Fish and Shellfish Immunology 93 (2019) 223–231

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Transcriptome analysis of oriental river Prawn(*Macrobrachium nipponense*) Hepatopancreas in response to ammonia exposure

Jielun Yu[a,b], Jian Sun[a], Shuo Zhao[a], Hui Wang[a,*], Qifan Zeng[c,**]

[a] Aquaculture Research Lab, College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Taian, 271018, China
[b] Experimental Center for Medical Research, Weifang Medical University, Weifang, 261053, China
[c] The Fish Molecular Genetics and Biotechnology Laboratory, School of Fisheries, Aquaculture, and Aquatic Sciences and Program of Cell and Molecular Biosciences, Auburn University, Auburn, AL, 36849, USA

ARTICLE INFO

Keywords:
Macrobrachium nipponense
Ammonia
Transcriptome
Immune response
Hepatopancreas

ABSTRACT

The oriental river prawn, *Macrobrachium nipponense*, is an economically and nutritionally important species of the Palaemonidae family of decapod crustaceans. Ammonia is a major aquatic environmental pollutant that negatively affects the health of prawns and their associated commercial productivity. Here, we used high-throughput sequencing techniques for detecting the effects of ammonia stress (22.1 mg/L ammonia-N for 48 h) on gene expression in the hepatopancreas of *M. nipponense*. We generated 176,228,782 high-quality reads after eliminating adapter sequences and filtering out low-quality reads, which were assembled into 63453 unigenes. Comparative analysis of the expression profiles of the ammonia-treated and control groups identified 887 differentially expressed genes ($P < 0.05$), including 481 upregulated genes and 406 downregulated genes. Analyses of the GO and KEGG databases revealed significant differences between the two groups in 32 pathways. Immune-related pathways under ammonia stress included Complement and coagulation cascades, Platelet activation, B cell receptor signaling pathway, Antigen processing and presentation, Chemokine signaling pathway, NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, T cell receptor signaling pathway and Toll-like receptor signaling pathway. Remarkably, ammonia stress altered the expression patterns of key immune genes (lectin3, syntenin, alpha-2-macroglobulin, cathepsin L, PIM3, serine protease inhibitor, suppressor of cytokine signaling-2 like protein), indicating that ammonia-stress induce immune response. These data provide new insights into the immune response of *M. nipponense* and pave a new way for fighting ammonia stress. The genes and pathways identified here represent valuable genetic resources for development of molecular markers and genetic breeding studies.

1. Introduction

Macrobrachium nipponense is an economically major species cultured by Chinese inland fisheries [1]. The annual production of farmed oriental river prawns approximates 20 billion tons (Bureau of Fisheries, Ministry of Agriculture, 2014). *M. nipponense* is relatively sensitive to ammonia exposure, which raises concerns because ammonia is a major environmental pollutant encountered during the aquaculture of prawns [2,3]. Ammonia can be removed by biological filtration or water exchange. However, a transient, sudden, and rapid increase in ammonia concentrations may have toxic effects on prawns [4]. For example, excess ammonia can increase susceptibility to pathogens, which inhibits growth, decreases osmoregulation, increases molting frequency, and causes high mortality [5]. However, the molecular mechanisms involved in ammonia detoxification remain unknown. Unfortunately, there are few physiological or molecular biological studies on the responses of *M. nipponense* to ammonia stress [6].

Ammonia is a common contaminant encountered during aquaculture and is the main limiting factor in the aquaculture of *Litopenaeus vannamei* [7], *Penaeus monodon* [8], *Farfantepenaeus paulensis* [9], and *Penaeus semisulcatus* [10]. Ammonia is typically present in water as ammonium ion (NH^{4+}) and unionized ammonia (NH_3), and the latter is more toxic than ammonium (NH^{4+}) because the former can diffuse through cell membranes [11]. Ammonia stress may reduce growth rates, suppress the immune system, and increase molting frequency, which can lead to high mortality [12–14]. Further, damage to the gills and the hepatopancreas of shrimp reduces the ability of hemolymph to transport oxygen, while increasing oxygen consumption [15,16].

* Corresponding author.
** Corresponding author.
E-mail addresses: wanghui2328@sdau.edu.cn (H. Wang), zengqifan@ouc.edu.cn (Q. Zeng).

https://doi.org/10.1016/j.fsi.2019.07.036
Received 25 February 2019; Received in revised form 7 July 2019; Accepted 13 July 2019
Available online 15 July 2019

Importantly, it has been revealed that increased ammonia in the water could inhibit the immune system and increase the susceptibility of shrimp to pathogens [17,18]. For example, the mortality of *Vibrio*-injected shrimp in the presence of ammonia for 48–168 h is significantly higher compared with *Vibrio*-injected and uninjected shrimp not treated with ammonia [15,16]. Mortality increases as a function of increasing ammonia concentrations and with time of exposure [15,16]. Shellfish such as abalone [19] and yellow catfish similarly suffer from exposure to excess ammonia [20].

Whole transcriptome shotgun sequencing (RNA-seq) is a highly sensitive technique that is frequently used to profile and annotate the transcriptome to identify the molecular mechanism of a specific physiological process through detection of genes and pathways involved in the corresponding physiological function [21–23]. Specifically, RNA-seq is widely applied to studies of the effects of stress on shrimp [24] and crabs [25].

High concentrations of ammonia inflict heavy damage to the hepatopancreas of shrimp. Consequently, here we employed RNA-seq to identify the genes and pathways that respond to ammonia stress in the hepatopancreas of *M. nipponense*. Comparative transcriptome analysis was performed using the Illumina sequencing platform to identify differences in gene expression between ammonia-challenged shrimp (treatment group) and shrimp exposed to normal environmental concentrations of ammonia (control group). This study represents the first, to our knowledge, to investigate the molecular responses to ammonia stress of the hepatopancreas of *M. nipponense* at the level of the entire transcriptome.

2. Materials and methods

2.1. Animals and ammonia-N exposure experiments

M. nipponense were collected from Weishan Lake, Shandong Province, China and acclimated in a 100 L tank at Shandong Agricultural University for 1 week. During acclimation, prawns were fed particulate commercial food twice daily (40% protein, 5.0% fat, 5.0% fiber and 16% ash, supplied by a commercial diet, China), and half the volume of water was changed daily to maintain water quality. The water temperature, pH, and dissolved oxygen in the tank were 20.0 ± 0.5 °C, 8.2 ± 0.05, and 6.5 ± 0.5 mg/L, respectively. The concentration of ammonia-N was 22.1 mg/L, which is the half-lethal dose for a 48 h exposure. This dose was optimal for measuring changes in gene expression when shrimp were exposed to ammonia stress. After acclimation, 200 prawns were divided into two groups and each group was separated to 3 tanks. 100 randomly selected shrimp maintained under normal conditions were assigned to the control group (CG), and the other 100 shrimp were exposed to ammonia stress for 48 h and designated the treatment group (TG). The desired concentration of ammonia was achieved by adding a stock solution of NH_4Cl to the water according to a published procedure [26]. We chose to focus on the hepatopancreas, because it is the most important immune organ in prawns. Hepatopancreatic tissue of shrimp subjected to 48 h of ammonia stress was selected for transcriptome analysis. The hepatopancreases of shrimp were dissected separately and immediately frozen in liquid nitrogen. Shrimp (n = 6) with similar sizes were assigned to each group.

2.2. Histopathology

The hepatopancreas of the *M. nipponense* was selected from the treatment group and control group. It was fixed in 0.1 M phosphate-buffered saline (pH 7.4) with 4% paraformaldehyde and fixed at room temperature for 24 h. Hepatopancreas tissue were dehydrated in ethanol with graded concentration and embedded in paraffin wax. The embedded tissue will be fixed on the microtome and crosssections of 5 mm were prepared. The sections were stained with hematoxylin and eosin and observed under the light microscopy (Olympus, Japan).

2.3. RNA extraction, library construction and sequencing

Total RNA was extracted from the hepatopancreas from CG/TG at 48 h of each sample using Trizol Reagent (Invitrogen, Shanghai, China) according to the manufact. RNA integrity was checked with 1.5% agarose gel electrophoresis. All RNA samples were high quality (OD260/280 = 2.10–2.15, OD260/230 ≧ 2.0).

