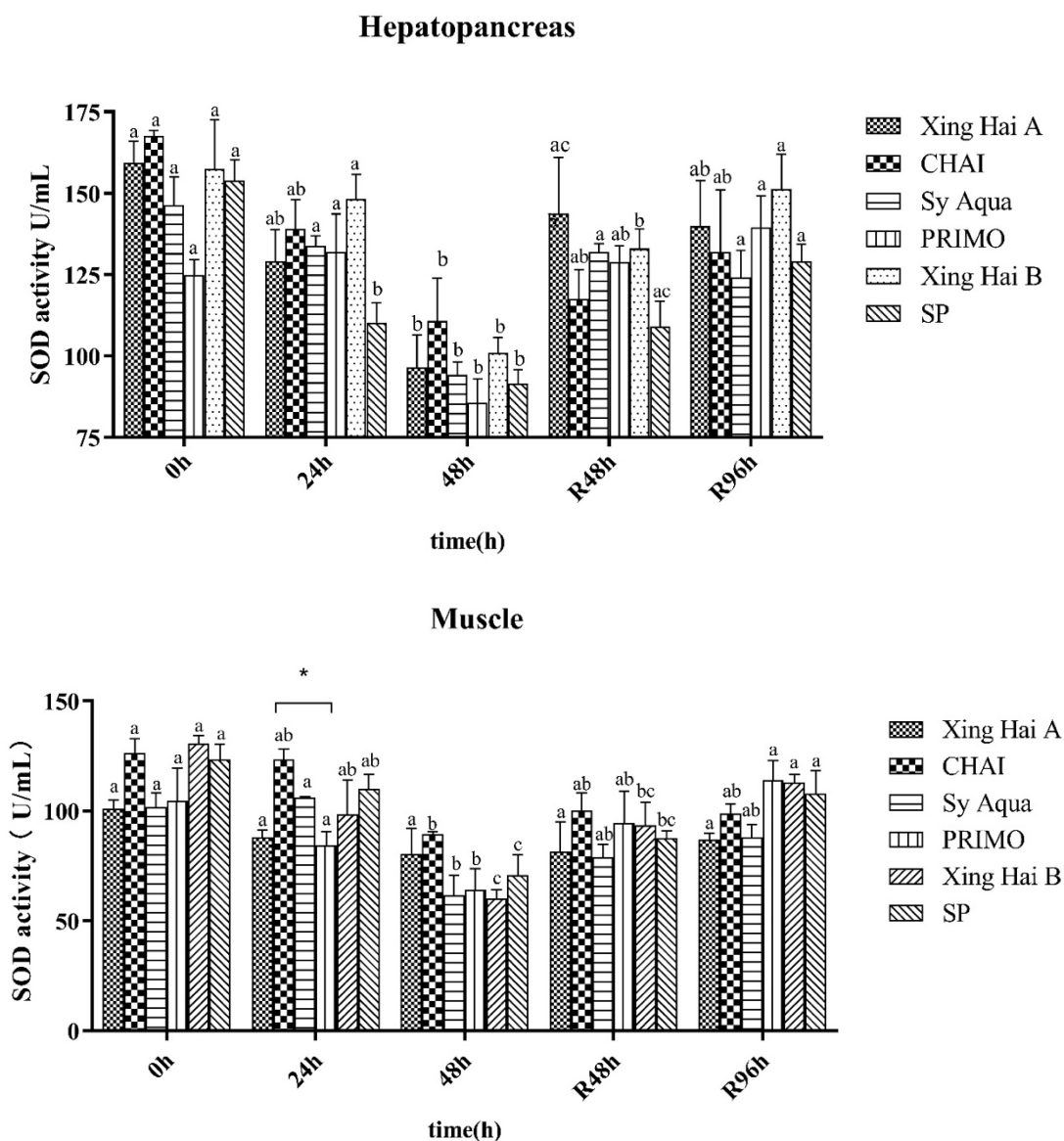


**Figure 1** The mortality of shrimp exposed to a 40% CL96h ammonia-N concentration.

\*Note: The experimental populations were Xing Hai No. 1 (A and B), CHAI, Sy Aqua, PRIMO, and the second-generation hybrid Sy Aqua-PRIMO population (SP).

#### *The change in SOD activity in the hepatopancreas and muscle of shrimps following ammonia exposure*

The variation in SOD activity in hepatopancreatic and muscle tissues are presented in **Figure 2**. There was no significant difference in SOD activity between populations in the normal state ( $P > 0.05$ ). After 48 h of ammonia exposure, SOD activity decreased significantly in the hepatopancreas compared with the control group ( $P < 0.05$ ). In muscle, SOD activity decreased significantly except for Xing Hai No.1 (A) muscle tissue ( $P < 0.05$ ). The SOD content in CHAI increased first and then decreased; it was significantly higher than that in PRIMO at 24h ( $P < 0.05$ ). In addition, SOD activity was not significant difference from that in the control group after 96 h of ammonia relief ( $P > 0.05$ ).

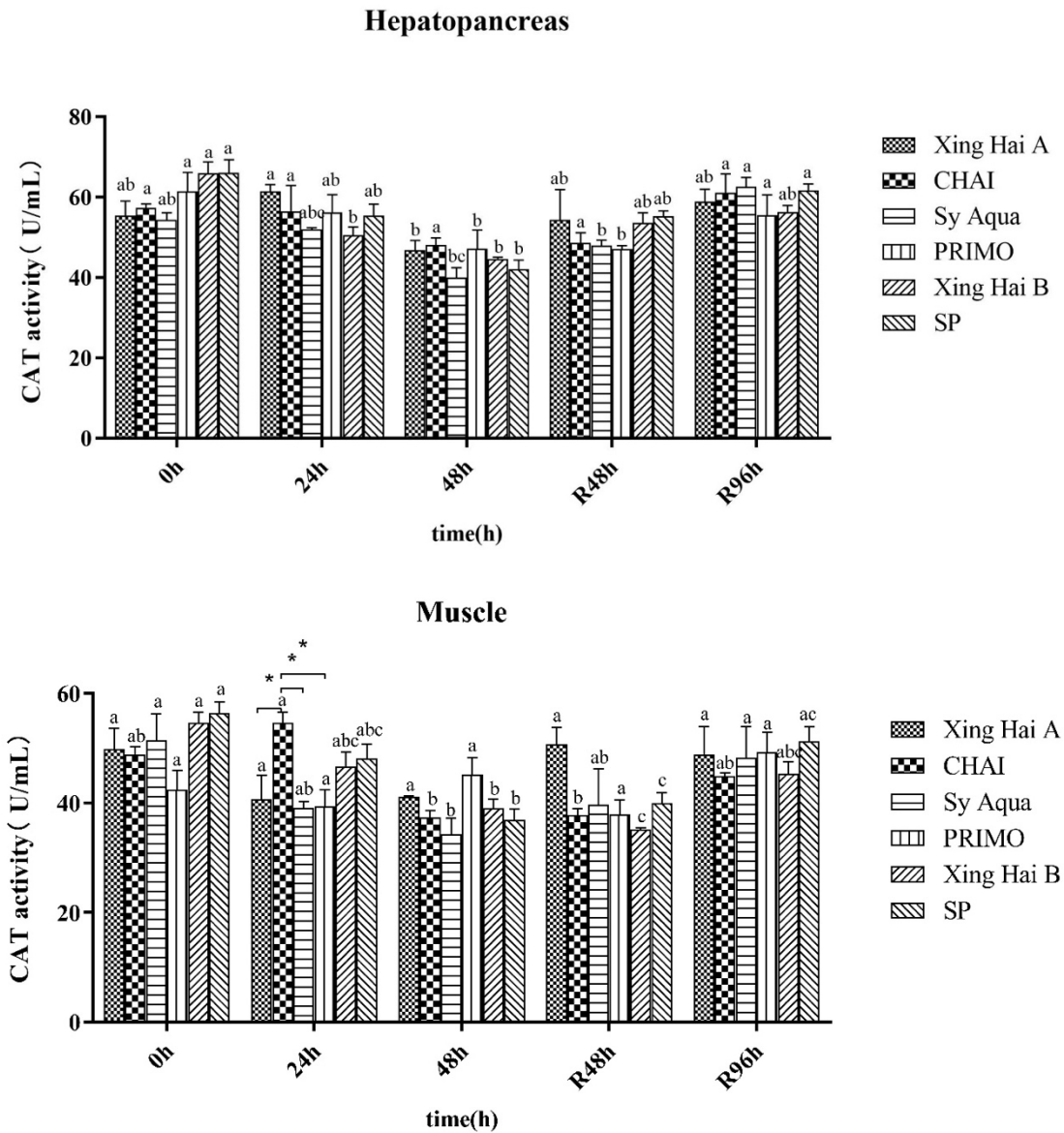


**Figure 2** Variation in SOD activity in muscle and hepatopancreatic tissues.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ), and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

