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# Exposure to acute ammonia stress influences survival, immune response and antioxidant status of pacific white shrimp (*Litopenaeus vannamei*) pretreated with diverse levels of inositol

Shijun Chen[a], Yingying Yu[a], Yujie Gao[b], Peng Yin[a], Lixia Tian[a,*], Jin Niu[a,**], Yongjian Liu[a]

[a] Guangdong Provincial Key Laboratory of Improved Variety Reproduction in Aquatic Economic Animals, Institute of Aquatic Economic Animals, School of Life Sciences, Sun Yat-Sen University, Guangzhou, 510275, PR China

[b] State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haikou, 570228, PR China

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ABSTRACT

The effect of acute ammonia challenge on survival, immune response and antioxidant status of *Litopenaeus vannamei* pretreated with diets containing different inositol levels was investigated. Shrimp (initial mean weight 0.40 ± 0.00 g) were randomly allocated in 18 tanks (30 shrimp per tank) and triplicate tanks were fed with a control diet without *myo*-inositol (MI) supplementation (242.6 mg inositol $kg^{-1}$ diet) or diets containing diverse levels of inositol (368.8, 459.7, 673.1, 993.8 and 1674.4 mg $kg^{-1}$ diet) as treatment groups for 8-week. Randomly selected 10 shrimp per tank (final mean weight approximately 11.1–13.8g) were exposed to ammonia stress (total ammonia-nitrogen, 60.21 mg $L^{-1}$) for 24 h after feeding trial. The results showed that after exposed to ammonia stress, survival rates of MI-supplemented groups were enhanced by 31–77% when compared with the control group. MI supplementation increased activities of alkaline phosphatase (AKP) and acid phosphatase (ACP) in plasma, and reduced its activities in hepatopancreas. It also enhanced activities of total antioxidant capacity (T-AOC), glutathione S-transferase (GST) and glutathione peroxidase (GPX) and content of reduced glutathione (GSH), and lowered malondialdehyde (MDA) and protein carbonyl (PC) content in plasma or hepatopancreas. In addition, mRNA expression levels of ferritin (FT), arginine kinase (AK), thioredoxin (Trx), heat shock protein 70 (Hsp70), catalase (CAT) and peroxiredoxin (Prx) were significantly differentially regulated in hepatopancreas owing to MI supplementation. Therefore, it suggested that *L. vannamei* pretreated with higher dietary inositol content may have better ammonia stress tolerance and antioxidant status after ammonia stress, and the optimum levels ranged from 459.7 to 993.8 mg inositol $kg^{-1}$ when total ammonia-nitrogen concentration was 60.21 mg $L^{-1}$.

## 1. Introduction

*Litopenaeus vannamei*, is a crucial penaeid species in China [1,2]. Owing to the limitation of water area, culturing density of shrimp has been strengthened [3]. However, intensive culture systems, being developed for economic and environmental reasons, have enhanced the ambient ammonia levels, thus may lead to detrimental effect on health condition of fish and shrimp [4–7]. Additionally, excessive ammonia could cause suppression on the immune response and an increase in susceptibility to infection of crustacean by pathogens [7–9]. Thus, natural and safe feed additives to improve immune capacity are necessary for aquatic animals.

Ammonia, deriving from excretion of cultured animals, microbial metabolism of nitrogenous compounds, agricultural runoff, industrial wastes and urban sewage effluents, are one of major pollutants in the aquatic ecosystem that threatens the survival and growth of aquatic animals [10–13]. Ammonia exists in two chemical forms (including ionised form and un-ionised form) in solution, and the latter is examined more toxic [14,15]. Enhancement of ammonia concentration may worsen quality of aquatic water, leading to reductions in production of cultured shrimp [10,16,17]. Li and Chen [18] reported that the 24 h LC50 values of total ammonia-nitrogen on juveniles *L. vannamei* were 59.72 mg $L^{-1}$ at 15‰, 66.38 mg $L^{-1}$ at 25‰ and 68.75 mg $L^{-1}$ at 35‰ (pH 8.05, 23 °C), which was lower than the value (79.97 mg $L^{-1}$ at 33‰, pH 7.94, 26 °C) obtained from the research on *Penaeus chinensis* [19]. Moreover, Ching et al. [20] found that the exposure to high

* Corresponding author.
** Corresponding author.
*E-mail addresses:* lixiatian2002@163.com (L. Tian), gzniujin2003@163.com (J. Niu).

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ammonia concentration for 12 or 48 h induced oxidative stress in gills and brain of *Boleophthalmus boddarti*. Excessive reactive oxygen species (ROS) generated from acute ammonia stress may lead to oxidative stress or even mortality in aquatic animals [21–23].

Inositol is a key nutrient for most aquatic animals [24]. Symptoms of inositol deficiency have been declared in fish and shrimp [25]. However, several aquatic species, such as sunshine bass (*Morone chrysops* × *Morone saxatilis*), channel catfish (*Ictalurus punctatus*), abalone (*Haliotis discus hannai*) and Nile tilapia (*Oreochromis niloticus*), did not require extra inositol for normal growth and development [26–29]. Most studies related to inositol have focused on growth, lipid metabolism, mediation of enzyme activity and regulation of signal transduction [30–32], and only a few researches have been carried out to investigate the antioxidant property of inositol [33–35]. So far, no report about the relationship between inositol and resistance to ammonia stress has been found in *L. vannamei*. It remains unknown whether pretreatment of dietary *myo*-inositol has any beneficial influence on survival rate and antioxidant status of *L. vannamei* under condition of ammonia stress.

Generally, we can investigate the protection of antioxidants against oxidative damage via placing the animals pretreated with antioxidants to oxidative situation induced by oxidants or toxic substances [36]. Thus, the purpose of the present study was to determine changes in antioxidant and immune indexes and survival rate of *L. vannamei* pretreated with different levels of dietary inositol after acute ammonia stress.