RNA from three individuals of each group was pooled for constructing a sequencing library. In total, four sequencing libraries were constructed in the present study, two libraries (K1, K2) from TG groups and other two (T1, T2) from CG groups. All RNA-seq procedures occurred in the Novogene Company (Beijing, China). The library was constructed and then checked in an Agilent 2100 Bioanalyzer (Agilent, 2100). Resultant cDNA libraries were sequenced on the Illumina HiSeq platform. The RNA-seq data was deposited into NCBI SRA database, and its accession is SRP186348.

2.4. Transcriptome assembly and gene function annotation

Sequencing of the original sequencing sequence (Sequenced Reads) or raw reads, which contain joints, low-quality reads, in order to ensure the quality of information analysis, the raw reads filter, get clean reads, the subsequent analysis is based on clean reads. The clean reads are spliced using Trinity [27]. Trinity is a highly efficient and stable transcriptome splicing software for RNA-seq data. The transcript sequence obtained by splicing Trinity was used as a reference sequence for subsequent analysis. The longest transcript in each gene was taken as Unigene for subsequent analysis.

All transcripts were compared with the NCBI non-redundant (nr) protein database, GO database, COG database, and KEGG database for functional annotation using BLAST software with an e-value cutoff of $1 \times e-5$. Functional annotation was performed with gene ontology (GO) terms (www.geneontology.org) that were analyzed using Blast2 GO software (http://www.blast2go.com/b2ghome) [28]. The COG and KEGG pathway annotations were performed using Blast software against the COG and KEGG databases.

2.5. Analysis of differentially expressed genes (DEGs) and functional enrichment

The input data for differential expression of genes is the readcount data obtained in the analysis of gene expression levels. For samples with biological replicates, we analyzed using DESeq [29] with a screening threshold of padj < 0.05. During the difference analysis of differential software, we will correct the p-value obtained from the original hypothesis test. Functional enrichment analysis (GO and KEGG) was performed in GOseq and KOBAS (2.0) [30,31], respectively. Candidate genes involved in the ammonia stress response were identified from DEGs to significantly enrich the GO terminology and the KEGG pathway.

2.6. Validation by quantitative real-time PCR (RT-qPCR)

RT-qPCR was used to validate the partial differential expressed genes (DEGs) identified using RNA-Seq. Primers were designed using Primer Premier 6 according to the sequencing data (Table S1). RT-qPCR reactions were carried out using the STRATAGENE MX3000 Real-time System (USA) and the β-actin gene was used as internal reference gene. The PCR was performed using SYBR Premix Ex Taq kit (TaKaRa, Dalian, China), and the PCR reaction systems (20 μl) consists of 10 μl SYBR Premix Ex Taq (2 ×), 0.4 μl of each gene specific primer (10 nmol), 2 μl cDNA, 0.4 μl ROX reference dye II. RT-qPCR was performed in a total volume of 20 mL, and cycling conditions were 95 °C for 1 min and followed by 40 cycles of 94 °C for 5s, 59 °C for 15s and 72 °C for 20s. Each

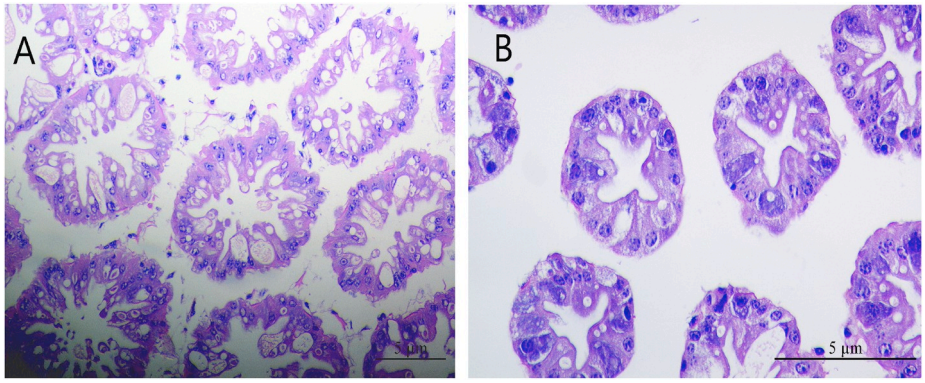

Fig. 1. The structure of hepatopancreas of *M. nipponense* after 48 h exposure to ammonia (22.1 mg/L). (A) Hepatopancreas from the treatment group of *M. nipponense* after 48 h ammonia stress; (B) Hepatopancreas from the control group.

Table 1
The RNA-Seq data of samples.

Samples	Read Number	GC Content	%≥Q30	Clean Reads
T1	43,203,648	49.34%	92.99%	41,426,642
T2	44,037,488	48.86%	92.78%	41,866,528
K1	46,317,414	49.39%	93.36%	44,613,488
K2	50,318,624	47.84%	93.49%	48,322,124

sample was run in triplicates. At the end of each PCR reaction, the dissociation curve analysis of the amplification products was done. Amplicons were verified through melting-curve analysis. Relative expression was determined with the comparative CT method ($2^{-\Delta\Delta CT}$).

3. Results

3.1. Histological changes of M. nipponense hepatopancreas after ammonia stress

To analyze the effects of ammonia stress on hepatopancreas morphology, the histological structure of *M. nipponense* from control and treatment groups was observed. After 48 h ammonia stress (22.1 mg/L), the paraffin sections showed that the hepatopancreas of the control group exhibited a well-organized glandular tubular structure. The lumen of the tubule is in the shape of an asterisk-like appearance. Epithelial cells are neatly arranged in the lining of the tubules (Fig. 1B). The gap between the adjacent glands becomes larger of the treatment group. Glandular epithelial cells of hepatopancreas under ammonia-nitrogen stress have different degrees of damage and partial shedding. A small number of fractures occur in the microvilli (Fig. 1A).

3.2. Transcriptome sequencing and assembly

The RNA-Seq results of the transcriptomic response to ammonia stress in *M. nipponense* is shown in Tables 1 and 2. We generated 183,877,174 raw reads, including 87,241,136 and 96,636,038 reads from the hepatopancreas of the CG group and the TG group respectively. After removing low-quality or contaminated reads, 176,228,782 clean reads (95.84% of total reads) were used for the following transcriptome assembly, with Q30 > 92.78%. We obtained 75,742 transcripts and 63,453 unigenes, and the N50 of transcripts and unigenes were 1810 and 1396, respectively.

Table 3
Summary of the annotations of the unigenes.

	Number of Unigenes	Percentage (%)
Annotated in NR	18556	29.24
Annotated in NT	5854	9.22
Annotated in KO	9043	14.25
Annotated in SwissProt	15291	24.09
Annotated in PFAM	17369	27.37
Annotated in GO	17611	27.75
Annotated in KOG	10921	17.21
Annotated in all Databases	2311	3.64
Annotated in at least one Database	24194	38.12
Total Unigenes	63453	100

3.3. Functional annotation and classification of transcriptome

To obtain comprehensive information on gene function, we performed gene function annotations of the databases as follows: Nr, Nt, Pfam, KOG/COG, Swiss-prot, KEGG, GO. Significant annotations of 63,453 unigenes were identified (Table 3). We identified 18,556 (29.24%), 17,611 (27.75%), 15,291 (24.09%), 10,921 (17.21%) and 9043 (14.25%), genes homologous to the sequences in the Nr, GO, Swiss-Prot, KOG and KEGG databases, respectively. All unigenes were functionally annotated, with 2311 (3.64%) matched to all databases and 21,194 (38.12%) matched to at least one database. Fig. 2 shows that 2858 unigenes are annotated in the top five databases.

The GO analysis revealed that the unigenes clustered according to molecular functions, biological processes, and cellular components (Fig. 3). The metabolic process (8694 unigenes), cellular process (9513 unigenes), and single-organism process (7429 unigenes) represented the majority category of biology process. The cell part (5366 unigenes), cell (5366 unigenes), organelle (3744 unigenes) and macromolecular complex (3489 unigenes) represented the majorities of cellular component. Catalytic activity (6976 unigenes) and binding (9503 unigenes) had the highest percentages in the molecular function category.

