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Effect of Ammonia Exposure on the Non-Specific Immunity of Fresh Water Pearl Mussel *Hyriopsis cumingii*

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Keywords: *Hyriopsis cumingii*; pearl mussel; ammonia-N; immune function; lysozyme activity

Abstract

Ammonia-N, the principal end-product of protein catabolism, is an important environmental toxic factor in ponds. Elevated environmental ammonia-N is very toxic to aquatic animals and has a deleterious effect on their immune system. However, its effect on their immune system remains unclear. In this study, the effects of ammonia-N (0, 5, 10 and 30 mg/L) on immune responses of Fresh Water Pearl Mussel *Hyriopsis cumingii* were determined. When exposed to 5 mg/L ammonia-N lysozyme activity increased significantly. Superoxide dismutase activity was highest in the 5 mg/L group followed by the 10 mg/L group. The marked decrease of Lysozyme activity of Fresh Water Pearl Mussel *Hyriopsis cumingii* in 10 mg/L and 30 mg/L ammonia-N groups suggests that the higher concentrations of ammonia-N reduces or inhibits their non-specific immunity. Compared to the control group, superoxide dismutase activity in 30 mg/L ammonia also decreased significantly. When exposed to 5mg/L ammonia-N, catalase, acid phosphatase and alkaline phosphatase activity enhanced. In the 10 mg/L and 30 mg/L group, ammonia-N decreased significantly The lysozyme, catalase, acid phosphatase and alkaline phosphatase activity showed a similar tendency. This suggests that in *H. cumingii* immunostimulatory response is enhanced at low ammonia-N concentrations. The results of this study provide a theoretical basis for disease prevention in the freshwater pearl mussel.

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Introduction

The bivalve mollusk *Hyriopsis cumingii* is the main freshwater pearl mussel species in China (Huang et al., 2016). Pearls produced by *H. cumingii* are smooth, bright in color, round in shape, and have high economic value (Wang et al., 2019). China is the largest producer of pearls in the world and more than 80% of these are obtained from *H. cumingii* (Bai et al., 2014). Like most invertebrates, *H. cumingii* lack a typical adaptive immune system and their ability to fend off pathogens relies mainly on humoral immune factors in the hemolymph, including a variety of enzymes, antibacterial peptides, opsonins, lectins, and complement components (Iwanaga and Lee, 2005).

The level of waterborne ammonia in natural waters and in culture systems has harmful effects on aquatic animals (Lemarie et al., 2004). Ammonia is produced as a metabolic waste product of protein catabolism and/or generated from the decomposition of organic material, industrial emission or produced by micro-organisms (Diricx et al., 2013). High environmental ammonia (HEA) not only hinders ammonia excretion in fish but also causes an uptake of ammonia from the environment (Diricx et al., 2013). Hence, during HEA, fish are confronted simultaneously with accumulation of endogenous ammonia and uptake of exogenous ammonia, causing adverse effects on their performance and welfare (Eddy, 2005; Randall and Tsui, 2002). Waterborne ammonia exists in two forms, as unionized ammonia (NH_3) and as an ionized form (NH_4^+) (Randall and Tsui, 2002). The sum of NH_3 and NH_4^+ comprises the total ammonia concentration. Throughout this paper, the term "ammonia-N" is used to refer to total NH_3 + NH_4^+, whereas these chemical symbols refer to the individual components of ammonia gas (NH_3) and ammonium ion (NH_4^+).

The health of aquatic animals is dependent on the complex interactions between the environment, pathogens, and the host (Dang et al., 2012). Ammonia is one of the major environmental pollutants in fish culture especially in recirculation systems (Cheng et al., 2015). It has been reported that the concentration of ammonia increases directly with culture period and might reach levels as high as 46 mg/L in intensive aquaculture systems (Chen et al., 1988). Elevated ammonia in aquaculture systems can be taken up through the gills by diffusion and cause very high concentrations in the body fluids (Eddy, 2005). Additionally, elevated concentration of ammonia in pond water can cause fish growth reduction, tissue erosion and degeneration, immune suppression, and high mortality (Benli et al., 2008; Lia et al., 2014). Many previous studies have demonstrated that suboptimal environmental conditions, such as elevated environment ammonia-N, decreases the immunity of some crabs and fish (Yue et al., 2010, Xia et al., 2018). However, the effect of ammonia-N on the immune system of bivalve mollusks remains unclear. This study aimed at determining the effect of ammonia exposure on the immune parameters of *Hyriopsis cumingii*.