#### *Changes in CAT activity in the hepatopancreas and muscle of shrimps after ammonia exposure*

The variation in CAT activity in hepatopancreatic and muscle tissues are presented in **Figure 3**. There were no significant differences in CAT activity between the populations in the normal state ( $P > 0.05$ ). After 48 h of ammonia exposure, in hepatopancreatic, CAT activity decreased significantly compared with the control population ( $P < 0.05$ ), however, there was no significant decrease in CHAI ( $P > 0.05$ ).



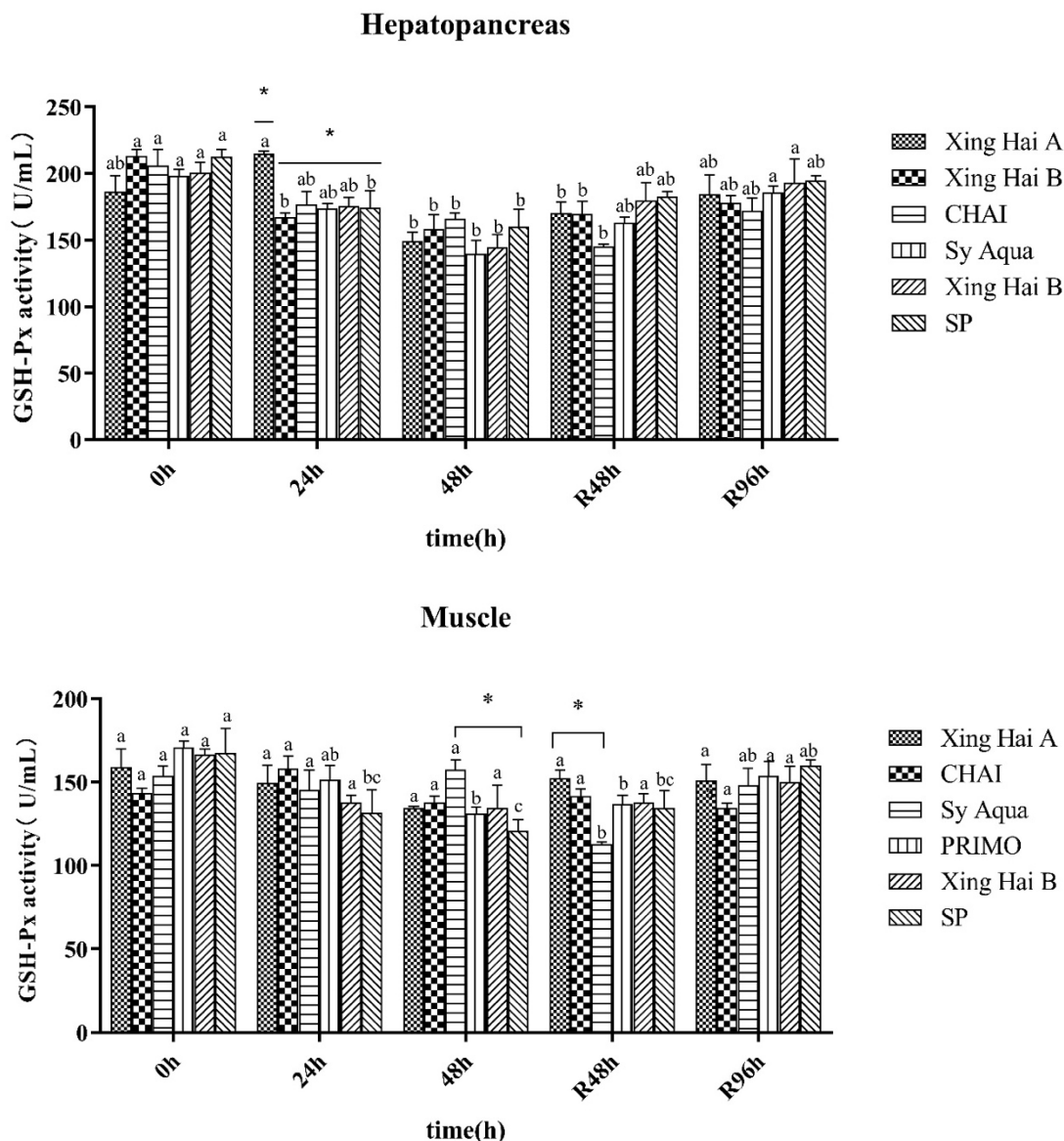
**Figure 3** Variations in CAT activity in muscle and hepatopancreatic tissues.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ), and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

CAT activity in CHAI was significantly higher than that in Xing Hai No.1(A), Sy Aqua, and PRIMO at 24h ammonia stress ( $P < 0.05$ ). CAT activity in muscle decreased significantly except for in Xing Hai No.1(A) and PRIMO ( $P < 0.05$ ). In addition, CAT enzyme activity in each population except for Sy Aqua and PRIMO returned to control population levels following 48 h of ammonia relief ( $P < 0.05$ ). After the ammonia was relieved for 48 h, Xing Hai No.1(B) and SP populations did not recover ( $P < 0.05$ ), but CAT activity was not significant difference from that in the control group after 96 h of ammonia relief ( $P > 0.05$ ).

### Changes in GSH-PX activity in the hepatopancreas and muscle of shrimps after ammonia exposure

The variations in GSH-PX activity in hepatopancreatic and muscle tissues are presented in **Figure 4**. In hepatopancreatic, GSH-PX activity of various experimental populations was significantly lower than that in the control population after 48 h ammonia stress ( $P < 0.05$ ). It was significantly higher in Xing Hai No.1(A) than that in other populations at 24 h ( $P < 0.05$ ). GSH-PX activity levels of CHAI and Sy Aqua were significantly inhibited after 48 h of ammonia relief ( $P < 0.05$ ). In muscle, GSH-PX activity did not decrease significantly in Xing Hai No.1 (A, B) and CHAI following ammonia exposure ( $P > 0.05$ ). GSH-PX activity level in Sy Aqua was still inhibited after 48 h of ammonia relief and was significantly lower than in the control population ( $P < 0.05$ ).

