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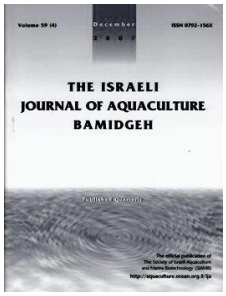

Effect of Acute Ammonia Stress on Antioxidant Enzymes and Digestive Enzymes in Barramundi *Lates calcarifer* Larvae

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Keywords: *Lates calcarifer*; larval fish; ammonia stress; antioxidant enzyme; digestive enzyme;

Abstract

The acute toxicity of ammonia to barramundi *Lates calcarifer* larvae (15 days post-hatching, DPH) was studied with a semi-static bioassay method to understand the physical response of fish larvae to ammonia stress. In this study, 15 and 20 mg/L ammonia were tested, and 0 mg/L ammonia (control) was used as control. The activity of antioxidant enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), acid phosphatase (ACP), glutathione peroxidase (GSH-Px), and digestive enzymes lipase (LPS), amylase (AMS), trypsin (TRYP) were used as biochemical indicators. With the increase of residue time, the SOD activity in barramundi was elevated at 15 mg/L ammonia and decreased at 20 mg/L ammonia. CAT activity was lower at 20 mg/L ammonia. GSH- PX activity was elevated at the low ammonia concentration and decreased at the high ammonia concentration. ACP activity was elevated after exposure to ammonia. LPS and AMS activities in the control were significantly higher than those of the other groups (P<0.05). TRYP activity was elevated after exposure to ammonia. The results indicate that acute ammonia stress significantly affects antioxidant capacity and digestive capacity, and barramundi exhibited a certain tolerance of ammonia in a short time (<6h). Results from the present study will improve our understanding of the physical response of fish to ammonia and may guide hatchery practice for *Lates calcarifer*.

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Introduction

Ammonia is the main end-product of protein metabolism in fish and is excreted via the skin (Handy and Poxton, 1993) and gills (Smith, 1929; Wood, 1958). In water, total ammonia in water exists mainly in two forms, NH_4^+ (the ammonium ion), and NH_3 (un-ionized ammonia). There is common agreement that NH_3 is more toxic than NH_4^+, as it readily diffuses through the epithelial membranes of aquatic animals (Le-Ruyet et al., 1997; Soderberg et al., 1984). Ammonia may exist in the toxic form in both semi-closed and closed intensive culture system and build up during live fish transportation (Fivelstad et al.1993). Rapid increase in ammonia may induce acute toxicity, as ammonia is toxic to aquatic organisms and can influence the central nervous system metabolism and cause damage to the kidney, gills, liver, thyroid, and spleen in fish (Colt and Armstrong, 1981). Much has been published relating to the acute effects of ammonia on fish and how environmental parameters such as pH, temperature, and salinity affect the concentration of NH_3 (Edison and Sonia, 2012; Xu et al., 2004; Hickey and Martin, 1999), but little is known about the physiological and biochemical responses of fish to ammonia stress.

To cope with oxidative stress, fish have developed antioxidant defense systems (Martinez-Alvarez et al., 2005). Antioxidant defense systems include a series of chemical systems to scavenge free radicals such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) (Li et al., 2011; Lyu et al., 2013; Ge et al. 2012). Digestive enzymes activity is expected to change due to external factors such as ammonia, pH and temperature that influence metabolic functions (Hochachka and Somero, 2002; Xu et al., 2004). Recent research has shown that ammonia stress can affect the activity of digestive enzymes in jade perch *Scortum barcoo* (Zhang et al., 2011) and swimming crab *Portunus trituberculatus* (Xu et al., 2011). These enzymes play an important role in the resistance of ammonia toxicity in fish, which can counteract stress via regulation of protein activity or abundance (Zhang et al., 2011; Xu et al., 2011).

Asian sea bass or barramundi *Lates calcarifer* (Bloch) is a euryhaline teleost found in tropical and subtropical estuarine areas and native to the Indo-Pacific region. This is a highly valued commercial and recreational species both for its flesh taste and rapid growth and one of the most important fish species in the Asia–Pacific region (Chou and Lee, 1997) for both saltwater and freshwater aquaculture. Aquaculture technology for this species is well established in cages, ponds, and tanks in Indonesia, Australia, Malaysia, Philippines, Thailand and Taiwan (Tian and Qin, 2003). Although considerable research has been done on this fish to improve production efficiency, little is known on the influence of ammonia concentration on antioxidant enzymes and digestive enzymes in barramundi larvae. Only one available publication on the acute effect of ammonia on juvenile barramundi was found in a literature search (Økelsrud and Richard, 2007), and there is no information regarding the acute toxicity of ammonia to barramundi larvae.

