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Effects of ammonia exposure on nitrogen metabolism in gills and hemolymph of the swimming crab *Portunus trituberculatus*

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ABSTRACT

The effects of ammonia-N (control, 1 and 5 mg L^{-1}) on nitrogen metabolism in gills and hemolymph of the swimming crab *Portunus trituberculatus* were investigated. The results showed that mRNA expression levels of Rh protein in gills were induced significantly by ammonia-N exposure. The levels of glutamine (Gln) and urea increased significantly in gills and hemolymph of *P. trituberculatus* exposed to high ammonia-N. After high ambient ammonia-N exposure, hemolymph ammonia increased significantly at 6 h and declined to control level at 12 h. The levels of Gln and urea in gills and hemolymph were significantly higher than control group during the whole exposure period and showed dose and time dependent effects. In addition, the activities of key-related enzymes involved in biosynthetic pathways of Gln and urea such as glutamate dehydrogenase (GDH), glutamine synthetase (GS) and Arginase (ARG) increased remarkably in the present study. This suggests that *P. trituberculatus* possesses some ammonia-detoxification strategies to cope with high ambient ammonia, including an up-regulation of Rh protein in gills and conversion ammonia to Gln and urea. The levels of Gln and urea in hemolymph could be considered as suitable physical indexes to evaluate ammonia toxicity to serve anti-ammonia selective breeding in the long run.

1. Introduction

Swimming crab *Portunus trituberculatus* has been one of the most popular aquatic products in Chinese seafood market with breeding area of 29,036 ha and output of 400,348 tons in 2012 according to the Chinese Fishery Statistical Yearbook (2013). In aquaculture, the crabs are usually fed with fresh fishes and shrimps. The residual baits accumulate in ponds, which can easily lead to water pollution. Especially, ambient ammonia content can increase to several mg L^{-1} due to the ammonization of organic matter by microorganism and the excretion of farmed crustaceans (Camargo and Alonso, 2006). It was reported that elevated ambient ammonia did harm to the immune system (Hong et al., 2007; Jiang et al., 2004; Le Moullac and Haffner, 2000; Rodríguez-Ramos et al., 2008; Verghese et al., 2007; Yue et al., 2010), and ionic balance (Harris et al., 2001; Rebelo et al., 1999; Romano and Zeng, 2007; Young-Lai et al., 1991), as well as decreased growth rates and even lead to death (Dosdat et al., 2003; Foss et al., 2004; Lemarie et al., 2004; Naqvi et al., 2007; Pinto et al., 2007) in aquatic animals.

Recently, more and more studies have been conducted on different aquatic animals to investigate their responses and detoxification mechanisms when exposed to ambient elevated ammonia. There have been many studies showing that ammonia content could be lowered by being excreted directly with the help of Rh proteins (Braun and Perry, 2010; Wright and Wood, 2009; Zimmer et al., 2014), or converted to urea via ornithine-urea cycle (OUC) (Chen and Chen, 1997) and glutamine (Gln) by coupling of glutamate dehydrogenase (GDH) and glutamine synthetase (GS) (Anderson et al., 2002). In crabs, a large amount of ammonia is excreted into the surrounding environment via gills rather than antennal gland (Cameron and Batterton, 1978). Meanwhile, there are many evidences for Rh proteins acting as ammonia transporter in mammals and fishes, including mediating NH_4^+ transportation (Nakhoul et al., 2005), facilitating NH_3 and NH_4^+ transportation (Bakouh et al., 2004; Benjelloun et al., 2005; Mak et al., 2006; Ripoche et al., 2004; Zidi-Yahiaoui et al., 2005) and accelerating NH_4^+/H^+ electroneutral exchange (Ludewig, 2004). Furthermore, Rh protein could also be expressed in gills of several crabs, such as the green crab *Carcinus maenas*, the blue crab *Callinectes sapidus*, the freshwater crab *Dilocarcinus pagei*, the Atlantic rock crab *Cancer irroratus* and the marine Dungeness crab *Metacarcinus magister* (Martin et al., 2011; Weihrauch et al., 2004). In spite of these recent advances in researches on Rh proteins transporting ammonia to cope with high environmental ammonia exposure in different species, to our knowledge, there is little information about the effects of ammonia on Rh protein in *P. trituberculatus*.

