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Digital gene expression analysis in the gills of *Ruditapes philippinarum* exposed to short- and long-term exposures of ammonia nitrogen

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ABSTRACT

Previous study revealed severe toxic effects of ammonia nitrogen on *Ruditapes philippinarum* including lysosomal instability, disturbed metabolic profiles, gill tissues with damaged structure, and variation of neurotransmitter concentrations. However, the underlying molecular mechanism was not fully understood yet. In the present study, digital gene expression technology (DGE) was applied to globally screen the key genes and pathways involved in the responses to short- and long-term exposures of ammonia nitrogen. Results of DGE analysis indicated that short-term duration of ammonia exposure affected pathways in Dorso-ventral axis formation, Notch signaling, thyroid hormone signaling and protein processing in endoplasmic reticulum. The long-term exposure led to DEGs significantly enriched in gap junction, immunity, signal and hormone transduction, as well as key substance metabolism pathways. Functional research of significantly changed DEGs suggested that the immunity of *R. philippinarum* was weakened heavily by toxic effects of ammonia nitrogen, as well as neurotransduction and metabolism of important substances. Taken together, the present study provides a molecular support for the previous results of the detrimental toxicity of ammonia exposure in *R. philippinarum*, further work will be performed to investigate the specific genes and their certain functions involved in ammonia toxicity to molluscs.

1. Introduction

Over the past decades, ammonia nitrogen has been a consistent pollutant in some marine fishery waters according to the annual reports on the state of Chinese marine environment. Of the two existing forms of ammonia in seawater, the un-ionized form (NH_3-N) is more toxic than the ion form (NH_4^+) because of its capacity to diffuse through cell membranes (Emmerson et al., 1975). The un-ionized ammonia nitrogen (UIA) can induce many adverse effects in fishes, crustaceans and molluscs, including severe histological changes, neurological dysfunction, growth restriction, respiration impairment and fecundity decrement (Armstrong et al., 2012; Keppler, 2007; Maas et al., 2012; Randall and Tsui, 2002; Smart, 1978). Although the invertebrates (such as molluscs) were generally found to be more tolerant to ammonia stress compared with the vertebrate (Arthur et al., 1987), toxicology studies indicated that ammonia nitrogen caused severe toxicity to the clams. Therefore, it is necessary to clarify the toxic mechanism of ammonia nitrogen on the molluscs in order to provide reliable data for practical treatment of reducing ammonia nitrogen pollution and environmental protection in shellfish aquaculture around the coastal area.

The toxic mechanism of ammonia has been studied more clearly in fishes than other aquatic organisms. It was found that ammonia depolarizes the neurons and activates the N-Methyl-D-aspartic acid (NMDA)-type glutamate receptor, leading to subsequent cell apoptosis in the

Abbreviations: DGE, Digital gene expression technology; UIA, un-ionized ammonia nitrogen; NMDA, N-Methyl-D-aspartic acid; DEG, differentially expressed genes; Nr, NCBI non-redundant protein sequences; Nt, NCBI non-redundant nucleotide sequences; Pfam, Protein family; KOG/COG, Clusters of Orthologous Groups of proteins; KOG, KEGG Ortholog database; GO, Gene Ontology; qRT-PCR, quantitative real-time PCR; 18S, 18S rDNA; Actin, beta actin; Tub, beta tubulin; EGF1α, elongation factor 1 alpha; Ubi, ubiquitin; Cyclo, cyclophilin A; ABC, ATP-binding cassette; MRP, multidrug resistance protein; ER, endoplasmic reticulum; MCD, Malonyl-CoA decarboxylase; IFI44L, Interferon-induced protein 44-like; A2M, Alpha-2 macroglobulin; EGF-like, epiderma-growth factor-like; GS, Glutamine synthetase; ABAT, 4-aminobutyrate aminotransferase

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brain by causing influxes of excessive Ca^{2+} and K^{+} (Randall and Tsui, 2002). However, the related study on molluscs is still in a primary stage. Such toxic studies basically focus on the changes of survival rates, energy

allocation, cellular and immune parameters after ammonia nitrogen exposure (Maas et al., 2012; Keppler, 2007; Wang et al., 2012; Widman et al., 2008). The underlying mechanism of ammonia toxicity in mollusc was not fully elucidated yet. In previous studies, we found that ammonia nitrogen exposure caused a series of adverse effects to the clam *Ruditapes philippinarum*, including decrease in lysosomal stability, disturbance of metabolic profiles, damage to the gill structure and variation of neurotransmitter concentrations (Cong et al., 2017). However, which genes and how many pathways were involved in the toxic reaction of *R. philippinarum* to ammonia nitrogen challenge? These questions were not answered clearly yet. In the present study, the expression profiles of related genes were detected by digital gene expression (DGE) analysis to preliminarily describe the genetic network of the *R. Philippinarum* clams to the ammonia exposure.

DGE is a kind of analysis approach widely used today to explore differentially expressed genes (DEGs) when the target organisms or their tissues are under a particular stress, with or without the background genome information. It has been quickly used in quite a few of aquatic organisms to detect their responses to external factors at gene level, such as *Oryzias melastigma* (Huang et al., 2012), *Crassostrea gigas* (Zhao et al., 2012) and *Chlamys farreri* (Hu et al., 2015). *R. philippinarum* is an important marine mollusc, distributed widely along the Chinese coastal area. As a sedentary habitant, *R. philippinarum* is more inclined to the environment pollutants including ammonia nitrogen. Among *R. philippinarum*'s organs, gill is the first one to contact with the environment and probably is the first target of external pollutants. In the present study, *R. philippinarum* received ammonia exposures for 1 and 30 days respectively to find out the differentially expressed genes involved in acute and subacute toxicities of ammonia nitrogen. Gill tissues were used to analyze the gene expression profiles by using DGE technology, and validated by quantitative real-time PCR to identify the genes and pathways which were involved in the molecular reaction of clams against ammonia nitrogen exposure.

2. Materials and methods

2.1. Animals and ammonia-N exposure experiments

Healthy clam *R. philippinarum* (averaging 3.56 ± 0.35 cm in shell length) were collected from Yangma Island (with an average background NH_3—N concentration of 0.0016 mg/L), Yantai city. The clams were acclimated for 15 days in aerated and filtered seawater (19 ~ 20 °C, pH 8.0, 32 psu), and fed with a mixture of *Isochrysis galbana* and *Chlorella vulgaris Beij*. The seawater in each tank was changed twice every day just before and 2-h after the feeding. Stock solution (1 mol/L) of high purity NH_4Cl was used as the source of the total ammonia. After the acclimation period, all of the clams were randomly divided into two groups as blank (0 mg/L) and ammonia nitrogen (0.1 mg/L) exposure groups with three replicate tanks respectively.

According to the annual reports on the environmental state of China, the actual concentration of ammonia nitrogen in the neritic China seas ranged from 0 to 19.32 mg/L with an average value of 1.82 mg/L. Based on the result of lysosomal destabilization which was an indicator of cellular damage (Keppler, 2007; Aguirre-Martínez et al., 2013), our previous study suggested that 1-day exposure of 0.1 mg/L ammonia nitrogen could cause toxic effects to the calms, and induced significant changes in neurotransmitter concentrations, lysosomal stabilization and metabolite concentrations (Cong et al., 2017). And a 30-day exposure of 0.1 mg/L ammonia nitrogen didn't cause massive mortality to the clams (data not shown). So 0.1 mg/L ammonia nitrogen was used in the present study to evaluate toxic reactions in clam *R. philippinarum* in an ammonia nitrogen polluted environment, and 1-day and 30-day durations of ammonia nitrogen were used to compare the effects of acute and subacute ammonia exposure in clams, respectively. During the 30-days' exposure experiment, gill tissues of the clams were collected into RNAlater® Stabilization Solution (Thermo Fisher) at the 0, 1st and 30th-day, and used as Blank (B), A1, A30 group samples respectively. In order to diminish the individual difference among the same group, five individuals were pooled as one sample. And three samples were prepared in each group at each sampling time-point. The transcriptome samples in the blank, 1-day and 30-day exposure groups were designated as RpB, RpA1 and RpA30 respectively. In addition, another five individual clams from each group were collected as five samples to be used in the real-time RT-PCR experiment.