Successful culture of fish larvae is critical. The primary objective of this work was to examine the response of barramundi larvae to ambient ammonia and the change of antioxidant enzymes and digestive enzymes. This study is unique in that a series of biochemical indicators were developed to detect ammonia stress on fish before symptoms occurred at the entire organism level. Results from the present study provide a scientific basis for the management of aquaculture water environment during larval fish aquaculture.

Materials and Methods

Fertilized eggs from the same batch of barramundi broodstock were obtained from the Tropical Fisheries Research and Development Center, Lingshui Town. All eggs were stocked into 500 L fiberglass incubators. On 3 days post hatch (DPH), fish larvae were transferred to 1000 L fiberglass tanks. The water temperature was maintained at 29.0± 1.0°C; PH was 8.0-8.2 and the salinity was 33 ± 0.8‰ throughout the experiment. The light regime was set to 14/10-h light/dark, and light intensity was maintained at 2000 lux. In each tank, two air stones were used to maintain dissolved oxygen >6.5 mg/L. The nitrite content was <0.03 mg/L. Water was changed twice a day at a daily exchange rate of 20% of the tank volume during the experiment.

Effect of acute ammonia stress on antioxidant enzymes and digestive enzymes in barramundi Lates calcarifer larvae

Rotifers (*Brachionus roundiformis*) at a density of 10-20 nauplii/mL were fed to the larvae from 2 DPH-9 DPH (4.50±0.10 mm). *Artemia nauplii* were first introduced at 20-30 nauplii/mL on 7 DPH. *Artemia nauplii* and rotifers were enriched with DHA Protein Selco (INVE Aquaculture, Salt Lake City, UT, USA) following the manufacturer's instruction before they were added into the larval rearing tanks. Instant microalgal paste (*Nannochloropsis* sp. Qingdao Hongbang Biotechnology Co. Ltd) was also added into larval fish tanks to create a green-water background.

The initial exploration of the ammonia range was conducted before the formal test to determine the level of ammonia concentrations. Ammonium chloride (NH_4Cl) crystals were used to prepare solutions with different concentrations. The behavior and survival conditions of larval barramundi were observed and recorded. The larvae were all dead in 96 hours (LC_{100}, 96h) at 20 mg/L, therefore this was chosen as the upper ammonia level in the test.

The acute effect of ammonia on larval barramundi was investigated in a semi-static 96 h experiment at pH 8.1 and temperature 29°C. All fish larvae were from the same batch. During the experimental period, no food was provided to the test fish to reduce the buildup of metabolic ammonia. The experiment consisted of three nonionized ammonia concentrations at 0, 15 and 20 mg/L. The experiment was conducted in 50 L aerated glass tanks. Each treatment had three replicates with 120 fish larvae each. Five fish were collected from every tank at 0, 6, 12, 24, 36, 48, 72, and 96 h. Water samples were collected from each tank every 3 h to adjust to the required ammonia concentrations (Science and Technology Department of the National Environmental Protection Bureau,1995).

Observations of abnormal behaviors of all treatments such as unusual swimming behavior, respiration rhythm, and any signs of abnormal organ changes were recorded. Fish were considered dead when the gills stopped moving for more than 15 sec.

Fish samples were anesthetized with an overdose of tricaine methanesulfonate (MS-222, Sigma, USA), and were thoroughly rinsed with distilled water to remove external salt and then immediately stored into liquid nitrogen. The whole frozen fish were thawed, weighed and homogenized for enzymatic assays, using a glass homogenizer on ice in 0.2 M NaCl (w/v) (Gawlicka et al., 2000). The homogenate was centrifuged at 13 000 g for 10 min at 2°C. Then, the aqueous supernatant was collected and incubated in the enzyme substrate at 25 °C or 37°C and read on a spectrophotometer (UV-1800BPC, LiuYi Biotechnology co., Ltd, China) at a specific wavelength. Enzyme activities were tested in triplicate.