## 2. Materials and methods

### 2.1. Experimental diets

The formulation and composition analysis of the basal diet is shown in Table 1. All diets contained approximately 42% crude protein and 7.8% crude lipid. Briefly, fish meal, soybean meal and peanut bran were utilized as the main protein sources. Fish oil and soy oil were chosen as dietary lipid sources. The basal diet was supplemented with *myo*-inositol (Guangzhou Chengyi Company Ltd, Guangzhou, China) at 0, 100, 200, 400, 800 and 1600 mg $kg^{-1}$ diets, respectively, resulting in final dietary inositol concentrations of 242.6, 368.8, 459.7, 673.1, 993.8 and 1674.4 mg $kg^{-1}$ diets, respectively. The dietary inositol contents were analyzed by gas chromatograph assay (Agricultural industry standard of the People's Republic of China, NY/T 1345–2007). The diets were manufactured by using the methods described by Chen et al. [37], and stored at −20 °C until used.

**Table 1**
Formulation and proximate composition of the basal diet (% dry matter).

| Ingredient | Content |
|---|---|
| Fish meal | 20.0 |
| Soybean meal | 25.0 |
| Peanut bran | 12.0 |
| Wheat flour | 24.3 |
| Shrimp meal | 5.0 |
| Beer yeast | 3.0 |
| Soy protein concentrate | 4.0 |
| Fish oil | 2.0 |
| Soy oil | 2.0 |
| Monocalcium phosphate | 1.0 |
| Choline chloride (50%) | 0.2 |
| Mineral mixture[a] | 0.5 |
| Vitamin mixture[b] | 1.0 |
| *Myo*-inositol (97%) | 0.0 |
| Proximate composition (% dry matter) | |
| Moisture | 10.14 |
| Crude protein | 42.09 |
| Crude lipid | 7.82 |
| Ash | 10.85 |
| Inositol (mg $kg^{-1}$ diet) | 242.6 |

[a] Mineral mixture (mg $kg^{-1}$ of diet): $FeSO_4 \cdot H_2O$, 30.41; $CuSO_4 \cdot H_2O$, 41.91; $ZnSO_4 \cdot H_2O$, 274.34; $MgSO_4 \cdot H_2O$, 284.47; Ca $(IO_3)_2$, 6.14; $Na_2SeO_3$, 0.44; $CoSO_4$, 2.89.

[b] Vitamin mixture ($kg^{-1}$ of diet): vitamin A, 12000 IU; riboflavin, 40 mg; cyanocobalamin, 0.02 mg; thiamin, 50 mg; menadione, 40 mg; folic acid, 10 mg; biotin, 1 mg; a-tocopherol, 120 mg; vitamin C, 250 mg; calcium pantothenate, 100 mg; nicotinic acid, 120 mg; vitamin $D_3$, 2000 IU; pyridoxine HCl, 60 mg.

### 2.2. Feeding trial and ammonia exposure

The experimental process is shown in Fig. 1. Healthy postlarvae (*L. vannamei*) were harvested from Guangdong Evergreen Group (Zhanjiang, China) and fed with commercial diet (Zhanjiang Yuehua Aquatic Feed Co., Ltd) for about 25 days, and then acclimated to laboratory condition for 2-week. After acclimation, 540 shrimp (mean initial body weight 0.40 ± 0.00 g) were randomly distributed into each of 18 500-L cylindrical fiberglass tank (triplicate tanks per each diet group, size of tank: height 80 cm, base diameter 90 cm) at a density of 30 shrimp per tank.

For the feeding trial, shrimp were fed the respective diets to apparent satiation four times a day for a period of 8-week. Uneaten feeds and faecal waste were eliminated by siphoning 2 h after feeding. Water temperature ranged from 28.4 to 30.2 °C. pH, salinity, total ammonia nitrogen and sulfur compounds was kept at 7.5–7.8, 27.0–30.0‰, less than 0.1 mg $L^{-1}$ and lower than 0.05 mg $L^{-1}$, respectively. Dissolved oxygen was more than 7.0 mg $L^{-1}$. Natural light-dark cycle was employed during the experiment (16th, May-10th, July).

According to Barbieri [3] and Lin et al. [18], after an 8-week feeding trial ended, 10 shrimp collected from each tank were moved to a new tank and prepared for ammonia challenge test. Ammonium chloride ($NH_4Cl$) was used as a source of total ammonia-nitrogen (TA-N, sum of $NH_3$ and $NH4^+$), which was employed to produce the desired final TA-N concentration according to the methods reported by Cheng et al. [23]. TA-N concentration was determined as 60.21 mg $L^{-1}$ according to the National Standard of the People's Republic of China (GB17378.4–2007, Part 4: Seawater analysis, the specification for marine monitoring). The stress trial went on for a period of 24 h without feeding supply. The TA-N levels were tested by the method mentioned above and adjusted by adding $NH_4Cl$ solution every 12 h. The mortality of shrimp was recorded every 6 h.

### 2.3. Sample collection

Four individuals were sampled from each tank after 24 h of ammonia exposure. Hemolymph was collected from the ventral sinus of each individual with a 1-mL disposable sterile syringe, containing an equal volume of precooled (4 °C) anticoagulant solution (27 mM trisodium citrate, 385 mM sodium chloride,115 mM glucose, pH 7.5) [38], then maintained at 4 °C for 12 h. Hemolymph was centrifuged at 7,100g for 10 min at 4 °C, and the plasma was separated for measuring the antioxidant indexes. Hepatopancreas was immediately homogenized in 10% (w/v) of ice-cold normal saline using an automatic sample rapid grinding instrument (Jingxin, China). The homogenate was centrifuged at 3,550g in pre-cooling centrifuge (Thermo Fisher Scientific, USA) at 4 °C for 20 min and the supernatant was saved at −80 °C until determined the enzyme activities. Meanwhile, hepatopancreas were immediately placed into RNAlater (Thermo Fisher Scientific, USA) and stored at −80 °C until extracted the total RNA.