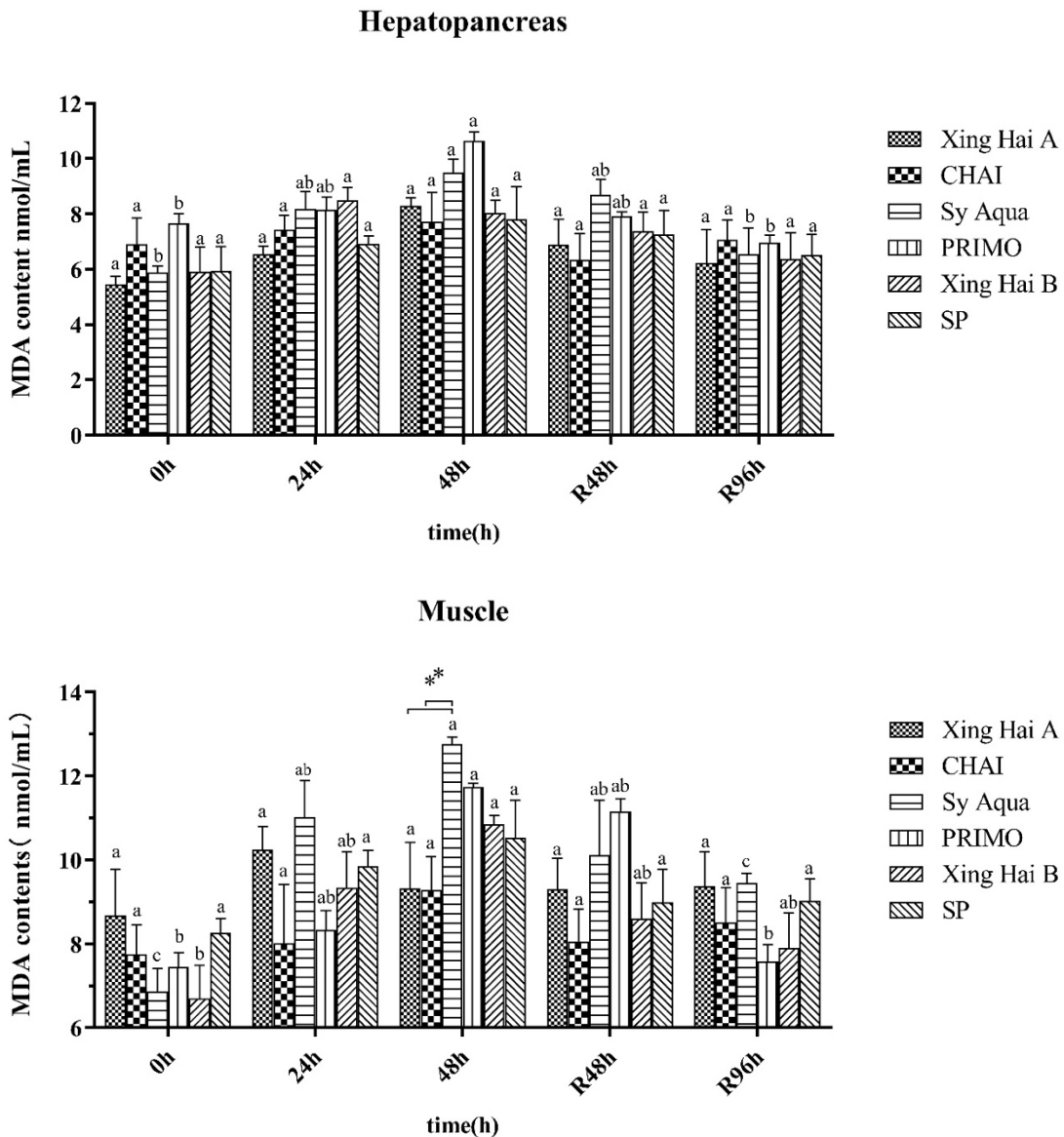


**Figure 4** Variations in GSH-PX activity in muscle and hepatopancreatic tissues.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ), and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

### Changes in the MDA contents of the hepatopancreas and muscle of shrimps after ammonia exposure

The variations in MDA content of the hepatopancreatic and muscle tissues are presented in **Figure 5**. There were no significant differences in MDA content among the populations in a normal state ( $P > 0.05$ ). MDA contents in Sy Aqua and PRIMO's hepatopancreatic and muscle tissues were significantly increased after 48 h of ammonia stress ( $P < 0.05$ ). And in muscle, MDA contents in Sy Aqua was significantly higher than that in Xing Hai No.1(A) and CHAI at 48 h ( $P < 0.05$ ). However, MDA contents recovered gradually after 48 h of ammonia relief and returned to control population levels after 96 h ( $P > 0.05$ ).

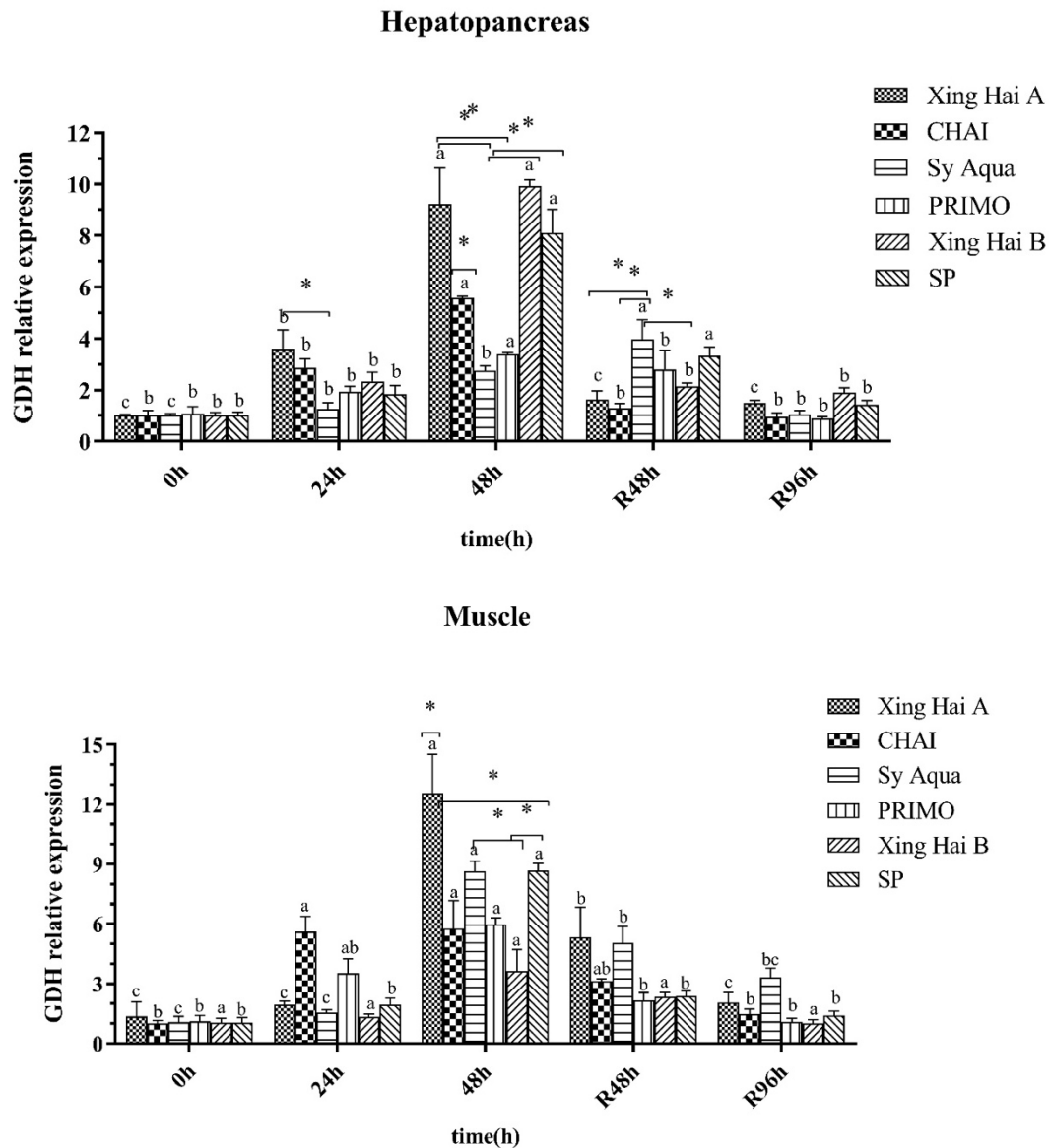


**Figure 5** Variations in the MDA contents of muscle and hepatopancreatic tissues.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ), and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

### Changes in *GDH* gene expression in shrimp hepatopancreatic and muscle tissue following ammonia exposure

The relative expression levels of *GDH* in the hepatopancreas and muscle are presented in **Figure 6**. In general, the relative expression level of *GDH* in hepatopancreas and muscle was significantly upregulated compared with that in the control population after 48 h of ammonia stress ( $P < 0.05$ ). The levels eventually returned to control levels after 96 h of ammonia relief ( $P > 0.05$ ). The expression levels of *GDH* were significantly lower in Sy Aqua's hepatopancreas and PRIMO's hepatopancreas than in the other populations. In contrast, the expression level of *GDH* in muscle in Xing Hai No.1(B) was significantly higher than that in the other populations after 48 h of ammonia stress ( $P < 0.05$ ).