Materials and Methods

Experimental layout

H. cumingii mussels, averaging 50 ± 2.5 g in body weight, were collected from Hanshou pearl breeding base. Before the beginning of the experiments, the mussels were acclimatized and quarantined in aerated freshwater plastic tanks kept at 26 ± 2°C for two weeks. The experiment was approved by the guidelines of Institutional Animal Care and Use Committees (IACUC) of Hunan University of Arts and Science, Changde, China.

Experimental design

The tested ammonia-N concentrations were: 0, 5, 10, and 30mg/L. The test solutions were prepared by dissolving ammonium chloride (NH_4Cl) in de-chlorinated tap water. Prior to the experiment, a total of 360 mussels were allocated into four groups and randomly stocked in 12 glass tanks ($0.95 \times 0.6 \times 0.5$ m^3). There were 30 individuals in each tank and three repetitions for each group.

During the experiment, ammonia-N concentration was measured every 12 h using an ammonia nitrogen detection kit purchased from Nanjing Jian Cheng Bioengineering Institute (China). Approximately one-third of the water was replaced every day and ammonium chloride solution was subsequently added to maintain a relatively constant concentration of ammonia-N. pH, water temperature, and dissolved oxygen were

controlled at levels of 7.5±0.2, 26 ± 2°C and 6.0±0.2 mg/L, respectively. The mussels were kept under a constant photoperiod (12 h light: 12 h darkness).

The experiment was conducted for 30 d. Sampling for various immunological parameters was carried out on the 30th day. 9 individuals were selected randomly from each group, three from each replicate. The livers of the mussels were collected and weighed, and 2 ml of normal saline was added. The homogenated tissues were then centrifuged 10 minutes at 4000 r/min. The supernatant was stored at -20°C for catalase (CAT), alkaline phosphatase (AKP), acid phosphatase (ACP), superoxide dismutase (SOD), and lysozyme activity tests.

Lysozyme activity

Lysozyme activity was measured using the turbidity assay. Lysozyme standard product powder (80,000 U/mg) was used as a standard, and 1 mg lyophilized micrococcus lysodeikticus with sodium phosphate buffer (pH 5.75) was used as substrate. 20 µL plasma sample was added to 2 mL of the substrate, and the reduction in the transmittance at 530 nm was determined after 20 s and 8 min incubation. One unit of lysozyme activity was defined as an increase in transmittance of 0.001 per min.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical-dependent reactions using the Ransod Kit (Randox, Crumlin, UK). A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units m/L.

Catalase, Alkaline phosphatase, and Acid phosphatase activity

In the experiments, we measured catalase (CAT), alkaline phosphatase (AKP), and acid phosphatase activity (ACP) of the liver. These were determined using the Diagnostic Reagent Kits purchased from Nanjing Jian Cheng Bioengineering Institute (China).

Statistical analysis

Data are presented as mean value ± standard error (SE); mean values of treatments were compared using the one-way analysis of variance by Duncan's test of STATISTICA software package (Version 6.0, Statsoft, Inc.). Differences between the control and the treatment groups were considered statistically significant at $P < 0.05$.

Results

Lysozyme activity

After the ammonia challenge, the lysozyme activity of tested mussels is shown in Fig. 1. Lysozyme activity of *H. cumingii* in the control group was 3.28 U/ml, 4.13 U/ml in 5mg/L ammonia-N group, 2.40 U/ml in 10 mg/L ammonia-N group and 2.25 U/ml in 30 mg/L ammonia-N group. *H. cumingii* when exposed to 5 mg/L ammonia-N had increased significantly the lysozyme activity but decreased significantly in 10 mg/L and 30 mg/L (Fig. 1).

