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Gender specific differences of immune competence in the sea cucumber *Apostichopus japonicus* before and after spawning

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ABSTRACT

The gender differences of immunity have been elucidated in many vertebrates and invertebrates. However, the information of this difference was still not clear in the sea cucumber *Apostichopus japonicus*, which is one of the most valuable aquaculture species and susceptible to diseases caused by pathogen infection. In the present study, the transcriptome of coelomocytes from female and male *A. japonicus* before and after spawning was obtained by RNA-sequencing technology. A total of 4,538 and 8,248 differentially expressed genes were identified between female and male *A. japonicus* before and after spawning, respectively, indicating that the gender differences of gene expression profiles in *A. japonicus* were more remarkable after spawning. Further KEGG enrichment analyses were conducted for both male and female up-regulated genes before and after spawning. The results revealed that the capacity to kill pathogens in female *A. japonicus* might be more powerful than that in males no matter before and after spawning; the antioxidant ability in male *A. japonicus* was probably stronger than that in females after spawning; the complement system in male *A. japonicus* might be more effective than that in females after spawning; and the apoptosis was likely to be more serious in male *A. japonicus* before spawning. Moreover, we speculated that the fatty acid composition might be one of the inducements for gender specific immune differences of *A. japonicus*. Overall, the results of our study illustrated the global gender specific immune differences of *A. japonicus* and contributed to understanding of the molecular mechanisms underlying sea cucumber immune regulation.

1. Introduction

The sea cucumber *Apostichopus japonicus* is one of the most valuable sea food in East Asian countries due to its superior nutritive and medicinal properties [1,2]. With increasing market demands for related products, sea cucumber culture along the coasts of China has developed rapidly in recent years and created enormous economic and social benefits [3]. However, various diseases that caused by bacteria, viruses, and protozoa were frequently observed during *A. japonicus* culture process, limiting the development of this industry [4]. As one of the invertebrates, *A. japonicus* depends on the innate immune system to defense against the invasion of pathogens [5]. Therefore, the adequate understanding of *A. japonicus* immune characteristics is vital for the more effective prevention and control of *A. japonicus* diseases.

The immune system of animals was reported to be affected by several genetic and environmental factors, among which, gender is the notable one [6–9]. In the immune system of vertebrates, gender specific differences of immune competence are well known. In general, the females commonly possess more powerful immune response than males [10]. However, the gender specific differences of immunity in invertebrates are ambiguous. For instance, in the echinoderm species *Paracentrotus lividus*, the females possess a significant higher number of immunocytes than males [11]. In the scorpionfly *Panorpa vulgaris*, lysozyme-like activity and phagocytosis activity in the hemolymph of females were higher than those of males [12]. On the contrary, acid phosphatase activity in haemocyte lysate from male *Ruditapes philippinarum* was significantly higher compared to females [13]. Furthermore, the immune system of animals was also reported to be affected by reproduction. Some studies suggested that there might be trade-offs between immunity and reproduction [14]. For example, both male and female *Gryllus texensis* showed a decline in immunocompetence after the reproduction behavior [15]. Similarly, lower cellular immunity was observed in reproductive male *Myotis daubentonii* compared to non-reproductive individuals [16]. In *A. japonicus*, although few kinds of

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immune-related factors were comparatively studied between females and males [17], the comprehensive difference in immunity between females and males before and after spawning is still not clear.

The coelomocytes are recognized as one of the most crucial immune tissues in *A. japonicus* due to their primary responsibility for cellular immunity [18]. Therefore, the coelomocytes are used to determining immune parameters to evaluate the immune condition or characteristics of *A. japonicus*. For example, the transcriptional expression of several immune-related factors, such as catalase, cathepsin D and complement 3, in coelomocytes of *A. japonicus* after bacterial challenges were determined to investigate the differential response against different pathogenic bacteria [19]. The activities of acid phosphatase, alkaline phosphatase, lysozyme, phenol oxidase, superoxide dismutase, catalase, and myeloperoxidase were evaluated in the coelomocytes of *A. japonicus* during a year cycle to assess the effects of seasonal change on the immunity [20]. In addition, the transcriptome sequencing was also applied to the coelomocytes of *A. japonicus* that challenged with lipopolysaccharide to identify novel immune effectors [21]. The transcriptome is a set of all transcripts produced in a population of certain types of cells at a particular stage in an organism, which can provide large amounts of data for understanding the mechanisms underlying various biological processes [22]. In recent years, the transcriptome analysis has been widely used in *A. japonicus* and proved to be effective for elucidating the comprehensive molecular characteristics and mechanisms of several immune or physiological processes [23].

