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Short communication

Effect of seasonal high temperature on the immune response in *Apostichopus japonicus* by transcriptome analysis

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ABSTRACT

The sea cucumber *Apostichopus japonicus* is a flourishing aquaculture species in China. However, there are challenges for sea cucumber aquaculture, one of which is the high temperature in summer. In this study, we explored the transcriptome expression profiles with seasons (APR, JUN and JUL) in the muscle tissue of *A. japonicus*. The temperature of the natural coast was 13 °C, 21 °C and 25 °C respectively when sampling. Compared with APR group, changes of expression profiles were more significant in JUL group than that in JUN group. A total of 46 differential expressed genes (DEGs) involved in both innate and adaptive immunity were highlighted, including 27 up-regulated and 19 down-regulated genes. They were further grouped into 10 subclasses: heat shock, coagulation cascades, antigen processing and presentation, inflammatory response, transporter activity, immunoglobulin, lectin C, cell adhesion, reactive oxygen species (ROS) scavenging, apoptosis and autophagy. The study will offer deep insights of the molecular mechanisms underlying the physiological responses to seasonal high temperature in *A. japonicus*. Particularly, knowledge about the immunological effects of seasonal temperature on the species is critical for the optimal management practices for both wild and aquaculture populations.

1. Introduction

There is overwhelming evidence that changes of global temperature occur rapidly [1]. It is reported that the global mean surface temperature (GMST) has increased by 0.85 °C from 1880 to 2012 [2]. The global mean sea surface temperature (SST) has increased by 0.67 °C over the last century [3]. Moreover, some coastal areas, which are ecologically and economically important, are strongly affected by global climate change with 2 °C temperature rise [4,5]. The warming trend has driven serious consequences on the marine organisms [6]. Temperature has a fundamental effect on biological processes on account of its influence on molecular kinetic energy, including enzyme reactions, diffusion and membrane transport. Metabolism of most marine organisms is temperature-dependent, which further alters their respiration, development, and other physiological processes [5]. A range of molecular response is evolved to confront the consequences of high temperature stress. The heat shock response (HSR), including the accumulation of heat shock proteins (HSPs) and other chaperones, is the predominant protective procedure [7]. Besides, physiological (e.g. reduce of metabolism) and behavioral adaptions (e.g. aestivation) are major strategies to suffer through the long-term adversity [8]. Beyond this range, however, populations decline or are driven to local extinction.

The echinoderms (e.g. sea cucumbers, sea urchins, starfish), together with the hemichordates (e.g. acorn worms), form the ambulacraria, a sister clade to the chordates in the deuterostome animals [9]. The echinoderms are of special interest for studies because they can provide insights on the evolutionary origins of immunology response and organism-environment interface that occur in vertebrates [10,11]. The sea cucumber *Apostichopus japonicus*, belonging to phylum Echinodermata, class Holothuroidea, is mainly distributed along the coast zones of northern China, south-eastern Russia, Japan, Republic of Korea and Democratic People's Republic of Korea (North Korea) [12]. *A. japonicus* has been an aquaculture species in China, and it becomes popular food owing to high value in nutrition and medicine. However, there are challenges for sea cucumber aquaculture, one of which is the high temperature in summer. According to the previous study, the optimal temperature for the growth of *A. japonicus* is around 14–15 °C, depending on the size of individuals [13]. In natural sea area of Shandong Province, the temperature usually increased up to 20 °C in June, which resulted in mild heat stress for *A. japonicus*. When the sea temperature rises to 25 °C or above in July and August, *A. japonicus* individuals entered to the aestivation state gradually [14]. Therefore, this temperature was regarded as a threshold of severe heat stress. On account of extreme high temperature, mortality risk of *A. japonicus*

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increases dramatically. It is estimated that about 80% mortality occurred in the coastal ocean and ponds in main distribution regions of *A. japonicus* in northern China in the summer of 2013 and 2016 [15]. Therefore, understanding the effects of high temperature on *A. japonicus* is of vital importance for studying HSR of the echinoderms, and also of commercially significance.

Our understanding about the effect of seasonal temperature variation on *A. japonicus* far lags behind that in fish species [16]. Most studies of HSR in *A. japonicus* were only carried out in a laboratory, under short-time acute heat stress [17,18]. The effects of elevating seasonal temperature, which reflects actual heat stress for *A. japonicus,* are not yet fully understood. In this study, we explored the transcriptome expression profile of *A. japonicus* respectively in April, June and July of the natural coast in Shandong Province of China. The aim of this study is to elucidate the potential molecular mechanisms underlying the physiological responses along with seasons in *A. japonicus*. Particularly, deep insights of the immunological effects of seasonal high temperature on the species will be offered, which is critical for the optimal management practices for both wild and aquaculture populations.

