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Comparative transcriptome analysis reveals molecular strategies of oriental river prawn *Macrobrachium nipponense* in response to acute and chronic nitrite stress

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ABSTRACT

Macrobrachium nipponense is an economically and nutritionally important species threatened by ambient superfluous nitrite. De novo RNA-Seq was used to explore the molecular mechanism in *M. nipponense* exposed to the acute nitrite stress (26.05 mg/L nitrite-N) for 24 h and the chronic nitrite stress (1.38 mg/L nitrite-N) for 28 d A total of 175.13 million reads were obtained and assembled into 58,871 unigenes with an average length of 1028.7 bp and N50 of 1294 bp. Under the acute and chronic nitrite stress trials, 2824 and 2610 unigenes were significantly expressed. In GO analysis and KEGG pathway analysis, 30 pathways were significantly different between the two treatments while four pathways were in common and the markedly altered pathways were divided into four sections as immunity, metabolism, cell and others. The immunity section revealing the different depth of immunity provoked by nitrite stress contained the most pathways including the important pathways as phagosome, folate biosynthesis, glycerolipid metabolism, glycine, serine and threonine metabolism, amino sugar and nucleotide sugar metabolism under the acute nitrite stress, and lysosome, alanine, aspartate and glutamate metabolism, arginine and proline metabolism under the chronic nitrite stress. This is the first report of responses of *M. nipponense* under acute and chronic nitrite stress through *de novo* transcriptome sequencing on the transcriptome level. The results of transcriptome analysis improve our understanding on the underlying molecular mechanisms coping with nitrite stress in crustacean species.

1. Introduction

Nitrite is one of the most important pollutants in recirculating aquaculture systems owing to incomplete oxidation of ammonia to nitrate during denitrification or nitrification [1]. Nitrite accumulation occurs in aquatic systems during the culture period and can reach as high as 20 mg/L in *Litopenaeus vannamei* ponds [2]. Furthermore, ambient nitrite is easily accumulated in fish and crayfish far above the concentration in the environment. Thus, high concentration of nitrite in water has become a potential factor triggering stress response in aquatic organisms [3]. The excessive nitrite in the culture system has incurred negative influence in growth, survival and product quality of oriental river prawn *Macrobrachium nipponense* [4], a member of the Palaemonidae family of decapod crustaceans [5], is one of the commercially important species in aquaculture throughout China [6] and other Asian countries [7–9]. Nevertheless, studies on the response of *M. nipponense* to nitrite stress were rare either in physiology or molecular biology. Considering the physiological response, it has been found that nitrite can break the balance between prooxidant

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forces and antioxidant defenses to injure *M. nipponense* [10]. However, little research has been published to reveal the partial and integrated molecular response of *M. nipponense* to nitrite stress.

Existing evidence on the toxicity of acute nitrite from physiological aspects in prawn reveals that stress can damage the respiratory system of prawn by absorbing nitrite in hemolymph and competitively binding oxyhemocyanin to reduce the level of oxyhemocyanin [11], influence nitrogenous excretion [12], inhibit immunologic function [13], increase susceptibility to bacterial infection [14] and induce apoptosis in shrimp hemocytes mediated by reactive oxygen species [1]. From molecular perspectives, transcriptome analysis of *L. vannamei* exposed to acute nitrite reveals that the stressor stimulates the high transcription of genes involved in immunity, detoxification and apoptosis promptly [15]. However, there still little research to reveal the integrated molecular mechanism of acute nitrite stress in prawn. Meanwhile, literature on chronic nitrite exposure of aquatic organisms mainly focuses on the combined effects of nitrite and chloride or potassium ions [16,17] and the complete molecular response of prawn under chronic nitrite stress is still unclear. Obviously, there is a knowledge gap on the acute and chronic nitrite stress of prawn in comparative physiology and biochemistry.

Recently, RNA sequencing (RNA-Seq) has been widely applied to the study of both mapping and quantifying transcriptome, which is superior to microarray technology [18]. Current research has verified that RNA-Seq is an adequate tool for investigating transcriptome response of aquatic animals to ambient stress [15,19,20]. RNA-Seq of *L. vannamei* exposed to nitrite stress has identified numerous candidate genes associated with immune response, detoxification and apoptosis pathway [15]. It is reported that the ability for an organism to respond to environmental stress is based on its ability to initiate changes in gene expression or transcriptome [21,22]. Thus, by using the RNA-Seq, we could find possible physiological and pathological differences in transcriptome responses of *M. nipponense* under acute and chronic nitrite stress.