Hence, in this study, in order to examine the global transcriptional expression characteristics of female and male *A. japonicus*, recognize the gender specific immune response of *A. japonicus,* and illustrate the differences of immune competence before and after spawning in *A. japonicus*, the RNA-Seq was applied to analyze the differences of transcriptome in coelomocytes of *A. japonicus* between females and males before and after spawning. Our study will provide new insight into *A. japonicus* immune strategy.

2. Materials and methods

2.1. Experimental animals

Sixty viripotent sea cucumbers *A. japonicus* with body weight of 257 ± 18.6 g were collected from Dalian, China in June. These sea cucumbers were acclimated in the laboratory for one week before use. The seawater used for temporary culture was filtered through sand, and then through 300-μm nylon sieves. The conditions of seawater aquaria were maintained at 16 °C, with salinity of 31‰, pH 8.2 and continuous aeration.

2.2. The collection of coelomocytes and determination of sex

Before spawning, the coelomic fluid was collected with a sterilized culture dish after the dissection of a sea cucumber at venter with a sterilized eye scissors. At the same time, the gender of these sea cucumbers was demonstrated by the phenotype of gonad (the gonad of female *A. japonicus* exhibits the color of orange red, while that of male *A. japonicus* shows a milk white color). For spawning, the remainder sea cucumbers were transported to a set of fishbowls so that there was only one individual in one fishbowl. The temperature in these fishbowls was immediately upgraded to 22 °C to simulate the spawning of *A. japonicus*. Subsequently, the seawater of each fishbowl was examined using microscope to determine the gender of sea cucumber through the morphology of egg and sperm. After spawning, the sex-determined sea cucumbers were continuously cultured one month at 16 °C, with salinity of 31‰, pH 8.2 and continuous aeration, and then the coelomic fluid was collected with the method mentioned above. All collected coelomic fluid was immediately transferred into 10 mL RNase-free centrifuge tubes and centrifuged at 3000 rpm for 10 min at 4 °C. The pellets, which were referred to as the coelomocytes, were suspended with RNA*later* RNA Stabilization Reagent (cat No. 76104, QIAGEN, USA) and stored at −20 °C before the further experiments. Totally, 9 female and 9 male sea cucumbers were dissected for coelomocytes collection before and after spawning, respectively.

2.3. RNA extraction, cDNA library construction and sequencing

The total RNA was extracted from coelomocytes by using RNAprep pure Tissue Kit (TIANGEN, China) according to the manufacturer's instructions. The quality and concentration of total RNA were measured by 1.5% agarose gel electrophoresis and NanoPhotometer N50 (IMPLE, Germany). The total RNA from three *A. japonicus* individuals having identical gender and being at the same stage were equivalently mixed to avoid individual differences. Finally, a total of twelve RNA samples were obtained for cDNA library construction, which were BF1-3 (females before spawning), BM1-3 (males before spawning), AF1-3 (females after spawning), and AM1-3 (males after spawning). A total amount of 3 μg RNA per sample was used as input material for the library preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Subsequently, the constructed libraries were purified (AMPure XP system) and the library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina Hiseq 2500 platform and 150 bp paired-end reads were generated.

2.4. Data processing and analysis

The raw data were firstly processed by removing reads containing adapter, reads containing ploy-N and low-quality reads (quality scores < 20) to generate clean data. All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from NCBI directly (PRJNA413998) [24]. Index of the reference genome was built using Bowtie v2.2.3 [25] and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12 [26]. HTSeq v0.6.1 [27] was used to count the reads mapped to each gene, and then FPKM, reads per kb per million reads, of each gene was calculated [28]. Differential expression analysis was performed using the DESeq package in R between samples of female and male *A. japonicus* before and after spawning, respectively [29]. Genes with a fold change > 1.5 and a p-value adjusted by FDR < 0.05 were assigned as differentially expressed. The enrichment analysis based on KEGG annotation was performed by KOBAS (http://kobas.cbi.pku.edu.cn/) with the entire transcriptome set as the background, and the KEGG pathways enriched cutoff was a p-value corrected by FDR < 0.05 [30]. The results of differential expression and enrichment analyses were visualized using ggplot2 package in R.