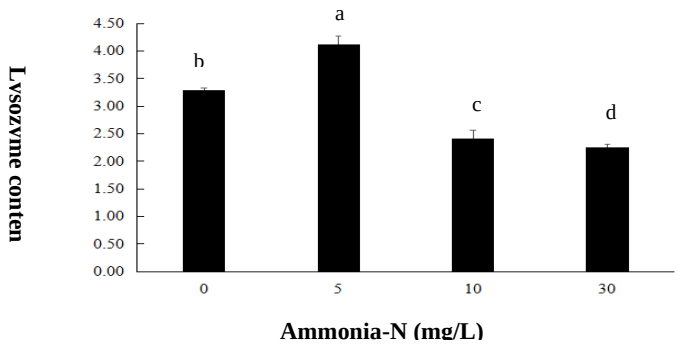

Fig. 1. Lysozyme activity of *H. cumingii* when exposed to ammonia-N (0, 5, 10 and 30 mg/L). Data are presented as mean ± SE (n =9). Differences were determined by one-way analysis of variance (ANOVA). Different letters above bars represented significant difference at the levels of $p<0.05$, and same letters above bars indicated no significant difference.

Superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) activity of *H. cumingii,* when exposed to ammonia is shown in Fig. 2. Superoxide dismutase activity was 0.78 U/ml in the 0mg/L group, 3.00 U/ml in the 5mg/L group, 1.05 U/ml in the 10 mg/L group and 0.64 U/ml in the 30 mg/L group. Compared with control group, this activity in the 30 mg/L group decreased significantly (Fig. 2).

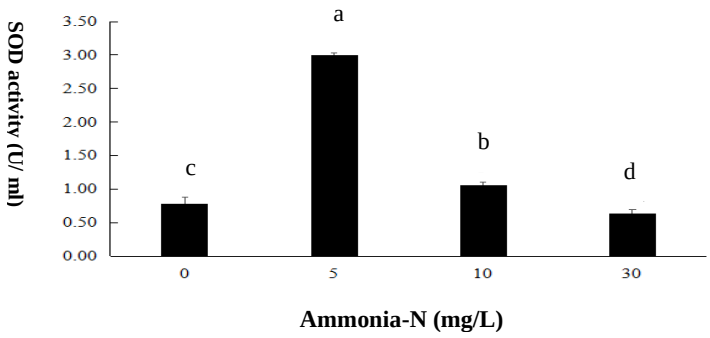

Fig. 2. Superoxide dismutase (SOD) activity of *H. cumingii* when exposed to ammonia-N (0, 5, 10 and 30 mg/L). Data are presented as mean ± SE (n =9). Differences were determined by one-way analysis of variance (ANOVA). Different letters above bars represented significant difference at the levels of $p<0.05$, and same letters above bars indicated no significant difference.

Catalase (CAT) activity

The catalase (CAT) activity of *H. cumingii* when exposed to ammonia-N is shown in Fig. 3.In the control group it was 31.96 U/ml, in the 5 mg/L group it was 37.70 U/ml followed by 20.42 U/ml in the 10 mg/L group and 4.74 U/ml in the 30 mg/L group. When exposed to 5 mg/L (Fig. 3). Catalase activity in the 10 mg/L and 30 mg/L groups decreased significantly (Fig. 3).

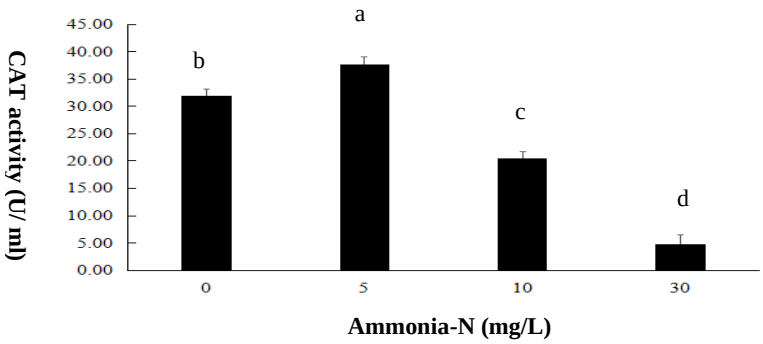

Fig. 3. Catalase activity (CAT) of *H. cumingii* when exposed to ammonia-N (0, 5, 10 and 30 mg/L). Data are presented as mean ± SE (n =9). Differences were determined by one-way analysis of variance (ANOVA). Different letters above bars represented significant difference at the levels of $p<0.05$, and same letters above bars indicated no significant difference.

Acid phosphatase (ACP) activity

The acid phosphatase (ACP) activity after ammonia-N exposure is shown in Fig. 4. In the control group it was 0.07 U/ml, 0.04 U/ml in the 0.10 U/ml group, and 0.03 U/ml in the 30 mg/L. When exposed to 5 mg/L ammonia-Nthe acid phosphatase activity enhanced significantly (Fig. 4) but decreased significantly in the 10 mg/L and 30 mg/L groups (Fig. 4).