2. Materials and methods

2.1. Samples

The sea cucumber samples (80–120 g) were collected by professional divers in Swan Lake (E122.6°, N37.4°), a marine lagoon located in Weihai, Shandong Province, China [19]. We collected the sea cucumber individual samples in three month groups, respectively in April, June and July of 2017. The three groups were named as APR, JUN and JUL respectively. All samples were collected in the afternoon. The temperature in the three groups was examined, which was 13 °C, 21 °C and 25 °C respectively.

2.2. cDNA library construction and RNA-Seq transcriptome

Sea cucumber individuals were collected respectively in APR, JUN and JUL groups. Three biological replications in each group were prepared in each group for the next transcriptomic sequencing. The muscle tissues were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

The total RNA was extracted from muscle tissues of every sea cucumber individual using the Trizol Kit (Invitrogen, USA) according to the manufacturer's instructions. RNA degradation and contamination was monitored on 1% agarose gels. RNA integrity number (RIN), was measured to determine RNA quality, using an Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). The RNA purity were determined using a Nanophotometer spectrophotometer (IMPLEN, CA, USA). The mRNA was purified from the total RNA and cut into fragments using the TruSeq RNA Sample Prep Kit (Illumina). Qualified RNA samples were prepared to build cDNA libraries. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. After purification with AMPureXP beads, the ligated products with about 250–300 bp were amplified to generate high quality cDNA libraries, which were then sequenced by an Illumina Hiseq platform at Novogene Bioinformatics Technology (Beijing, China).

2.3. Sequence assembly and Gene Ontology (GO) annotations

Clean reads were obtained from the raw reads by removing the adaptor sequences, low quality sequences and reads containing poly-N. All the downstream analyses were based on the clean data with high quality. Reference genome of *A. japonicus* were downloaded from at NCBI with the accession number PRJNA354676 [20]. Index of the reference genome was built and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. Gene Ontology (GO) annotations were determined using Blast2GO (http://www.BLAST2go.org/) to obtain a functional classification of the genes.

2.4. Differential gene identification

Gene expression profiling was based on the number of reads. FPKM (fragments per kb of exon model per million mapped reads) values were used to estimate the expressed values and transcript levels [21]. Briefly, FPKM value was calculated based on the number of reads mapping to each gene and the length of the gene. The resulting *P*-values were corrected using the Benjamini and Hochberg's approach for controlling the false discovery rate (FDR). The hierarchical heat map of all expressed genes was shown based on the centered and normalized $\log_{10}(\text{FKPM}+1)$, and the subclusters was analyzed based on the centered and normalized $\log_2(\text{FKPM}+1)$ [22]. Genes with an adjusted *P*-value (padj) < 0.05 found by DESeq2 were assigned as differentially expressed genes (DEGs).

2.5. Real-time PCR validation

Muscle tissues of five individuals were sampled in each group for real-time PCR validation. The mRNA extraction was followed by the method mentioned above. First strand cDNA was synthesized using reverse transcriptase (Takara, Japan). The mRNA expression validation were examined by a SYBR Green® real-time PCR assay (SYBR PrimeScrip™ RT-PCR Kit II, Takara) with an Eppendorf Mastercycler® ep realplex (Eppendorf, Hamburg, Germany).The gene β-actin was used as a control gene for internal standardization [17]. Six genes chosen for validation included heat shock protein 26 (gene30047, hsp26), heat shock protein 70 (gene22609, hsp70), sperm-associated antigen 17-like isoform X3 (gene6758, saa17), major yolk protein 1 (gene24412, myp1), superoxide dismutase (gene8830, sod) and deleted in malignant brain tumors 1 protein-like isoform X1 (gene29473, dmbt).The specific primers were listed in Supplemental Material Table S1. The amplification cycling was performed in: (1) 5 s at 95 °C for 1 cycle (2) 10 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C for 40 cycles.

The $2^{-\triangle\triangle CT}$ method was used to analyze the comparative mRNA expression levels. One-way analysis of variance with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical test. The level of significance was set with $P < 0.05$. All the data were presented as mean ± SD (standard deviation of the mean) ($N = 5$).