2.5. Expression validation using qRT-PCR

To validate the results of RNA-Seq, 9 differentially expressed genes (DEGs) was selected for qRT-PCR on an Applied Biosystems 7500 Real Time PCR system (ThermoFisher Scientific, USA). The total RNA that used in the RNA-Seq was reverse-transcribed into cDNA with the PrimeScript™ RT reagent Kit (TAKARA, Dalian, China) according to the manufacturer's instruction. Based on the sequence information in the *A. japonicus* genome, primers were designed using Primer 5.0 software and the primer information is provided in Table 1. The cytochrome b (Cytb) gene was chosen as the reference gene based on previous report [21]. The qRT-PCR amplification was conducted in a volume of 20 μL containing 10 μL of 2 × SYBR Premix Ex Taq™ II (Tli RNaseH Plus, TaKaRa,

Table 1
The primers used for qRT-PCR validation.

Gene	Primer Sequence (5′-3′)
Cytochrome b (Cytb)	Cytb-F: TGAGCCGCAACAGTAATC
	Cytb-R: AAGGGAAAAGGAAGTGAAAG
Serine/threonine-protein kinase (TBK1-like)	TBK1-F: AGATGATGTTGTCCATTCTCG
	TBK1-R: ACAGGAGGAAGTGATGTGCT
mitogen-activated protein kinase kinases 3 (MKK3)	MKK3-F: CCGAGGAGAAAGGATCAAGAGA
	MKK3-R: TTATGACAGGGTAGCCACACAA
Complement 3	C3-F: GCGTTGTTTCGTTCAACAAGGGGA
	C3-R: GCCATTCACTGGAGGTGTGCCA
Caspase-3	Caspase-3-F: TCAGGGACTACTTTGATGGATGG
	Caspase-3-R: TGTGTTGGTGGGGTTGGAATG
Caspase-6	Caspase-6-F: AGAATGAACAGGAGAGTCGGAAC
	Caspase-6-R: TGAGTGAGAAAAGCACACAGGAA
Glutathione S-transferases (GST)	GST-F: CAACCCACGGAAAAAGTTACCTG
	GST-R: TTGCTGTCTGTTTAGTGTCTGGG
Toll-like	Toll-F: ACGAAAGCGATTTAGCC
	Toll-R: GAGCCCGTGGTGAGATG
Heat shock protein 70 (HSP 70)	HSP70-F: AAGAGCACAGGCAAAGAG
	HSP70-R: TGATGATGGGTTGGCACA
Heat shock protein 90 (HSP 90)	HSP90-F: TATGAAAGCCTGACAGACGCAAGC
	HSP90-R: TAACGCAGAGTAAAAGCCAACACC

Dalian, China), 0.4 μL of ROX Reference Dye I, 1 μL of cDNA template, and 0.4 μM of each primer. Thermal cycling was as follows: 95 °C for 30 s, 40 cycles at 95 °C for 5 s, 55 °C for 35 s and 72 °C for 25 s. All reactions were run in triplicates and the specificity of primers were checked by the melting curve. Relative Expression Software Tool 384 v.2 (REST) (Technical University of Munich, Munich, Germany) [31] was used to calculate the expression differences between female and male *A. japonicus* before and after spawning, respectively. The correlation between the results of RNA-Seq and qRT-PCR was displayed using Origin 8.0 software.