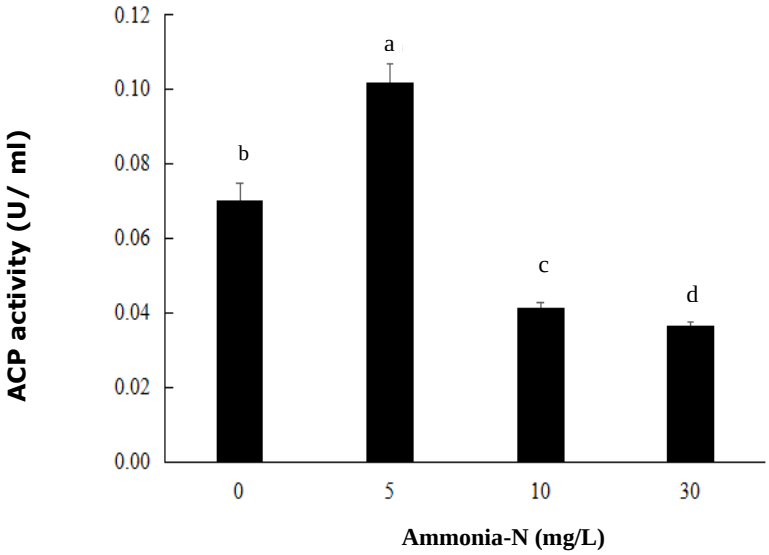

Fig. 4. Acid phosphatase activity (ACP) of *H. cumingii* when exposed to ammonia-N (0, 5, 10 and 30 mg/L). Data are presented as mean ± SE (n =9). Differences were determined by one-way analysis of variance (ANOVA). Different letters above bars represented significant difference at the levels of $p<0.05$, and same letters above bars indicated no significant difference.

Alkaline phosphatase (AKP) activity

The alkaline phosphatase (AKP) activity after ammonia-N exposure is shown in Fig. 5. In the control group it was 0.14 U/ml, 0.19 U/ml in the 5mg/L group, 0.03 U/ml in the 10 mg/L group and 0.03 U/ml in 30 mg/L ammonia-N group. *H. cumingii* when exposed to 5 mg/L ammonia-N increased significantly alkaline phosphatase activity (Fig. 5). Alkaline phosphatase activity of *H. cumingii* in 10 mg/L and 30 mg/L ammonia-N had decreased significantly (Fig. 5).

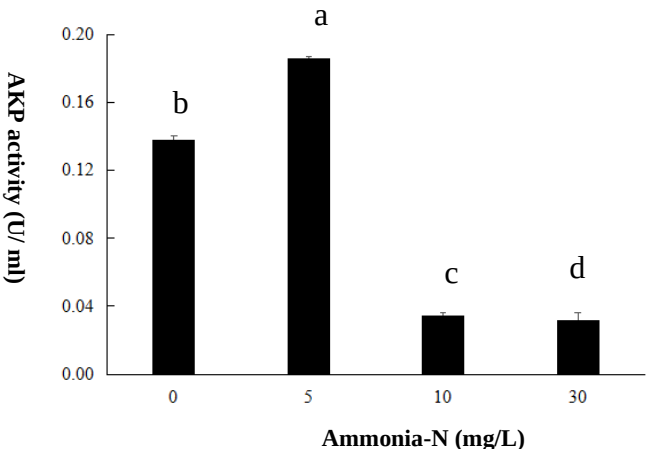

Fig. 5. Alkaline phosphatase activity (AKP) of *H. cumingii* when exposed to ammonia-N (0, 5, 10 and 30 mg/L). Data are presented as mean ± SE (n =9). Differences were determined by one-way analysis of variance (ANOVA). Different letters above bars represented significant difference at the levels of $p<0.05$, and same letters above bars indicated no significant difference.

Discussion

The present study determined, for the first time, the effect of ammonia exposure on the non-specific immunity of *H. cumingii*. The activities of lysozyme, superoxide dismutase, catalase, acid phosphatase, and alkaline phosphatase were assessed after exposure of *H. cumingii* to various ammonia-N concentrations in water after 30 days. The

activities of these five enzymes increased at the lowest ammonia-N concentration tested (5 mg/ml) and decreased at higher ammonia-N concentrations (10 mg/ml and 30 mg/ml). These results suggest that high ammonia levels could influence the expression or activity of key enzymes associated with non-specific immunity functions in *H. cumingii*.