3. Results

3.1. Summary of transcriptomic sequencing data

We acquired a total of 260,420,628 raw reads for the studied 9 sea cucumber individuals (Table 1). After data filtration, we obtained

Table 1
Summary of transcriptomic sequencing data.

	Raw reads	Clean reads (rate)	Total map rate	Unique map rate	Multi map rate
APR1	27547441	27124100(98.46%)	69.27%	63.66%	5.61%
APR2	27481729	26718002(97.22%)	69.21%	63.0%	6.21%
APR3	34332052	33402143(97.29%)	70.25%	63.29%	6.96%
JUN1	26652457	25878399(97.10%)	68.75%	62.32%	6.44%
JUN2	30868621	29522639(95.64%)	69.46%	62.62%	6.84%
JUN3	27483864	26517678(96.48%)	68.28%	62.39%	5.89%
JUL1	31632955	30693971(97.03%)	68.18%	62.5%	5.68%
JUL2	28947990	27998544(96.72%)	69.68%	64.13%	5.54%
JUL3	25473519	25079663(98.45%)	68.92%	63.96%	4.97%

252,935,139 clean reads, accounting for an average rate of 97.13% of raw reads. These clean reads were applied in the following analysis. On average, 69.11 (± 0.66) % of the RNA-Seq reads were mapped onto the reference genome of *A. japonicus* [20]. Around 63.10 (± 0.69) % reads were mapped uniquely while 6.01% (± 0.65) % reads have multiple mapping positions. The sequence data from this study have been submitted to the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) with the accession number **PRJNA529308**.

3.2. Annotation of predicted proteins and GO analysis

Annotation of the transcripts was performed against the genomic data and NCBI non-redundant (Nr) database. A total of 73,183 genes were annotated in the studied 9 individuals. We quantified the expression levels of all genes by determining FPKM values (Supplemental Material Table S2).

GO analysis is an international standardized gene functional classification system that defines genes according to three ontologies: molecular function (MF), cellular component (CC) and biological process (BP). In this test, we applied GO analysis for the annotated genes. The results showed that we annotated the genes to 20,513 GO terms. In the biological process category, the major subcategories were"cellular process" (11,813, 58%) and "metabolic process" (9,664, 47%) **(**Fig. 1**)**. In the cellular component subcategory, a significant proportion of the clusters were classified as "cell" (5,643, 28%) and "cell part" (5,642, 28%). In the molecular function category, the largest proportion subcategory was "binding" (11,633, 57%), followed by "catalytic activity" (8,169, 40%).

3.3. Differential expression gene (DEGs) among groups

In order to visualize the expression patterns of transcripts along with different temperature, we performed hierarchical clustering using the FKPM values. The group cluster results illustrated that the JUN group and JUL group were clustered together depending on the gene expression profiles **(**Fig. 2A**)**. Four subclusters, according to expression patterns of genes, were characterized **(**Fig. 2B**)**. Genes in subcluster 2 had an increasing trend with APR, JUN and JUL groups. Conversely, genes in subcluster 3 had a decreasing expression tendency. The detailed genes involved in every subcluster were listed in Supplemental Material Table S3.

To better understand the biological mechanism resulting in transcriptomic changes depending on different environmental temperature, we identified the DEGs among the three groups (APR, JUN and JUL), with the standard of adjusted *P*-value (padj) < 0.05. A total of 455 (288 up-regulated and 173 down-regulated), 383 (164 up-regulated and 219 down-regulated) and 1153 (604 up-regulated and 549 down-regulated) DEGs were detected in APR vs JUN, JUN vs JUL, and APR vs JUL comparison respectively **(**Fig. 3**)**. This result showed that the number of DEGs was the most abundant in APR vs JUL comparison. Of these DEGs, 122 DEGs were shared between both APR vs JUN and APR vs JUL comparison. Remarkably, solute carrier organic anion transporter family 1A4 (novel.6633, oatp1A4) was differentially expressed in all the three comparison groups.