### 2.4. Enzyme activity assays

The content of PC and GSH and activities of AKP, ACP, T-AOC, GST, total superoxide dismutase (T-SOD), copper/zinc superoxide dismutase (Cu/ZnSOD) and GPX were determined by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The MDA was measured by utilizing the Lipid Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology, China).

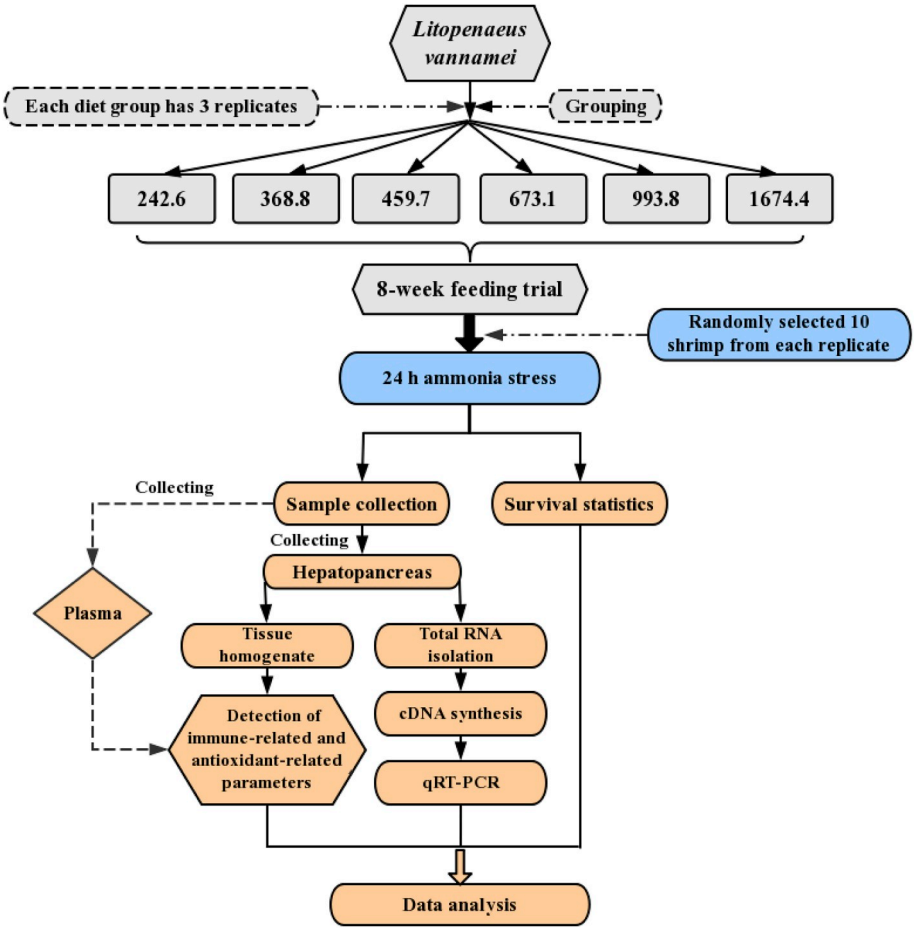


**Fig. 1.** The framework to illustrate the analysis procedure of the present study.

### 2.5. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the hepatopancreas tissue by using TRIzol reagent (Invitrogen, USA) and then dissolved in DEPC treated water. The quantity and quality of isolated RNA were assessed by detecting their absorbance at 260 and 280 nm using a NanoDrop2000c spectrophotometer (NanoDrop Technologies, USA) and by electrophoresis in 1% agarose gel, respectively. The first-strand cDNA was synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer's protocol. The cDNA was then saved at −20°Cfor real-time quantitative polymerase chain reaction (qRT-PCR).

### 2.6. Real-time quantitative polymerase chain reaction (qRT-PCR)

The mRNA expression levels of heat shock protein 70 (Hsp70, 5′F:TCTTGATCTTCGATCTTGGC; R:TCTCACTTGGGTCCTTCTTG, 183bp), arginine kinase (AK, 5′F:CAACACCAGAGTCCAGGTTC; R:TGAGTCGAGGAGGTGTTAGG, 241bp), peroxiredoxin (Prx, 5′F:GAAGAGCAATGCCATACGTT; R:CTTGAGCTCACGGAACTCTC, 159bp), ferritin (FT, 5′F:CCACAGAATTTGGATGGAAG; R:ATGATTACCAAGCTGAAGCG, 293bp), thioredoxin (Trx, 5′F:TTCATGAAGAATGGCCAGAA; R:TCCATCTTTCATGCTCTTGG, 128bp) and catalase (CAT, 5′F:AGAGGGTTGTGCATGCTAAG; R:CAGCTGATCCACTCTCACCT, 159bp) in hepatopancreas were detected by qRT-PCR in an ABI 7900 real-time fluorescence quantitative PCR System (Applied Biosystems, USA) using SYBR® Green Realtime PCR Master Mix Kit (TOYOBO, Japan). The β-actin gene (5′F: CCACGAGACCACCTACAAC; R: AGCGAGGGCAGTGATTTC, 142bp) was invoked as a housekeeping gene for internal standardization. The specificity and efficiency of the primers above were checked before the prescribed experiments. Amplifications were performed in a 384-well plate with a 10 μL reaction volumes containing 5 μL of SYBR® Green Realtime PCR Master Mix, 0.2 μL of each primers (10 mM), 0.5 μ L of cDNA sample and 4.1 μL of RNase Free $dH_2O$. The PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 20 s. The threshold cycle (Ct) values were collected from each sample after finishing the process. The relative expression levels of the above genes were calculated by using the $2^{-\Delta\Delta Ct}$ method [39].