3. Results and discussions

3.1. The global transcriptional differences between female and male A. japonicus

To achieve a comprehensive gene expression profile for the female and male *A. japonicus*, twelve libraries were constructed for RNA-Seq that represented the stages before and after spawning. A range of 39,733,394 to 55,594,720 raw reads were generated from RNA-Seq and deposited in the NCBI SRA database (Accession number: SRP173420). After quality control and mapping to the reference genome, the gene expression levels among different samples were obtained. Subsequently, the correlation between different samples was performed by heatmap based on the pearson correlation coefficient (Fig. 1). The gene expression profiles of samples from the same group were more correlated between each other and distinct with samples in other groups, indicating that the global transcriptional differences exist in *A. japonicus* between different genders as well as before and after spawning.

The DEGs between female and male *A. japonicus* before and after spawning were respectively recognized to evaluate the gender differences of gene expression levels (Fig. 2). Before the spawning, a total of 4,538 DEGs were identified, in which 1,767 genes were up-regulated in females and 2,771 genes were up-regulated in males. Meanwhile, 8,248 DEGs were established between the samples after spawning, in which 4,476 genes were up-regulated in females and 3,772 genes were up-regulated in males. Such a huge number of DEGs found in female and male *A. japonicus* also indicated that there were significant differences in gene expression profiles between different genders of sea cucumbers. In addition, compared to the samples before spawning, the samples after spawning had higher number of DEGs, revealing that the gender differences of gene expression profiles in *A. japonicus* were more remarkable after spawning.

Nine DGEs were selected to validate the reliability of the RNA-Seq results for the reasons that these genes were provided with full-length cDNA sequence information, widely distributed in different tissues, and involved in diverse and representative immune pathways of *A. japonicus*. Moreover, the expression of these genes was detected in all samples and differed at least in the comparison (female vs male before or after spawning). Among these genes, *complement 3* is a key component of complement system and directly involved in the lysis of pathogen cells [32]; *Toll* plays crucial role in recognition of pathogens and initiation of immune reactions in the innate immunity of invertebrates [33]; *caspase-3* and *-6* are a family of protease enzymes playing essential roles in apoptosis [34]; heat shock protein (*HSP*) *70* and *90* are a family of proteins that are produced by cells in response to exposure to stressful conditions [35]; *mitogen-activated protein kinase kinases 3* (*MMK3*) gene is a member of the MAPK signaling cascade and negatively regulated the cell proliferation and apoptosis [36]; *glutathione S-transferases* (*GST*) is best known for their ability to catalyze the conjugation of the reduced form of glutathione to xenobiotic substrates for the purpose of detoxification [37]; *serine/threonine-protein kinase* (*TBK1*), a member of Toll signaling pathway, interacts with the *TRAF2* and *IKK* to degrade the *IκB* and activate the NF-κB [38]. The validation results indicated that the relative expression levels of selected genes from qRT-PCR were similar to those from RNA-Seq (Fig. 3). As shown in Fig. 3, the relative expression level of these nine genes varied significantly and diversely before and after spawning, suggesting that the effects of reproduction process on the immunity of *A. japonicus* are very complicated. Maybe in *A. japonicus*, the reproduction process not only has different effects on different gender, but also has different effects on different immune factors that in the same gender. Further studies are required to reveal the mechanisms that reproduction process affects immune factors in gender-specific *A. japonicus*.

3.2. The differences of enriched pathways between female and male A. japonicus

In order to further assess the immune and physiological characteristics of *A. japonicus* with different gender, the KEGG enrichment analyses of up-regulated expression patterns in different samples were carried out. Three enriched pathways associated with nucleic acid synthesis, including purine metabolism, pentose phosphate pathway and glycosaminoglycan degradation, which probably related to the spermatogenesis, were found in male *A. japonicus* before spawning (Fig. 4A). In addition, several pathways concerned with energy consumption were enriched in male *A. japonicus* after spawning (Fig. 4B), implying more carbohydrates supplement were required for male *A. japonicus* during the reproductive recovery period. But most importantly, multiple metabolic pathways associated with resistance to oxidative stress, containing proteasome, N-Glycan biosynthesis and glutathione metabolism, were enriched in male *A. japonicus* after spawning (Fig. 4B). This finding meant that male *A. japonicus* might have stronger antioxidant capacity than female after spawning. Consistent result was also obtained in our previous study, in which the activities of catalase and peroxidase in *A. japonicus* coelomocytes from males were significantly higher than those from females [17].