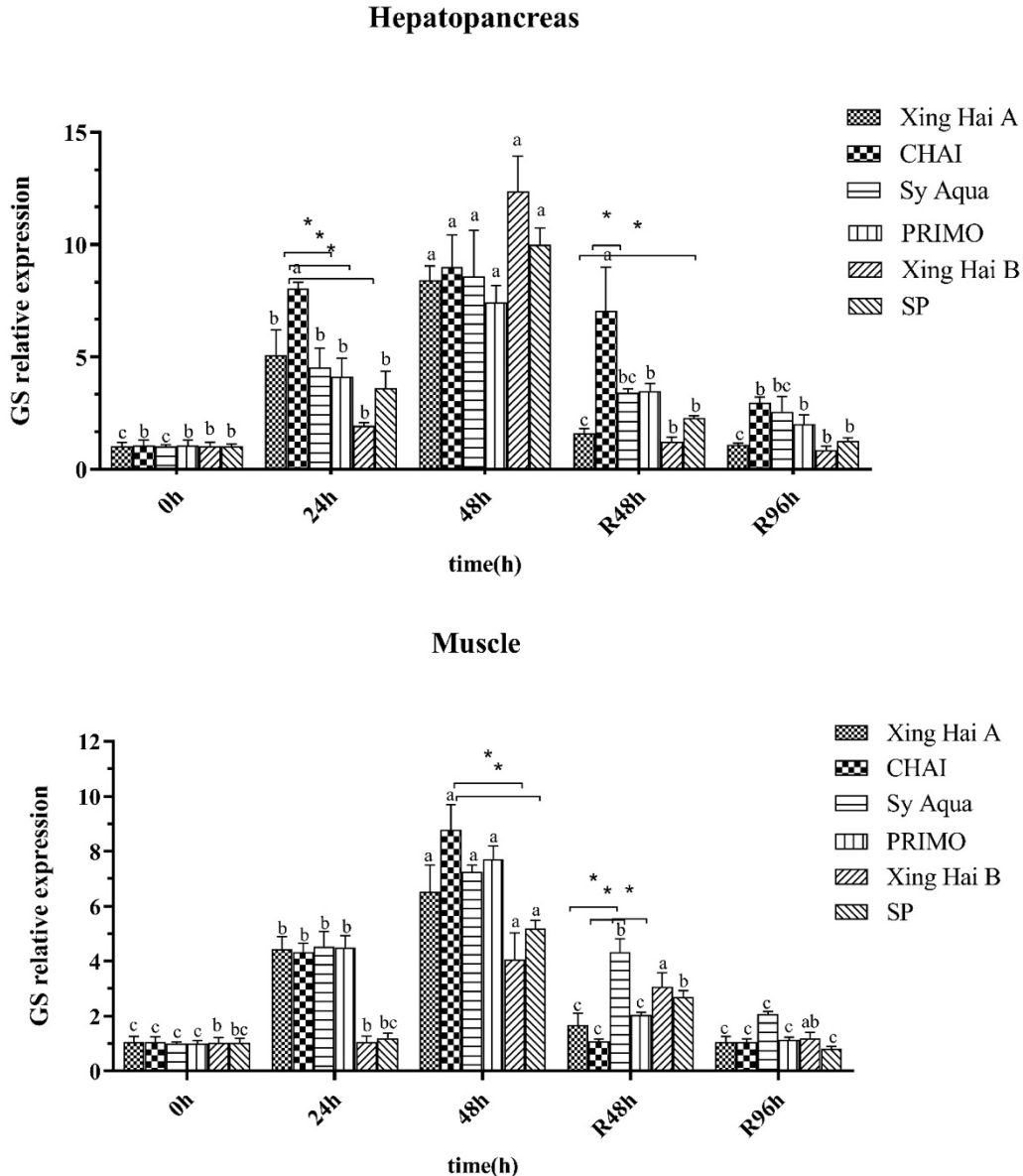


**Figure 6** Variations in the relative expression levels of *GDH* in hepatopancreas and muscle.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ) and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

### Changes in GS gene expression in shrimp hepatopancreatic and muscle tissues following ammonia exposure

The relative expression levels of GS in the hepatopancreas and muscle are presented in **Figure 7**. The expression levels of GS in the hepatopancreas and muscle of the three populations (Xing Hai No.1 A, CHAI, and Sy Aqua) were significantly higher than those in the control population after 24 h of ammonia stress. The expression in the ammonia-treated populations was higher than that in the control population after 48 h of ammonia stress ( $P < 0.05$ ). Moreover, the expression levels of GS in the ammonia-treated populations returned to the control level after 96 h of ammonia stress relief ( $P > 0.05$ ).