The unigenes were mapped into the KEGG pathway database to predict the significantly enriched pathways. There 9043 unigenes

Table 2
Summary of de novo assembly results.

	Min Length	Mean Length	Median Length	Max Length	N50	N90	Total Nucleotides
Transcripts	201	891	402	16209	1810	306	67481289
Unigenes	201	745	359	16209	1396	270	47277135

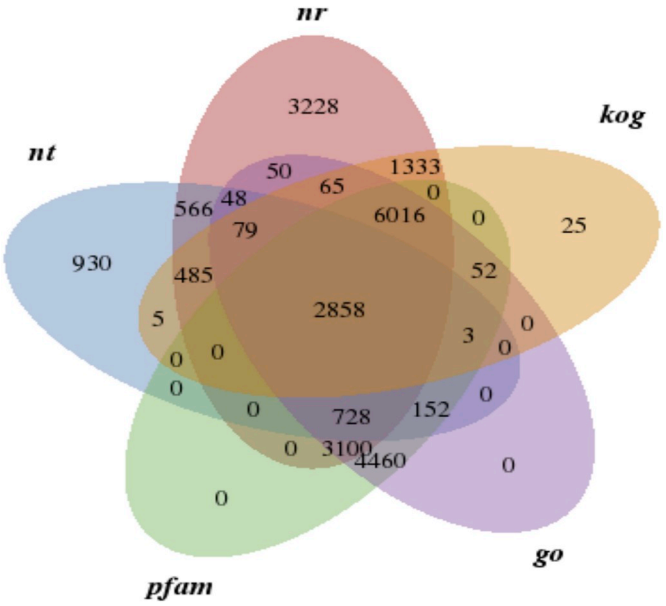

Fig. 2. Venn diagram showing overlap of NR, NG, KOG, GO, and PFAM; numbers in each circle indicate the amount of annotated unigenes.

assigned to different pathways in five major groups of KEGG pathways, including Metabolism, Organismal Systems, Cellular Processes, Genetic Information Processing and Environmental Information Processing. The 32 most abundant KEGG pathways are shown in Fig. 4. The top five KEGG pathways are signal transduction, translation, transport and catabolism, immune system and endocrine system. Among these genes, 1203 were related to signal transduction and 575 were related to immune system.

3.4. Analysis of differential gene expression

We identified 887 significantly differential genes (q-value < 0.005 and |log2.Fold_change| > 1), which included 481 upregulated and 406 downregulated genes. A heatmap depicting the majority of differentially expressed genes (DEGs) was presented in Fig. 5.

According to nr annotations, DEGs were implicated in immunity and apoptosis. Upregulated genes associated with immunity and apoptosis related were linked to lectin 3, syntenin, G-protein coupled receptor, heat shock protein (HSP90A), alpha-2-macroglobulin (A2M), cathepsin C, serine protease inhibitor, suppressor of cytokine signaling-2 like protein (SOCS-2), cathepsin L, PIM1, ADP-ribosylation factor, cytochrome *c*, caspase 2, ATF-b. These genes were significantly expressed at higher levels after 48 h of ammonia stress (Fig. 6).

Many DEGs were contribute to detoxification processes and metabolic pathways, such glutathione S-transferase, L-lactate dehydrogenase, aldehyde dehydrogenase type III, hexokinase, fructose 1,6-biphosphate-aldolase. These genes will be the focus of further research (Table 4). These genes specifically respond to ammonia stress, indicating that they stimulate or induce the immune system of prawns to cope with environmental stress. The remainder of the proteins were associated with osmoregulation and the growth response to stress.

3.5. Gene ontology and KEGG analyses of DEGs

DEGs were further analyzed using GO and KEGG annotations to identify their potential functions and metabolic pathways. All DEGs were successfully mapped to GO terms, revealing 24,782 enriched GO terms in biological process, cellular component, and molecular function. For biological process, metabolic process (375), single-organism

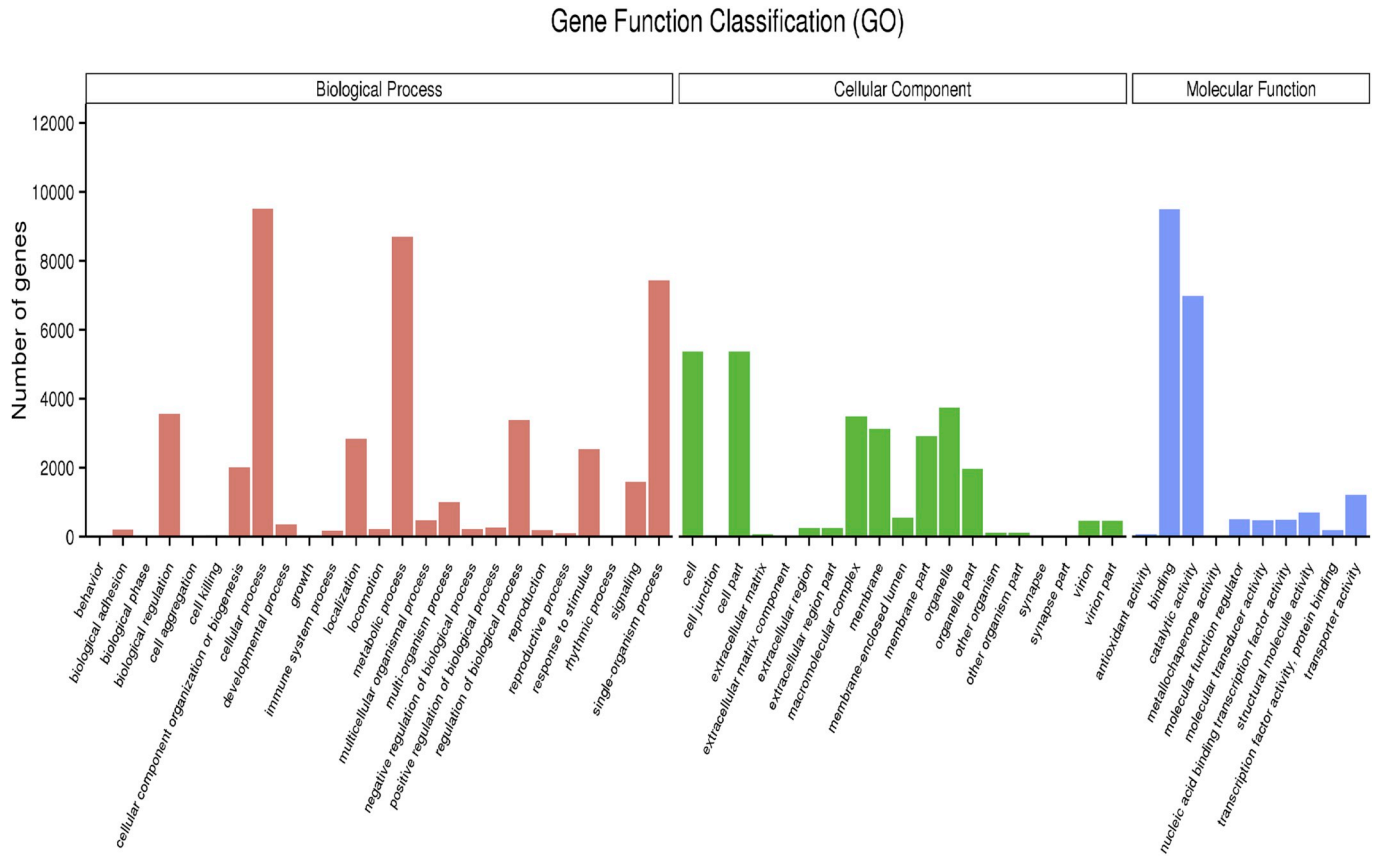

Fig. 3. GO annotation of transcripts. Three major GO categories were enriched: biological process, cellular component, and molecular function.