All enzyme measurement kits in this study were purchased from Jiancheng Bioengineering Institute, Nanjing, China. Peroxidase (E.C. 1.11.1.7) activity was assayed using a peroxidase assay kit (Catalog No. A084). In the assay, peroxidase can catalyze the reaction of hydrogen peroxide, and the enzyme activity of peroxidase was obtained by measuring the change of absorbance at 420 nm. Superoxide dismutase (E.C. 1.15.1.1) activity was measured using a superoxide dismutase activity assay kit (Catalog No. A001-1). Superoxide dismutase activity was determined by the xanthine oxidase method (hydroxylamine). Catalase (E.C. 1.15.1.1) activity was measured using a catalase activity assay kit (Catalog No. A007-1). Catalase can decompose H_2O_2 and this reaction can be quickly suspended by adding ammonium molybdate. The rest of H_2O_2 combined with ammonium molybdate to produce a pale yellow complex compound, which was detected at 405 nm. Glutathione peroxidase (E.C. 1.11.1.9) activity was measured using a glutathione peroxidase activity assay kit (Catalog No. A005). In the assay, glutathione peroxidase could catalyze the reaction of reduced glutathione to hydrogen peroxide. Acid phosphatase (E.C. 3.1.3.2) activity was measured using an acid phosphatase activity assay kit (Catalog No. A060-2). Acid phosphatase could catalyze the decomposition reaction of disodium phenyl phosphate, free phenol and phosphoric acid produced. In an alkaline condition, the reaction of phenol and 4-aminoantipyrin generated red quinone derivatives. Acid phosphatase enzyme activity was detected according to the red shades at 520 nm.

Trypsin (E.C. 3.4.21.4) activity was measured using a trypsin activity colorimetric assay kit (Catalog No. A080-2). In the assay, trypsin could hydrolyze the ester chain of arginine ethyl ester and increase the absorbance value at 253 nm. Trypsin activity can

be calculated according to the change of absorbance. The activity of α-amylase (E.C. 3.2.1.1) was measured using an amylase activity assay kit (Catalog No.C016). In the assay, iodine solution combined with unhydrolyzed starch into blue complex. Amylase activity could be calculated according to the absorbance value at 660 nm. Lipase (E.C. 3.2.1.1) activity was assayed using a lipase assay kit (Catalog No. A054-2). This assay was done according to methyl halide substrate method measured at 570 nm. The specific activity was expressed as units per milligram of protein (U/mg protein). Soluble protein of crude enzyme extracts was quantified by the bicinchoninic acid method (Walker J.M., 2002) using the bicinchoninic acid protein assay kit (Catalog No. A045-4).

All data were expressed as mean ± SD and were analyzed with SPSS19.0 using ANOVA to determine whether there was any significant difference between the concentrations. When ANOVA was significant, the Duncan test was performed to determine where the difference was.

Results

During the experimental period, fish in the 15 and 20 mg/L groups exhibited stress behaviors which were not seen in the control group. At the beginning of the test, individuals under ammonia stress increased swimming activity and respiratory frequency. Mucus secretion on the body surface increased and gill filaments became partially hyperemic. Blood capillaries at the base of pectoral fins were fractured and hemorrhaging was observed. After 24 h exposure, individuals adapted to the high ammonia environment and the behavior became normal. Test fish in the elevated ammonia concentration swam to tank corners and remained inactive prior to death. Fish body color in the 15 and 20 mg/L groups became paler than the fish in the control. Some fish died at 48 h in the 20 mg/L group. Gill covers and mouth were wide open, and the body was bent and rigid. One percent of larval barramundi died with 96 h exposure to the 20 mg/L ammonia group, but no fish died in the other two groups.

Ammonia stress significantly affected the SOD activity of larval barramundi ($P < 0.05$, Fig. 1). At 24 and 48 h, the lowest SOD activity was observed in 20 mg/L treatment, and the SOD activity of fish exposed in 0 and 15 mg/L ammonia was not significantly different ($P > 0.05$). At 72h, the highest SOD activity was observed in fish exposed to 15 mg/L ammonia, and the lowest SOD activity was observed in fish exposure to 20 mg/L ammonia. At 96h, the highest SOD activity was observed in 0 mg/L ammonia group, and the lowest SOD activity was observed in 20 mg/L ammonia group.