In natural environment, aquatic animals could not only excrete waste nitrogen mainly as ammonia, but could also produce small amounts of urea (Claybrook, 1983). It is well known that urea is produced via OUC, uricolysis and argininolysis generally, and it was reported that urea had played an important role in ammonia detoxification when hemolymph ammonia was high (Anderson, 2001; McDonald et al., 2006; Spaargaren, 1982). Arginase (ARG) acts as the key enzyme in the OUC and it has been found in crustaceans, such as shore crab *C. maenas*,

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Atlantic rock crab *C irroratus* (Hanlon, 1975), *Penaeus japonicus* (Chen and Chen, 1997) and *Marsupenaeus japonicus* (Lee and Chen, 2003). Besides, there have been many reports indicating that hemolymph urea increased during exposure to high ambient ammonia in crustaceans, such as in *Penaeus monodon* (Chen et al., 1994), *Pacifastacus leniusculus* (Harris et al., 2001), *M. japonicus* (Lee and Chen, 2003), *Scylla serrata* (Chen and Chia, 1996), *C. maenas* (Durand et al., 1999), *Eriocheir sinensis* (Hong et al., 2007) and *Metacarcinus magister* (Martin et al., 2011). In addition, elevated ammonia levels could be reduced by the synthesis of glutamine as temporary intermediary nitrogen store, such as in *Porcellio scaber* (Wright et al., 1994), *Oncorhynchus mykiss* (Wright et al., 1995), *Clarias gariepinus* (Terjesen et al., 1997), *Bostrichthys sinensis* (Ip et al., 2004a), *Armadillidium vulgare* and *Ligidium lapetum* (Wright and Pen˜a-Peralta, 2005) and *E. sinensis* (Hong et al., 2007). As we all know, Gln is formed from glutamate and NH_4^+ while glutamate from α-ketoglutarate and NH_4^+, which is involved with GS and GDH (Murray et al., 2003; Wright et al., 1994, 2007). However, there have been little researches on the effects of ammonia on the contents of urea and glutamine along with activities of ARG, GS and GDH in *P. trituberculatus*.

In this study, we examined the effects of ammonia-N on the mRNA expression level of Rh protein, and the synthesis pathways of Gln and urea in the gills and hemolymph of *P. trituberculatus*. The activities of GDH, GS, ARG and the levels of Gln and urea were analyzed. These results will not only offer more information about ammonia detoxification mechanisms of *P. trituberculatus* but also offer suitable physical indexes for serving anti-ammonia selective breeding in the long run.

2. Materials and methods

2.1. Animals and culture management

Healthy *P. trituberculatus* (mean weight 100 ± 7.5 g) were obtained from a commercial crab farm in Qingdao, China and acclimated in tanks (60 cm × 50 cm × 40 cm) with 90 L of seawater for one week before the exposure experiment. Tanks were filled with sand-filtered seawater (salinity 32 g L^{-1}, pH 8.2) at 25 ± 0.5 °C and aerated continuously using air-stones. The tank was separated into six cubicles with plastic plate, and one crab was placed in one cubicle. During the acclimation period, one half of the water in the tanks was replaced with new water every morning and evening, and the photoperiod was maintained on a 12:12 h light–dark cycle. The crabs were fed at 18:00 with fresh clam *Ruditapes philippinarum* daily by 10% wet weight of crabs. One hour after feeding, the uneaten food was removed to keep the water clean. All crabs were starved for 2 days prior to exposure experiment. During the entire period of the exposure experiment, the crabs were starved.