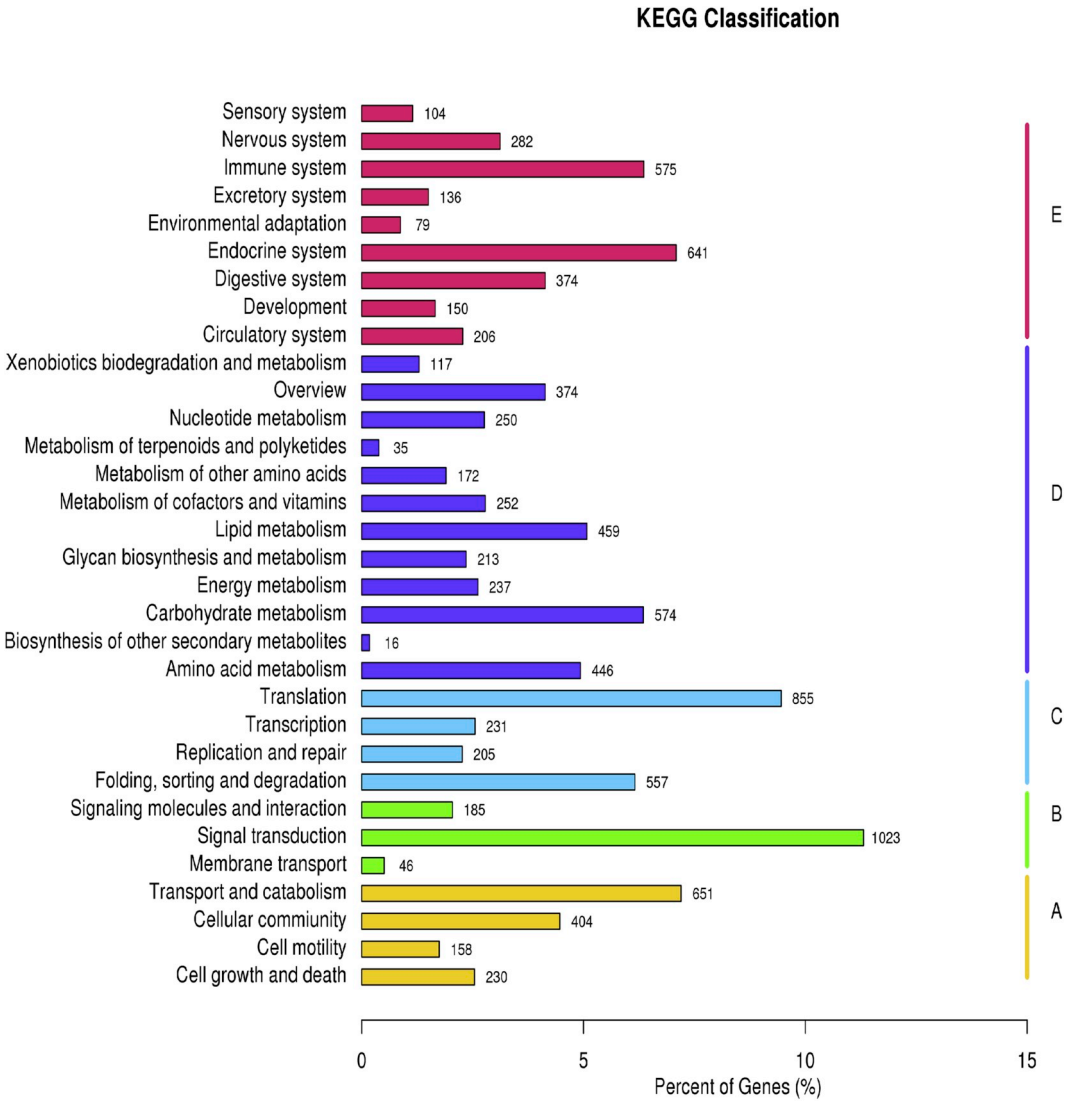

Fig. 4. KEGG annotation of transcripts. Five major pathways were enriched: A, Cellular Processes; B, Environmental Information Processing; C, Genetic Information Processing; D, Metabolism; E, Organismal Systems.

process (344) and cellular process (333) had the most abundant GO function, and cell (179), cell part (179) and membrane (177) were most abundant in cellular component. In molecular function, catalytic activity (331) and binding (310) comprised more GO items than the others.

The DEGs were mapped to 219 pathways. According to the threshold of P value < 0.05, the top 10 KEGG significant pathways were list in Table S2. They are Complement and coagulation cascades, Drug metabolism-cytochrome P450, Chemical carcinogenesis, Platelet activation, Ascorbate and aldarate metabolism, Propanoate metabolism, Metabolism of xenobiotics by cytochrome P450, Protein digestion and absorption, Glycolysis/Gluconeogenesis, PPAR signaling pathway. Among those pathways, some closely related to immunity and apoptosis, metabolism and Cell, such as Complement and coagulation cascades, Platelet activation and PPAR signaling pathway.

3.6. Validation of RNA-seq with RT-qPCR

We randomly selected 15 genes for RT-qPCR validation from those with different expression patterns based on our analysis of functional enrichment and pathway results to validate the accuracy and reliability of DEGs identified using RNA-Seq. The expression patterns of these 15 genes were consistent with the RNA-seq results (Fig. 7), which suggest that the results of the RNA-seq experiments were accurate and reliable.

4. Discussion

M. nipponense is an economically and nutritionally important aquaculture species in China, Japan, and South-East Asian countries [32]. Increased ammonia in the water has heavy detrimental effects on prawns. However, limited information was available for the molecular mechanism of the detrimental effects of ammonia stress to *M. nipponense*. Hence, the present findings are highly significant for understanding the molecular mechanism underlying the response of *M. nipponense* to ammonia stress. This present study is the first RNA-seq analysis, to our knowledge, that identifies the key genes and complex pathways associated with the response to ammonia stress by *M. nipponense*. This information contributes new insights into the mechanisms through which shrimp, and likely other organisms, adapt to ammonia stress at the molecular level.

The study generated 176,228,782 clean reads (95.84%), and identified 887 DEGs. GO annotation and KEGG pathways analysis revealed that the DEGs were involved in metabolic process, single-organism process and cellular process of biological process, as well as the cell, cell part and membrane of cellular component, the catalytic activity and binding of molecular function. Numerous key genes and numbers of

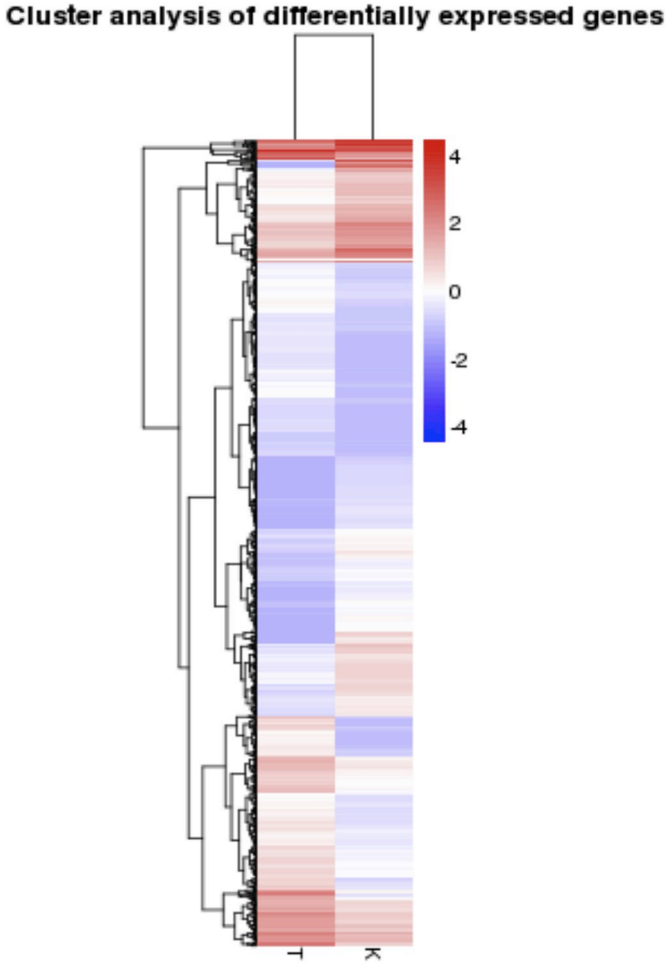

Fig. 5. Hierarchical clustering for the differentially expressed genes between Treatment group and Control group. Different columns in the figure represent different samples, and different rows represent different genes (T:TG. K: CG). The color represents the logarithm of the expression level of the gene in the sample FPKM. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