3.4. Key DEGs associated with immune response

In the present study, we focused on DEGs related to the immune responses. The response of immunological genes was complicated, with inconsistent expression tendency. In general, there were much more DEGs involved in immunological functions in APR vs JUL comparison than that in APR vs JUL comparison. We highlighted 46 DEGs involved in both the innate and adaptive immunity, including 27 up-regulated and 19 down-regulated genes **(**Table 2**)**. They were further grouped into 10 sub-classes as follows: heat shock, coagulation cascades, antigen processing and presentation, inflammatory response, transporter

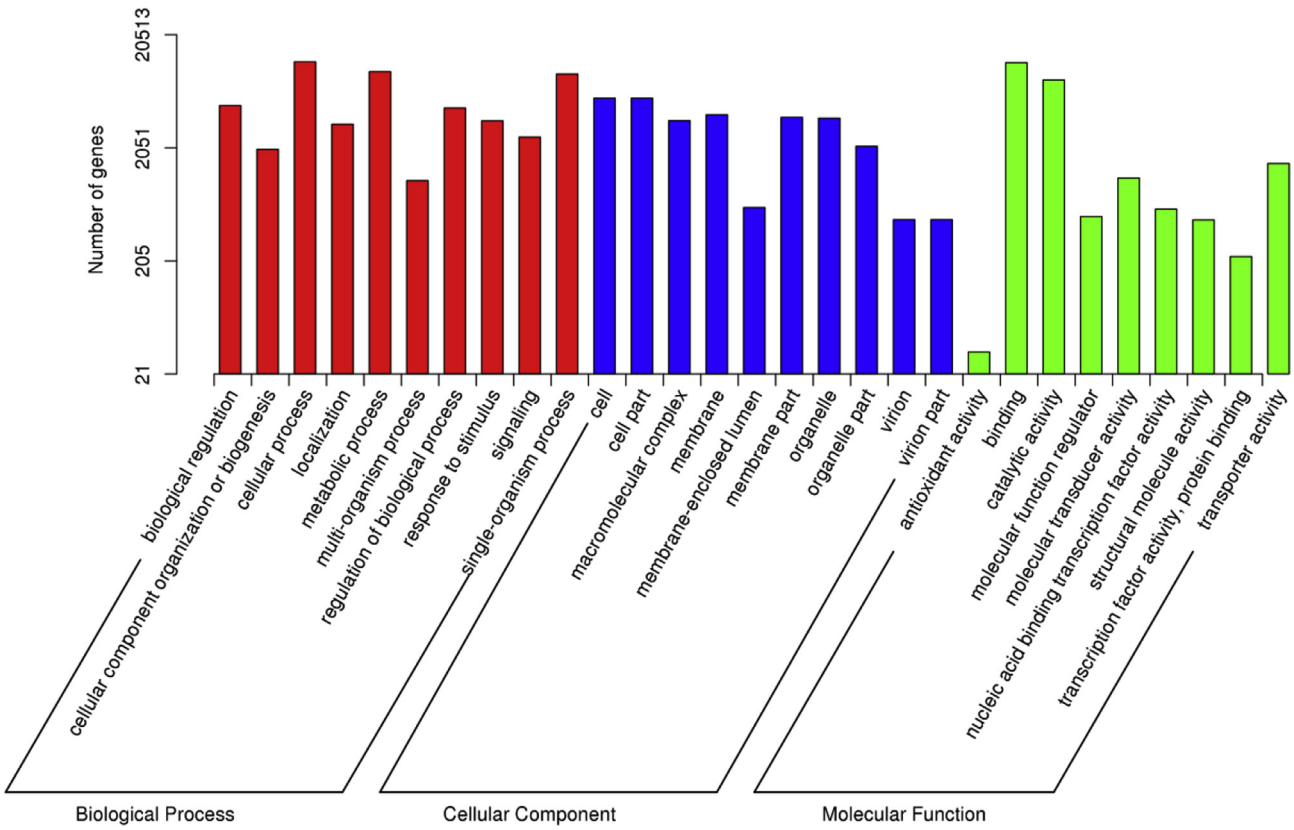

Fig. 1. Gene Ontology (GO) annotation of genes in Biological Process (BP), Cellular Component (CC) and Molecular Function (MF).