2.2. Experimental design

In the present study, ammonia exposure experiment was set up by three different ammonia concentrations: 0.07 mg L^{-1} (control), 1 mg L^{-1} and 5 mg L^{-1}. The seawater used for the control group was the same as the acclimated period. The ammonia concentrations of 1 and 5 mg L^{-1} were prepared by adding 10 g L^{-1} NH_4Cl stock solution into the seawater. Crabs were randomly divided into three groups and each group contained three replicates. Each replicate contained 18 individuals. 144 crabs were originally collected and no death during the exposure experiment. One half of the water was replaced by the seawater with same ammonia concentration every 12 h to keep ammonia concentrations constant. During the pre-experiment, the addition of 1 and 5 mg L^{-1} NH_4Cl caused small changes in pH of the seawater which were considered negligible and no measures were taken for correction during exposure period. During the exposure experiment, ammonia-N concentrations were measured every 12 h and varied at 0.07 ± 0.02, 1.10 ± 0.06 and 5.17 ± 0.20 mg L^{-1} respectively by hypo-bromate oxidimetry method (Li, 1995). Three crabs from each replicate were sampled randomly at 0, 2, 6, 12, 24 and 48 h respectively after ammonia exposure.

Table 1
GenBank accession numbers, qPCR primer sequences and products size of all genes used in the present study listed. F: forward, R: reverse.

Target gene	Accession no.	Sequence (5′–3′)	Product size (bp)
β-actin	FJ641977.1	F: TCACCAACTGGGACGACAT R: GCGGGAGTGTTGAAGGTTT	166
Rh protein	KJ126844	F: CGTGGACCATGTCAAACTTCT R: CATGATAGGCACCGTATTTCTG	179

2.3. Hemolymph collection

Hemolymph sample was obtained from the arthrodial membrane at the third walking leg using a sterilized syringe with an equal volume of the anti-coagulant (450 mmol L^{-1} NaCl, 100 mmol L^{-1} glucose, 30 mmol L^{-1} trisodium citrate, 26 mmol L^{-1} citric acid, 10 mmol L^{-1} EDTA, pH 7.45) modified from the anti-coagulant devised by Söderhäll and Smith (1983). After collection, the sample was immediately centrifuged in a refrigerated centrifuge at 700 *g* for 10 min at 4 °C and the supernatant was collected as plasma sample and frozen at −80 °C until analysis.

2.4. Tissue preparation

Gill sample was collected after the crabs were placed on ice for about 30 min. Sample used for RNA extraction was dissected using RNase-treated scissors and forceps and then was lysed with RNAiso Plus reagent (TaKaRa, Dalian, China). After that, the lysed samples were centrifuged at 12,000 *g* for 15 min at 4 °C and stored at −80 °C until RNA isolation and mRNA expression analysis. For nitrogenous compound contents and related enzyme activity assays, sample was collected quickly and stored at −80 °C immediately.

2.5. Measurement of nitrogenous compound levels

Hemolymph ammonia was determined directly using hemolymph ammonia assay kit (no. A086, Nanjing Jiancheng Bioengineering Institute, China). In order to measure Gln content, equal volume of acetonitrile (HPLC grade) was pipetted into the plasma sample and then the sample was shaken for a while. After that, the sample was centrifuged at 3000 *g* for 10 min at 4 °C and the supernatant was retained. Gln content was assayed by High Performance Liquid Chromatographic (HPLC) (Shimadzu, LC-20A, Japan) equipped with Zorbax Eclipse XDB-C18 column (4.6 × 250 mm, Agilent, USA) at 35 °C (Liang et al., 2003). Gln content in gills was measured as described previously except that the sample was homogenized in equal volume of 40 mmol L^{-1} Borate buffer (pH 9.5) before adding acetonitrile. As for the determination of urea content, hemolymph was added in equal volume of ice cold 10% trichloroacetic acid solution and then centrifuged at 10,000 *g* for 15 min at 4 °C to obtain the supernatant. Urea content was measured by adding diacetylmonoxime to form pink hydroxylamine which has the largest photoabsorption at 525 nm (Rahmatullah and Boyde, 1980). Urea content in gills was measured as described previously except that sample was homogenized in 5 times volume of ice cold 10% trichloroacetic acid solution.

2.6. Enzyme assay

To measure the activities of GDH, GS and ARG, gill sample was homogenized in 5 times volume of ice-cold 25 mmol L^{-1} Tris buffer (pH 7.5) containing 1 mmol L^{-1} dithiothreitol (DTT), 1 mmol L^{-1} Na_2EDTA and 0.2 mmol L^{-1} phenylmethylsulfonyl fluoride (PMSF) at 2400 rpm for 60 s at 4 °C. Prior to use, 2 μL of PMSF stock (0.1 mmol L^{-1}) was added