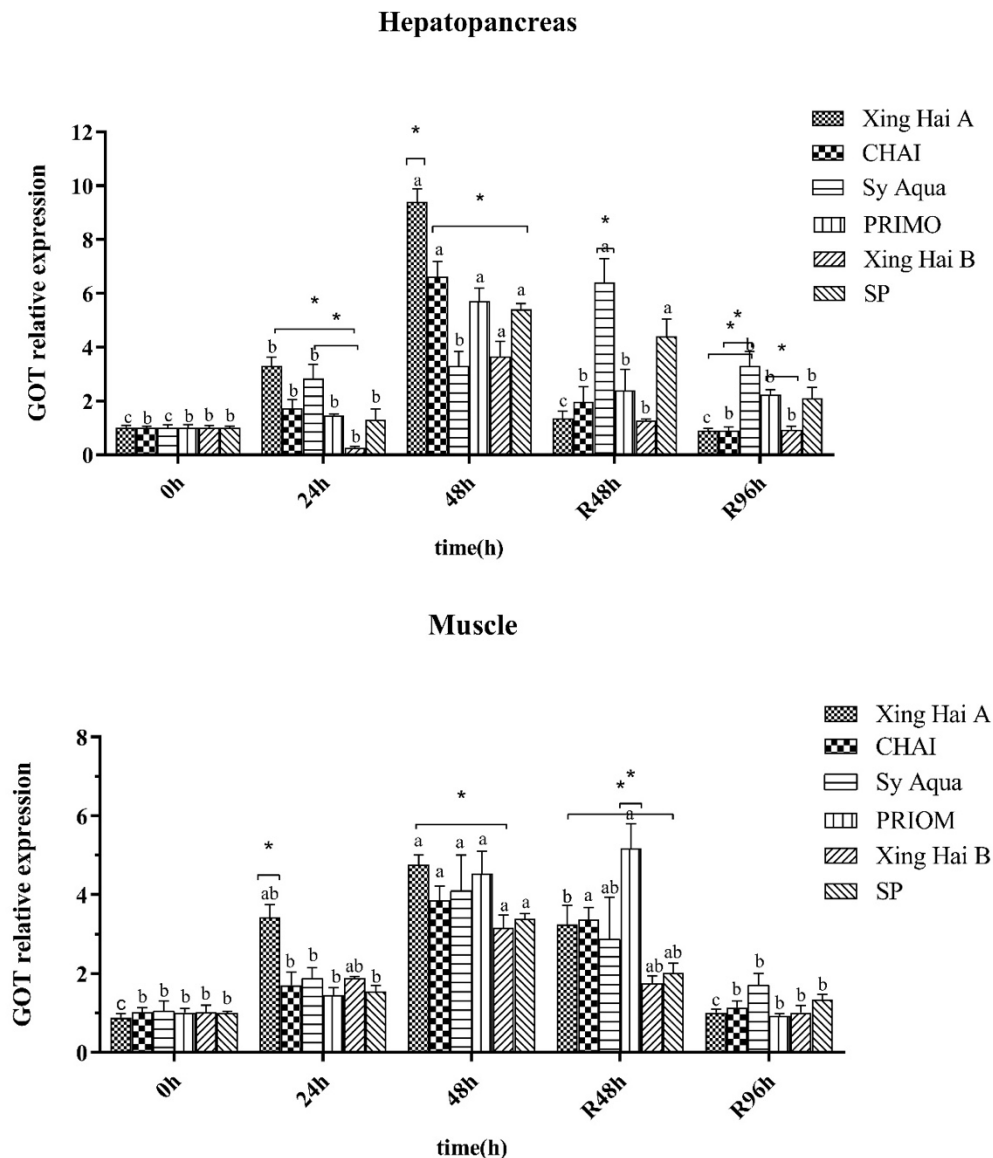


**Figure 7** Variations in the relative expression levels of GS in hepatopancreas and muscle.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ), and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

### Changes in GOT gene expression in the hepatopancreas and muscle of shrimps after ammonia exposure

The expression levels of GOT in shrimp hepatopancreas and muscle are presented in **Figure 8**. In general, the expression level of GOT in hepatopancreas and muscle was significantly upregulated compared with that in the control population after 48 h of ammonia stress ( $P < 0.05$ ). The expression of GOT in the hepatopancreas of Sy Aqua remained upregulated after 48 h of ammonia relief, and it remained significantly higher than that in the control population and significantly higher than in Xing Hai No.1 (A, B) and CHAI after 96 h of ammonia relief ( $P < 0.05$ ). Moreover, the expression of GOT in the muscle of PRIMO remained upregulated after 48 h of ammonia relief, and it eventually returned to the control level after 96 h of ammonia relief ( $P > 0.05$ ).



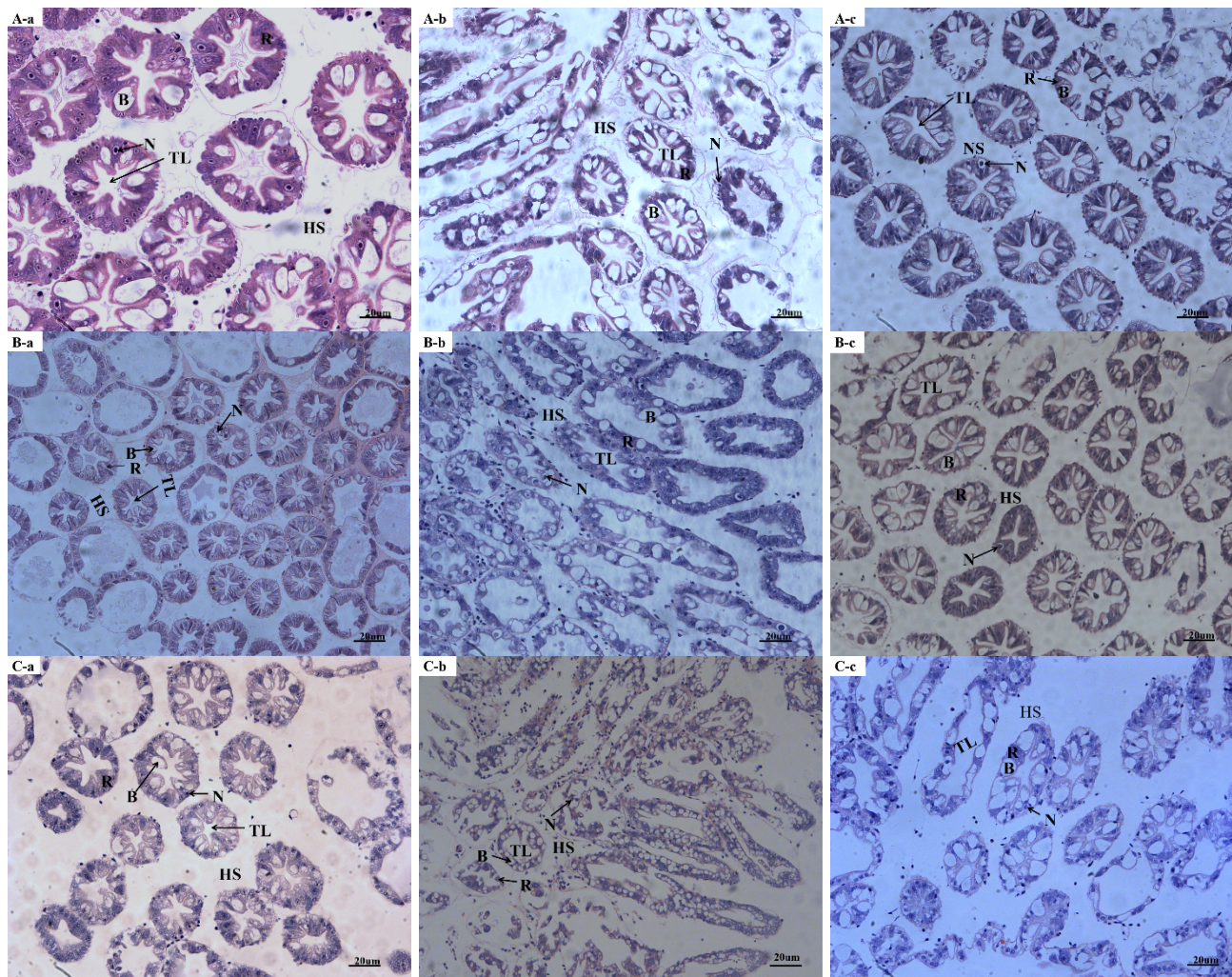
**Figure 8** Variations in the relative expression levels of GOT in hepatopancreas and muscle.

\*Note: Different lowercase Journal Pre-proof letters represent significance at different exposure times ( $P < 0.05$ ), and the asterisk (\*) represents significance among different groups at the same exposure time ( $P < 0.05$ ).