Lysozyme is an antimicrobial enzyme and an important humoral factor that enhances non-specific immunity in animals. Lysozyme activity can be enhanced at relatively low concentrations of pollutants (Low and Sin, 1996). The lysozyme serum activity significantly increased in the low dosage in the MC groups. In the present study when exposed to 5 mg/L ammonia-N it increased significantly due to the enhanced release of lysozyme from the phagocytes that were activated in damaged immune organs (Qiao et al., 2013). However, in the high dose exposure group, the effect of immunotoxicity exceeded the regulation response of the fish, thus weakening their ability to synthesize lysozyme (Kong et al., 2011). The marked decrease of the Lysozyme activity in the 10 mg/L and 30 mg/L ammonia-N the high dosage groups reduced or inhibited its non-specific immunity function.

Superoxide dismutase (SOD) is an enzyme that catalyzes the conversion of the superoxide (O_2^-) radical into oxygen and hydrogen peroxide. Superoxide and hydrogen peroxide play important microbicidal roles in non-specific disease resistance (Song and Hsieh, 1994; Downs et al., 2001). As such, SOD is an important antioxidant defense. Several other studies have observed ammonia exert an influence on superoxide anion production or SOD activity in aquatic animals. The production of superoxide anion in the freshwater prawn (*Macrobrachium rosenbergii*) and whiteleg shrimp (*Litopenaeus vannamei*) was stimulated following exposure of the animals to ammonia-N (Cheng and Chen, 2002; Liu and Chen, 2004). In studies that examined bighead carp (*Hypophthalmichthys nobilis*) larvae, Chinese mitten crab (*Eriocheir sinensis*), and submerged *Vallisneria natans*, low NH_3-N concentrations were shown to elevate SOD activity, whereas high NH_3-N levels caused reduced SOD activity (Sun et al., 2012; Hong et al., 2007; Wang et al., 2008). SOD activity in the liver and white muscle of Nile tilapia (*Oreochromis niloticus*) exposed to ammonia-N was significantly increased, which was proposed to manage the elevated formation of reactive oxygen species (ROS) (Hegazi et al., 2010). In the present study, superoxide dismutase activity of *H. cumingii* in 5 mg/L and 10 mg/L ammonia-N increased significantly while decreased significantly at 30 mg/L ammonia-N, which is in line with the results found in previous studies (Sun et al., 2012; Hong et al., 2007; Wang et al., 2008). It indicates that a lower dose of ammonia-N could induce organs to produce increased amounts of ROS, and SOD activity was elevated to deal with excessive ROS (Hegazi et al., 2010); however, under higher doses of ammonia-N, SOD showed lower activity, possibly due to the inability of SOD to overcome extremely high levels of ROS, and excessive ROS could in turn inactivate SOD activity (Bagnyukova et al., 2006).

Catalase breaks down the harmful by-product hydrogen peroxide into water and oxygen and is a key enzyme that protects cells from oxidative damage by ROS (Pandey et al., 2003). A similar result to the present study was observed in bighead carp, where catalase activity increased significantly when exposed to 0.06 mg/L NH_3-N, and decreased when exposed to 0.264 mg/L NH_3-N (Sun et al., 2012).

Increased phosphatase activity indicated a higher breakdown of the energy reserve, which was utilized for fish growth and survival (Sahu et al., 2008). Various effects have been observed to influence phosphatase activities in fish. Increased acid and alkaline phosphatase activities were observed following *Aeromonas hydrophilia* infection of blunt snout bream (*Megalobrama amblycephala*) and climbing perch (*Anabas testudineus*) (Xia et al., 2017, Das et al., 2009). Another study showed that alkaline phosphatase activity was increased in *Labeo rohita* fish that were fed turmeric (Sahu et al., 2008).

The findings of this study indicated that high ammonia exposure caused significant reductions in the activity or production of enzymes participating in the non-specific immune response of *H. cumingii*. Further research into the mechanisms of these interactions will help provide a better understanding of the effects that ammonia has on the health and disease prevention of *H. cumingii*, and possibility other aquatic animals.

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The authors declare no conflict of interests.

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