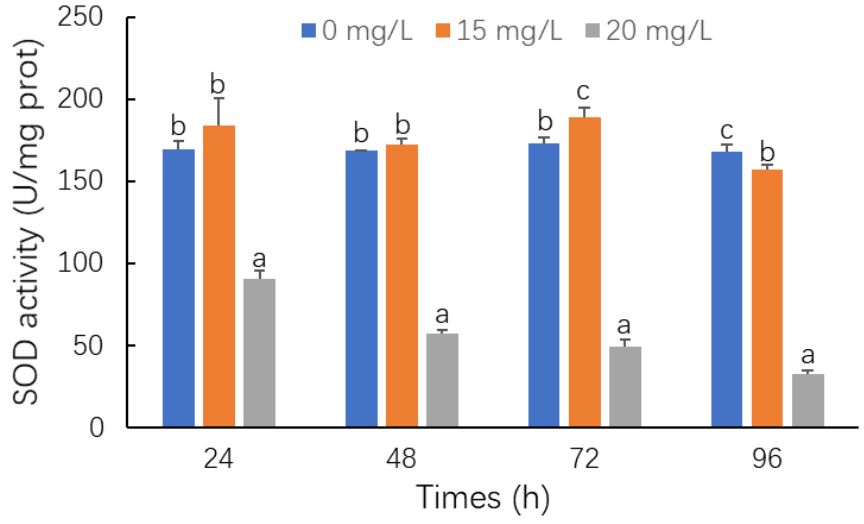

Fig.1 Effect of ammonia on SOD activities of larval *L. calcarifer*.

Ammonia stress significantly affected POD activity ($P < 0.01$, Fig. 2). At 24h, the lowest POD activity was observed in fish exposed to 20 mg/L ammonia, and the highest POD was observed in fish exposed in 15 mg/L ammonia. Starting from 48h, the lowest POD activity was observed in fish exposed in 15 mg/L ammonia, and the POD activity in fish was not significantly different when exposed to 0 and 20 mg/L ammonia ($P > 0.05$).

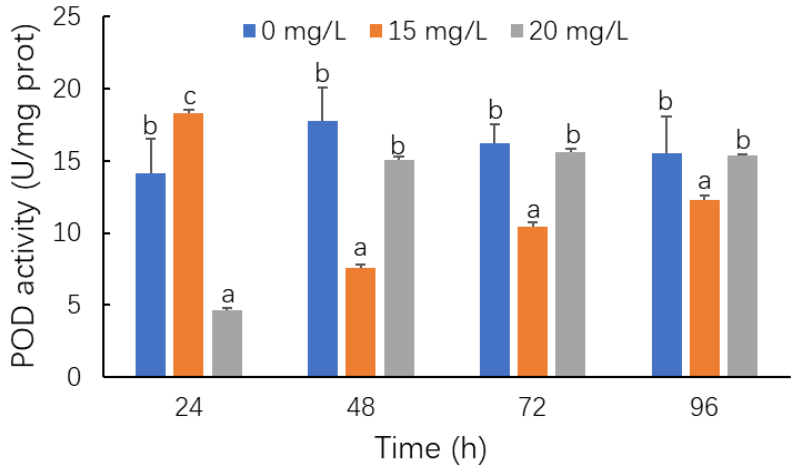

Fig.2 Effect of ammonia on POD activities of larval *L. calcarifer*.

CAT activities of fish were significantly affected by ammonia stress ($P < 0.05$, Fig. 3). At 24h, the highest CAT activity was observed in fish exposed to 0 mg/L ammonia ($P < 0.05$), and the lowest CAT activity was observed in fish exposed to 20 mg/L ammonia. At 48h, the highest CAT activity was observed in fish exposed to 0 mg/L ammonia ($P < 0.05$), and the lowest CAT activity was observed in fish exposed to 15 mg/L ammonia. Starting from 72h, the highest CAT activity was observed in fish exposed to 15 mg/L ammonia, and the lowest CAT activity was observed in fish exposed to 20 mg/L ($P < 0.05$).