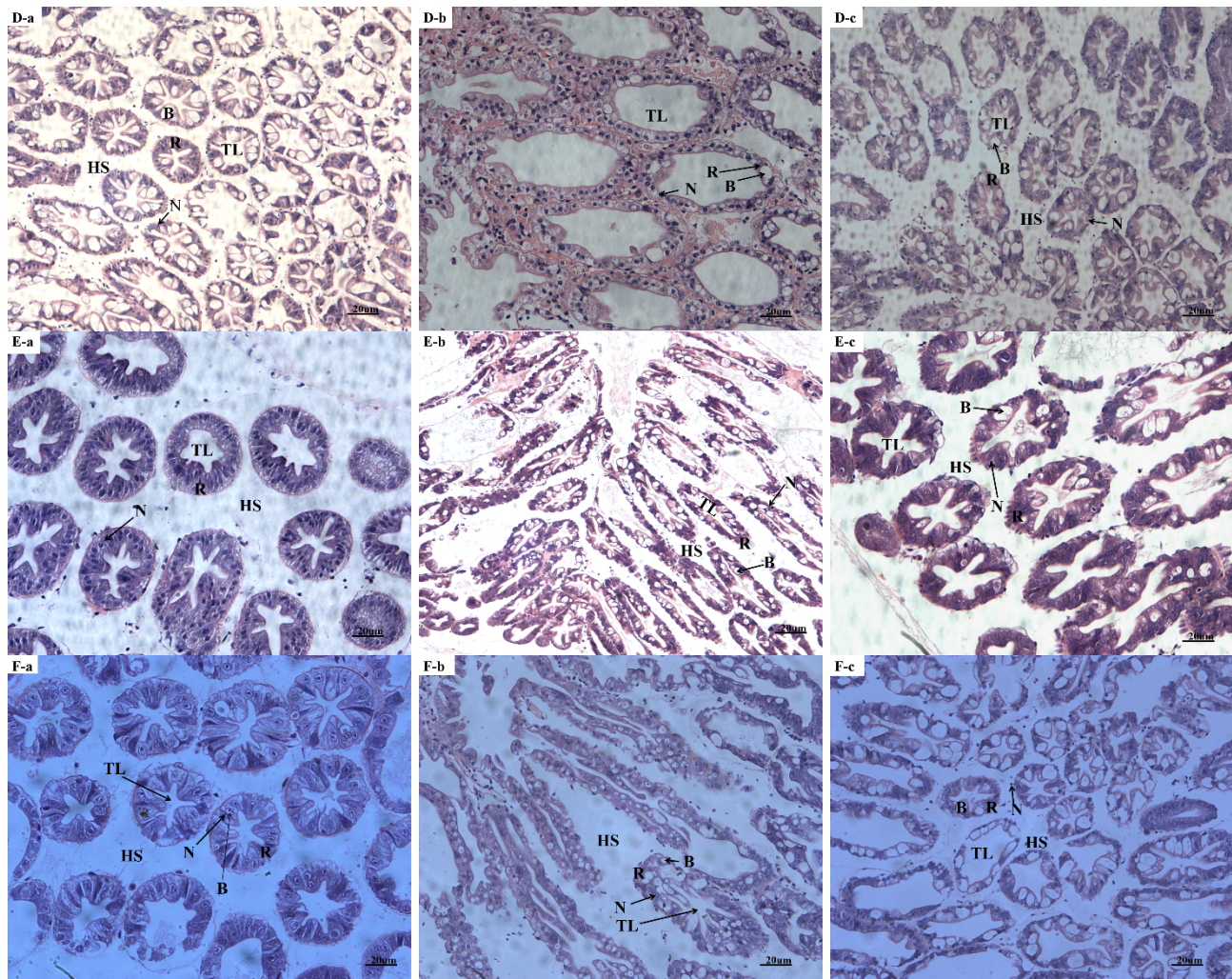


### The histological structure changes of hepatopancreas after ammonia exposure

The microscopic observation results are in **Figure 9**. Bubble cells (B) and absorber cells (R) are the main components of the hepatic tubules. Compared with the control group, the hepatic tubules were disordered, the lumen was enlarged, and the composition of B cells and R cells changed after ammonia stress. Moreover, the stellate lumen (TL) was deformed; the cell tissue was ruptured. Compared with Xing Hai No.1(A), the stellate lumen of the other populations was more seriously deformed, among which Sy Aqua and PRIMO populations were the most serious, and the nuclei (N) were broken and scattered. Although the morphology of hepatocytes recovered gradually after 96 h was relieved, we found that the vacuolization remained. There was still obvious vacuolization of hepatic tubules, and the broken nucleus can be seen in Sy Aqua, PRIMO and SP populations.







**Figure 9** The structure of the hepatopancreas of *L. vannamei* from six populations. The numbers represent the processing time: a. control shrimp, b. 48 h of exposure to ammonia; c.96 h of recovery.

\*Note: The experimental populations were Xing Hai No. 1 (A), CHAI (B), Sy Aqua (C), PRIMO (D), Xing Hai No. 1 (E), and the second-generation hybrid Sy Aqua-PRIMO population (F). Transverse section of hepatopancreas tubules showing R cells (R), B cells (B); TL: tubular lumen; HS: hemolymph sinus; N: nucleus.

## Discussion

### *Effects of acute ammonia stress on survival rate*

For a long time, researchers have used a series of criteria to selectively breed shrimp to improve their resistance to stress (Luo et al., 2018). Mortality is an essential indicator of shrimp stress tolerance in research and breeding efforts (Romano and Zeng 2013); researchers have used mortality to evaluate shrimp tolerance to ammonia, with similar studies investigating WSSV resistance and other environmental tolerances (Zhang et al., 2020). We compared this experiment's six populations to identify the best ammonia tolerance. The results suggest that the Xing Hai No.1 (A) has significant survival advantages compared to the introduced populations after ammonia stress. We speculate that this result came about because Xing Hai I was produced through directional breeding under ammonia stress. The aim was to produce shrimp that grow fast despite high ammonia levels (Yuan et al., 2018). However, Huang(2012) found that improved ammonia resistance could prolong survival time but not increase the survival rate. Even for groups with the

same survival or mortality rate, the ammonia resistance indices possibly differed due to the different survival times of individual shrimp. Thus, the survival rate should not be the only indicator used to assess the effectiveness of selective breeding. We suggest using the antioxidant enzyme index and expression of metabolic genes with survival rate measurements to more accurately evaluate resistance.

#### *Effects of acute ammonia stress on the immune-related enzyme activities*

Ammonia exists mainly as ammonium ions ( $\text{NH}_4^+$ ) or nonionic ammonia ( $\text{NH}_3$ ) in water. The latter form is more toxic due to its ease of entry into cultured animals where it causes oxidative stress, which is one of the toxic mechanisms of environmental stress to aquatic animals (Wang et al., 2021). An increase in the concentration of ammonia in aquaculture water causes the production of large amounts of ROS, which can bind with unsaturated fatty acids and cholesterol in the cell membrane and induce lipid peroxidation, resulting in reduced cell membrane mobility, increased permeability and a disordered distribution of membrane proteins (including receptor proteins and enzymes). This leads to a loss of cell membrane function and apoptosis (Liang et al., 2016). SOD, CAT, and GSH-PX act as interrelated antioxidant enzymes in living organisms that jointly scavenge ROS. In this study, the activity levels of the antioxidant enzymes SOD, CAT, and GSH-PX in hepatopancreatic and muscle of different populations of *L. vannamei* were significantly inhibited after 48 h of ammonia stress; this trend is similar to that in the work of Long (2021) and Liang (2020).

The difference is that the SOD activity level was significantly inhibited and then rose. CAT and GSH-PX activity was delayed in Sy Aqua and PRIMO (at 48 h after the resumption of treatment). Thus, we speculate that the catalysis rates of Sy Aqua and PRIMO may be slower than in the other populations due to the low antioxidant enzyme activity levels. This is because the ROS content was abnormally increased, the SOD uses superoxide radicals as substrates to generate  $\text{H}_2\text{O}_2$ , and then a series of antioxidant catalytic enzymes such as CAT and GSH-PX generate  $\text{H}_2\text{O}$  and  $\text{O}_2$  (Ighodaro and Akinloye 2017). In addition, the chart showed that the MDA content in the muscle of Sy Aqua was significantly higher than that in the other populations. It is well known that MDA is a sensitive and stable biomarker of whole-body ammonia exposure. When the antioxidant enzyme activity level in the body is reduced after shrimp are stressed by ammonia, the accumulation of oxygen radicals can cause lipid peroxidation and form lipid peroxides (MDA) (Long et al., 2021), which exacerbates cellular damage through increased rigidity of cellular membranes. We speculate that this may result from combining two classes of enzymes, which causes the population Sy Aqua to be more heavily damaged by ammonia and exhibit poor resistance to ammonia and high mortality.