In *A. japonicus* after spawning, various development-related pathways were enriched in females compared to males, such as Wnt signaling pathway, Notch signaling pathway, mTOR signaling pathway, etc (Fig. 5B). This result indicated that female *A. japonicus* might experience a stage of rapid development after spawning. Moreover, multiple pathways associated with immune competence of killing pathogen, containing phosphatidylinositol signaling system, Jak-STAT signaling pathway, inositol phosphate metabolism and endocytosis, were enriched in female *A. japonicus* whether or not spawning (Fig. 5). This result suggested that, compared with the immune system of males,

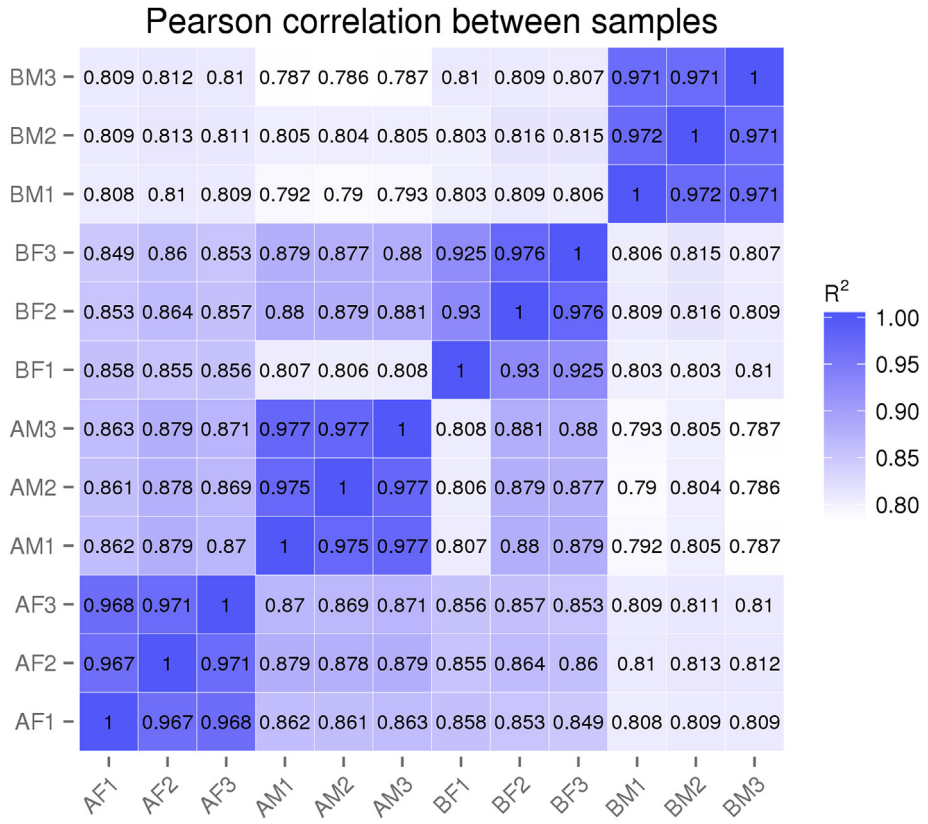

Fig. 1. Heatmap exhibits the pearson correlation of gene expression profiles between female and male *A. japonicus* before and after spawning.

that of female *A. japonicus* might be more efficient in pathogen killing. Similar results were also found in a variety of vertebrates and invertebrates, including human beings [10], *P. lividus* [11], *P. vulgaris* [12], and *Gryllus texensis* [15].

In addition, the metabolism pathways of hormones, including arachidonic acid and its precursor linoleic acid, were enriched in male *A. japonicus* before spawning (Fig. 4A). Meanwhile, some fatty acid metabolism pathways, including synthesis and degradation of ketone bodies, sphingolipid metabolism, pyruvate metabolism, and histidine metabolism, were enriched in female *A. japonicus* before spawning (Fig. 5A). Many previous studies have proved the relationship between the fatty acid composition of immune cells and their function. For example, eicosanoids produced from arachidonic acid have roles in inflammation and regulation of T and B lymphocyte functions [39]. Short-chain fatty acids, such as butyrate, can regulate the skin immune system via expansion of regulatory T cells [40]. Glycosylated sphingolipids participate in the initiation of receptor mediated signaling transduction and regulate several immune events [41]. Moreover, the sexual hormones have been demonstrated to be the crucial factors for the immune differences between females and males in vertebrates [42]. Therefore, the differences in fatty acid composition might be one of the important reasons that leading to the differences in immune competence between female and male *A. japonicus*.