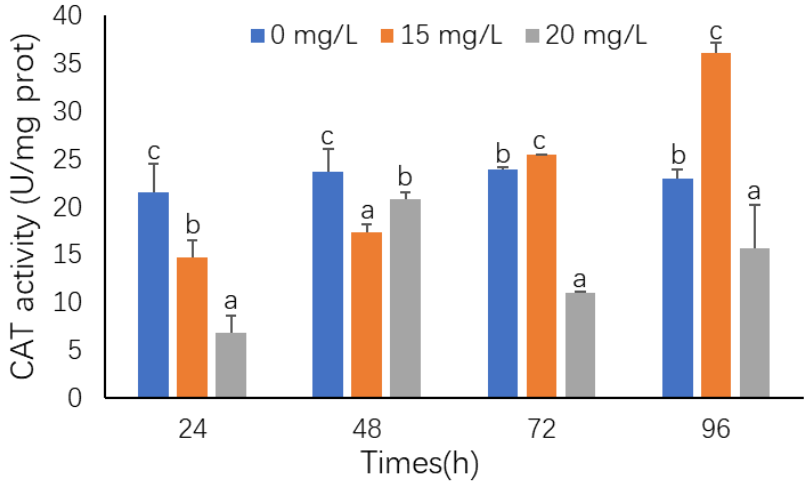

Fig.3 Effect of ammonia on CAT activities of larval *L. calcarifer*.

Ammonia stress significantly ($P < 0.05$) affected ACP activity of larval *Lates calcarifer*, (Fig. 4). At 24h, the highest ACP activity was found in fish exposed to 15 mg/L ammonia ($P < 0.05$), and the lowest ACP activity was observed in fish exposed to 20 mg/L ammonia. At 48h, the lowest ACP activity was observed fish exposed to 0 mg/L ammonia, and the ACP activity of fish was not significantly different when exposed to 15 and 20 mg/L ammonia ($P > 0.05$). At 72h, the highest ACP activity was observed in fish exposed to 20 mg/L ammonia, and the lowest ACP activity was observed in fish exposed to 0 mg/L ammonia. At 96h, the highest ACP activity was observed in fish held in 20 mg/L ammonia, and the lowest ACP activity was observed in fish exposed to 15 mg/L ammonia.

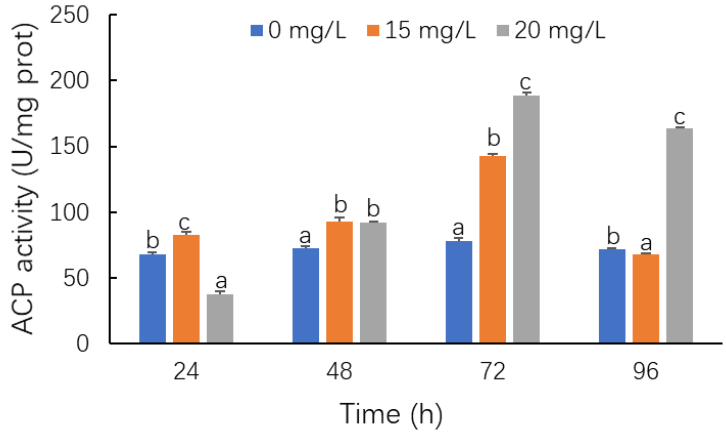

Fig.4 Effect of ammonia on ACP activities of larval *L. calcarifer*.

Ammonia stress significantly affected the GSH-Px activity of larval barramundi (P<0.01, Fig. 5). At 24 and 72h, the highest GSH-Px activity was observed in fish held in 15 mg/L ammonia, and the lowest GSH-Px activity was observed in fish exposed to 20 mg/L ammonia. At 48 and 96h, GSH-Px activity of fish was not significantly different when held in 0 and 15 mg/L ammonia but was significantly higher than those held in 20 mg/L ammonia (P < 0.05).