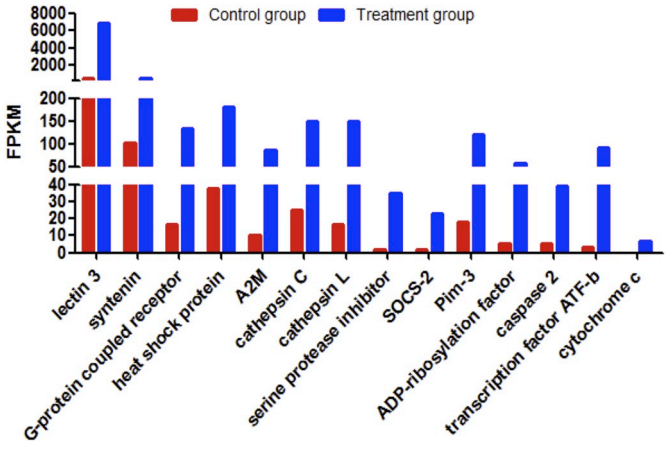

Fig. 6. Expression profiles of 14 genes involved in immune, displayed on the basis of their FPKM values from RNA-seq data from the control–treatment comparison. The FDR values of all comparisons are < 0.05.

important pathways that may be related to the immunity and metabolic process were obtained in the current transcriptome data.

In the current study, DEGs upregulated by ammonia stress that are associated with immune-related pathways including Complement and

Table 4
DEGs related to immunity and detoxification processes.

Gene ID	Gene Name	Up or Down
c16662_g1	lectin 3	up
c22523_g1	syntenin	up
c23735_g1	G-protein coupled receptor	up
c23092_g1	heat shock protein	up
c26319_g1	A2M	up
c7786_g1	cathepsin C	up
c18100_g1	cathepsin L	up
c18521_g1	serine protease inhibitor	up
c22215_g1	SOCS-2	up
c824_g1	Pim-3	up
c20264_g1	ADP-ribosylation factor	up
c25462_g3	caspase 2	up
c25343_g1	transcription factor ATF-b	up
c11459_g1	cytochrome *c*	up
c23655_g1	glutathione S-transferase	up
c24532_g2	L-lactate dehydrogenase	up
c25957_g1	aldehyde dehydrogenase type III	up
c26322_g2	hexokinase	up
c6178_g1	fructose 1,6-biphosphate-aldolase	up
c26164_g1	glucose transporter 1	down
c21895_g2	aquaporin	down
c26825_g1	integrin	down
c16631_g1	glutathione S-transferases	down
c3745_g1	hemocyanin	down
c25045_g1	ecdysteroid-regulated protein	down
c26866_g1	JHE-like carboxylesterase 1	down
c20785_g1	macrophage migration inhibitory factor	down

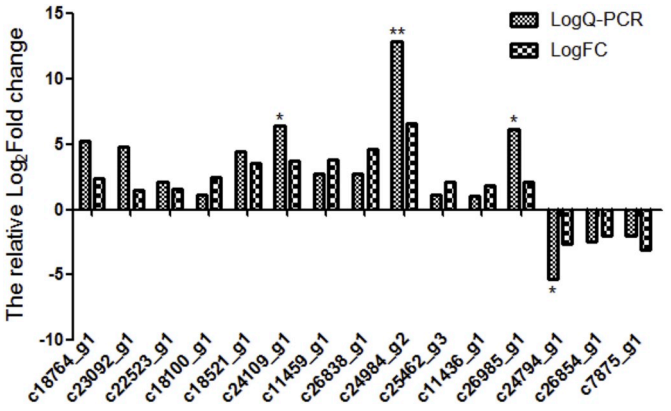

Fig. 7. Candidate unigene expression levels revealed by qRT-PCR and RNA-seq. They are showing the same expression trend.

coagulation cascades, Platelet activation, B cell receptor signaling pathway, Antigen processing and presentation, Chemokine signaling pathway, NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway, T cell receptor signaling pathway and Toll-like receptor signaling pathway under the ammonia stress were upregulated in this study. The Complement and coagulation cascades pathway is a central component of innate immunity [33,34] that plays multiple roles in immune responses, such as chemotaxis, cell activation, lysis of antigens and opsonization. The complement system, which was discovered in 1896 by Jules Bordet [35], is present in vertebrates and invertebrates, and complement-like activity is present in diverse invertebrates [36,37]. Ammonia exposure can lead to oxidative stress in aquatic animals [38], leading to the induction of an immune response, extensive DNA damage, and even apoptosis [2,39]. Complement proteins play an important role in mediating tissue injury and inflammatory process after oxidative stress https://www.sciencedirect.com/science/article/pii/S1050464811003585 [40,41]. Moreover, increasing evidence indicates that reactive oxygen species (ROS) activate the complement system https://www.sciencedirect.com/science/article/pii/S1050464811003585 [42].

The hepatopancreas of crustaceans serves as the key metabolic centre for the production of ROS, plays a major role in immune defenses https://www.sciencedirect.com/science/article/pii/S1050464811003585 [43], and mediates the synthesis of digestive enzymes and the detoxification of xenobiotics https://www.sciencedirect.com/science/article/pii/S1050464811003585 [44]. Therefore, we investigated the hepatopancreas of *M. nipponense*. Alpha-2-macroglobulin (A2M), an abundant and multifunctional protein is a broad-spectrum protease inhibitor that belongs to the ThioEster containing Protein (TEP) super-family [45]. A2M is an important effector of the complement and coagulation cascades pathway, which is involved in immune responses in invertebrates https://www.sciencedirect.com/science/article/pii/S1050464816307914 [46]. A2M has been reported its presence in shrimp such as *Marsupenaeus japonicus* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [47], *P. monodon* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [48], *L. vannamei* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [49], *Macrobrachium rosenbergii* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [50], *Fenneropenaeus chinensis* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [51], *Farfantepenaeus paulensis* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [52], and *Fenneropenaeus indicus* https://www.sciencedirect.com/science/article/pii/S1050464816307914 [53], as well as from the crab *Scylla serrata* https://www.sciencedirect.com/science/article/pii/S0145305X10000273?via=ihub [54]. The expression of A2M gene is highly upregulated upon bacterial, fungal and viral infection [51,52]. The research on complement system is rare in crustaceans. In our study, there was a significant increase in the expression of A2M in the hepatopancreas of *M. nipponense* after exposure to ammonia stress, indicating that A2M is sensitive to ammonia stress, which may represent a response that has evolved to neutralize harmful free radicals generated by stressors. The mechanism whereby ammonia stress stimulates A2M transcription is unknown. Our results suggest that environmental stress such as caused by ammonia, may induce the expression of A2M, after which the complement system components cooperate to help organisms to resist stress and survive. The RIG-I-like receptor and Toll-like receptor, as innate immune-recognition receptors that recognize molecular patterns associated with microbial pathogens, and each receptor plays a critical role in antimicrobial immune responses. The activities of the RIG-I-like receptor are regulated by post-translational modifications, such as ubiquitination and phosphorylation [55]. The Toll-like receptor play a unique and essential function the immune systems of animals and belong to the family of type I transmembrane receptors [56]. These receptors are expressed by *L. vannamei* https://www.sciencedirect.com/science/article/pii/S0165247813001958 [57], *F. chinensis* https://www.sciencedirect.com/science/article/pii/S0165247813001958 [58], *M. japonicus* https://www.sciencedirect.com/science/article/pii/S0165247813001958 [59]. Therefore, we speculate that the immunity of shrimp is impaired under ammonia stress, thereby increasing the risk of infection by bacterial and viruses.