2.2. Total RNA extraction and DGE sequencing

RNA extraction from each sample was carried out following the manual instructions of the Trizol reagent. The purity, concentration and integrity of RNA samples were checked by using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA), Qubit® RNA Assay Kit in Qubit® Library preparation for Transcriptome sequencing 2.0 Flurometer (Life Technologies, CA, USA) and RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively. A total amount of 3 μg RNA (A_{260}/A_{280} = 1.8) per sample was used as input material for further analysis. Sequencing libraries were generated using NEB Next® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations, and library quality was assessed on the Agilent Bioanalyzer 2100 system. Then the clustered library was sequenced on an Illumina Hiseq 2500 platform and 125 bp/50 bp paired/single-end reads were generated as raw data in fastq format.

2.3. Bioinformatics analyses and in silico gene expression analyses

Clean data were got by removing reads containing adapter, ploy-N and other low quality reads from raw data. Q20, Q30, GC-content and sequence duplication level of the clean data were all calculated. After that, transcriptome was assembled by pooling the clean data with high quality from all treatments together. Then gene functions were annotated based on several databases, including Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (A manually annotated and reviewed protein sequence database), KOG (KEGG Ortholog database) and GO (Gene Ontology). SNP calling was performed by GATK2 software with distance > 5.

Gene expression levels were estimated by RSEM (Li and Dewey, 2011) for each sample. Clean data were mapped back onto the assembled transcriptome and readcount for each gene was obtained from the mapping results. Differential expression analyses of the blank and the ammonia-nitrogen exposed groups were performed by the DESeq R package (1.10.1). The resulting *P* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P* value < 0.05 found by DESeq were assigned as differentially expressed. In addition, a pheatmap was constructed according to the relative expression levels of the differentially expressed genes among the blank, 1-day and 30-day exposure groups (designated as RpB, RpA1 and RpA30, respectively).

2.4. GO and KEGG analysis of the DGEs

GO enrichment analysis of the DEGs was implemented by the GOseqR packages based on Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length bias in DEGs. KEGG (Kanehisa et al., 2008) pathway analysis was performed by KOBAS (Mao et al., 2005) software to enrich the differential expression genes. In addition, interaction between proteins from the DEG

sequences in the transcriptome was predicted.

2.5. Quantitative analysis by real-time RT-PCR

To confirm that ammonia-nitrogen exposure induced genes differentially expressed in *R. philippinarum*, quantitative real-time PCR (qRT-PCR) was performed using Prime Script RT reagent kit (TaKaRa, Dalian, China). Identification of ammonia nitrogen-response genes were carried out by BLASTX search against Nr, Nt, Pfam, Swiss-Prot, GO and KEGG database. Ten significantly expressed genes (with adjusted P value $<$ 0.05) were selected as the targeted genes from membrane binding pathway, nervous and signal transduction pathway, transcriptional regulation pathway and glutamine metabolism pathway. Six reference candidate genes, including *18S rDNA* (*18S*), *beta actin* (*Actin*), *beta tubulin* (*Tub*), *elongation factor 1 alpha* (*EGF1α*), *ubiquitin* (*Ubi*) and cyclophilin A(*Cyclo*), were screened for qPCR validation by geNorm and Normfinder softwares. The corresponding primers were designed by Primer Premier 5.0. Each primer was defined to a usual length of 20 ~ 25 bp, with the GC content between 40–60% and the 3′ of a primer ending in C or G.

Total RNA was extracted from the gill tissues of clams in the blank and ammonia exposed groups, and 1 μg of total RNA (A260/A280 = 1.8) in each sample was reversely transcripted to get the first-strand cDNA according to the protocols (PrimeScript™ RT-PCR Kit with gDNA Eraser, Takara). The qRT-PCR was carried out in an ABI 7500 Real-Time Detection System (Applied Biosystems) to find out the expression profiles of related genes. Gene-specific primers of six candidate genes and ten targets genes were shown in Table 1. The PCR amplifications were carried out in triplicate in a total volume of 20.0 μl, containing 10.0 μl of 2 × SYBR Green Master Mix (Applied Biosystems), 5.0 μl of 1:50 diluted cDNA, 0.6 μl of each primer (10 μmol/L), 3.8 μl of PCR-grade DEPC-water. The PCR program was 50 °C for 2 min and 95 °C for 5 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C. At the end of each PCR amplication, dissociation curve analysis of final products was carried out to confirm that only one product was amplified and detected. Data were analyzed automatically with the ABI 7500 SDS software (Applied Biosystems). The comparative CT method was used to analyze the expression level of each gene (Livak and Schmittgen, 2001). The gene expression levels were given in terms of mean ± S. D. (n = 5). The final data were subjected to one-way analysis of variance (one-way ANOVA) followed by least significant difference (LSD) analysis. Statistical significance was defined at $P < 0.05$. In order to compare the variation trends of different genes detected by Illumina and qRT-PCR techniques, the final results were all expressed as $\log_2$ (fold change of An vs. B, n = 1 or 30).

3. Results

3.1. Analysis of sequenced data quality

Because we haven't got the genome of *R. philippinarum* clam yet, a transcriptome was used as a reference to identify the differentially expressed genes induced by ammonia nitrogen. Therefore, a mixed RNA pool from the samples of the blank and 0.1 mg/L ammonia nitrogen exposed groups at 1st and 30th day was sequenced as the reference transcriptome. The data qualities from each sample were shown in Table 2. After filtering low quality sequences by trimming sequencing adapters/poly-N and removing poor quality reads, there were 40,029,424 clean reads (97.17% of raw data) were obtained in the reference transcriptome. The longest transcript of each gene was regarded as its unigene, which was the reference to the following analysis. There were 219,534 transcripts with a mean length of 713 base pairs, and 151,283 unigenes with a mean length of 637 base pairs in the transcriptome.

Table 1
Primer sequences for housekeeper genes and targeted genes.

Item	Gene Name		Sequence(5′-3′)	Length of amplicon (bp)
Housekeeper genes	*18S*	Forward	ACTCAAGGGGAACCTC	125
		Reverse	TTAACCAGACAAATCGCTCCAC	
	Actin	Forward	CTCCCTTGAGAAGAGCTACGA	121
		Reverse	GATACCAGCAGATTCCATACCC	
	Tub	Forward	AACACCGACGAGACATACTGC	160
		Reverse	CGTTCAACTGACCTGGGAAT	
	EGF1α	Forward	GAATGGTTGTTACCTTTGCTCC	214
		Reverse	ACGATGACCTGGGCATAGA	
	Ubi	Forward	ACTCTTCATCTTGTGCTCCGTC	240
		Reverse	AAGATGGAGAGTGGACTCTTTCTG	
	Cyclo	Forward	AACGGTACTGGAGGCAAGAG	196
		Reverse	TACCCTCAACTACTGAACCAAACA	
Targeted unigenes	*TMDP*	Forward	TGGTTGGTGGACTTGTTTCG	155
		Reverse	GACATCCGAGGATTCCGAGA	
	ABCA	Forward	GTACCGAGGCCAATAACCTAC	200
		Reverse	TGACAGAACTAAGCCGACCACT	
	TRP	Forward	GGCAATGTTCTAATGTAGCGTC	108
		Reverse	TTGCGTCTCTACTCAATGTGTTAC	
	CYP450-like	Forward	AGAGGGCGAAGAGTCAGAAA	161
		Reverse	GTTTTGCCAACGAGTATAAGC	
	Dat-like	Forward	ATCATTTCGGCGTTACAGAGA	102
		Reverse	CCATTGCAGCGTGAACTATC	
	GS	Forward	GCTGAAGTTATGCCAGGACA	151
		Reverse	CTTCCATAGGTTTCGGGTCT	
	FOX	Forward	TGCATGGCTATGAAAGAGACTAAGA	200
		Reverse	TCTACGTTTCTTGAACACACCATTT	
	Apo X1	Forward	GGAACAATGACACGCACGA	108
		Reverse	GTCACTGACCACCTCAAACCTA	
	TG 1	Forward	GCACATAATACAGCGTTCCAAC	111
		Reverse	ATAACAACATATCTTAGGTCACGC	
	TG 2	Forward	GCTGAAGTTATGCCAGGACA	151
		Reverse	CTTCCATAGGTTTCGGGTCT	

Table 2
Summary of the DGE data collected from the gills of *R. philippinarum* in response to ammonia-N.