### 2.7. Statistical analysis

Results are presented as means ± standard error (standard error of the mean, SEM). All data were firstly examined for homogeneity of variance using SPSS Statistic 20.0 software (IBM, New York, USA). One-way ANOVA was used to test the main effect of dietary MI inclusion when the data had homogeneous variance. The tukey test was used to determine significant differences among treatment groups, and probability values of $P < 0.05$ were deemed to be statistically different. The Kruskal-Wallis ANOVA test (non-parametric test) was applied when inhomogeneous variance appeared, followed by pairwise comparisons using Kruskal-Wallis. Broken-line regression analysis and quadratic regression method were utilized to evaluate the dietary inositol requirement of *L. vannamei* according to Shiau and Su [40] and Chen et al. [38].

## 3. Results

### 3.1. Growth and survival after 8-week feeding trial

As shown in Table 2, although FBW of the control group was lower than the other experimental groups, there was no significant difference between them ($P > 0.05$). Survival rate showed no obvious difference among all groups ($P > 0.05$).

**Table 2**
Effects of dietary inositol levels on growth performance and survival rate of *L. vannamei* for 8-week.

| | Dietary inositol levels (mg/kg diet) | | | | | |
|---|---|---|---|---|---|---|
| Parameters | 242.6 | 368.8 | 459.7 | 673.1 | 993.8 | 1674.4 |
| IBW | 0.40 ± 0.00 | 0.40 ± 0.00 | 0.40 ± 0.00 | 0.40 ± 0.00 | 0.40 ± 0.00 | 0.40 ± 0.00 |
| FBW | 11.17 ± 1.04 | 12.35 ± 0.91 | 13.75 ± 0.05 | 11.55 ± 1.16 | 11.68 ± 1.00 | 12.44 ± 1.13 |
| Survival | 98.9 ± 1.1 | 97.8 ± 2.2 | 98.9 ± 1.1 | 97.8 ± 2.2 | 98.9 ± 1.1 | 98.9 ± 1.1 |

Results are mean ± SEM (n = 3).
IBW, initial mean body weight (g shrimp$^{-1}$).
FBW, final mean body weight (g shrimp$^{-1}$).
Survival (%) = 100 × (final number of shrimps)/(initial number of shrimps).

### 3.2. Survival rate after ammonia stress

No obvious differences were found among all groups in the first 12 h of stress trial ($P > 0.05$) (Fig. 2). When the experiment was performed to 18 h, shrimp fed 368.8 mg kg$^{-1}$ diet had the highest survival rate, and the lowest in shrimp fed control diet ($P < 0.05$). Shrimp fed control diet had a lower survival rate than the shrimp fed 368.8, 459.7 and 1674.4 mg kg$^{-1}$ diets when the 24 h ammonia stress experiment ended ($P < 0.05$).

### 3.3. Immune response-related parameters in the plasma and hepatopancreas of shrimp after ammonia stress

AKP and ACP activities in plasma and hepatopancreas of shrimp pretreated with different dietary inositol levels are illustrated in Fig. 3. AKP activities in plasma for shrimp fed 993.8 and 1674.4 mg kg$^{-1}$ diets were considerably higher than shrimp fed control diet ($P < 0.05$), while the others were in between ($P > 0.05$). All MI-supplemented groups (except 368.8 mg kg$^{-1}$ group) had significantly lower AKP activity in hepatopancreas than the shrimp fed control diet ($P < 0.05$). Although MI-supplemented groups had higher ACP activity in plasma, but only 459.7 and 1674.4 mg kg$^{-1}$ groups were significantly greater than the control group ($P < 0.05$). Oppositely, ACP activity in hepatopancreas decreased with increasing dietary inositol levels up to 368.8 mg kg$^{-1}$ diet ($P < 0.05$), and plateaus thereafter ($P > 0.05$).

### 3.4. Antioxidant-related parameters in the plasma of shrimp after ammonia stress

All MI-supplemented groups had higher T-AOC activity than the shrimp fed control diet ($P < 0.05$) (Table 3). GPX activity significantly

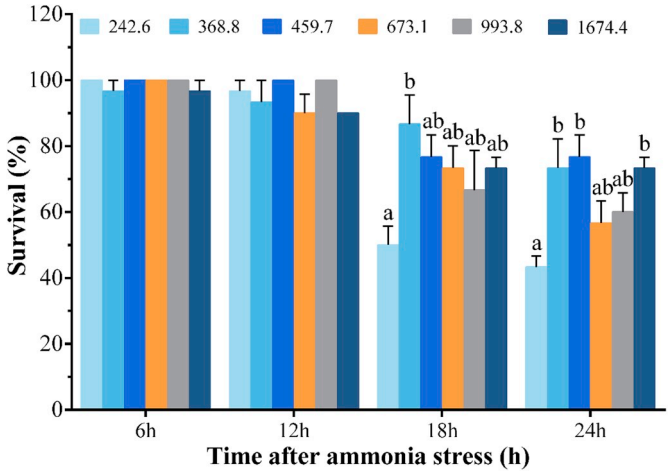


**Fig. 2.** Survival rate of *L. vannamei* pretreated with different dietary inositol levels after 24 h ammonia stress. Results are mean ± SEM (n = 3). The column with different superscripts manifests significant differences ($P < 0.05$).