Besides the above key genes enriched in the significant KEGG pathways, many other DEGs between CG and TG were potentially involved in immune and have been previously reported in other crustaceans, such as hemocyanin, serine proteinase inhibitors, crustin, lysozyme, G protein-coupled receptor, prophenoloxidase-2, and peritrophin etc [38,60,61]. In addition to genes associated with immune processes mentioned above, several genes also exhibited significant differential expression upon ammonia exposure, such as cathepsin L, Pim-3, lectin 3, syntenin. Lectin 3 is a kind of C-type lectins (CTL). In recent years, CTLs have been reported to participate in innate immunity against pathogens of shrimp. For example, LvCTL3 from the *L. vannamei*, significantly reduces mortality associated with infection by *Vibrio parahaemolyticus* white spot syndrome virus (WSSV) [62]. MnCTLDcp2 and MnCTLDcp3 function as pattern recognition receptors involved in the innate immunity of the *M. nipponens*e [63]. Four CTLs from the giant freshwater prawn *M. rosenbergi*i play vital roles in innate immunity against WSSV challenge [64]. Syntenin is a multifunctional cytosolic adaptor protein that contributes to cell migration, proliferation, attachment, and apoptosis, as well as immune response to viruses by invertebrates [65,66]. Research has shown that syntenin was upregulated in infected shrimp, and its levels rapidly decline during infection [66]. Pim-3 exhibits serine/threonine kinase activity. The previous study reported that the overexpression of PIM kinases leads to tumor progression, and Pim-3 is aberrantly expressed in numerous malignant tumors [67]. Cathepsin L is involvement in the innate immunity. Furthermore, cathepsin gene has been reported in some invertebrates, such as *Haliotis discus hannai*, *Procambarus clarkii*, *L. vannamei* and *P. monodon* [68–70].

The results in the present study reveal the gene and pathway responses ammonia stress, and the expression levels of the genes of the prawn would be changed under the ammonia stress (up or down). Generally, defense mechanisms are activated when organisms are exposed to environmental toxicants, among which cell death/apoptosis is usually triggered for defensing against stress or pathogen. Consequently, we deduce that the ammonia stress can induces prawns to mount a immune response that mitigates the adverse effects of environmental stress and helping prawn recover the damages from environmental stress. These speculations merit further investigation in future studies. From these results, we can conclude that further research on the DEGs and pathways related to immunity and apoptosis has great potential for improving the density, yield and economic benefits of intensive production of *M. nipponense*. Our findings provide some clarification of the immune mechanisms that engage during ammonia exposure. We believe that these findings will benefit future research to fully understand the mechanism of the response of *M. nipponense* to ammonia stress.

5. Conclusions

This study is the first to provide transcriptome-level analysis of key genes and metabolic pathways in *M. nipponense* ammonia-stress response. A total of 887 unigenes changed significantly after 48 h of ammonia exposure. Immunization, cellular activity, metabolism, signaling, and genetic material-related transcripts are clearly detected. We observed differentially expressed genes and pathways related to immune function, metabolism, and the like. Our data indicate that ammonia exposure induces oxidative stress, thus triggering related pathway, such as complement and coagulation cascades pathways. Complement system products cooperatively helping organisms resist stress, thereby enabling individual to survive ammonia stress. These genes and pathways are involved in defense responses under ammonia stress, and these DEGs can be used as molecular immunological indicators under crustacean ammonia stress. Transcriptome analysis also produced a large number of new transcripts that laid the foundation for further genomic research in the future. Well-categorized and annotated *M. nipponense* transcriptome could serve as important and valuable resources for gene identification and functional analysis.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Authors' contributions

The first authors Jielun Yu, Jian Sun contributed equally to this work.

Acknowledgements

This work was financially supported by the key project of Agricultural Fine Breeding of Shandong Province (Identification and analysis of genes involved in growth rate and stress tolerance of *Macrobrachium nipponense* for germplasm resource exploitation), the Modern Agricultural Industry Technical System of Shandong Province

(SDAIT-13-02). The Postgraduate education innovation projects of Shandong Province(SDYY11083; SDYC12048), Agricultural expert advisory group of Shandong Province, and the "Double First-Class Discipline" Construction Foundation of Shandong Province.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.036.

References

[1] H.T. Fu, S. Jiang, Y. Xiong, Current status and prospects of farming the giant river prawn (*Macrobrachium rosenbergii*) and the oriental river prawn (*Macrobrachium nipponense*) in China, Aquacult. Res. 43 (2012) 993–998.

[2] Z.X. Liang, R. Liu, D.P. Zhao, L.L. Wang, M.Z. Sun, M.Q. Wang, et al., Ammonia exposure induces oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*), Fish Shellfish Immunol. 54 (2016) 523–528, https://doi.org/10.1016/j.fsi.2016.05.009.

[3] W. Cheng, J.C. Chen, The virulence of Enterococcus to freshwater prawn *Macrobrachium rosenbergii* and its immune resistance under ammonia stress, Fish Shellfish Immunol. 12 (2002) 97–109.

[4] J.C. Chen, P.C. Liu, S.C. Lei, Toxicities of ammonia and nitrite to *Penaeus monodon* adolescents, Aquaculture 89 (2) (1990) 127–137, https://doi.org/10.1016/0044-8486(90)90305-7.

[5] G.J. Jiang, R.C. Yu, M.J. Zhou, Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus, Aquaculture 241 (2004) 61–75, https://doi.org/10.1016/j.aquaculture.2004.08.020.

[6] Z. Ding, Y. Kong, Y. Zhang, J. Li, F. Cao, J. Zhou, J. Ye, Effect of feeding frequency on growth, body composition, antioxidant status and mRNA expression of immunodependent genes before or after ammonia-N stress in juvenile oriental river prawn, *Macrobrachium nipponense*, Fish Shellfish Immunol. 68 (2017) 428–434, https://doi.org/10.1016/j.fsi.2017.07.045.

[7] María de Lourdes Cobo, S. Sonnenholzner, M. Wille, P. Sorgeloos, Ammonia tolerance of *Litopenaeus vannamei* (Boone) larvae, Aquacult. Res. 45 (3) (2014) 470–475, https://doi.org/10.1111/j.1365-2109.2012.03248.x.

[8] J.C. Chen, S.C. Lei, Toxicity of ammonia and nitrite to *Penueus monodon* juveniles, J. World Aquac. Soc. 21 (4) (2010) 300–306, https://doi.org/10.1111/j.1749-7345.1990.tb00543.x.

[9] A. Ostrensky, W. Wasielesky, Acute toxicity of ammonia to various life stages of the São Paulo shrimp, *Penaeus paulensis* Pérez-Farfante, Aquaculture 132 (3) (1967) 339–347, https://doi.org/10.1016/0044-8486(94)00343-M 1995.

[10] N. Wajsbrot, M.D. Krom, T.M. Samocha, A. Gasith, Effect of dissolved oxygen and the molt stage on the acute toxicity of ammonia to juvenile green tiger prawn *Penaeus semisulcatus*, Environ. Toxicol. Chem. 9 (4) (2010) 497–504, https://doi.org/10.1002/etc.5620090413.

[11] K. Emerson, R.C. Russo, R.E. Lund, R.V. Thurston, Aqueous ammonia equilibrium calculations: effect of pH and temperature, J. Fish. Res. Board Can. 32 (12) (1975) 2379–2383.

[12] J.C. Chen, Y.Z. Kou, Effects of ammonia on growth and molting of *Penaeus japonicus* juveniles, Aquaculture 104 (3–4) (1992) 249–260, https://doi.org/10.1016/0742-8413(92)90068-I.

[13] A. Maas, B.A. Seibel, P.J. Walsh, Effects of elevated ammonia concentrations on survival, metabolic rates, and glutamine synthetase activity in the Antarctic pteropod mollusk *Clione limacina* Antarctica, Polar Biol. 35 (7) (2012) 1123–1128, https://doi.org/10.1007/s00300-012-1158-7.

[14] Y. Cui, X. Ren, J. Li, Q. Zhai, Y. Feng, Y. Xu, L. Ma, Effects of ammonia-N stress on metabolic and immune function via the neuroendocrine system in *Litopenaeus vannamei*, Fish Shellfish Immunol. 64 (2017) 270–275, https://doi.org/10.1016/j.fsi.2017.03.028.

[15] I.S. Racotta, R. Hernández-Herrera, Metabolic responses of the white shrimp, *Penaeus vannamei*, to ambient ammonia, Comp. Biochem. Physiol. Mol. Integr. Physiol. 125 (4) (2000) 437–443.

[16] U. Wongsasak, S. Chaijamrus, S. Kumkhong, S. Boonanuntanasarn, Effects of dietary supplementation with β-glucan and synbiotics on immune gene expression and immune parameters under ammonia stress in Pacific white shrimp, Aquaculture 436 (2015) 179–187, https://doi.org/10.1016/j.aquaculture.2014.10.028.