Summery	Blank group			1st −day group			30rd-day group		
	RpB_1	RpB_1	RpB_1	RpA1_1	RpA1_1	RpA1_1	RpA30_1	RpA30_1	RpA30_1
Raw data	11,293,884	10,521,712	12,837,253	10,759,221	11,550,358	12,843,888	12,166,006	11,339,469	13,062,181
Clean data	10,712,980	10,461,708	12,627,883	10,441,740	11,297,511	12,810,505	11,912,659	11,117,448	12,740,691
Error rate (%)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Q20	98.64	98.64	98.64	98.63	98.65	98.52	98.65	98.63	98.65
Q30	97.34	97.33	97.35	97.33	97.37	97.11	97.29	97.24	97.29
GC content (%)	37.57	38.24	38.44	38.91	38.98	39	38.77	38.4	38.5
Total mapped (%)	8,244,355 (76.96%)	8,114,696 (77.57%)	9,899,238 (78.39%)	8,216,239 (78.69%)	9,034,676 (79.97%)	9,970,928 (77.83%)	9,342,031 (78.42%)	8,645,563 (77.77%)	9,939,857 (78.02%)

3.2. Functional annotation and classification of the transcriptome

Putative functions of proteins encoded by the 151,283 genes were predicted by Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KO and GO database. The results showed that there were 38,479 (25.43%), 2884 (1.9%), 13,078 (8.64%), 26,252 (17.35%), 35847 (23.69%), 36,654 (24.22%), 18,434 (12.18%), 1772 (1.17%), 49,888 (32.97%) genes homologous to the sequences in the Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KO and GO databases, respectively (Table 3). There were 1772 unigenes (1.17%) were annotated in all databases and 49,888 unigenes (32.97%) were annotated in at least one database. In total, all of the 151,283 unigenes (100%) were functionally annotated.

By Blast X search, we found out that the genes in *R. philippinarum* transcriptome showed homologies to the sequences from other organisms. Among them, 30.1%, 13.2%, 6.4% and 6.2% of the genes exhibited significant homologies to the sequences in *Crassostrea gigas*, *Lottia gigantean*, *Aplysia californica* and *Opisthorchis viverrini*, respectively (Fig. 1a). The *E*-value distribution analysis revealed that 40.2% sequences were highly conserved with an *E*-value $< 10^{-45}$, and 52.8% sequences showed strong homology with an *E*-value $< 10^{-30}$ (Fig. 1b). According to the similarity distribution, 19.6% of the sequences had strong match (> 80%) with the deposited sequences, 45.2% sequences showed 60–80% match, and 34.7% had 40–60% match (Fig. 1c).

Based on the annotation results of Nr and Pfam, 36,654 (24.22%) of the mapped genes could be assigned to GO categories of biological process, cellular component, molecular function (Fig. 2). The category of biological process was made up to 21 subcategories, of which the major one was macromolecule metabolic process with 11,630 (31.73%) genes. The category of cellular component contained 15 subcategories, among which cellular components was the biggest one with 9628 (26.26%) genes. The category of molecular function was consisted of 12 subcategories, of which the major one was cation binding with 5760 (15.71%) genes (Fig. 2a). And 18,434 (12.18%) of the mapped genes were assigned into 26 groups in KOG database (Fig. 2b), of which the (T) signal transduction mechanism was the largest group with 3942 (21.38%) genes, followed by (R) endocrine system with 1019 (5.5%) genes, translation with 970 (5.3%) genes, and transport and catabolism with 921 (5.0%) genes. A total of 13,078 genes were characterized into 5 special KEGG pathways (Fig. 2c), including cellular process (13.28%, A), environmental information processing (19.64%, B), genetic information processing (12.58%, C), metabolism (23.06%, D), organismal systems (31.44%). The transcriptome and the following DGE data have been deposited at Genbank under the accession number SRP116794.

Table 3
Functional annotation of the *R. philippinarum* transcriptome.

Annotated Database	Number of Unigenes	Percentage (%)
NR	38479	25.43
NT	2884	1.9
KO	13078	8.64
SwissProt	26252	17.35
PFAM	35847	23.69
GO	36654	24.22
KOG	18434	12.18
Total Unigenes	151283	100

3.3. Digital gene expression (DGE) analysis

DGE analysis was conducted to identify the related genes in response to ammonia nitrogen exposure. All of the tagged sequences in each sample were mapped to the assembled transcriptome library of *R. philippinarum* using RSEM software (Li and Dewey, 2011). To mitigate the effect of transcript length on the calculation of gene differential expression, FPKM method (expected number of Fragments Per Kilo base of transcript sequence per Millions base pairs sequenced) was introduced in the calculation. If the FPKM values are over 0.3, the genes are regarded expressed. The number of raw reads produced for blank and experiment groups ranged from 10.5 to 13.0 million (Table 2). Among the clean data in each group, the percentages of sequences mapped to genes ranged from 76.96% to 79.97%, and the error rates of base sequencing were all 0.1%. In conclusion, all the data were suitable for further gene expression analysis.

Compared with the blank group, DGE analysis revealed that there were 65 genes significantly up-regulated and 35 genes significantly down-regulated in the A1 group. There were 106 unigenes up-regulated and 143 genes down-regulated in the A30 group. However, long duration of ammonia nitrogen exposure caused 161 genes differentially regulated in the A30 group compared with the A1 group, of which 94 genes were up-regulated and 67 genes were down-regulated (Fig. 3). The hierarchical clustering pheatmap displayed globally and intuitively on the DEGs expression patterns (Fig. 4). It can be seen that the expression patterns of both the A1 and the A30 groups were distinguishable from the blank group.

3.4. GO enrichment and KEGG pathways analysis of DEGs

The DEGs were assigned to the GO database by GO seq to characterize the involved biological processes upon ammonia nitrogen exposure. No significant enriched GO terms were found in A1 or A30 groups compared with the blank group. The first five GO terms in A1 group included substance binding (GO: 0005488, 20genes), cellular process (GO: 0009987, 18genes), single-organism process (GO: 0044699, 14 genes), membrane (GO: 0016020, 14 genes), metabolic process (GO: 0008152, 14 genes). However, the GO terms of the DEGs in A30 group dispersed in a wider range, of which the first five were cellular process (GO: 0009987, 70 genes), substance binding (GO: 0005488, 59 genes), catalytic activity (GO: 0003824, 58 genes), metabolic process (GO: 0008152, 56 genes), single-organism process (GO: 0044699, 53 genes).