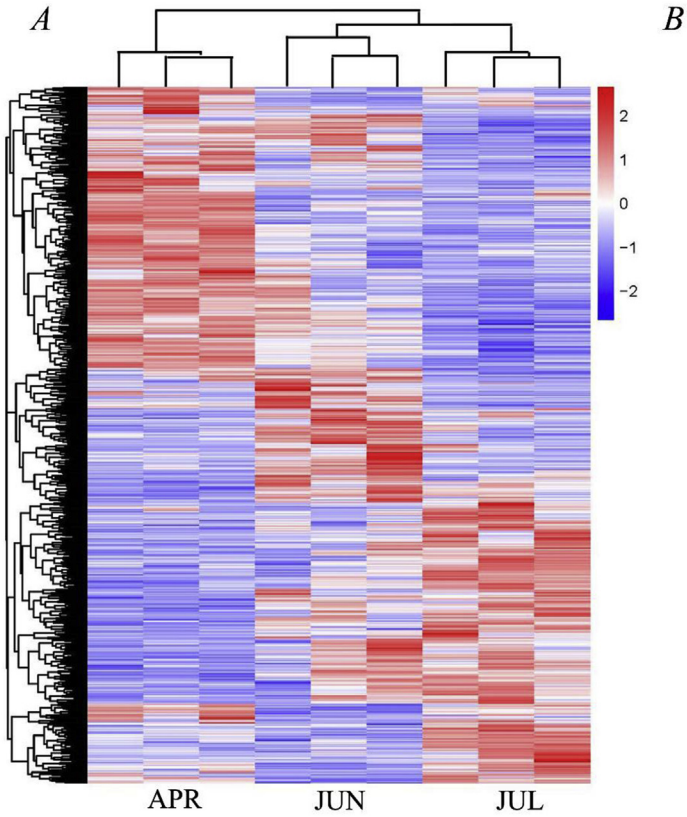

Fig. 2. Expression profiles of genes in three groups. (A) Hierarchical heat map of all expressed genes based on the centered and normalized $\log_{10}$(FKPM +1). Red color represents high expression value while blue color represents low one. (B) Expression patterns of four clustered profiles based on the centered and normalized $\log_2$(FKPM+1). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

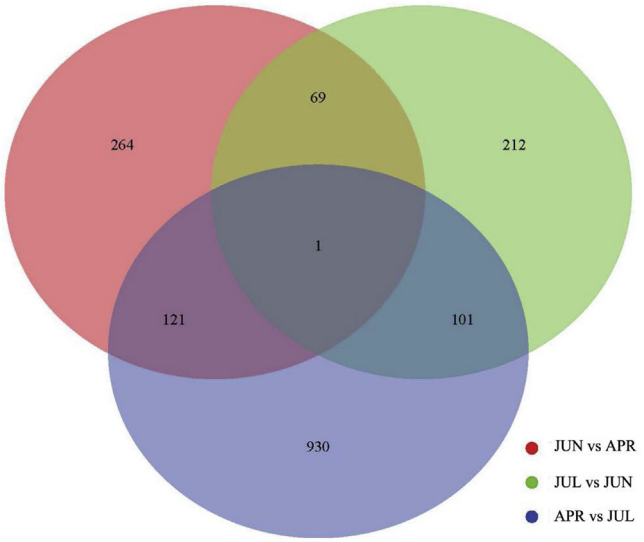

Fig. 3. Venn diagram showing the number of DEGs among three groups.

activity, immunoglobulin, lectin C, cell adhesion, Reactive oxygen species (ROS) scavenging, apoptosis and autophagy.

Various genes associated with heat shock were examined in *A. japonicus*. After expression normalization and significance analysis, we found that the expression of hsp26 increased significantly in both JUN and JUL group, compared with APR group (padj < 0.05) **(**Table 2**)**. Besides, the expression of hsp70 and aha1 were only up-regulated in JUL group. In the "coagulation cascades" category, alpha-2-macroglobulin family N-terminal region and two fibrinogen genes were up-regulated. In the "antigen processing and presentation" category, the expression of three sperm-associated antigen genes and paraneoplastic antigen rose significantly in JUL group while the expression of IgGFc-binding protein increased in JUN group. Besides, bacterial extracellular solute-binding proteins and B-cell lymphoma 9 were up-regulated. The genes in the category "inflammatory response" and "transporter activity" showed an increasing expression as well.

Our results also presented a diversity of immune genes with decreasing expression in JUN and JUL groups **(**Table 2**)**. The expression of genes involved in "immunoglobulin" and "lectin C" were significantly down-regulated in JUL group. Six genes associated with "cell adhesion" were down-regulated as well, such as cadherin-23 and collagen. Besides, superoxide dismutase (sod), glutathione S-transferase and glutathione peroxidase, which play roles in "ROS scavenging", were down-regulated in JUL group. Regarding to the category "apoptosis and autophagy", we found that apoptosis regulator genes, including Beclin-2 and dmbt, showed declining expression in the high temperature groups. By contrast, the expression of autophagy-related protein 27 presented rising expression. Additionally, four proteases were involved in the DEGs, including metalloprotease, OTU-like cysteine protease, papain family cysteine protease and gag-polyprotein aspartyl protease.