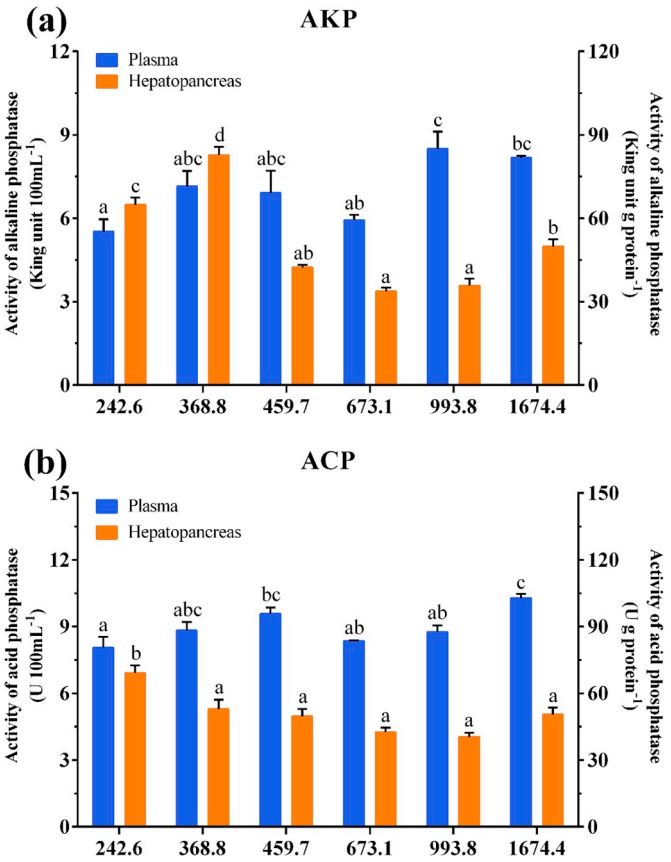


**Fig. 3.** Alkaline phosphatase (a) and acid phosphatase (b) activities in plasma and hepatopancreas for *L. vannamei* pretreated with different dietary inositol levels after 24 h ammonia stress. Results are mean ± SEM (n = 3). The column with different superscripts manifests significant differences ($P < 0.05$).

increased with increasing dietary inositol levels up to 993.8 mg kg$^{-1}$ diets ($P < 0.05$), and no difference was observed with a further increase of dietary inositol level ($P > 0.05$). However, no significant differences of T-SOD activity were found among all treatment groups ($P > 0.05$). GSH content significantly increased with increasing dietary inositol levels up to 993.8 mg kg$^{-1}$ diets ($P < 0.05$), and decreased thereafter ($P < 0.05$). Compared with the control group, MDA content substantially reduced in plasma, but significant decrease of MDA content were only found in shrimp fed 459.7 and 993.8 mg kg$^{-1}$ diets ($P < 0.05$). The dietary inositol requirement was estimated to be 673.5 mg kg$^{-1}$ diet based on GPX activity in plasma using broken-line analysis ($y = 1.1099x + 350.07$, $R^2 = 0.9791$; $y_{max} = 1097.63$) (Fig. 4a). The dietary inositol requirement was estimated to be 1207.5 mg kg$^{-1}$ diet based on GSH content in plasma using quadratic regression method ($y = -0.00006x^2 + 0.1449x - 27.322$, $R^2 = 0.7635$) (Fig. 4b).

**Table 3**
Changes in antioxidant-related parameters of plasma for *L. vannamei* pretreated with different dietary inositol levels after 24 h ammonia stress.

| Parameters | Dietary inositol levels (mg/kg diet) | | | | | |
|---|---|---|---|---|---|---|
| | 242.6 | 368.8 | 459.7 | 673.1 | 993.8 | 1674.4 |
| T-AOC | 1.97 ± 0.12$^{a}$ | 3.70 ± 0.20$^{b}$ | 3.58 ± 0.20$^{b}$ | 3.45 ± 0.09$^{b}$ | 4.19 ± 0.14$^{b}$ | 4.18 ± 0.36$^{b}$ |
| T-SOD | 84.51 ± 3.86 | 90.56 ± 4.18 | 83.78 ± 5.18 | 88.44 ± 1.92 | 82.07 ± 0.84 | 86.83 ± 2.74 |
| GPX | 605.8 ± 37.99$^{a}$ | 747.7 ± 18.42$^{ab}$ | 904.1 ± 31.72$^{bc}$ | 1078.4 ± 55.32$^{cd}$ | 1141.7 ± 37.3$^{d}$ | 1072.8 ± 34.2$^{cd}$ |
| GSH | 11.31 ± 1.19$^{a}$ | 13.69 ± 2.54$^{ab}$ | 27.56 ± 1.77$^{bc}$ | 25.89 ± 1.03$^{abc}$ | 69.94 ± 4.87$^{d}$ | 39.35 ± 5.19$^{c}$ |
| MDA | 2.42 ± 0.17$^{b}$ | 2.20 ± 0.03$^{ab}$ | 1.82 ± 0.13$^{a}$ | 2.01 ± 0.14$^{ab}$ | 1.81 ± 0.09$^{a}$ | 2.22 ± 0.04$^{ab}$ |

Results are mean ± SEM (n = 3). Values within the same line having different superscripts indicate significant differences ($P < 0.05$). T-AOC, total antioxidant capacity (U mL$^{-1}$); T-SOD, total superoxide dismutase (U mL$^{-1}$); GPX, glutathione peroxidase (U); GSH, reduced glutathione (μmol L$^{-1}$); MDA, malondialdehyde (nmol mL$^{-1}$).

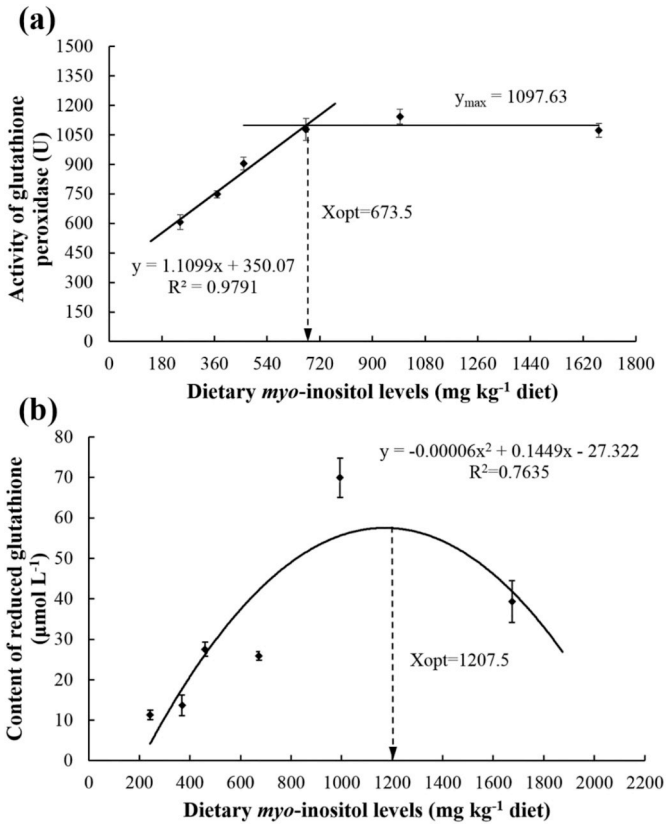


**Fig. 4.** Relationship between GPX (a) and GSH (b) of plasma after 24 h ammonia stress and dietary inositol levels based on quadratic regression method and two slope broken-line regression analysis, respectively.