#### *Effects of acute ammonia stress on the metabolism*

When exposed to an enriched ammonia environment, the tissues in shrimps have unique metabolic pathways for ammonia metabolism (Jie et al., 2019). To further evaluate and verify ammonia tolerance among different populations, we investigated the expression levels of three key genes (*GDH*, *GS*, and *GOT*) involved in the ammonia metabolism (Li et al., 2014; Wang et al., 2021). *GDH* plays a key role in amino acid catabolism and the maintenance of ammonia levels in the body, which catalyzes ammonia assimilation with  $\alpha$ -KG to address endogenous ammonia poisoning in tissues. When maintained in ammonia-N-rich water, high-level *GDH* expression was recorded (Qiu et al., 2018). Similar to the present results, an increase in *GDH* expression was found in the muscles and hepatopancreas of almost all populations. The expression of *GDH* in hepatopancreas in Xing Hai No.1 (A, B) was significantly higher than in the other populations. *GDH* viability has a similar trend with ammonia discharge, which can be used as an indicator of the ammonia removal (Qiu et al., 2018). We speculate that Xing Hai No.1 (A, B) respond more rapidly to ammonia exposure and excrete ammonia more quickly, which may explain their higher survival rates.

Moreover, we found *GS* and *GOT* expression in *L. vannamei* were significantly increased, whereas the hepatopancreas in Sy Aqua and PRIMO expressed less *GDH* after ammonia stress. Furthermore, *GOT* and *GS* were upregulated after 48 h of ammonia relief, suggesting that a stressed state remained. These results are consistent with the changes in mortality after ammonia exposure in different populations. *GS* can catalyze the generated glutamate and excess ammonia to form non-toxic glutamine after ammonia is decomposed by *GDH* (Gomez-Jimenez et al., 2004). As the first member of the aminotransferase family to be purified, *GOT* catalyzes the reaction of 2-oxosuccinic acid with glutamic acid to form aspartic acid, which is required for the synthesis of urea. This reduces ammonia concentration in the body, resulting in eventual detoxification (Chen et al., 2017). This suggests that the Sy Aqua and PRIMO were less efficient than the local dominant Xing Hai No.1 in ammonia detoxification. This may cause result in lower ammonia excretion in Sy Aqua and PRIMO and poorer ability to dissolve ammonia.

#### *Analysis of the reasons for the difference in the ammonia-tolerance index*

Multiple comparisons of survival rates and the ammonia resistance indexes from six populations showed that the expression of the immunity and metabolic indicators varied. This indicates that introduced and selective populations adapt differently to the domestic ammonia culture environment. We speculate that this is related to the breeding background of the shrimp. Known data indicate that the Sy Aqua is introduced bloodstock from Thailand; the Sy Aqua and PRIMO breeding was based on American pathogen-free shrimp (WSSV, YHV, TSV, and IHNV) and selective breeding. They used a specific pathogen-free model selection strategy based on shrimp survival in replicated laboratory-based viral challenges to select for rapid growth and disease resistance (Wyban 2019). This differs from the Xing Hai No.1 (A), as an indigenous population, which was further selected based on the introduced populations. These shrimp have undergone multiple generations of selective breeding for resistance to ammonia, and, as such, significant success has been achieved (Yuan et al., 2018).

The muscle tissue of population Xing Hai No.1 (B) did not show significant advantages when compared with the muscle tissues of the other populations. The differences in indicators in the hepatopancreas of different populations were more regular and prominent. One reason for this result may be that the economic traits of the selected population may not be completely stable (Huang et al., 2012; Qiu et al., 2018), the use of these advantages to stabilize genetic improvements in useful traits should be considered in the future. As a second-generation hybrid population, the population was superior to Sy Aqua and PRIMO in terms of lower mortality, and the antioxidant enzyme changes were more minor than in Sy Aqua and PRIMO; MDA content was relatively low. Moreover, the SP population exhibited greater and faster expression of metabolic genes in the hepatopancreas; the results show the potential advantages of the hybridization (Wyban 2019).

#### *Recovery of shrimp after ammonia stress relief*

Ammonia is one of the prevailing harmful factors in aquatic environments for industrial culturing (Ip and Chew, 2010). Ammonia concentrations may abruptly increase, negatively impacting various physiological, developmental, and immunological processes in aquatic animals (Ye et al., 2015). In practice, ammonia can be converted by microbial nitrification and denitrification, absorbed by algae and aquatic plants, assimilated by microorganisms, or removed by volatilization or substrate adsorption when environmental ammonia becomes excessive (Gao et al., 2020). In previous research, recovery treatment positively affected organisms; the physiological indicators gradually recovered after relieving the ammonia (Liang et al., 2020). Similar to the previous results, we found that some of the shrimp's antioxidant and metabolic indicators can be gradually restored after ammonia relief. The physiological indicators of the Xing Hai No.1 (A) all returned to control levels, whereas Ry Aqua and PRIMO recovery was slower. In addition, we observed tissue slices of six populations and found that tissue damage in each population significantly improved after the stress



was relieved. However, when the physiological indicators gradually adapt to the average level, there is still stress damage to the shrimp tissue, and the maybe recovery may take longer. In addition, compared with physiological indicators and survival rate, tissue sectioning shows the more intuitive performance of body damage and recovery; however, it has limitations and subjectivity in observing differences between the six populations.

Overall, our find shows that the introduced populations have selective potential and could be used to produce base populations in breeding programs. The results of this experiment will benefit shrimp production. We also found that farmers can prevent the toxic effects of ammonia on shrimp if excess ammonia is found in time and disposed of within 48 h.

### Conclusion

At the tissue and physiological levels, studying stress resistance in aquatic organisms is crucial; studying ammonia resistance in the founding population provides a reference for selected breeding and healthy farm breeding of shrimp fry. The results of the present study suggest that the Xing Hai population (A) has a good ammonia tolerance index; thus, breeding methods to improve ammonia resistance are feasible. The introduced shrimp populations also have the selective potential for hybridization. The differences in ammonia resistance observed among *L. vannamei* populations indicate that breeding programs have been successful; however, the use of population-specific advantages to stabilize genetic improvements in valuable traits should be considered in the future. In addition, the concentration of ammonia increases with the culture period in intensive aquaculture systems, but our experimental data show that shrimp undergo self-healing within 48 h of ammonia relief; thus, timely monitoring and treatment can reduce the toxic effects of ammonia stress.

### Acknowledgments

The authors would thank all the colleagues in the lab for helpful discussion and technical advice. The research was funded by the National Key R&D Plan "Blue Granary Science and Technology Innovation" key special project in 2020 (2020YFD0900205) and Modern Seed Industry Park for Whiteleg Shrimp of Guangdong Province.

### References

- Chatvijitkul S, Boyd C E, Davis D A and Mcnevin A A.** (2017). Pollution potential indicators for feed-based fish and shrimp culture. *Aquaculture*. S0044848617308244. <https://doi.org/10.1016/j.aquaculture.2017.04.034>
- Chen J, Zhou F, Huang J, Ma Z and Jiang S.** (2016). Ammonia and salinity tolerance of *Penaeus monodon* across eight breeding families. *Springerplus*.
- Chen Y, Ye B, Niu D and Li J.** (2021). Changes in metabolism and immunity in response to acute salinity stress in Chinese razor clams from different regions. *Aquaculture Reports*. 19, 100624. <https://doi.org/10.1016/j.aqrep.2021.100624>
- Fan Y, Tan J, Luan S, Luo K, Sui J, Meng X, Chen B, Cao J and Kong J.** (2021). Morphological Differences in Four Introduced Populations of *Litopenaeus vannamei*. *Progress in Fishery Sciences*. 42, 62-69. <https://doi.org/10.19663/j.issn2095-9869.20200413002>
- Fao.** (2020). 2020 The State of World Fisheries Aquaculture 2020. . Food and Agriculture Organization of the United Nations. <https://doi.org/> Rome <https://doi.org/10.4060/ca9229e>
- Gao J, Zhu Y, Guo Z, Xu G and Xu P.** (2020). Transcriptomic analysis reveals different responses to ammonia stress and subsequent recovery between *Coilia nasus* larvae and juveniles. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 230, 108710-. <https://doi.org/10.1016/j.cbpc.2020.108710>
- Gomez-Jimenez, Silvia, And, Urias-Reyes, Abel, Antonio, And, Vazquez-Ortiz, Francisco and And.** (2004). Ammonia efflux rates and free amino acid levels in *Litopenaeus vannamei* postlarvae during sudden salinity changes - ScienceDirect. *Aquaculture*. 233, 573-581. <https://doi.org/10.1016/j.aquaculture.2003.09.050>