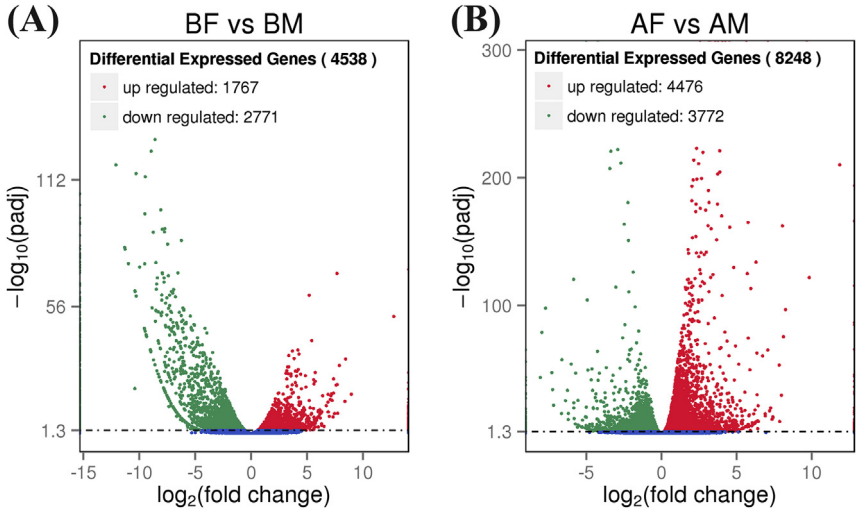

Fig. 2. The results of differentially expression genes recognition between female and male *A. japoncus* before (A) and after (B) spawning, respectively.

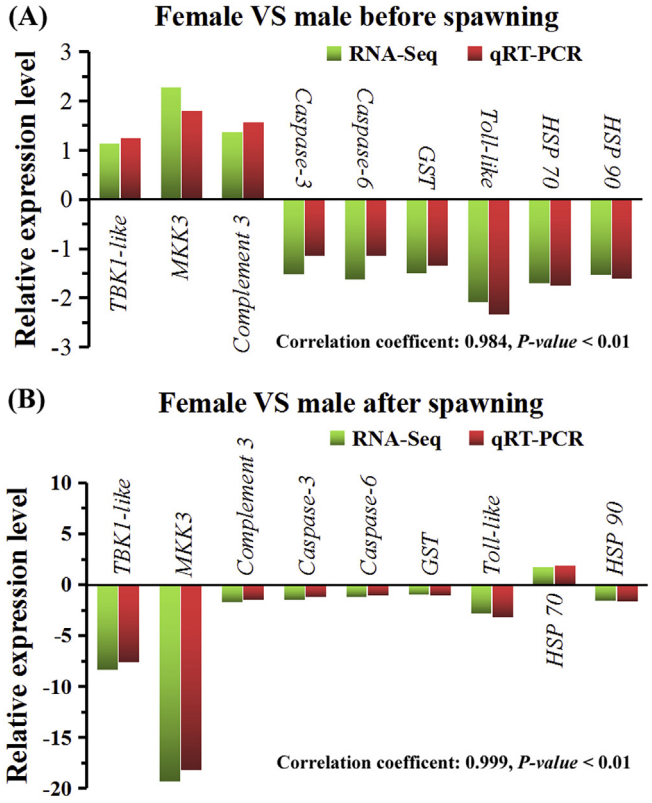

Fig. 3. Validation of RNA-Seq results using qRT-PCR.