[17] D.Q. Qiu, X.J. Zhou, M.S. Qiu, Study on Anti-disease ability of *Litopenaeus vannamei* and the biological control of *vibrio* parahaemolyticus bacteriophage under stresses of ammonia nitrogen, Acta Hydrobiol. Sin. 32 (4) (2008) 455–461, https://doi.org/10.3724/SP.J.1035.2008.00455.

[18] C.H. Liu, J.C. Chen, Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* alginolyticus, Fish Shellfish Immunol. 16 (2004) 321–334, https://doi.org/10.1016/S1050-4648(03)00113-X.

[19] W. Cheng, I.S. Hsiao, J.C. Chen, Effect of ammonia on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*, Fish Shellfish Immunol. 17 (2004) 193–202, https://doi.org/10.1016/j.fsi.2004.03.004.

[20] M. Li, S.Y. Gong, Q. Li, L.X. Yuan, F.X. Meng, R.X. Wang, Ammonia toxicity induces glutamine accumulation, oxidative stress and immunosuppression in juvenile yellow catfish *Pelteobagrus fulvidraco*, Comp. Biochem. Physiol., C 183 (2016) 1–6, https://doi.org/10.1016/j.cbpc.2016.01.005.

[21] S.K. Kim, Z. Pang, H.C. Seo, et al., Effect of bioflocs on growth and immune activity of Pacific white shrimp, *Litopenaeus vannamei* postlarvae, Aquacult. Res. 45 (2014) 362–371, https://doi.org/10.1186/s40064-015-1234-x.

[22] J. Xu, P. Ji, B. Wang, et al., Transcriptome sequencing and analysis of wild Amur Ide (*Leuciscus waleckii*) inhabiting an extreme alkaline-saline lake reveals insights into stress adaptation, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0059703 e59703.

[23] J.H. Xia, P. Liu, F. Liu, et al., Analysis of stress-responsive transcriptome in the intestine of Asian seabass (*Lates calcarifer*) using RNA-Seq, DNA Res. 20 (2013) 449–460, https://doi.org/10.1093/dnares/dst022.

[24] K. Chen, E. Li, T. Li, C. Xu, X. Wang, H. Lin, J.G. Qin, L. Chen, Transcriptome and molecular pathway analysis of the hepatopancreas in the Pacific White Shrimp *Litopenaeus vannamei* under chronic low-salinity stress, PLoS One 10 (7) (2015), https://doi.org/10.1371/journal.pone.0131503 e0131503.

[25] J. Lv, P. Liu, Y. Wang, B. Gao, P. Chen, J. Li, Transcriptome analysis of *Portunus trituberculatus* in response to salinity stress provides insights into the molecular basis of osmoregulation, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0082155 e82155.

[26] J. Jin, Y. Wang, Z. Wu, A. Hergazy, J. Lan, L. Zhao, X. Liu, N. Chen, L. Lin, Transcriptomic analysis of liver from grass carp (*Ctenopharyngodon idellus*) exposed to high environmental ammonia reveals the activation of antioxidant and apoptosis pathways, Fish Shellfish Immunol. 63 (2017) 444–451, https://doi.org/10.1016/j.fsi.2017.02.037 doi: 10.1016/j.fsi.2017.02.037.

[27] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B.W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (2011) 644–652, https://doi.org/10.1038/nbt.1883.

[28] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T.L. Madden, BLAST+: architecture and applications, BMC Bioinf. 10 (2009) 421, https://doi.org/10.1186/1471-2105-10-421.

[29] S. Anders, W. Huber, Differential expression analysis for sequence count data, Genome Biol. 11 (2010) 106, https://doi.org/10.1186/gb-2010-11-10-r106.

[30] M.D. Young, M.J. Wakefield, G.K. Smyth, A. Oshlack, Gene ontology analysis for RNA-seq: accounting for selection bias, Genome Biol. 11 (2010) R14, https://doi.org/10.1186/gb-2010-11-2-r14.

[31] C. Xie, X. Mao, J. Huang, Y. Ding, J. Wu, S. Dong, L. Kong, G. Gao, C.Y. Li, L. Wei, KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases, Nucleic Acids Res. 39 (2011) 316–322, https://doi.org/10.1093/nar/gkr483.

[32] Y. Yang, S.Q. Xie, W. Lei, X.M. Zhu, Y.X. Yang, Effect of replacement of fish meal by meat and bone meal and poultry by-product meal in diets on the growth and immune response of *Macrobrachium nipponense*, Fish Shellfish Immunol. 17 (2) (2004) 105–114, https://doi.org/10.1016/j.fsi.2003.11.006.

[33] C. Kemper, M.K. Pangburn, Z. Fishelson, Complement nomenclature 2014, Mol. Immunol. 6 (2014) 56–58, https://doi.org/10.1016/j.molimm.2014.07.00.

[34] D.G. Calame, S.L. Mueller-Ortiz, R.A. Wetsel, Innate and adaptive immunologic functions of complement in the host response to Listeria monocytogenes infection, Immunobiology 221 (2016) 1407–1417, https://doi.org/10.1016/j.imbio.2016.07.004.

[35] M. Nonaka, A. Kimura, Genomic view of the evolution of the complement system, Immunogenetics 58 (2006) 701–713, https://doi.org/10.1007/s00251-006-0142-1.

[36] N.K.B. Day, H. Gewurz, R. Johannsen, J. Finstad, R.A. Good, Complement and complement-like activity in lower vertebrates and invertebrates, J. Exp. Med. 132 (1970) 941–950, https://doi.org/10.1084/jem.132.5.941.

[37] J.L. Hall, D.T. Rowlands, U.R. Nilsson, Complement-unlike hemolytic activity in lobster hemolymph, J. Immunol. 109 (1972) 816–882.

[38] Y. Zhang, C. Ye, A. Wang, X. Zhu, C. Chen, J. Xian, Z. Sun, Isolated and combined exposure to ammonia and nitrite in giant freshwater pawn (*Macrobrachium rosenbergii*): effects on the oxidative stress, antioxidant enzymatic activities and apoptosis in haemocytes, Ecotoxicology 24 (2015) 1601–1610, https://doi.org/10.1007/s10646-015-1477-x.

[39] C.H. Cheng, F.F. Yang, R.Z. Ling, S.A. Liao, Y.T. Miao, C.X. Ye, A.L. Wang, Effects of ammonia exposure on apoptosis, oxidative stress and immune response in pufferfish (*Takifugu obscurus*), Aquat. Toxicol. 164 (2015) 61–71, https://doi.org/10.1016/j.aquatox.2015.04.004.

[40] M. Holcik, N. Sonenberg, Translational control in stress and apoptosis, Nat. Rev. Mol. Cell Biol. 6 (2005) 318–327, https://doi.org/10.1038/nrm1618.

[41] C.D. Collard, R. Lekowski, J.E. Jordan, A. Agah, G.L. Stahl, Complement activation following oxidative stress, Mol. Immunol. 36 (1999) 941–948, https://doi.org/10.1016/S0161-5890(99)00116-9.

[42] M.L. Hart, M.C. Walsh, G.L. Stahl, Initiation of complement activation following oxidative stress. *In vitro* and in vivoobservations, Mol. Immunol. 41 (2004) 165–171, https://doi.org/10.1016/S0161-5890(04)00075-6.

[43] K. Soderhall, L. Cerenius, Role of the prophenoloxidase-activating system in invertebrate immunity, Curr. Opin. Immunol. 10 (1998) 23–28, https://doi.org/10.1016/S0952-7915(98)80026-5.

[44] G. Vogt, Life-cycle and functional cytology of the hepatopancreatic cells of *Astacus astacus* (Crustacea, Decapoda), Zoomorphology 114 (1994) 83–101, https://doi.org/10.1007/BF00396642.

[45] S. Blandin, E.A. Levashina, Thioester-containing proteins and insect immunity, Mol. Immunol. 40 (2004) 903–908, https://doi.org/10.1016/j.molimm.2003.10.010.