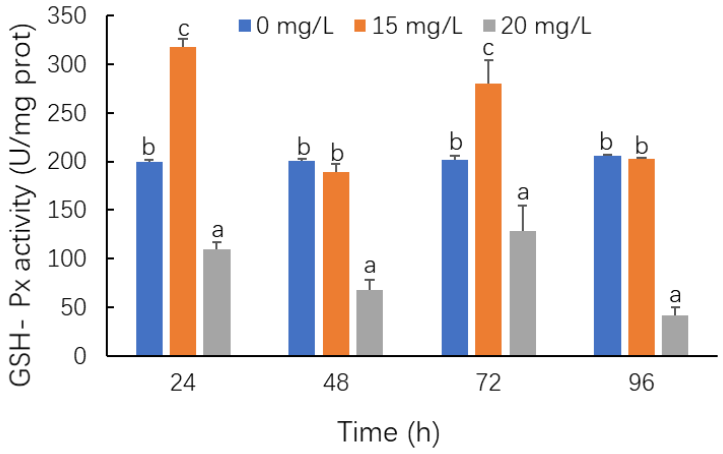

Fig.5 Effect of ammonia on GSH-Px activities of larval *L. calcarifer*.

Ammonia stress significantly affected the LPS activities of larval barramundi (P<0.01, Fig. 6) in this study. At 24 and 96h, the lowest LPS activity was observed in fish held in 20 mg/L ammonia (P < 0.05), and the highest LPS activity was observed in fish held in 0 mg/L ammonia. At 48h, the highest LPS activity was observed in fish held in 15 mg/L ammonia, and the lowest LPS activity of fish was observed in 20 mg/L ammonia. At 72h, LPS activity of fish was not significantly different when exposed to 0 and 15 mg/L ammonia but was significantly higher than those fish held in 20 mg/L ammonia (P<0.05).

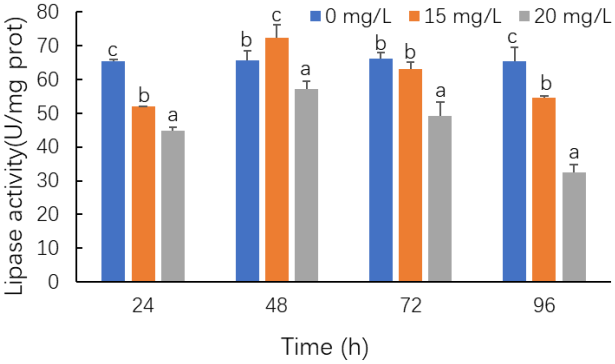

Fig.6 Effect of ammonia on LPS activities of larval *L. calcarifer*.

Ammonia stress significantly affected the AMS activity of larval barramundi (P<0.01, Fig. 7). At 24 and 96h, the lowest AMS activity of fish was observed in 20 mg/L ammonia (P < 0.05), and the highest AMS activity of fish was observed in 0 mg/L ammonia. At 48 and 72h, the AMS activity of fish was not significantly different when held in 0 and 15 mg/L ammonia but was significantly higher than those fish held in 20 mg/L ammonia (P<0.05).

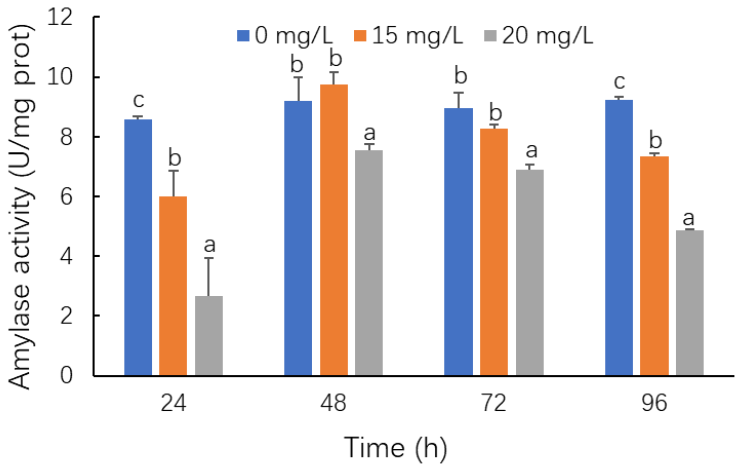

Fig.7 Effect of ammonia on AMS activities of larval *L. calcarifer*.

Ammonia stress significantly affected TRYP activity of larval barramundi (P<0.05, Fig. 8). At 0, 48, and 72h, the highest TRYP activity of fish was observed in 15 mg/L ammonia treatment, and the lowest TRYP activity of fish was observed in 0 mg/L group (P < 0.05). At 96h, the highest TRYP activity of fish was found in 20 mg/L treatment, and the lowest TRYP activity of fish was observed in 0 mg/L treatment.