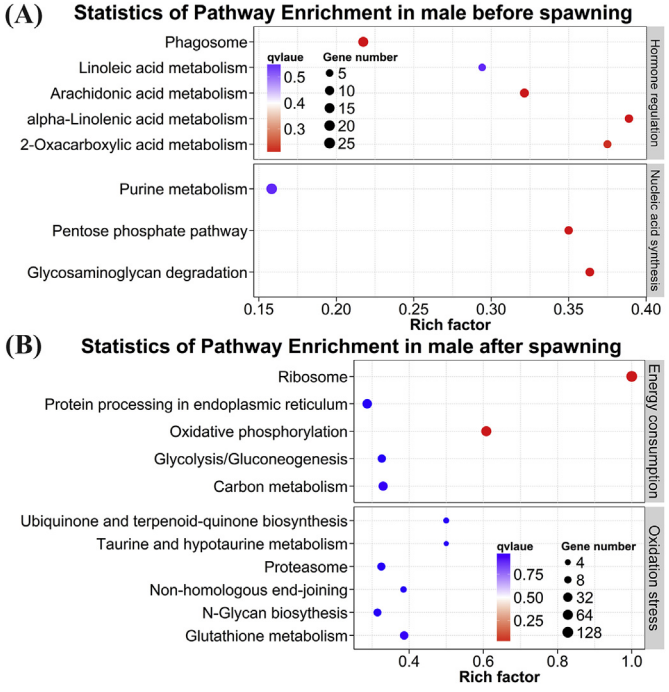

Fig. 4. The results of KEGG enrichment analyses based on the up-regulated genes in male *A. japonicus* compared to females before (A) and after (B) spawning.

3.3. The difference in complement system and anti-apoptosis between female and male A. japonicus

During the progression of breeding seedling and culturing, immune regulation was an important event in *A. japonicus* resistance to diseases.

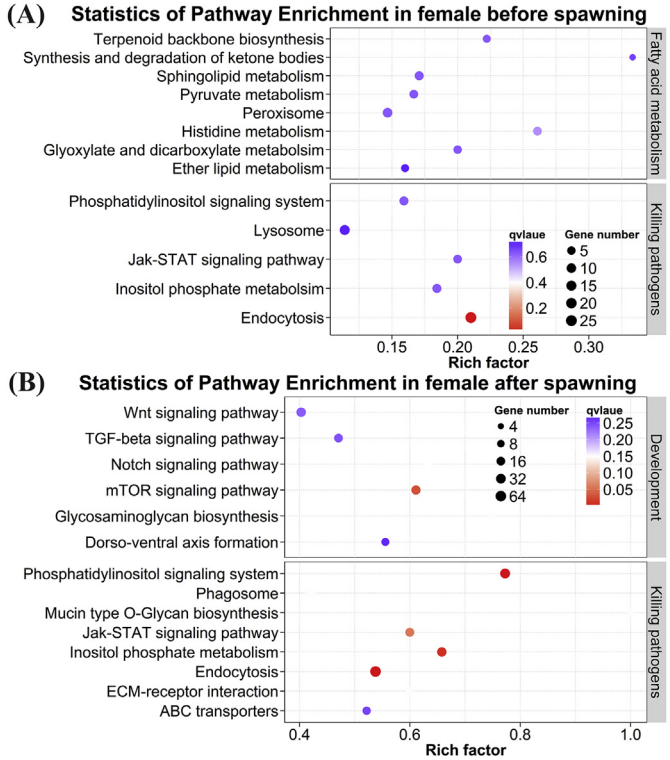

Fig. 5. The results of KEGG enrichment analyses based on the up-regulated genes in female *A. japonicus* compared to males before (A) and after (B) spawning.

In this study, many DEGs involved in immune regulation were attained, in which the complement system and apoptosis were more remarkable. In complement system, the pattern recognition receptors, filcolins and mannan-binding lectins (MBLs), recognize the pathogens and transmit signals to promote the decomposition of complement 3, which activates the production of cytokines [32]. During this process, the factor B positively while the factor H negatively regulate the cleavage of complement 3 [43]. The investigation of DEGs belong to the complement system in this study showed that (Fig. 6), in *A. japonicus* before spawning, the genes of ficolins and MBLs were up-regulated in females compared to males, suggesting the female *A. japonicus* might have more powerful capacity of pathogen recognition. However, the expression level of *A. japonicus* complement 3 was down-regulated in females compared to males before spawning, which meant that the complement

