The Effects of Ammonia Stress Exposure on Protein Degradation, Immune Response, Degradation of Nitrogen-containing Compounds and Energy Metabolism of Chinese Mitten Crab.

Dan Tang

Yancheng Teachers University

Ya Wu

Yancheng Teachers University

Lu Wu

Yancheng Teachers University

Yuze Bai

Yancheng Teachers University

Ying Zhou

Yancheng Teachers University

Zhengfei Wang (✉ wangzf@yctu.edu.cn)

Yancheng Teachers University <https://orcid.org/0000-0003-1918-6282>

Research Article

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Abstract

The Chinese mitten crab is one of the most economically important crab that is widely farmed in China. Ammonia, which is a main physiological challenge for crab culture, grow rapidly in the intensive culture system over time, but little information is available with Chinese mitten crab on the molecular mechanisms. Therefore, to understand the mechanism of response to ammonia stress in *Eriocheir japonica sinensis*, comparative transcriptome analysis was used to identify the key genes and pathways in hepatopancreas challenged with ammonia stress (325.07 mg/L NaCl). By sequencing the transcriptome hepatopancreas of *E. j. sinensis* treated with ammonia, 366,472 unigenes were obtained and annotated into several public libraries for later analyses. Subsequently, 1,775 differentially expressed genes (DEGs) were identified according to comparative transcriptome analysis, of which 307 were up-regulated and 1,468 were down-regulated. According to the DEGs of GO and KEGG enrichment analyses, we focused on four aspects of significant enrichment in this study: protein degradation, immune response, degradation of nitrogen-containing compounds and energy metabolism. The genes involved in protein degradation and energy metabolism process showed a significant decrease which was consisting of overall biological activity of *E. j. sinensis* decreased. In addition, five genes involved in high concentration of ammonia were discovered and validated by qRT-PCR. Therefore, this study will help us understand the molecular mechanisms of *E. j. sinensis* under high ammonia exposure.

Introduction

The Chinese mitten crab (*E. j. sinensis*) is one of the most economically aquaculture species in China, and has become a traditional dish in China because of its high nutritive value [1]. This species is one of fundamental species contributing to fresh-water fishery in China [2]. The total farming area of *E. j. sinensis* was about 670,000 ha in 2020 (<http://www.moa.gov.cn/>). In order to pursue more production value, the semi-intensive and intensive culture systems were used to culture the Chinese mitten crab. However, intensive culture often led to the degradation of the pond water from uneaten protein feeds and heterotrophic bacterial metabolites [3], but also the nitrifying bacteria have reduced ability to nitrify bacterial metabolites due to the low level of dissolved oxygen (DO). Therefore, the degraded pond water factors, such as ammonia, play an important role in crab culture.

In aquaculture, ammonia is one of the main environmental limiting factors that give rise to a series of adverse effects in crabs, such as, the low growth rate, the damage of tissues, and even the high mortality [3, 4]. In the last decade, the intensive culture system in aquaculture has become a high trend which will lead to the accumulation of ammonia. The more intensively farmed, the higher level of the ammonia content produced. For crustaceans, the metabolic wastes will mainly excrete through the gills, which depends on the difference between the concentration of ammonia in the environment and organism [3]. If the concentration of ammonia in the environment rises, it prevents crustaceans from getting rid of the metabolites. In addition, toxic nitrogen-containing compounds are digested in hepatopancreas, which is a significant organ for digestion and detoxification process in crabs [5, 6].

Generally, ammonia comes in two forms ionic form (NH_4^+) and in its unionized form (NH_3). Moreover, in most cases, the unionized ammonia is more toxic to aquatic organisms [3]. As ionic ammonia cannot get into the cell while the unionized ammonia can pass through cell membranes, the ionic form of ammonia can convert to the toxic form through the change of some other factors, such as salinity, pH and temperature. Low salinity can increase the toxicity of ammonia (NH_3) to *Litopenaeus vannamei*[7], *Penaeus chinensis* juveniles[8], *P.*

semisulcatus juveniles[9]. It has reported that high pH and high temperature increase the relative proportion of NH_3 in *Rhamdia quelen* and *Piaractus mesopotamicus*[10], [11].