2. Materials and methods

2.1. Animals and nitrite exposure

Healthy juvenile *M. nipponense* (1.12 ± 0.06 g) were obtained from a commercial farm in Zhejiang, China. The *M. nipponense* were acclimated for one week prior to the experiment in four plastic circular tanks (2826 cm^2 × 70 cm) and were fed a commercial diet with 5% of the body weight (40% protein, 5.0% fat, 5.0% fiber and 16% ash, China) twice daily at 08:00 h and 16:00 h. After acclimation and food deprivation for 24 h, the animals were divided into four groups in 12 tanks (50 × 60 × 60 cm) with triplicate each. Two groups of prawn in freshwater served as acute control (AC) and chronic control (CC), respectively, and the rest two groups were challenged with 26 mg/L nitrite-N in acute stress test (AT) and 1.33 mg/L nitrite-N in chronic stress test (CT) respectively [23]. The water was changed daily and sodium nitrite (Sinopharm Chemical Reagent, Cat. no. 20,140,218) was added to distilled water to make the stock solution and gradually diluted to 20 mg/L NO_2^--N. The actual nitrite concentration of exposed groups were 26.05 ± 0.01 mg/L and 1.38 ± 0.01 mg/L respectively, measured and corrected according to Yang et al. [24] and the nitrite solution was renewed daily. Each group was in triplicate with 50 prawns in each tank. Feeding was ceased during the acute nitrite stress while the animals were fed routinely during the chronic nitrite stress. During acclimation and experimentation, the environment was maintained at 25 ± 1 °C, 7.78 ± 0.23 pH, 12:12 dark/light and >5.8 mg/L dissolved oxygen. Eighteen prawns (six prawns per tank) from the AC and the AT groups respectively were randomly selected and sampled at 24 h while the prawns in the CC group and the CT group were sampled after 28 d. Hepatopancreas tissues from both the controls and treatments were extracted and frozen immediately in liquid nitrogen and then stored at −80 °C prior to RNA extraction. All handlings of prawns were conducted in accordance with the standard code of protocol for the care and use of laboratory animals in China. This research protocol was approved by the Animal Ethics Committee of East China Normal University.

2.2. Total RNA extraction

Total RNA extraction from hepatopancreas was produced by the Trizol method (Invitrogen) according to the manufacturer's protocol. The integrity of every single RNA sample was examined by 1% agarose gel electrophoresis to ensure that the RNA sample for RNA-Seq was integral with three distinct and bright stripes. To make sure the veracities and universalities of RNA-Seq, nine qualified RNAs from each treatment were mixed with the same amount of RNA. Further confirmation of the quantity and integrity of mixed RNA was conducted on a Bioanalyzer 2200 (Agilent Technologies, Santa Clara, CA, USA) with a RNA integrity number (RIN) value > 6.2. The mRNA was purified using the Dynabeads® mRNA Purification Kit (Life tech, Cat. no. 1,264,684).

2.3. cDNA library construction and sequencing

The RNA-Seq was conducted by Novel Bioinformatics Co., Ltd with Hiseq2500. The construction of cDNA library required 5 μg total RNA using the Ion Total RNA-Seq Kit v2 (Life technologies, Cat. no. 4,479,789) following the manufacturer's instructions. Briefly, the poly(A)-containing mRNA was isolated using Dynabeads (Life technologies, USA), fragmented with RNaseIII and then purified. After being added and ligated with ion adaptor, the fragmented RNA was reverse-transcribed and amplified to double-stranded cDNA. Double-stranded cDNA was purified by the magnetic bead based method. Then, the molar concentration of the purified cDNA was detected for each cDNA library. Emulsion PCR was produced using the template of cDNA library [25]. The filtering of the raw reads was produced with FAST-QC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to screen out the substandard reads. The qualified cDNA were processed on a OneTouch 2 instrument and enriched on a OneTouch 2 ES station for preparing the template-positive Ion PI™ Ion Sphere™ Particles according to the Ion PI™ Template OT2 200 Kit (Life tech, Cat. no. 4,482,286). The mixed template-positive Ion PI Ion Sphere particles of four samples were enriched and loaded onto a 1 P1v2 Proton Chip, then sequenced on Proton Sequencers according to the Ion PI™ Sequencing 200 Kit (Life tech, Cat. no. 4,482,283).

2.4. Quality control and trinity assembly

The raw sequencing data were likewise evaluated by FAST-QC. The evaluation metrics scrutinized the nature of the data to make sure the reliability of the subsequent various evaluations. To filtering unqualified reads and adaptor sequences, the quality evaluation of raw reads with <10 would be controlled <10%, quality evaluation <13 would be <14% and quality evaluation <20 would be controlled <30% to process the clean reads. In the absence of the genome of *M. nipponense*, assembly from the reads of the *de novo* RNA-Seq was performed using Trinity software [26,27] which is developed by the Broad Institute containing inchworm, chrysalis and butterfly. The three independent modules dealt with the large-scale reads of RNA-Seq in turns. Upon the overlaps of each two reads, the inchworm was used to build the k-mer library (k = 25)