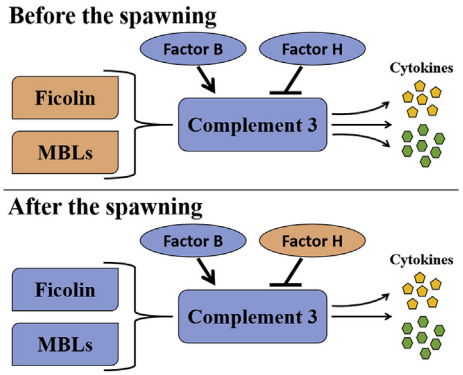

Fig. 6. The differences in expression levels of genes involved in complement systems from the female and male *A. japonicus* before and after spawning. The color of orange represents up-regulated in females and the color of blue represents up-regulated in males. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

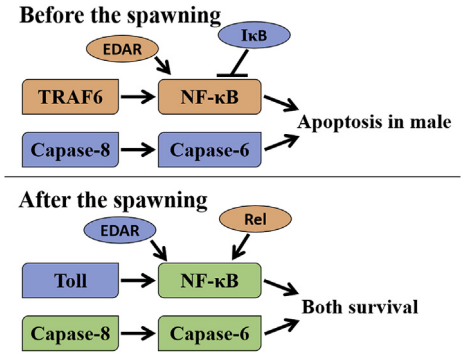

Fig. 7. The differences in expression levels of genes associate with apoptosis from the female and male *A. japonicus* before and after spawning. The color of orange represents up-regulated in females and the color of blue represents up-regulated in males. The color of green represents no significant difference in gene expression between the female and male *A. japonicus*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

system was relatively inefficient in female *A. japonicus* before spawning. In male *A. japonicus* before spawning, although the expression of complement 3 was up-regulated, the genes of factor B and H were also up-regulated. This result indicated that the complement system of male *A. japonicus* might be in a controlled state. Furthermore, the expression of ficolins, MBLs, complement 3, and factor B were up-regulated meanwhile the expression of factor H was down-regulated in male *A. japonicus* compared with females after spawning. This finding implied that the complement system functioned effectively in male *A. japonicus* after spawning.

Apoptosis is a form of programmed cell death, which is a form of traumatic cell death that resulted from acute cellular injury [44]. Caspases play the central role in the transduction of apoptotic signals and degrade intracellular proteins to carry out the cell death program [45]. Meanwhile, many families of proteins act as negative regulators that inhibit the cell death signaling pathway, such as NF-κB [46]. Some DEGs related to apoptosis were detected in *A. japonicus* before and after spawning (Fig. 7). The genes of caspases and *IκB*, coding a protein family of NF-κB inhibitor, were up-regulated in male *A. japonicus* before spawning. On the contrary, the up-regulation of *TRAF6* and *EDAR* genes was detected in female *A. japonicus* before spawning, which might result in activating the NF-κB through the TLR and TNF signaling pathways, respectively. These outcomes illustrated that the apoptosis might be more serious in male *A. japonicus* before spawning. On the other hand, no significant difference was found in the expression of caspases between female and male *A. japonicus* after spawning. At the same time, the genes of *Toll* and *EDAR* were significantly up-regulated in male *A. japonicus*, which might lead to activating the NF-κB through the TLR and TNF signaling pathways, respectively. Besides, the gene of *Rel*, which can form NF-κB heterodimer with p50, was significantly up-regulated in female *A. japonicus*. Therefore, male and female *A. japonicus* might have similar anti-apoptosis capacity after spawning.

4. Conclusions

Taken together, our study demonstrated the gender specific of immune competence in *A. japoncus* before and after spawning. The female *A. japonicus* might be more efficient in pathogen killing than male no matter before and after spawning. But stronger antioxidant ability was observed in male *A. japonicus* than that in females after spawning. Moreover, the complement system was more effective in male *A. japonicus* after spawning and more serious apoptosis could be presented in males before spawning. The findings of our study demonstrated that both gender and reproduction had remarkable effects on the immunity of *A. japoncus*. In addition, the complex gender-related immune differences in *A. japoncus* indicated that the effects of gender on immunity had a close relationship with species in invertebrates.

Acknowledgments

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