Although no significant enriched GO terms were found after ammonia nitrogen exposure, the genes with significantly changed profiles (adj $P < 0.05$) were analyzed according to their GO terms to describe

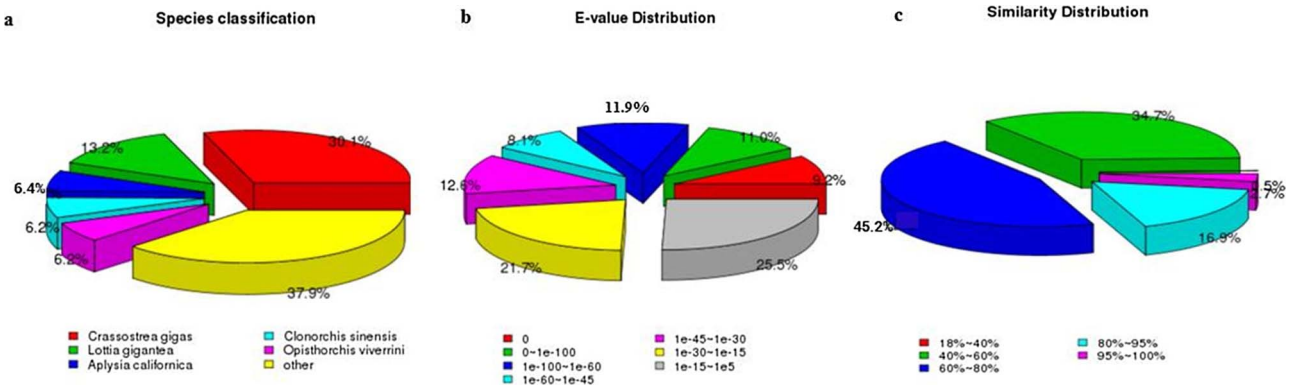

Fig. 1. Functional classification of the transcriptome. (a) Species classification. (b) E-value distribution. (c) Similarity distribution.

the toxicological network of ammonia nitrogen on *R. philippinarum*, which were shown in Table 4 (1-day duration) and Table 5 (30-day duration) respectively. According to Table 4, 1-day duration of ammonia-nitrogen exposure could significantly down-regulate the genes related to stimulus response, binding and transmembrane transportation of metal ion, and up-regulated the genes involved in stress-response, ion-binding, signal transduction, receptors, protein processing, cell adhesion, conjugation and membrane formation. A 30-day duration of ammonia nitrogen exposure caused significant down-regulation of the genes related to stimulus response, immune response, protein processing (synthesis, modification, folding and transporting), substance transporting, cellular component organization, chaperone and oxidoreductase activities, as well as binding. In addition, the 30-day duration of ammonia nitrogen significantly up-regulated six kinds of genes which were involved in response to stress/stimulus, signal transduction, important cellular compounds metabolism, catalytic reaction, intracellular and transmembrane transporting, and ion/receptor/organic substance binding.

The DEGs were also assigned to KEGG database to find out the correlated pathways of biochemical metabolite and signal transduction, with corrected *P* value of 0.05 as the cutoff. One-day ammonia nitrogen exposure caused the DEGs enriched in 13 pathways, of which the first 4 included Dorso-ventral axis formation (ko04320), Notch signaling pathway (ko04330), thyroid hormone signaling pathway (ko04919) and protein processing in endoplasmic reticulum (ko05206). KEGG analysis also found that 30-day exposure caused the DGEs significantly enriched in gap junction (ko04540), pathogenic *Escherichia coli* infection (ko05130), bile secretion (4ko04976), GABAergic synapse (ko04727), alanine, aspartate and glutamate metabolism (ko00250), phagosome (ko04145), ABC transporters (ko02010). Between A1 and

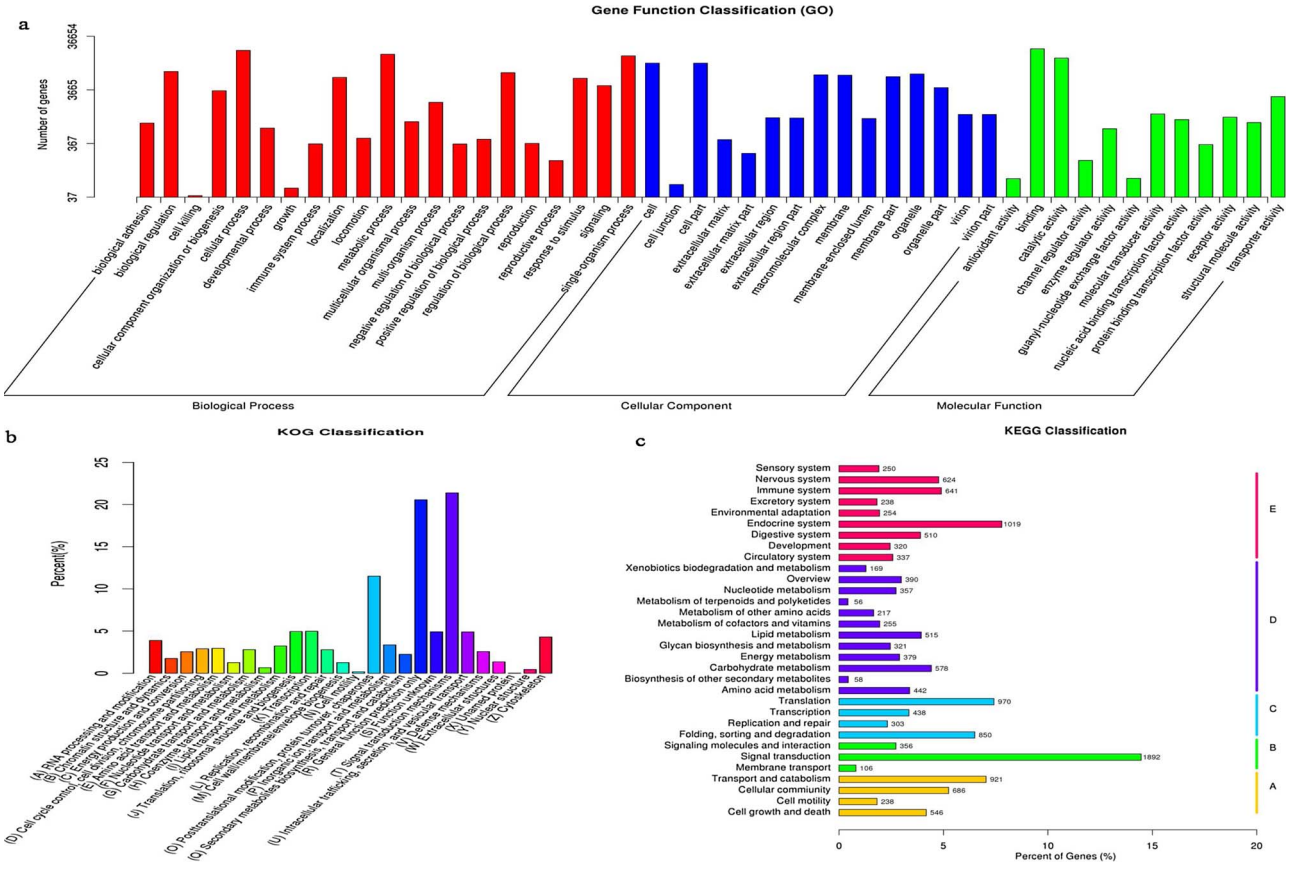

Fig. 2. Functional annotation of the transcriptome. (a) GO classification of the transcriptome from the de novo assembly of the blank, A1 and A30 groups. (b) KOG annotation of the putative proteins. (c) KEGG classification of the transcriptome.