Previous studies have shown that the high concentration of ammonia stress will cause the increase of oxygen consumption, the decrease of osmotic regulation ability, affecting the molting frequency of crustacean, and even cause the damage of hepatopancreas and gill tissue, contributing to the high mortality of crustacean [7, 12]. Additionally, the high levels of ammonia exposure also impact on immune responses and metabolic processes of crustacean, which have been confirmed in *E. j. sinensis* [13]. A lot of studies have been done on aquatic animals exposed to high levels of ammonia, such as *Marsupenaeus japonicas*[12], *L. vannamei*[14], *Dicentrarchus labrax*[15, 16], *M. carcinus*[17], *Portunus trituberculatus* and *E. j. sinensis* [13, 18]. However, most of them, the underlying molecular basis of the hepatopancreas in crabs on ammonia stress is still limited. In order to discover and investigate the complex molecular responses of *E. j. sinensis* under high concentrations of ammonia stress, the technology of Illumina sequence was used to identify genes and pathways involved in the response to ammonia stress. The results will provide a useful insight into immune mechanisms of the *E. j. sinensis* response to high level of ammonia.

Materials And Methods

Animals and ammonia stress experiment

E. j. sinensis used in this experiment were obtained from Yancheng (Jiangsu, China). Experimental crabs were acclimated in the plastic crate (30cm × 18cm × 20cm) for one week, constantly filled with filtered, aerated water (temperature:~17°C; PH:~7.5). During these days, the water was changed at 9:30 am and experimental crabs were fed at 12:00 am on a daily basis. Ammonia concentration was determined according to the study of Hong ML, finally the concentration of ammonia was set to 325.07 mg/L. Several healthy crabs were randomly divided into two groups after domestication (three crabs in each group); One group was only kept in clean water as control group; another group was added 325.07 mg/L NH_4Cl and exposed to ammonia for 24h as the experimental group (Fig. 1). Eventually, three crabs in each group were dissected on ice bath and the hepatopancreas tissue were obtained. The hepatopancreas were immediately frozen in liquid nitrogen for later RNA extraction.

RNA extraction, library construction and sequencing

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to extracted the total RNA of samples' hepatopancreas. Magnetic bead with Oligo (dT) and poly A probes were used to separate mRNA from total RNAs. Then, mRNAs were fragmented (approximately 200 bp) by a fragmentation buffer. The purity and concentration of them were confirmed via a Nanodrop 2000 spectrometer (Thermo Fisher Scientific Inc. Waltham, MA, USA). Random-hexamer primers were prepared to synthesize the first strand cDNA, subsequently the second strand cDNA was synthesized to form a stable double-stranded structure. 2% agarose gel electrophoresis was used to purify the fragments, followed by amplification of PCR for 15 cycles. After this a cDNA library was established. Finally, the mRNA database was established with the Illumina TruSeq™ RNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA).

Transcriptome assembly and annotation

The raw reads were contained the adaptor sequences, contamination, ambiguous nucleotides or low quality reads (< 20bp reads). The raw reads were filtered by the SeqPrep software (<https://github.com/jstjohn/SeqPrep>) and Sickle software (<https://github.com/najoshi/sickle>). The nucleotide sequences obtained by above method were assembled to form longer fragments and transcripts with Trinity software (<http://trinityrnaseq.sourceforge.net/>). Transcripts, representing significant parts of individual isoforms, were clustered on the basis of the same gene sequence. The longest transcripts of each gene were pooled as “unigenes”. BlastX (criterion: e-value of $<10^{-5}$) was performed to compare the transcripts with the following databases: NCBI-nr, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG), Swiss-Prot protein, String, and Pfam.

Analysis of differentially expressed genes (DEGs)

The expressed values and transcription levels were calculated via fragments per kilobase per million mapped reads (FPKM) method, reducing the influence of gene lengths. Differences in gene expression abundance between the ammonia treatment and control groups were statistically analyzed. DEGs were determined from a $FDR < 0.05$ and $\log_2|FC| \geq 1$. Then all DEGs were mapped into GO functional enrichment and KEGG pathway functional enrichment analysis. Goatools (<https://github.com/tanghaibao/GOatools>) and KOBAS (<http://kobas.cbi.pku.edu.cn/home.do>) were applied for GO function analysis and KEGG pathway analysis respectively. The DEGs function and candidate genes involved in ammonia stress were selected via GO functional and KEGG pathway enrichment analyses. Corrected p value ≤ 0.05 sets as thresholds for indicating significantly enriched.