- Huang Y, Yin Z, Weng S, He J and Li S.** (2012). Selective breeding and preliminary commercial performance of *Penaeus vannamei* for resistance to white spot syndrome virus (WSSV). *Aquaculture*. 364-365, 111-117. <https://doi.org/10.1016/j.aquaculture.2012.08.002>
- Ighodaro O M and Akinloye O A.** (2017). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*. S2090506817301550. <https://doi.org/10.1016/j.ajme.2017.09.001>
- Ip Y K and Chew S F.** (2010). Ammonia Production, Excretion, Toxicity, and Defense in Fish: A Review. *Frontiers in Physiology*. 1, 134. <https://doi.org/10.3389/fphys.2010.00134>
- Jie X A, Qyl B, Jpt A, Xlc B, Xhc B, Qyl B, Hong L A, Xyz A, Yzz B and Hlw A.** (2019). Stress response and tolerance mechanisms of ammonia exposure based on transcriptomics and metabolomics in *Litopenaeus vannamei*. *Ecotoxicology and Environmental Safety*. 180, 491-500. <https://doi.org/10.1016/j.ecoenv.2019.05.029>
- Li S, He Y, Li J, Li J, Liu P and Ge Q.** (2014). Cloning and expression analysis of aspartate aminotransferase c DNA in *Fenneropenaeus chinensis* following ambient ammonia stresses. *Journal of Fishery Sciences of China*. 21, 1125-1133. <https://doi.org/10.3724/SP.J.1118.2014.01125>
- Liang, Zhongxiu, Liu, Rui, Zhao, Depeng, Wang, Lingling, Sun and Mingzhe.** (2016). Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of pacific white shrimp (*Litopenaeus vannamei*). *Fish & Shellfish Immunology*. 54, 523-528. <https://doi.org/10.1016/j.fsi.2016.05.009>
- Liang L, Huang Z, Li N, Wang D and Hong M.** (2020). Effects of ammonia exposure on antioxidant function, immune response and NF- $\kappa$ B pathway in Chinese Strip-necked Turtle (*Mauremys sinensis*). *Aquatic Toxicology*. 229, 105621. <https://doi.org/10.1016/j.aquatox.2020.105621>
- Liu S, Pan L, Liu M and Yang L.** (2014). Effects of ammonia exposure on nitrogen metabolism in gills and hemolymph of the swimming crab *Portunus trituberculatus*. *Aquaculture*. 8, 351-359. <https://doi.org/10.1016/j.aquaculture.2014.05.029>
- Liu S, Pan L, Liu M and Yang L.** (2014). Effects of ammonia exposure on nitrogen metabolism in gills and hemolymph of the swimming crab *Portunus trituberculatus*. *Aquaculture*. 432, 351-359. <https://doi.org/10.1016/j.aquaculture.2014.05.029>
- Long J, Cui Y, Wang R, Chen Y, Zhao N, Wang C, Wang Z and Li Y.** (2021). Combined effects of high salinity and ammonia-N exposure on the energy metabolism, immune response, oxidative resistance and ammonia metabolism of the Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture Reports*. 20, 100648. <https://doi.org/10.1016/j.aqrep.2021.100648>
- Luo K, Kong J, Meng X, Luan S, Cao B and Chen B.** (2018). Comparisons of Growth and Survival Performance Among Selected Families and Wild Populations of *Fenneropenaeus chinensis*. *Journal of Ocean University of China*. 17, 407-412. <https://doi.org/CNKI:SUN:QDHB.0.2018-02-021>
- Qiu L, Shi X, Yu S, Han Q, Diao X and Zhou H.** (2018). Changes of Ammonia-Metabolizing Enzyme Activity and Gene Expression of Two Strains in Shrimp *Litopenaeus vannamei* Under Ammonia Stress. *Front Physiol*. 9, 211. <https://doi.org/10.3389/fphys.2018.00211>
- Romano N and Zeng C.** (2013). Toxic Effects of Ammonia, Nitrite, and Nitrate to Decapod Crustaceans: A Review on Factors Influencing their Toxicity, Physiological Consequences, and Coping Mechanisms. *Reviews in Fisheries Science*. 21, 1-21. <https://doi.org/10.1080/10641262.2012.753404>
- Wang T, Li W, Shan H and Ma S.** (2021). Responses of energy homeostasis and lipid metabolism in *Penaeus vannamei* exposed to ammonia stress. *Aquaculture*. 737092. <https://doi.org/10.1016/j.aquaculture.2021.737092>
- Wang W-N, And, Yue, Wang, And, An-Li and Wang.** (2006). Effect of supplemental l-ascorbyl-2-polyphosphate (APP) in enriched live food on the immune response of *Penaeus vannamei* exposed to ammonia-N. *Aquaculture*. 256, 552-557. <https://doi.org/10.1016/j.aquaculture.2006.02.017>
- Wicks B J and Randall D J.** (2002). The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. *Comparative Biochemistry & Physiology Part A Molecular & Integrative Physiology*. 132, 275-285. [https://doi.org/10.1016/S1095-6433\(02\)00034-X](https://doi.org/10.1016/S1095-6433(02)00034-X)
- Wyban J.** (2019). Selective Breeding of *Penaeus vannamei*: Impact on World Aquaculture and Lessons for Future. *Journal of Coastal Research*. 1-5. <https://doi.org/10.2112/SI86-001.1>
- Yang L, Yang Q, Jiang S, Li Y, Zhou F, Li T and Huang J.** (2014). Metabolic, immune responses in prawn (*Penaeus monodon*) exposed to ambient ammonia. *Aquaculture International*. 23, 1049-1062. <https://doi.org/10.1007/s10499-014-9863-6>

- Ye, Chao-Xia, Yang, Fang-Fang, Miao, Yu-Tao, Ling, Ren-Zhi, Wang and An-Li.** (2015). Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (*Takifugu obscurus*). *Aquatic Toxicology*. 164, 61-71. <https://doi.org/10.1016/j.aquatox.2015.04.004>
- Yuan R, Hu Z, Liu J and Zhang J.** (2018). Genetic parameters for growth-related traits and survival in pacific white shrimp, *litopenaeus vannamei* under conditions of high ammonia-N concentrations. *Turkish Journal of Fisheries and Aquatic Sciences*. 18, 37-47. [https://doi.org/10.4194/1303-2712-v18\\_1\\_05](https://doi.org/10.4194/1303-2712-v18_1_05)
- Yuan R, Liu J, Zhang J and Hu Z.** (2015). A comparative study of hypoxia and high-ammonia resistance between *Litopenaeus vannamei* inbred and hybrid offspring at different stages. 22, 410-417. <https://doi.org/10.3724/SP.J.1118.2015.14305>
- Zhang Z, Huang Z, Yang Z, Chen S and Ge H.** (2020). Comparative study on the low salt tolerance in larval offspring of *Litopenaeus vannamei* breeding populations. *Journal of Fisheries Research*. 42, 366-370. <https://doi.org/10.14012/j.cnki.fjsc.2020.04.008>