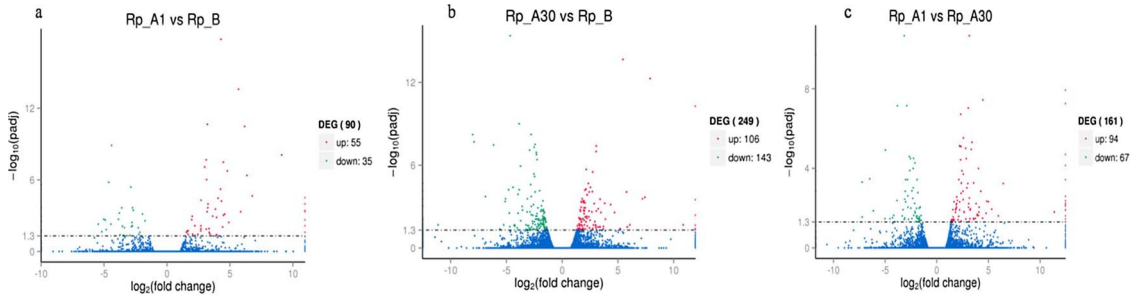

Fig. 3. DEG Volcanoplots among different treatments.

A30 groups, there was no significant difference in their enriched pathways.

From the enriched KEGG pathways of different exposure durations, it can be seen that one-day exposure to ammonia nitrogen significantly affected the expression profiles of genes involved in neuron formation (Dorso-ventral axis formation, up-regulated), signal transduction in cell proliferation and apoptosis (Notch signaling, up-regulated), hormone signaling (thyroid hormone signaling, up-regulated) and protein processing (protein processing in endoplasmic reticulum, down-regulated) pathways. However, 30-day exposure led to significant regulation of genes in junction (gap junction, up-regulated), immunity (pathogenic *Escherichia coli* infection and phagosome, up-regulated), signal and hormone transduction (ABC transporters and GABAergic synapse, both up-regulated), as well as key material metabolism (bile, alanine, aspartate and glutamate, up-regulated).

3.5. QRT-PCR validation of differentially expressed genes

In order to confirm the results of transcriptome, ten of the differentially expressed genes were selected from DGE libraries to detect their expression levels by qRT-PCR (Fig. 5). These genes included TM2 domain-containing protein (*TMDP*), ATP-binding cassette sub-family A member (*ABCA*), transcriptional regulatory protein (*TRP*), cytochrome P450-like (*CYP450-like*), dopamine N-acetyltransferase-like (*Dat-like*), glutamine synthetase (*GS*), forkhead box protein (*FOX*), apolipoprotein-like isoform X1 (*Apo X1*), transglutaminase (*TG1*, *TG2*). RNA samples from another five biological replicate sets were used as the templates for qRT-PCR. Each unigene was found to be a single product by the melting-curve analysis. According to qRT-PCR results of the target genes validated by the reference gene VpEGF1α (GenBank NO JK845897), long-term exposure of ammonia nitrogen caused 4 unigenes

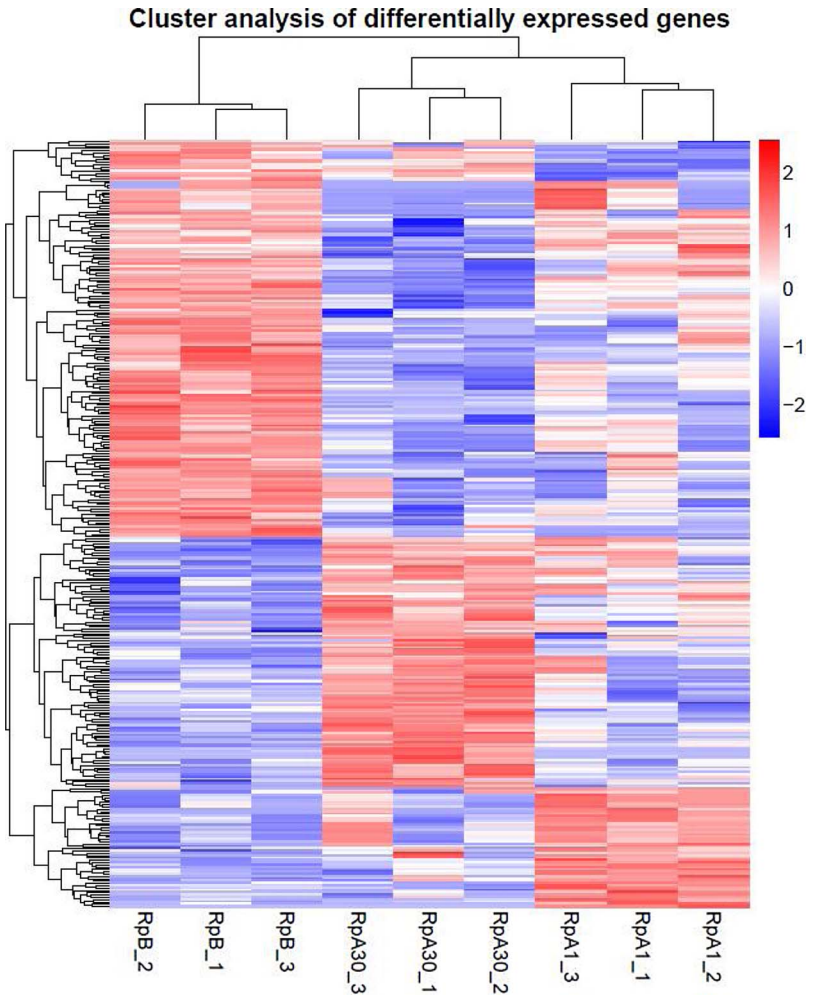

Fig. 4. In silico gene transcription analysis of different exposed groups, each with three replicates (RpB, the blank groups; RpA1, the NH_3-N exposed groups for one day; RpA30, the NH_3-N exposed groups for thirty days). Red color suggests high expression and blue color represents down expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
List of genes with significant changed GO terms after NH_3-N exposure for 1 day.

Description	Readcounts		FC	Function
	A1	B		
Significantly down-regulated genes				
PIF1-like helicase	0	78.46	-inf	response to stimulus (telomere maintenance)
DSBA-like thioredoxin	0	34.97	-inf	response to stimulus (oxidoreductase activity)
Growth-arrest specific micro-tubule	12.61	152.75	−3.60	response to stimulus (regulation of cell death)
STAT protein	0	24.83	-inf	DNA binding
nucleotide exchange factor SIL1	212.95	823.43	−1.95	binding
Fibronectin type III domain	0	26.02	-inf	binding
GRP78	188.96	1750.39	−3.21	binding
ZIP Zinc transporter	43.96	282.23	−2.69	zinc transmembrane transport
Ferrous iron transport protein B	228.21	1522.46	−2.74	ferrous iron transmembrane transporter activity
Significantly up-regulated genes				
DNA double-strand break repair and V(D)J recombination protein XRCC4	888.74	95.73	3.21	response to stress
Coleoptericin	32.89	0	Inf	response to stimulus
DNA repair protein Ercc1	108.74	0	Inf	response to DNA damage stimulus
Cytochrome P450	842.88	88.67	3.25	oxidoreductase activity
EGF-like domain-containing protein (EGFL)	205.75	4	5.69	calcium ion binding
Sushi-repeat-containing protein (SRP)	288.18	9.54	4.92	calcium ion binding
Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein (SVEP)	148.66	6.66	4.48	calcium ion binding
Sushi repeat-containing protein 2 (SRPX2)	235.14	8.45	4.80	calcium ion binding
Calcium-binding EGF (cbEGF)	296.61	72.21	2.04	calcium ion binding
fibulin 1/2	677.24	47.05	3.85	ion binding
Neurogenic locus notch like protein 2 (NOTCH2)	66.56	3.11	4.42	calcium activated cation channel activity
Rabaptin	191.60	21.97	3.12	Neuro-signal transduction
Transcriptional regulatory protein	291.66	36.59	3.00	signal transduction
Nine Cysteines Domain of family 3 GPCR	60.73	3.02	4.33	receptor activity
ATP-binding cassette sub-family A member	846.08	271.10	1.64	ion transmembrane transport
Multidrug resistance protein 1	440.93	109.69	2.01	transporter activity
Oligosaccharyltransferase subunit Ribophorin II	61.21	0	Inf	macromolecule modification
Sequestosome-1	5378.96	1817.93	1.57	ubiquitin-dependent protein catabolic process
Acyltransferase	584.91	181.87	1.69	transferase activity
Lysosomal aspartic protease	3557.96	1214.47	1.55	hydrolase activity
SAM dependent carboxyl methyltransferase	88.37	3.69	4.58	exocytosis
basement membrane-specific heparan sulfate proteoglycancore protein-like 1	370.17	36.49	3.34	cell adhesion
Neural cell adhesion molecule 2	303.80	35.00	3.12	Neural cell adhesion
basement membrane-specific heparan sulfate proteoglycan core protein-like 2	73.99	6.94	3.41	cell adhesion
Claudin tight junction	451.40	115.51	1.97	membrane