Validation of gene expression data using quantitative real-time PCR (qRT-PCR)

In order to validate the Illumina sequencing data, five DEGs (*CTSB*, *CTSA*, *RPA2*, *UCEE2-17*, and *D5-TN*) were randomly selected to be quantified by qRT-PCR. Primer Premier 5 software was performed to design the specific primers of selected five DEGs and β -actin was used as the reference gene to normalize the levels of target gene expression (Table S1). The qRT-PCR analysis was performed on an Applied Biosystem 7500 real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The volume of the reaction mixture was 25 μ L, made up of 12.5 μ L of 2 $\times$ SYBR qPCR Mix, 1 μ L of cDNA, 1 μ L of forward and reverse primers, and 10.5 μ L RNase-free H₂O. The PCR program was set into four steps: 95°C for 3min, 40 cycles at 95°C for 15s, 60°C for 15s and 72°C for 25s. Each sample and the internal control gene repeated three times to make sure only one PCR product. The relative expression level of gene was calculated and converted to fold differences by the comparative CT method ($2^{-\Delta\Delta CT}$ method).

Results

Transcriptome sequencing and *de novo* assembly

Illumina sequencing were performed on the hepatopancreas of *E. j. sinensis* in control groups (QS-1, QS-2) and ammonia groups (NH₃-1, NH₃-2, NH₃-3). Due to the poor quality of QS-3, it was removed and it was difficult to obtain samples for supplement in this season, there were therefore only two sets of QS. All raw-sequence reads data have been deposited in the NCBI Sequence Read Archive (SRA) database under the accession number SRR15882015, SRR15882014, SRR15882013, SRR15882012, SRR15882011. After removing the low-quality, short or contaminated reads, clean reads were finally generated for control and experiment group libraries, respectively

(Table 1). These sequences were used for subsequent analyses. Clean bases of transcriptome data previously obtained in our laboratory were subjected to de novo assembly using Trinity software and assembled into 366,472 unigenes. The length of the unigenes ranged from 201 to 32,740 bp with an average length of 555.44 bp and the length of N50 is 679, with a 42.42 percentage of GC (Table 2).

Function annotation and classification

To further understand the comprehensive gene function information, all unigenes were subjected to annotation analysis by matching sequences against public databases, including the NR, Swiss-Prot, String, KEGG and GO databases. 20,648 unigenes can be matched in NR database, 13,503 matched in GO database (Table 3). According to the GO analysis, a total of 13,503 unigenes were identified into three major function categories: biological process (51.54%), cellular components (37.30%) and molecular functions (11.13%). The biological process (BP) category comprised 31 subcategories, among which cellular process and metabolic process predominated, followed by biological regulation, regulation of biological process and response to stimulus. The cellular component (CC) category comprised 20 subcategories, among which cell and cell part predominated. The molecular function (MF) category comprised 15 subcategories, among which binding and catalytic activity predominated (Table 4).

11,362 unigenes were identified to the KEGG database, which can be classified into six categories: Cellular Processes, Environmental Information Processing, Metabolism, Genetic Information Processing Human Disease, and Organismal Systems. Among them, Metabolism was the most enriched (24.10%), followed by Human Diseases (23.75%) and Organismal Systems (19.80%). In Metabolism category, Amino acid metabolism and Energy metabolism was enriched. In Organismal systems, endocrine system was the most enrichment term, followed by the Immune system (Fig. 2).

Identification of DEGs

To identify the DEGs under the ammonia treatment to *E. j. sinensis*, comparative transcriptome analysis of *E. j. sinensis*' hepatopancreas was performed between ammonia group and control group. FPKM method was used to compare relative transcript abundance in each unigene. A total of 1,775 DEGs were identified between NH₃ VS QS on the basis of $|\log_2 FC| > 1.0$ and $FDR < 0.05$, containing 307 up-regulated genes and 1,468 down-regulated genes (Fig. 3).