[46] V. Buresova, O. Hajdusek, Z. Franta, D. Sojka, P. Kopace, IrAM-An alpha2-macroglobulin from the hard tick Ixodes ricinus: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*, Dev. Comp.

Immunol. 33 (2009) 489–498, https://doi.org/10.1016/j.dci.2008.09.011.
[47] A. Rattanachai, I. Hirono, T. Ohira, Y. Takahashi, T. Aoki, Molecular cloning and expression analysis of alpha 2-macroglobulin in the kuruma shrimp, *Marsupenaeus japonicus*, Fish Shellfish Immunol. 16 (2004) 599–611, https://doi.org/10.1016/j.fsi.2003.09.011.
[48] Y.C. Lin, B. Vaseeharan, C.F. Ko, T.T. Chiou, J.C. Chen, Molecular cloning and characterisation of a proteinase inhibitor, alpha 2-macroglobulin (alpha2-M) from the haemocytes of tiger shrimp *Penaeus monodon*, Mol. Immunol. 44 (2007) 1065–1074.
[49] Y.C. Lin, B. Vaseeharan, J.C. Chen, Molecular cloning and phylogenetic analysis on alpha2-macroglobulin (alpha2-M) of white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 32 (2008) 317–329, https://doi.org/10.1016/j.molimm.2006.08.002.
[50] P.Y. Ho, C.H. Cheng, W. Cheng, Identification and cloning of the alpha2-macroglobulin of giant freshwater prawn *Macrobrachium rosenbergii* and its expression in relation with the molt stage and bacteria injection, Fish Shellfish Immunol. 26 (2009) 459–466, https://doi.org/10.1016/j.fsi.2009.01.007.
[51] H. Ma, B. Wang, J. Zhang, F. Li, J. Xiang, Multiple forms of alpha-2 macroglobulin in shrimp *Fenneropenaeus chinesis* and their transcriptional response to WSSV or *Vibriopathogen* infection, Dev. Comp. Immunol. 34 (2010) 677–684, https://doi.org/10.1016/j.dci.2010.01.014.
[52] L.M. Perazzolo, E. Bachère, R.D. Rosa, P. Goncalves, E.R. Andreatta, S. Daffre, M.A. Barracco, Alpha2-macroglobulin from an Atlantic shrimp: biochemical characterization, sub-cellular localization and gene expression upon fungal challenge, Fish Shellfish Immunol. 31 (2011) 938–943, https://doi.org/10.1016/j.fsi.2011.08.011.
[53] S. Shanthi, B. Vaseeharan, Alpha 2 macroglobulin gene and their expression in response to GFP tagged *Vibrio parahaemolyticus* and WSSV pathogens in Indian white shrimp *Fenneropenaeus indicus*, Aquaculture (2014) 48–54, https://doi.org/10.1016/j.aquaculture.2013.10.003.
[54] B. Vaseeharan, Y.C. Lin, C.F. Ko, T.T. Chiou, J.C. Chen, Molecular cloning and characterisation of a thioester-containing alpha2-macroglobulin (alpha2-M) from the haemocytes of mud crab *Scylla serrata*, Fish Shellfish Immunol. 22 (2007) 115–130, https://doi.org/10.1016/j.fsi.2006.03.017.
[55] C. Chiang, M.U. Gack, Post-translational control of intracellular pathogen sensing pathways, Trends Immunol. 38 (2017) 39–52, https://doi.org/10.1016/j.it.2016.10.008.
[56] P.R. Rauta, M. Samanta, H.R. Dash, Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses, Immunol. Lett. 158 (1–2) (2014) 14–24, https://doi.org/10.1016/j.imlet.2013.11.013.
[57] L.S. Yang, Z.X. Yin, J.X. Liao, X.D. Huang, C.J. Guo, S.P. Weng, A Toll receptor in shrimp, Mol. Immunol. 44 (2007) 1999–2008, https://doi.org/10.1016/j.molimm.2006.09.021.
[58] C. Yang, J. Zhang, F. Li, H. Ma, Q. Zhang, T.A. Jose-Priya, A Toll receptor from Chinese shrimp *Fenneropenaeus chinensis* is responsive to *Vibrio anguillarum* infection, Fish Shellfish Immunol. 24 (2008) 564–574, https://doi.org/10.1016/j.fsi.2007.12.012.
[59] T. Mekata, T. Kono, T. Yoshida, M. Sakai, T. Itami, Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus*, Fish Shellfish Immunol. 24 (2008) 122–133, https://doi.org/10.1016/j.fsi.2007.10.006.
[60] X. Lu, J. Kong, S. Luan, P. Dai, X. Meng, B. Cao, K. Luo, Transcriptome analysis of the hepatopancreas in the pacific white shrimp (*Litopenaeus vannamei*) under acute ammonia stress, PLoS One 10 (2016), https://doi.org/10.1371/journal.pone.0164396 e0164396.
[61] J.M. Chen, J.C. Chen, Study on the free amino acid levels in the hemolymph, gill, hepatopancreas and muscle of *Penaeus monodon* exposed to elevated ambient ammonia, Aquat. Toxicol. 50 (1–2) (2000) 27–37, https://doi.org/10.1016/s0166-445x(99)00095-8.
[62] N. Li, C. Li, C. Ma, H. Li, H. Zuo, S. Weng, X. Chen, D. Zeng, J. He, X. Xu, Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 46 (2014) 231–240, https://doi.org/10.1016/j.dci.2014.04.014.
[63] Y. Xiu, L. Hou, X. Liu, Y. Wang, W. Gu, Q. Meng, W. Wang, Isolation and characterization of two novel C-type lectins from the oriental river prawn, *Macrobrachium nipponense*, Fish Shellfish Immunol. 46 (2015) 603–611, https://doi.org/10.1016/j.fsi.2015.07.011.
[64] Q. Ren, M. Li, J. Du, C.Y. Zhang, W. Wang, Immune response of four dual-CRD C-type lectins to microbial challenges in giant freshwater prawn *Macrobrachium rosenbergii*, Fish Shellfish Immunol. 33 (2012) 155–167, https://doi.org/10.1016/j.fsi.2012.03.009.
[65] J.M. Jimenez-Guardeño, J.L. Nieto-Torres, M.L. DeDiego, J.A. Regla-Nava, R. Fernandez-Delgado, C. Castaño-Rodriguez, L. Enjuanes, The PDZ-binding motif of severe acute respiratory syndrome coronavirus envelope protein is a determinant of viral pathogenesis, PLoS Pathog. 10 (2014), https://doi.org/10.1371/journal.ppat.1004320 e1004320.
[66] P. Bangrak, P. Graidist, W. Chotigeat, K. Supamattaya, A. Phongdara, A syntenin-like protein with postsynaptic density protein (PDZ) domains produced by black tiger shrimp *Penaeus monodon* in response to white spot syndrome virus infection, Dis. Aquat. Org. 49 (2002) 19–25, https://doi.org/10.3354/dao049019.
[67] L. Brault, C. Gasser, F. Bracher, K. Huber, S. Knapp, J. Schwaller, PIM serine/threonine kinases in the pathogenesis and therapy of hematologic malignancies and solid cancers, Haematologica 95 (2010) 1004–1015, https://doi.org/10.3324/haematol.2009.017079.
[68] K.L. Glenn, L. Grapes, T. Suwanasopee, D.L. Harris, Y. Li, K. Wilson, SNP analysis of AMY2 and CTSL genes in *Litopenaeus vannamei* and *Penaeus monodon* shrimp, Anim. Genet. 36 (3) (2005), https://doi.org/10.1111/j.1365-2052.2005.01274.x 235-23.
[69] J.D. Shen, Q.F. Cai, L.J. Yan, C.H. Du, G.M. Liu, W.J. Su, C. Ke, M.J. Cao, Cathepsin L is an immune-related protein in Pacific abalone (*Haliotis discus hannai*) - purification and characterization, Fish Shellfish Immunol. 47 (2015) 986–995, https://doi.org/10.1016/j.fsi.2015.11.004.
[70] L.S. Dai, S.H. Chu, X.M. Yu, Y.Y. Li, A role of cathepsin L gene in innate immune response of crayfish (*Procambarus clarkii*), Fish Shellfish Immunol. 71 (2017) 246–254, https://doi.org/10.1016/j.fsi.2017.10.021.