(*TMDP*, *ABCA*, *CYP450-like*, *TG2*) significantly down-regulated compared with those after acute exposure. However, another 5 unigenes (Dat-like, GS, FOX, Apo X1 and TG1) were found significantly up-regulated after 30-day exposure compared with those after 1-day exposure. Of the ten tested genes, only the regulation trend of TRP gene detected by qRT-PCR was not consistent with that detected by Illumina. It indicated that most of the expression profiles of unigenes detected by Illumina RNA-seq technology were consistent with those qualified by qRT-PCR, and the transcriptome results were reliable for further analysis of ammonia-responsive genes.

4. Discussion

Our previous study revealed that ammonia exposure could significantly reduce the integrity of lysosome membrane, decrease the contents of glutamate and branched chain amino acid, decrease ATP supply, increase apoptosis ratios, decrease contents of neurotransmitters, reduce basal layer inside the filaments with the emergence of xenobiotic substances (Cong et al., 2017). It suggested that ammonia nitrogen exposure brought detrimental toxicity to *R. philippinarum*. Given that gill tissue is important for the clam in feeding and respiration functions, the aberrant gill structures caused by ammonia exposure is supposed to be detrimental for the survival of *R. philippinarum*. However, little information was obtained to explain the molecular mechanism of ammonia nitrogen on the clams. In order to well elucidate the genetic mechanisms underlying severe damage of ammonia nitrogen exposure on the gill tissues of *R. philippinarum*, DGE analysis technique was applied and yielded a number of expressed sequences in the present study. After assemblage, these sequences were annotated and developed into a database for *R. philippinarum* gill tissue. The annotated functional sequences would be searched and further investigated. It is of particular importance for well understanding toxic mechanism of ammonia on a mollusc without complete genome background and also for pollution management in mollusc aquaculture.

Differentially expressed genes after ammonia nitrogen exposure were established into a DEG library, with considerate homologies to the known sequences of other molluscs, such as *C. gigas* and *L. gigantean*. It is convenient to discriminate the involved pathways of *R. philippinarum* after encountering ammonia nitrogen. In order to describe the toxicological overview of ammonia nitrogen on *R. philippinarum*, the differentially changed DEGs after 1- or 30-day exposures were performed with GO enrichment and KEGG pathway analysis. And the main functions of DEGs would lay a good foundation to understand the potential mechanism of ammonia toxicity.

After systematical GO analysis, some toxic effects of ammonia nitrogen exposure could be predicted according to the genes with differential expression patterns. First of all, it can be seen that the immune system of *R. philippinarum* would be heavily weakened according to the variation profiles of immune-related genes. These genes included coleoptericin, interferon-induced protein 44-like (IFI44L), alpha-2 macroglobulin (A2M), C-type lectin 9a, complement component C3, tumor necrosis factor-like protein and ligand superfamily member 10, coleoptericin, sialic acid binding lectin and so on. In Table 4, coleoptericin gene was significantly up-regulated after short-term exposure of ammonia nitrogen. Since coleoptericin is a kind of antibacterial peptide in invertebrate immune system (Zhu et al., 2014). The significant up-

Table 5
List of the genes with significant changed GO terms after NH_3-N exposure for 30 day.

Description	Readcounts		FC	Function
	A30	B		
Significantly down-regulated genes				
suppressor of cytokine signalling-2	582.19	2208.44	−1.92	response to stimulus
C-type lectin 9a	75.10	321.12	−2.1	response to stimulus
inhibitor of apoptosis protein 1 and 2	86.66	267.77	−1.63	response to stimulus
tumor necrosis factor-like protein	488.39	1399.82	−1.52	Immune response
Tumor necrosis factor ligand superfamily member 10	462.81	1177.93	−1.35	Immune response
interferon-induced protein 44-like (ILL44)	934.18	4226.34	−2.18	Immune response against virus
Alpha-2-macroglobulin	37.36	136.15	−1.87	Immune response
complement component C3	68.17	221.09	−1.7	retrograde vesicle-mediated transport, Golgi to ER
FKBP-type peptidyl-prolyl *cis*-trans isomerase	74.64	229.78	−1.62	protein folding
Protein arginine N-methyltransferase 1	433.12	1221.55	−1.5	protein methyltransferase activity
Tryptophanyl-tRNA synthetase	191.26	599.96	−1.65	catalytic activity
Pterin 4 alpha carbinolamine dehydratase	531.59	1494.99	−1.49	dehydratase activity
asparaginyl-tRNA synthetase	20.03	87.09	−2.12	tRNA metabolic process
E3 ubiquitin-protein ligase	90.87	261.97	−1.53	cation transport
Na^+ dependent nucleoside transporter	68.67	209.81	−1.61	organic substance transport
sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1-like	462.15	2491.41	−2.43	carbohydrate transmembrane transporter activity
Phospholipase A2 isozymes PA3A/PA3B/PA5	272.71	725.12	−1.41	cellular lipid metabolic process
Malonyl-CoA decarboxylase	298.61	980.50	−1.72	cellular lipid metabolic process
Pescadillo-like protein	98.17	295.69	−1.59	cellular component biogenesis
Cartilage intermediate layer protein 1	114.66	793.31	−2.79	cellular macromolecule catabolic process
Mucolipin-3	1.19	23.73	−4.31	cellular component organization or biogenesis
Ankyrin repeat domain-containing protein	84.16	319.08	−1.928	cell adhesion
Hypoxia up-regulated protein 1	699.93	2161.30	−1.63	Molecular chaperones GRP170/SIL1
heat shock 70kDa protein 5	3142.59	10394.61	−1.73	HSP70 superfamily
cysteine dioxygenase	2574.68	13466.01	−2.39	oxidoreductase activity
tyrosinase-like protein tyr-1	42.74	192.05	−2.19	oxidoreductase activity
Fibropellin-3	92.16	254.34	−1.46	chitin binding
Sushi domain-containing protein 1	6.17	81.77	−3.73	Ca^{2+}-binding
Significantly up-regulated genes				
Coleoptericin	19.02	0	Inf	response to stress
Cytochrome P450	64.84	8.10	3	cellular response to stimulus
Acetyltransferase (GNAT) family	282.61	99.27	1.51	response to stress
catalase	3421.72	1324.85	1.37	response to stimulus
selenium binding protein	2818.95	631.42	2.16	response to stimulus
CYP356A1	81.29	9.66	3.07	response to stimulus
Perlucin	263.67	78.89	1.74	response to stimulus
sialic acid binding lectin	367.14	72.12	2.35	response to stimulus
Kappa-type opioid receptor	194.26	23.43	3.05	signal transduction
neurotransmitter transporter	184.13	34.37	2.42	neurotransmitter transporter activity
dopamine N-acetyltransferase-like	323.20	38.63	3.06	N-acetyltransferase activity
transglutaminase 1	478.45	166.29	1.52	cellular protein modification process
Pyridine nucleotide-disulphide oxidoreductase	211.26	44.63	2.24	cellular aromatic compound metabolic process
GPI ethanolamine phosphate transferase	134.91	32.25	2.06	macromolecule biosynthetic process
Dolichyl-phosphate-mannose-protein mannosyltransferase	122.42	0	Inf	glycoprotein biosynthetic process
Interferon-induced very large GTPase	405.16	92.41	2.13	fatty acid metabolic process
P-glycoprotein	3084.00	883.44	1.8	organonitrogen compound metabolic process
Sulfotransferase family cytosolic 1B member 1	162.34	11.51	3.82	catalytic activity
multidrug resistance protein 1A-like	1260.02	404.39	1.64	catalytic activity
phenylalanine-4-hydroxylase	625.28	221.48	1.5	catalytic activity
arginine kinase	674.75	171.80	1.97	catalytic activity
glutaminase	106.46	28.60	1.9	catalytic activity
aryl sulfotransferase	29.58	2.82	3.39	catalytic activity
Glutamine synthetase	132.65	33.97	1.96	catalytic activity
Interferon-inducible GTPase	200.50	3.65	5.78	catalytic activity
para-nitrobenzyl esterase	250.37	60.15	2.06	catalytic activity
sigma class glutathione S-transferase	1968.26	551.57	1.84	catalytic activity
Prolyl oligopeptidase	439.70	116.93	1.91	catalytic activity
Proprotein convertase subtilisin/kexin type 5/6	1364.22	437.26	1.64	proteolysis
Ileal sodium/bile acid cotransporter	307.64	77.15	1.99	bile acid and bile salt transport
Sodium/calcium exchanger protein	177.00	26.39	2.74	transmembrane transport
Growth-arrest specific micro-tubule binding	48.31	5.13	3.23	transmembrane transport
Sodium/glucose cotransporter	242.09	82.44	1.55	transmembrane transport
heat shock protein 22 isoform 2	8117.61	2458.01	1.72	passive transmembrane transporter activity
Sodium/glucose cotransporter 4	92.83	23.09	2.01	transmembrane transport
Excitatory amino acid transporter 1	7807.72	2371.61	1.72	ion transmembrane transporter activity
Phosphatidylinositol-glycan biosynthesis class S protein	14.02	0	Inf	intracellular protein transport
forkhead box protein J1	267.90	94.13	1.51	binding
beta tubulin	4622.11	1118.41	2.05	binding
Fibronectin type III	118.88	11.23	3.4	metal ion binding
Transient receptor potential cation channel	67.87	11.94	2.51	cation binding