Analysis of DEGs

A total of 1,775 DEGs were mapped to GO, to understand the functions of DEGs. According to the DEGs of GO analysis, among the five items with significant enrichment, four were subjected to metabolic pathways. DNA metabolism process term (GO:0006259) was the dominant. Nucleic acid metabolic process (GO:0090304), heterocycle metabolic process (GO:0046483), nucleobase-containing compound metabolic process (GO:0006139) and nucleic acid binding (GO:0003676) were followed. In addition to many metabolic pathways being most enriched, such as TCA cycle (ko00020), Oxidative phosphorylation (ko00190), Pentose phosphate pathway (ko00030), and Glycolysis/Gluconeogenesis (ko00010), we also noted enrichment in immune-related items, such as immune response (GO:0006955), antioxidant activity (GO:0016209), and regulation of innate immune response (GO:0045088). Moreover, according to the KEGG analysis of DEGs, we observed that the

Proteasome pathway (ko03050) was the most enriched. In addition, we also focused on immune-related pathways: Lysosome (ko04142) and common immune genes: C-type lectin, and serine/threonine-protein kinase.

Verification of transcriptome data by qRT-PCR

To understand the pathways affected by ammonia stress, five DEGs (Cathepsin B, Cathepsin A, DNA-directed RNA polymerase I subunit RPA2-like, ubiquitin-conjugating enzyme E2-17 kDa-like, deoxyuridine 5'-triphosphate nucleotidohydrolase) were selected randomly for further confirmation by qRT-PCR results. With β -actin gene serving as the reference gene, qRT-PCR analysis was performed. The results showed that the expression patterns of the selected DEGs were consistent with the sequencing results indicating the results were reliable (Fig. 4).

Discussion

Ammonia exposure is one of the most important ambient stressors influencing the survival rate of crustaceans during cultivation process. Although studies on the effects of ammonia in crustaceans are available [5, 7-12, 14, 15, 19, 20], the studies on *E. j. sinensis* treated with a high concentration of ammonia are limited. Under a high concentration of ammonia, there is no difference between the concentration of ammonia in the organism and that in the environment, therefore the toxic nitrogen-containing compounds excreta cannot be disposal. Moreover, as a homologous organ of the mammalian liver and pancreas, hepatopancreas is responsible for major metabolic processes and plays a significant role in immune response [5, 6].

Therefore, comparative transcriptome analysis of hepatopancreas was used in this study and focused on several relevant and relatively enriched parts of the data, protein degradation, immune response, the degradation of nitrogen-containing compounds and energy metabolism.

Protein degradation

In order to adapt a high level of ammonia, aquatic animals tend to clean the damaged proteins in the body to adapt to the new environment [21-23]. In addition, the ubiquitin-proteasome system also plays a key role in many biological processes such as cell cycle control, apoptosis, inflammation, transcription, signal transduction, and protein quality control [24]. In our results, 14 DEGs were involved in Proteasome pathway and all of them were down-regulated (Fig. 5). Moreover, as a member of the Ras superfamily[25], ADP-ribosylation factor activates ADP-ribosyltransferase, which is involved in DNA repair, gene regulation, material transport, signal transduction and protein ubiquitination and degradation[26]. In our study, the expression of ADF was down-regulated. Down-regulated of 14 DEGs involved in Proteasome and ADP-ribosylation factor indicates that protein degradation process was suppressed. The hepatopancreas of *E. j. sinensis* were severely damaged and could not carry out normal metabolism to maintain homeostasis. Therefore, it was further speculated that the overall biological activity of *E. j. sinensis* decreased under this stress because of the high concentration of ammonia.

Immune response

The ammonia stress can contribute to an immune response in crustaceans. However, crustaceans lack acquired immunity and only can rely on innate immunity to cope with environmental stress and pathogen invasion [27-31]. In our study, many immune-related pathways and genes were clearly expressed according to the enrichment of GO analysis. Here, we found in immune response subcategory (GO:0006955), the expression of 14 genes involved were down-regulated and only the remaining five genes were up-regulated. In the antioxidant activity

entry (GO:0016209), the two genes involved were also down-regulated, Hematopoietic prostaglandin D synthase and GST-theta, which were important parts of the antioxidant reaction. According to the DEGs of GO enrichment analysis, the activities of many immune-related subcategories were down-regulated indicating that the overall activity of the immune system of *E. j. sinensis* was decreased and damaged after being exposed to high concentration stress. However, Lysosome (ko04142), important components of innate immunity and strong antimicrobial properties was up-regulated [6, 32]. Moreover, several important immune-related genes were also up-regulated, such as C-type lectin, Cathepsin B, and Cathepsin. C-type lectin, as a pattern recognition receptor, plays an important role in recognizing and eliminating pathogens [6, 14]. The up-regulation of C-type lectin indicated that the immune system of *E. j. sinensis* was damaged partly, which leads to the invasion of foreign pathogens of bacteria and viruses. Thus it was up-regulated to fight against the invasion. Based on the analysis of both GO enrichment and KEGG enrichment, we can conclude that the immune system was partly damaged.