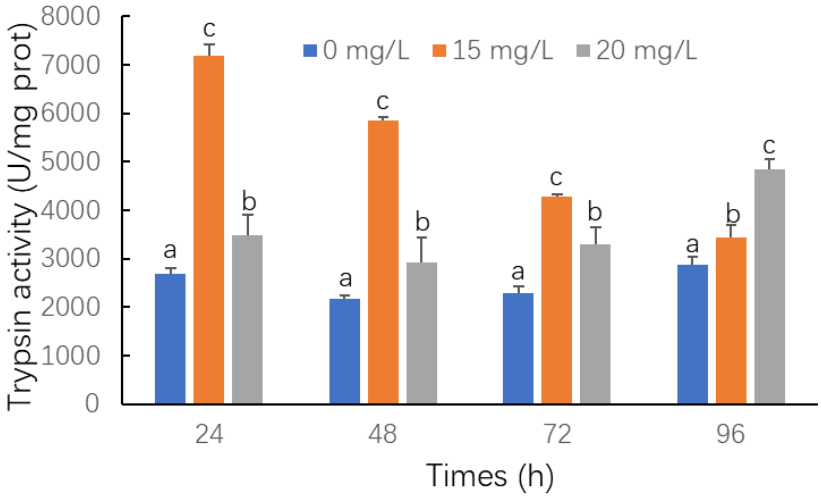

Fig.8 Effect of ammonia on TRYP activities of larval *L. calcarifer*.

Discussion

Gills represent the primary site for ion regulation and oxygen exchange (Lease et al., 2003). They are well-known target organs in fish, being the first site to react to unfavorable environmental conditions (Aysel, 2008), and are the first site of exposure involved in ammonia detoxification (Randall and Tsui, 2002). When Tilapia (*Oreochromis niloticus* L.) larvae and fingerlings were under acute toxicity of ammonia, the mucous secretion increased in the gills, and appeared darker in color in both eye and skin (Benli et al. 2005). Similarly, when rainbow trout (*Salmo gairdneri*) were exposed to high doses of ammonia, mucous secretion increased, and swimming behavior changed significantly. Microscopic observation of the gills revealed some thickening of the lamellar epithelium and increase in mucus production. In the present study, when exposed to high

concentrations of ammonia, larval barramundi displayed gill damage. The results indicate that the histopathological effect of acute ammonia exposure on the gills was hemorrhaging and mucus production. Hemorrhage is a result of blood channel disruption and is indicative of severe physical damage (Mallat, 1985). The observation of gill hemorrhage in the present study is very similar to those gill structure changes reported in Benli (2005). There is evidence that the number of recognizable mucous cells always stays constant, but the time required for the formation and discharge of the mucous cells varies under the influence of NH3 (Lang et al 1987). The structure of rainbow trout epidermis and the data yielded by the mucous cell counts provided no evidence of ammonia having an impact on mucus production. The fish may have adapted the formation and release of mucus to the ammonia level during the experiment.

Swimming performance is the physiological process affected by ammonia (Lease et al., 2003). Fish at 15 mg/L and 20 mg/L ammonia, swam more actively at the beginning of exposure but returned to normal after 24h exposure. A possible explanation may be that the tested fish adapted to the elevated concentrations. It is possible that ammonia stress causes severe damage to structures and organs of fish and affects their swimming performance. Behavioral abnormalities and unbalanced swimming during chronic ammonia exposure are well documented in literature (Knoph, 1992; Benli, 2005; Suski et al. 2006). In the present study, fish moved very rapidly, lost equilibrium, and swam sideways in the early stage of ammonia stress. This may suggest that acute increase of ammonia in an intensive culture system causes significant physiological changes of barramundi larvae.