(*continued on next page*)

Table 5 (*continued*)

Description	Readcounts		FC	Function
	A30	B		
4-aminobutyrate aminotransferase	4256.42	1107.99	1.94	metal ion binding
sarcoplasmic calcium-binding protein	472.41	111.98	2.07	transition metal ion binding
Down syndrome cell adhesion molecule	235.50	60.59	1.96	receptor binding
Metabotropic glutamate receptor 7	2215.52	885.49	1.32	carbohydrate binding

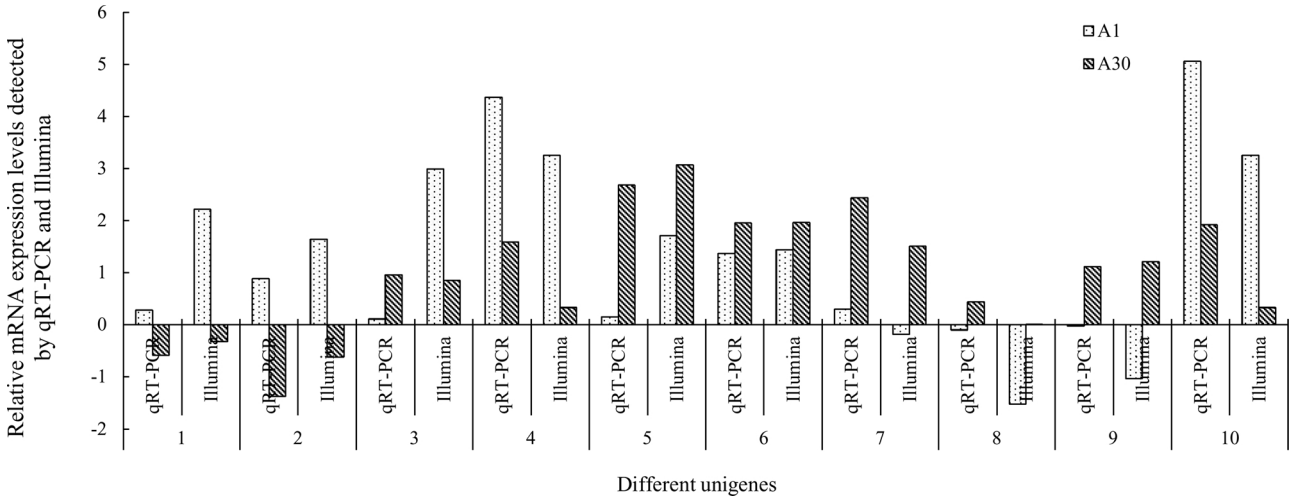

Fig. 5. Validation RNA-seq profiles by qRT-PCR for the samples after 1 and 30 days' exposure of ammonia nitrogen.
Note: 1. *TMDP*; 2. *ABCA*; 3. *TRP*; 4. *CYP450-like*; 5. *Dat-like*; 6. *GS*; 7. *FOX*; 8. *Apo X1*; 9. *TG1*; 10. *TG2*.

regulation of coleoptericin gene suggested that a short-term exposure of ammonia nitrogen would increase the opportunities of bacterial infection to *R. philippinarum*. In addition, immune-related genes were significantly changed after a 30-day exposure of ammonia nitrogen (Table 5). Among them, IFI44L is a kind of critical protein in host immune response to viral infections and can be used as molecular signature for viral infection diagnosis (Ngo et al., 2015). C-type lectin 9a belongs to the NK cell receptor group of C-type lectins and plays important roles in apoptosis and immune response against pathogens (Sancho et al., 2008). Complement component C3 is a stable circulating complement component that can facilitate both phagocytosis and bacterial killing but decreased after trauma (Shatney and Benner, 1985). Tumor necrosis factor-like protein and tumor necrosis factor ligand superfamily member 10 were both important immune factors (Ślebioda and Kmieć, 2014; Šedý et al., 2015). Alpha-2 macroglobulin (A2 M) is a major component of the innate immune system, as a modulator of prophenoloxidase system against bacterial infection (Ponprateep et al., 2017). Therefore, the significant down-regulations of the immune-related genes suggested that the immune abilities of *R. philippinarum* clam were significantly attenuated especially against virus and bacteria. Of the significantly up-regulated genes after 30-day exposure, coleoptericin gene continuously kept a significantly higher increment. Besides, sialic acid binding lectin gene was similarly significantly up-regulated. It is a defense related protein in mollusc with affinity for bacterial lipopolysaccharide and facilitates their phagocytic uptake (Tunkijjanukij and Olafsen, 1998). The significant up-regulation of these immune-related genes implied that long-term exposure of ammonia nitrogen would increase the opportunities of *R. philippinarum* to bacterial infection.

Secondly, ammonia nitrogen exposure might cause some disturbances in membrane stability of the clams. Significantly variations of gene expression in membrane (GO: 0016020) might affect the membrane integrity. Phospholipase A2 isozymes (PA3A/PA3B/PA5) are key enzymes for phospholipid hydrolysis and generate free arachidonic acids. It has been suggested as a potential mediator for phospholipidosis during lysosome dysfunction (Balsinde et al., 2002). The significant variation of phospholipase A2 isozymes (PA3A/PA3B/PA5) gene after ammonia nitrogen exposure would attribute to the reduced integrity of lysosome membrane which we found in the previous study (Cong et al., 2017).