### 3.5. Antioxidant-related parameters in the hepatopancreas of shrimp after ammonia stress

As showed in Fig. 5, T-AOC activities in the hepatopancreas of shrimp fed 368.8, 459.7 and 1674.4 mg kg$^{-1}$ diets significantly increased when compared with control group ($P < 0.05$). GST activity was the highest for shrimp fed 368.8 mg kg$^{-1}$ diet, and the lowest for shrimp fed control diet. In addition, GPX and Cu/ZnSOD activities substantially enhanced in hepatopancreas compared with the control group, but no obvious difference was observed between MI-supplemented and control groups ($P > 0.05$). MDA and PC showed a semblable trend (Fig. 6). MDA content was the highest for shrimp fed the control diet, and the lowest for shrimp fed 368.8 mg kg$^{-1}$ diet ($P < 0.05$). PC content was the highest for shrimp fed the control diet, and decreased with increasing dietary inositol levels up to 368.8 mg kg$^{-1}$ diet ($P < 0.05$), and no significant differences were present with increasing inositol levels from 368.8 to 1674.4 mg kg$^{-1}$ diet ($P > 0.05$).

### 3.6. Relative expression of immune-related and antioxidant-related genes in hepatopancreas of shrimp after ammonia stress

As showed in Fig. 7, after 24 h ammonia stress, the highest expression level of FT and Trx was found in the hepatopancreas of shrimp fed 993.8 and 1674.4 mg kg$^{-1}$ diet, respectively, and the lowest expression level of both genes was observed in control group. Relative expression of CAT for shrimp fed the control diet was lower than the MI-supplemented treatments ($P < 0.05$), except for that of shrimp fed 673.1 mg kg$^{-1}$ diet ($P > 0.05$). Shrimp fed control diet induced the highest AK mRNA expression, which was considerably higher than that of the other three treatments (459.7, 673.1, and 1674.4 mg kg$^{-1}$) ($P < 0.05$). The expression level of Hsp70 in shrimp fed the 368.8, 459.7 and 673.1 mg kg$^{-1}$ diets were significantly lower than those of the control ($P < 0.05$). Relative expression of Prx down-regulated significantly in the hepatopancreas of shrimp fed MI-supplemented diets when compared with shrimp fed control diet ($P < 0.05$).

## 4. Discussion

Ammonia may cause physical stress in fish [41]. Survival rate of aquatic animals reduced with increasing levels of ammonia and exposure time, and diverse aquatic species exhibited remarkable ammonia tolerance [42–44]. Elevated resistance against ammonia stress was found in shrimp treated with immunostimulants, such as astaxanthin and mannan oligosaccharide [11,45]. The present study showed that the survival rate of shrimp fed MI-supplemented groups was higher than that of the control group after acute ammonia challenge, suggesting that *L. vannamei* pretreated with higher dietary inositol levels may have higher resistance to ammonia challenge.

AKP and ACP, involved in various metabolic processes, have been identified as indicators to reflect health status and immune state of aquatic animals [46–48]. Fish and shrimp treated with immunostimulants (including vitamin C, α-tocopherol, polysaccharides and soybean isoflavones) often have higher AKP and ACP activities in serum compared with untreated group [49–52]. However, reduction of AKP activity in serum due to exposure to pH stress has been observed in *Megalobrama amblycephala* [53]. After exposed to acute ammonia stress, AKP and ACP activities of MI-supplemented groups generally increased in the plasma when compared with control group, which is similar with the research conducted by Diao et al. [32]. On the contrary, MI supplementation reduced activities of AKP and ACP in hepatopancreas. AKP is also a vital marker to reflect liver function of fish [54]. The decrease in AKP and ACP activities may be due to the involvement of inositol in reducing the deleterious effects caused by ammonia stress in hepatopancreas. Further studies are required to decipher the mechanism behind the results.

AK plays a major role in cellular energy metabolism in invertebrates [55,56], which can mediate the synthesis and breakdown of phosphagen [57]. Exposure to stress response, including ammonia stress,