Excessive ammonia is known to induce oxidative stress of fish due to increased production of reactive oxygen species (ROS). Levels of ROS are controlled by the collaborative action of antioxidant defense mechanisms under normal conditions (Hegazi et al.2010). Therefore, activities of antioxidant enzymes are the potential indicators of oxidative stress and adaptive responses to remove the excessive ROS. Previous studies have claimed that ammonia exposure induced the production of ROS (Cheng et al.,2015). In response to excessive ROS, SOD and CAT provide the first line of defense to clean up ROS (Farombi et al., 2007). In this study, SOD activity of barramundi was elevated and also scavenged the excess ROS after exposure to lower concentrations of ammonia. Previous studies in Nile tilapia (Hegazi et al., 2010), Rockfish (Kim et al.,2015) and *Cyprinus carpio* (Hari and Neeraja, 2012) found that SOD activities clearly increased under ammonia stress but decreased under higher concentration of ammonia. These findings are consistent with our results. It is possible that high concentrations of ammonia cause irreversible damage to SOD enzyme system so that it fails to scavenge extremely high levels of ROS (Bagnyukova et al., 2006). CAT is one of the key antioxidant enzymes for the removal of superoxide anion produced by SOD. Our results show that ammonia exposure leads to a decrease of CAT activity. Furthermore, CAT activity was lower under higher concentration of ammonia. This may suggest that CAT activity was inhibited when animals were exposed to high levels of ammonia.

In this study, the GSH-Px activity of larval barramundi was elevated to scavenge excess ROS after exposure to lower concentrations of ammonia. However, it decreased under higher concentrations of ammonia. Similarly, GSH-Px activity declined in the Pacific white leg shrimp (*Litopenaeus vannamei*) (Liu et al. 2007). GSH-Px activity declined in juvenile yellow catfish *Pelteobagrus fulvidraco (*Zhang et al. 2012). These physical responses are probably caused by oxidative stress that enhances the activity of GSH-Px in the early-stage exposure to ammonia. Nevertheless, after prolonged stress, reduced its activity. GSH-Px possibly plays a key role in protection against ROS after ammonia stress that specifically catalyzes the reduction of glutathione (GSH) and hydrogen peroxide to protect cell membrane structure and function. We deduced that individuals activate GSH-Px to remove excessive ROS under lower concentrations of ammonia but may fail to scavenge extremely high levels of ROS that cause irreversible damage under higher concentrations of ammonia.

In this study, POD activities whose main biological role is to protect the organism from oxidative damage decreased significantly compared to the control group under ammonia stress. This suggests that other elements of the antioxidant system may be responsible for the barramundi tolerance of ammonia when exposed to ammonia stress.

POD activity increased at the beginning, and then declined with increasing ammonia concentration, which differs from our findings (Zhao 2006). This may suggest that the physical response of POD to ammonia is species-dependent. ACP activity plays an important role in the body defense system, immune regulation, ion secretion and other important physiological functions (Foss et al., 2009; Paust et al., 2011). It opens the phosphate ester bond and modifies surface structure of exogenous substances so that blood cells can modify exogenous substances more precisely. In this study, ACP activity of larval barramundi was enhanced in relation to the levels of ammonia. This suggests that individuals produce a higher level of ACP activity under ammonia stress.

The fish digestive system is highly susceptible to ROS. Digestive enzyme activity is a good indicator of digestive capacity and directly reflects the nutritional status of fish (Deng et al., 2010). The increase of digestive enzyme activity is known to be related to enzyme synthesis and secretion in fish (Beccaria et al., 1991). The functional changes of the digestive system are often associated with food assimilation of fish (Gisbert et al., 2004). In *Charybdis japonica*, digestive enzyme activity can be induced at low ammonia concentrations but is inhibited at higher levels of ammonia stress (Xu, 2014). In this study, LPS, primary function of which is to digest fats and lipids in order to sustain healthy functioning of the gallbladder, acted according to the rule of "induce first and inhibit later" under lower concentration of ammonia stress. This may indicate that long-term exposure of ammonia may affect the absorption of carbohydrates in fish larvae. In the present study, TRYP affected the absorption efficiency and metabolic levels of protein substance (Xu et al., 2011). This suggests that more TRYP is required to absorb additional albuminoidal food to supply the energy consumed under ammonia stress. On the other hand, the decline of LPS and AMS activities may also be caused by feed deprivation in this study.

In summary, high level of ammonia exposure (15mg/L, 20mg/L) significantly affected antioxidant enzymes and digestive enzymes activity of larval barramundi. Fish larvae exhibited a certain tolerance of ammonia in a short period of time, but long-term exposure of ammonia can cause physical damage. In the process of intensive aquaculture and practical production, acute ammonia stress should be avoided in *L. calcarifer* larvae. Results from the present study provide evidence to further understanding of the physical response of fish larvae to ammonia and a scientific basis for the management of aquaculture water environment.

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