Thirdly, ammonia nitrogen exposure would probably affect neurotransduction from several aspects. The significantly down-regulation of glutamine synthetase (GS) and glutaminase (GLS) genes involved in biosynthesis of amino acids would result in decreased contents of glutamate and branched chain amino acids. It is known that GS is exclusively located in astrocytes to synthesize glutamine from glutamate and ammonia (Jayakumar and Norenberg, 2016). GLS is a key enzyme in cancer cell metabolism, growth and proliferation by converting glutamine to glutamate (Xiang et al., 2015). And branched amino acids are proved to be involved in the process of glutamine formation to alleviate the toxicity of ammonia (Holecek, 2013). Therefore, the reduced expression of glutamate and branched chain amino acid genes might both be involved in the detoxification of ammonia nitrogen. In addition, the significantly changed expression of many neuro-related genes would affect significantly the concentrations of neurotransmitters, including rabaptin, neural cell adhesion molecule, kappa-type opioid receptor, dopamine N-acetyltransferase-like, neurogenic locus notch like protein, neurotransmitter transporter, 4-aminobutyrate aminotransferase, glutaminase, transglutaminase, dopamine N-acetyltransferase-like, metabotropic glutamate receptor 7 and so on. Rabaptins are a family of proteins involved in EGF receptor-mediated signal transduction during endocytosis (Barbieri et al., 2004; Disanza et al., 2009). Neural cell adhesion molecule is a kind of membrane glycoprotein which acts as a ligand in adhesion among neural cell bodies or neurites (Sadoul et al., 1983). Kappa-type opioid receptor is reported to be involved in signal transduction in central nervous system (Rácz and Halasy, 2002). Dopamine N-acetyltransferase-like is a kind of N-acetyltransferase to catalyze the N-acetylation of dopamine into N-

acetyldopamine, which is the major precursor for quinone and subsequent pigment (Noh et al., 2016). 4-aminobutyrate aminotransferase (ABAT) catalyzes the first step of the principal inhibitory neurotransmitter γ-aminobytyric acid (GABA) to degrade within the mitochondrial matrix (Iftikhar et al., 2017). Moreover, GABA has been implicated in modulating the opioid mechanisms. For example morphine and beta-endorphin administration increases GABA levels in the globus pallidus and naloxone, at high dosages, acts as a GABA blocker (Stella et al., 2005). Significant variations of these genes suggested that neuro-signal transduction was obviously changed after the short-/long-term exposure of ammonia nitrogen.

Fourthly, ammonia nitrogen exposure would lead to some kind of tumor through several pathways. In previous study, we found some xenobiotic substances in the gill tissue after ammonia nitrogen exposure. It probably signified some kind of tumor. Although the mechanism of tumor formation has not been well elucidated, tumor has been found in invertebrate animals (Domazet-Lošo et al., 2014; Muehlenbachs et al., 2015) with similar transcriptome shifts of genes in vertebrate, affecting cell cycle, apoptosis, genomic stability and metabolism (Domazet-Lošo et al., 2014). In the present study, we also found alterations of gene expression involved in apoptosis and genomic stability. There were 7 overexpressed binding-related protein genes significantly up-regulated after 1-day exposure, including EGFL, SRP, SVEP, SRPX2, cbEGF, fibulin 1/2 and NOTCH2 (Table 4). It is known that calcium is an essential element for the cells to function correctly, and its concentration is maintained within a narrow range. Additional calcium influx was approved to induce cell death by activating Ca^{2+}-dependent enzymes, after NMDA-receptors were activated by ammonia nitrogen (Randall and Tsui, 2002). All of the 7 binding-related genes were found to contain EGF domain (Weinmaster et al., 1992; Knott et al., 1996), which were responsible for the high-affinity calcium binding (Selander-Sunnerhagen et al., 1992). EGF was proved to activate calcium signaling in vertebrate tumor cells (Bryant et al., 2004). So the 7 overexpressed calcium-binding protein genes were anticipated to take part in stress-related responses especially apoptosis after ammonia nitrogen exposure. Besides, there were two apoptosis inhibitors significantly down-regulated after 30-day exposure. The decrement of apoptosis inhibitor 1 and 2 would help to change the fate of cell death after ammonia nitrogen. Meanwhile, three genes responsible for DNA stability were found to be significantly changed their expressions after ammonia exposure. From Table 4, it can be seen that the expression level of PIF1-like helicase gene was significantly decreased and two DNA repair protein (XRCC4 and Ercc1) genes were significantly increased after one-day exposure. PIF1-like helicase is a conserved DNA helicase in organisms to keep the maintenance of genomic stability (Boulé and Zakian, 2006). XRCC4 and Ercc1 were responsible for repair of DNA double-strand break. Significant variations of the three genes suggested that genome of the clams were not stable after ammonia nitrogen exposure. It may be a hallmark of tumor formation like that in vertebrate (Domazet-Lošo et al., 2014). However, detailed mechanisms of these genes contribute to the formation of the tumor-like substances in clam requires more effort in the future.

In another aspect, KEGG enrichment analysis pointed out the key pathways affected by ammonia nitrogen exposure. Simply, 1-day exposure of ammonia nitrogen caused DEGs significantly enriched in four pathways, including Dorso-ventral axis formation, Notch signaling pathway, Thyroid hormone signaling pathway, Protein processing in endoplasmic reticulum (ER). According to the KEGG description, the first three pathways were all related to cancer formation. It seemed that short time (1 day) of ammonia stress of low concentration (0.1 mg/ml) was toxic enough to interfere with hormone signal and trigger cancer reaction of clam *R. philippinarum*. And turbulence in the fourth pathway would promise mistake in protein folding and induce ER stress to the gill tissue. After a long term (30 day) exposure of ammonia nitrogen, seven KEGG pathways were detected to enrich significantly. Three of the enriched KEGG pathways were involved in the immune response against pathogen infection, including the phagosome (ko04145), gap junction (ko04540) and pathogenic *Escherichia coli* infection (ko05130) pathways. Two of the enriched KEGG terms were responsible for fat and oil digestion and cancer development, including bile secretion (ko04976) and ABC transporters (ko02010) pathways. The rest two enriched KEGG pathways were involved in the metabolisms of carbohydrate and amino acids, including arginine biosynthesis (ko00220), alanine, aspartate and glutamate metabolism (ko00250). So the analysis of enriched KEGG pathway indicated that a long-term exposure of ammonia exposure would significantly change the metabolisms of lipid, carbohydrate and amino acid, and affect the immune system of clam to increase the opportunities of pathogen infection and cancer formation.

Therefore, it can be seen that ammonia nitrogen exposure disturbed the expression of genes in quite a few of pathways, and genes in different pathways might work together leading to a certain kind of toxicological endpoint. The present study provides a backbone description of gene and pathway responses towards ammonia nitrogen. Further study will be laid on the correlation of certain genes expression, protein function and its cellular location with a specific toxicological effect.

5. Conclusion

The present research provided the analysis for the molecular identification of key genes and main metabolic pathways of clam *R. philippinarum* under short- and long-term exposures of ammonia nitrogen at transcriptome level. Digital gene expression analysis in the gills revealed 90 significant DEGs under a short duration period and 161 significant DEGs under a long duration period. No significantly enriched GO terms were found in the short-term and the long-term exposure groups compared with the blank group. KEGG pathway analysis of DEGs indicated that short duration of ammonia exposure affected the Dorso-ventral axis formation, Notch signaling pathway, thyroid hormone signaling pathway and protein processing in endoplasmic reticulum pathway. The 30-day exposure led to DEGs significantly enriched in gap junction, immunity, signal and hormone transduction, as well as key substance metabolism. Functional research of significantly changed DEGs suggested that toxic effects of ammonia nitrogen on *R. philippinarum* clam was correlated with quite a few of pathways and mainly concerned with response to stimulus, binding and transporting of calcium ion, immune response, neuro-transduction, and metabolism of important substances such as fatty acid, protein, nucleotide acids and carbohydrate. The present study might provide a preliminary genetic mechanism about the detectable toxicological endpoints in previous study.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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