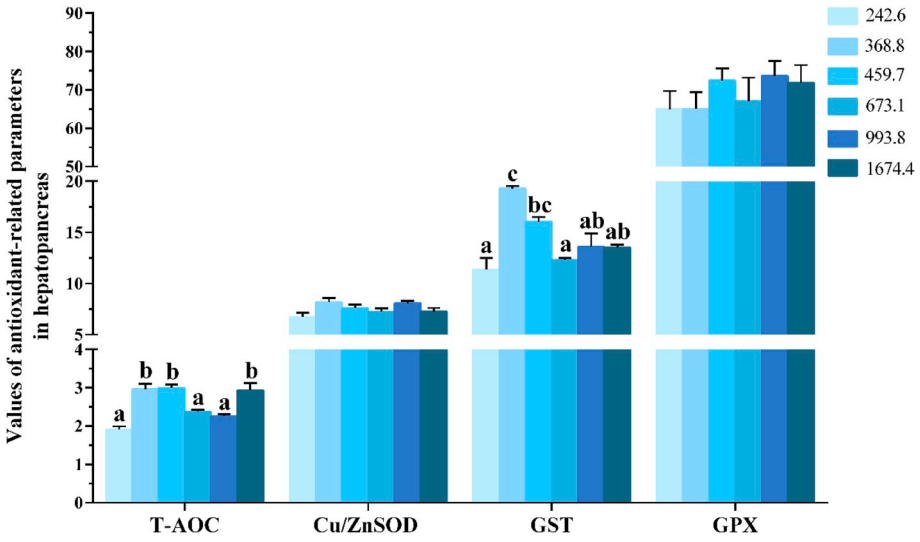


**Fig. 5.** Activities of T-AOC, GPX, GST and Cu/ZnSOD in hepatopancreas of *L. vannamei* pretreated with different dietary inositol levels after 24 h ammonia stress. Results are presented as the mean ± SEM (n = 3). The column with different letters indicate significant differences ($P < 0.05$). T-AOC, total antioxidant capacity (U mg protein$^{-1}$); GPX, glutathione peroxidase (U); GST, glutathione S-transferase (U mg protein$^{-1}$); Cu/ZnSOD, copper/zinc superoxide dismutase (U mg protein$^{-1}$).

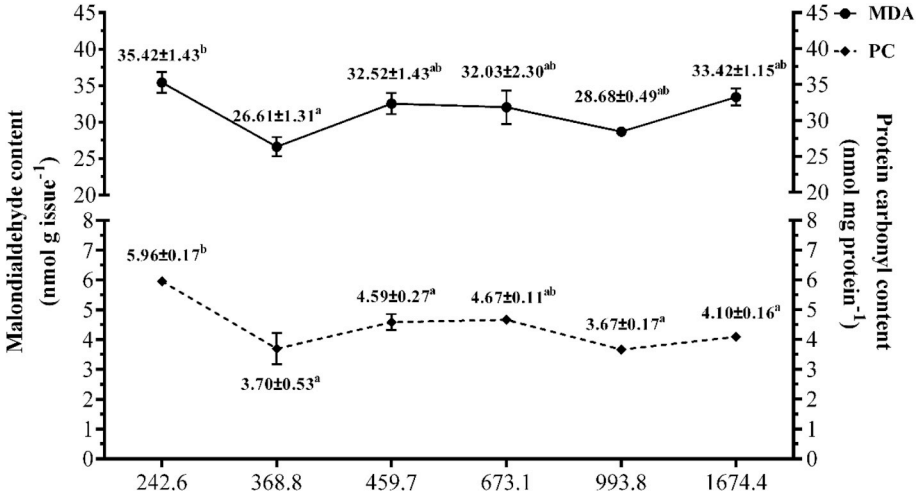


**Fig. 6.** MDA and PC contents in hepatopancreas of *L. vannamei* pretreated with different dietary inositol levels after 24 h ammonia stress. Results are presented as the mean ± SEM (n = 3). Values within the same line holding different letters indicate significant differences ($P < 0.05$). MDA, malondialdehyde (nmol g issue$^{-1}$); PC, protein carbonyl (nmol mg protein$^{-1}$).

can result in an elevated energy demand for fish [58–60]. Compared to control group, down-regulation of AK due to MI supplementation, was found in *L.vannamei* after ammonia stress in the present study. Previous studies showed that AK was up-regulated in shrimp after viral infection or environmental changes [61,62], indicating that expression of AK was closely connected with the immune response of shrimp [63]. Hsp70, as a dominant form of heat shock proteins [64], has been proven to participate in the response against the nutrient deprivation, osmotic stress, thermal stress, ammonia stress, nitrite stress, oxidant injury, heavy metal pollution and bacterial infection in aquatic animals [65–68]. When organisms are subjected to stress conditions mentioned above, Hsp70 expression increase significantly [69,70]. However, only few studies have investigated the relationship between Hsp70 expression and ammonia stress in *L. vannamei*. In a previous study of Sinha et al. [71], common carp could deal with the proteotoxicity induced by high environmental ammonia due to enhancement of Hsp70 expression. Hsp70 may adjust stress tolerance via stopping protein denaturation, refolding damaged proteins or ensuring degradation of irreversibly damaged proteins [72]. In this study, administration of MI reduced the expression levels of Hsp70 in hepatopancreas which is the principal immune site in crustaceans [73]. This finding did not agree with the research of Duan et al. [74] who found that Hsp70 expression in intestine of *L. vannamei* treated with *Clostridium butyricum* increased significantly under ammonia stress (ammonia 35 mg $L^{-1}$). Previous studies reported that dietary MI could improve non-specific immune response and antioxidant status in Jian carp *Cyprinus carpio* [35,75]. Down-regulation of Hsp70 expression may be linked to fact that MI supplementation could mitigate the damage caused by ammonia stress. In addition, the chaperone activity of Hsp70 is mostly dependent on its ability to bind and hydrolyze ATP [76]. Changes in AK expression, related to energy metabolism [56], may affect Hsp70 expression. The lower mRNA expression of AK and Hsp70 in *L.vannamei* could be speculated that MI may be beneficial for immune response of shrimp against ammonia stress. However, further researches are still required to elucidate above phenomenon.

A balance is located between the production of ROS and the antioxidant defense system under normal situations [77]. The changes of environmental factors, such as salinity, temperature, oxygen level, ammonia, nitrite and pH, could disturb the dynamic equilibrium and induce the excessive production or accumulation of ROS, leading to oxidative stress [78–81]. Cheng et al. [23] reported that significant increase of ROS was observed in pufferfish *Takifugu obscurus* after exposed to ammonia. To counteract the deleterious effects caused by excessive ROS, aquatic animals employ the antioxidant system to protect cells against oxidative stress which can damage DNA, proteins and lipids [82]. Improving the levels of antioxidant enzymes, such as SOD, GPX, CAT and glutathione reductase (GR), is considered a major defense mechanism for removing the ROS [83]. Exposure to ammonia could induce alterations in antioxidant enzyme levels [84]. T-AOC is recognized as a vital indicator of the antioxidant enzyme system for evaluating antioxidant status [11]. SOD, including Cu/ZnSOD, catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide

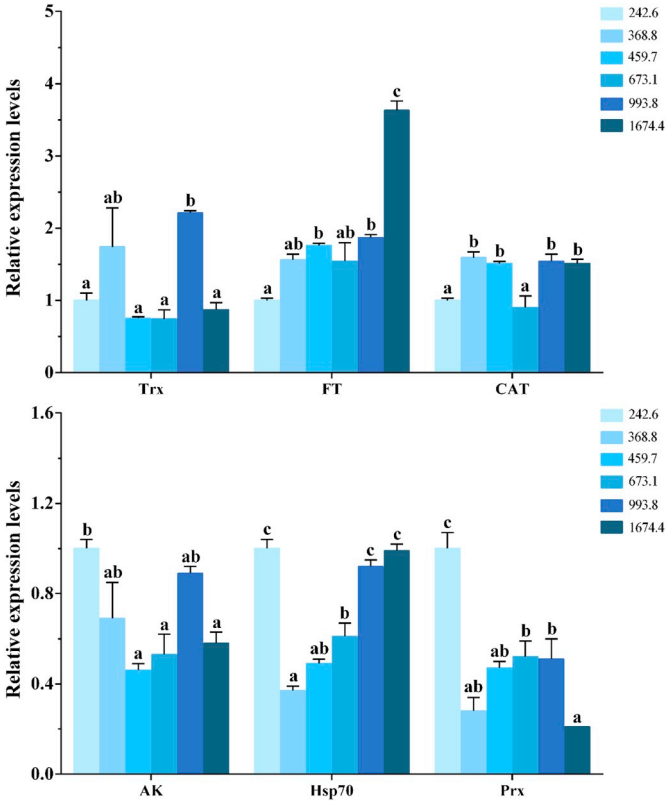


**Fig. 7.** Relative expression of immune-related and antioxidant-related genes in hepatopancreas of *L. vannamei* pretreated with different dietary inositol levels after 24 h ammonia stress. Results are mean ± SEM (n = 3). The column with different superscripts manifests significant differences ($P < 0.05$). Trx, thioredoxin; FT, ferritin; CAT, catalase; AK, arginine kinase; Hsp70, heat shock protein 70; Prx, peroxiredoxin.

($H_2O_2$), and $H_2O_2$ can be decomposed into oxygen and water via GPX which can use GSH as an electron donor to catalyze this reaction [85,86]. In this study, changes in GPX activity and GSH content among all groups showed a similar variation tendency, demonstrating that there is a close relationship between them. GST is also an antioxidant enzyme related to the conjugation and elimination of xenobiotics [87]. In addition, GSH is a non-enzymatic antioxidant which could directly eliminate ROS under oxidative stress [88]. Therefore, mentioned antioxidant parameters were determined to evaluate the antioxidant status of *L. vannamei* in this study. In the experiments reported by Sun et al. [89] and Li et al. [59], fish exposed to low ambient ammonia had higher SOD activity than those exposed to elevated ambient ammonia. The activity of antioxidant enzyme can be impaired under higher concentrations of ammonia [90]. Oppositely, MI numerically increased Cu/Zn SOD activity of hepatopancreas in this study, although no statistically prominent difference between control group and MI-supplemented groups could be established. Compared with control group, after faced with ammonia stress, content of GSH and activities of T-AOC, GPX and GST in plasma or hepatopancreas increased significantly. MDA can be used as a sensitive index of oxidative injury in organisms [91,92]. A higher level of MDA may result in higher toxicity to organisms [59]. Liang et al. [90] reported that elevated MDA concentration was detected in *L. vannamei* after exposed to high ambient ammonia. In the present study, lower MDA concentration was found in MI-supplemented groups than the control group, suggesting that MI could reduce the impact of lipid peroxidation on shrimp, by evaluating the MDA concentration. Lipid peroxidation may cause protein damage by its end products [93], while the ROS can also directly impair the protein [94]. PC is utilized as an indicator of oxidative damage to proteins [34]. PC content in hepatopancreas of shrimp was depressed by MI supplementation, which showed the parallel variation trend with MDA. Overall, elevated survival rate after acute ammonia stress may be related to increasing the activities of above antioxidant enzymes and decreasing the lipid peroxidation and protein oxidation.

Genes FT, Trx, CAT and Prx, have been reported to be involved in the antioxidant system [95–99]. Some ecological factors, such as the pH, nitrite stress and temperature, may induce oxidative stress, while activating the expression of antioxidant genes mRNA [97,100,101]. FT could inhibit the generation of ROS derived from Fenton reaction to mitigate damage induced by excessive iron [102]. Trx and Prx have been shown to play a vital role in scavenging ROS [100,103]. In the present study, the up-regulation in expression of FT, Trx and CAT genes and down-regulation of Prx were found in shrimp when exposed to ammonia challenge. Prx is known to remove hydroperoxide [97], and the same function is also present in CAT and GPX [104]. Hu et al. [105] and Jiang et al. [35] demonstrated that MI could improve the scavenging capacity of the hydroxyl radical (OH•). MI also could prevent Jian carp *Cyprinus carpio* against Cu-induced oxidative stress via mediating antioxidant system (including CAT and GPX) [106]. Decreased expression of Prx in MI-supplemented groups may be related to the main role of CAT and GPX in removing ROS.

In summary, the study indicated that *L. vannamei* pretreated with higher dietary inositol levels had better resistance against ammonia stress. MI supplementation decreased the levels of lipid peroxidation and protein oxidation and enhanced activities of antioxidant enzymes after acute ammonia stress. Based on survival rate, immune-related and antioxidant-related indexes, the concentration of 459.7–993.8 mg inositol $kg^{-1}$ in practical diet are recommended when *L. vannamei* are exposed to high ammonia stress. Our findings suggested that increasing dietary inositol levels may be an effective way to assist shrimp overcome environmental stresses. Further researches are required to clarify mechanism of action of inositol.

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