Metabolism of nitrogen compounds

Hepatopancreas is the main metabolic organ, and toxic nitrogen-containing compounds cannot be metabolized causing harm to living organism [12, 33, 34]. According to GO significant enrichment analysis of DEGs, we focused on many metabolic processes that significantly enriched after ammonia stress treatment. Most of genes in these entries were down-regulated. Among metabolism-related items, Nucleic acid metabolic process and Nucleobase-containing compound metabolic process were related to ammonia metabolism. In the Nucleic acid metabolic process item, 181 genes were found to be down-regulated and only 12 genes were up regulated. In the nucleobase-containing compound metabolic process, 195 genes down regulated, while only 14 genes were up regulated. According to KEGG enrichment analysis, Purine metabolism was paid attention[12]. Since all 12 genes were down-regulated in the purine metabolism pathway. Moreover, we found that an enzyme, NAD-specific glutamate dehydrogenase, which catalyzes the conversion of glutamate to A-ketoglutarate accompanied by the production of NH_4^+ in low energy [35, 36]. In our data, the expression of this enzyme was down-regulated, which may be due to the excessive toxic nitrogen content in the body and hence, inhibiting the generation of NH_4^+ . Furthermore, it related to the physiological activity of the Chinese mitten crab.

Energy metabolism

In response to environmental changes, aquatic animals adjust their metabolic process to adapt to the new energy requirements [8], and all life activities are inseparable from the supply of energy. Therefore, we focused on several common pathways involved in energy metabolism: TCA cycle (ko00020), Oxidative phosphorylation (ko00190), Pentose phosphate pathway (ko00030) and Glycolysis/Gluconeogenesis (ko00010). Oxidative phosphorylation and TCA cycle are the main ways in which an organism obtains energy. In addition, although glycolysis produces ATP much less efficiently than the aerobic metabolism of sugar, it is also the primary means of energy synthesis for eukaryotes. Moreover, pentose phosphate pathway is not directly involved in energy synthesis, but the NADPH synthesized by it can generate NADH under the action of enzymes and enter the respiratory chain and hence generating ATP indirectly [37]. Hexokinase type 2, the key enzyme in Glycolysis [38], was down-regulated after high levels ammonia exposure. Also, Glucose-6-phosphate 1-dehydrogenase, the key enzyme of Pentose phosphate pathway [39], was down-regulated in the group of NH_3 . Additionally, both of them involved in the TCA cycle and Oxidative phosphorylation were downregulated (Table 5). As energy is the basis of all life activities in an organism, the down regulation of most key genes and pathway suggested that the Chinese mitten crab were in short supply when facing to ammonia stress. Therefore, the life activities of *E. j.*

sinensis which is consist of reduced feeding rate, oxygen consumption, food absorption efficiency, and scope for growth.

In our study, four parts were investigated, including protein degradation, immune response and metabolism of nitrogen compounds, and energy metabolism. All of which were inhibited. This result was consistent with the results of Meiling Hong's study on the energy metabolism and immune process of juvenile *E. j. sinensis* under ammonia stress [13]. Our study further illustrates the mechanism of the immune response with *E. j. sinensis* after the high level of ammonia exposure

Conclusion

In conclusion, the present study explored the effects of high ammonia concentration on the hepatopancreas mechanism of *E. j. sinensis* at transcriptome level. We demonstrated the molecular mechanism of the Chinese mitten crab response to high ammonia exposure (Fig.6). All the results demonstrated that ammonia exposure led to decreased proteasome activity which can remove misfolded and damaged proteins, mostly impaired immune function, which reflected with related key genes and pathways involved in the basis of all physiological activities, such as degradation of related nitrogen-containing compounds, and significantly down-regulation of energy metabolism. Thus, these results revealed that biological function in the hepatopancreas of *E. j. sinensis* was impaired and its activity in response to high ammonia concentration was decreased, which facilitate the future study on molecular mechanism related to ammonia exposure.

Declarations

Authors' contributions

DT, YW and ZFW designed and conceived the experiment. YW, DT collected and processed the samples. DT, YW, LW, YZ and YZB performed the data analysis and draft the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data supporting the results of this article are included within the article and in its supplementary files.

Competing interests

We confirm that we have read Molecular Biology Reports's guidance on competing interests and have included a statement in the manuscript on any competing interests. The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Tables

Table1 Basic statistics of RNA-seq in *E. j. sinensis*

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Error(%)	Q20(%)	Q30(%)	GC(%)
NH3-1	57007186	8608085086	56955610	8439931659	0.0261	97.36	93.33	50.18
NH3-2	54387044	8212443644	54320304	8017487278	0.0258	97.52	93.62	47.97
NH3-3	55670068	8406180268	55612848	8237447563	0.0271	97.08	92.32	48.31
QS-01	55562130	8389881630	55523076	8249214103	0.0258	97.49	93.58	49.68
QS-02	56991300	8605686300	56935020	8422153754	0.0257	97.61	93.62	43.54
QS-03	56778266	8573518166	56734398	8410663482	0.026	97.46	93.32	45.37

Table2 Summary of assembled RNA-seq results

Type	unigenes	transcripts
Total sequence num	366472	661846
Total sequence base	203552416	478411538
Percent GC	44.04	44.73
Largest	32740	32740
Smallest	201	177
Average	555.44	722.84
N50	679	1067
N90	252	294

Table3 Summary of database search results

type	unigene	transcript
GO	13503	29837
KEGG	11362	25241
NR	20648	46772
PFAM	8927	25314
STRING	14418	33121
SWISSPROT	13634	30120

Table4 The five items with the most enrichment in BP,CC and MF

Term Type	Term	Number
biological process	cellular process	12332
biological process	metabolic process	11166
biological process	biological regulation	8924
biological process	regulation of biological process	8436
biological process	response to stimulus	8374
cellular component	cell	12434
cellular component	cell part	12428
cellular component	organelle	11070
cellular component	organelle part	9346
cellular component	membrane	8268
molecular function	binding	11222
molecular function	catalytic activity	8435
molecular function	structural molecule activity	1589
molecular function	molecular function regulator	1547
molecular function	transporter activity	1257

Table5 Expression of genes in four energy-related KEGG pathways

KEGG	Gene Title	up/down	log2FC(NH3/QS)
Pentose phosphate pathway	glucose-6-phosphate 1-dehydrogenas	down	-17.1483625
Glycolysis / Gluconeogenesis	hexokinase type 2	down	-10.37859381
	ADP-dependent glucokinase	up	7.185408463
TCA cycle	uncharacterized protein LOC106128880	down	-14.27603813
	iron regulatory protein 1	down	-12.17670888
Oxidative phosphorylation	V-type proton ATPase	down	-17.29975304

Figures

Figure 1

Establishment of ammonia response in *E. j. sinensis*.

Figure 2

KEGG pathway enrichment analysis of DEGs.

Figure 3

Analysis of DEGs in the hepatopancreas of *E. j. sinensis* following exposed to the ammonia. The change pattern is displayed as a Scatter plot. The X axis represents the FPKM value [$\log_2$ (fold change)]. Red points represent up-regulated genes and blue points represent down-regulated genes based on the discriminative significance values ($|\log_2FC| > 1.0$ and $FDR < 0.05$) adopted in this study.

Figure 4

Comparison of relative fold change of DGE and qRT-PCR results between ammonia treatment and control group in hepatopancreas of *E. j. sinensis*. Gene abbreviations are as follows: CTS B, cathepsin B; CTS A, cathepsin A; RPA2, DNA-directed RNA polymerase I subunit RPA2-like; UCEE2-17, ubiquitin-conjugating enzyme E2-17 kDa-like; D5-TN, deoxyuridine 5'-triphosphate nucleotidohydrolase.

Figure 5

KEGG pathway mapping of the Proteasome pathway in *E. j. sinensis*. The blue mark represents down-regulated genes during the ammonia stress response.

Figure 6

Effects of ammonia on the expression of protein degradation, immune response, metabolism of nitrogen compounds and energy metabolism related genes in the hepatopancreas.