3.5. Real-time PCR validation

The mRNA expression profiles of six genes were examined by real-time PCR. Consistent with the transcriptome data, the expression of hsp26, hsp70, myp1 and saa17 increased significantly (Fig. 4). Particularly, the expression of hsp26 was 74.51-fold higher in JUL than that in APR group. The expression of sod declined significantly in JUL, and the expression of dmbt declined significantly in both JUN and JUL groups.

4. Discussion

4.1. Seasonal variation of immune response in A. japonicus

Though there were 455 DEGs in JUN compared with APR group,

Table 2
DEGs involved in immune responses in JUN and JUL groups compared with APR group.

ID	Gene description	Diff.	log_2Fold JUN	log_2Fold JUL
Heat shock				
gene30047	heat shock protein 26	up	2.85	6.64 *
gene20270	heat shock protein 26	up	4.43 *	4.06 *
gene20271	heat shock protein 26	up	2.96	3.43 *
gene20272	heat shock protein 26	up	4.84 *	3.09 *
gene13647	heat shock protein 26	up	4.45 *	1.92
gene22609	heat shock protein 70	up	3.66	4.65 *
gene23962	heat shock protein 70	up	3.44	3.96 *
gene23963	heat shock protein 70	up	3.04	3.80 *
gene28699	activator of heat shock protein 90 ATPase 1	up	1.52	2.54 *
Coagulation cascades				
novel.28725	alpha-2-macroglobulin family N-terminal region	up	3.11	4.10 *
novel.3950	fibrinogen beta and gamma chains, C-terminal globular domain	up	2.70*	1.81 *
novel.49183	fibrinogen beta and gamma chains, C-terminal globular domain	up	2.17	3.09 *
Antigen processing and presentation				
gene6757	sperm-associated antigen 17-like isoform X3	up	0.39	1.95*
gene6758	sperm-associated antigen 17-like isoform X3	up	0.58	2.26 *
gene5724	sperm-associated antigen 8-like	up	0.34	1.61 *
gene17552	paraneoplastic antigen Ma1-like	up	1.57	2.44 *
gene18945	IgGFc-binding protein	up	4.62*	3.10
novel.1558	bacterial extracellular solute-binding proteins	up	2.32*	3.63*
novel.41005	B-cell lymphoma 9	up	1.56	2.26*
Inflammatory response				
novel.5637	phosphoinositide-specific phospholipase C	up	2.31	2.36 *
novel.5639	phospholipase C delta isoform	up	0.95	1.52 *
Transporter activity				
gene24412	major yolk protein 1	up	2.47 *	3.00
novel.6633	solute carrier organic anion transporter family member 1A4	up	3.73 *	6.58 *
Immunoglobulin				
novel.36651	immunoglobulin I-set domain	down	1.21	−2.65 *
novel.36649	immunoglobulin I-set domain	down	1.32	−2.85*
novel.40558	immunoglobulin I-set domain	down	−5.11*	−3.50 *
Lectin C				
novel.1402	lectin C-type	down	−0.83	−1.89 *
novel.26322	lectin C-type	down	−0.96	−1.43 *
novel.44161	lectin C-type	down	−0.84	−1.93 *
Cell adhesion				
novel.23590	cadherin-23	down	−0.15	−2.94*
gene21102	alpha-1 collagen isoform X4	down	−0.03	−2.03 *
novel.3565	fibrillar collagen C-terminal domain	down	−0.65	−2.29*
novel.5852	collagen triple helix repeat	down	0.13	−1.81 *
novel.36555	integrin beta-1	down	1.29	−2.16*
novel.11569	annexin	down	−0.85	−2.49 *
ROS scavenging				
gene8830	superoxide dismutase	down	−0.39	−1.95 *
gene29940	glutathione S-transferase alpha-4 isoform X1	down	−1.27	−1.97*
novel.20216	glutathione peroxidase	down	−0.95	−2.86*
Apoptosis and autophagy				
novel.15491	Beclin-2 family	down	−1.23	−1.74*
novel.35873	Beclin-2 family	down	−2.56	−2.59 *
gene29473	deleted in malignant brain tumors 1 protein-like isoform X1	down	−6.94*	−4.92 *
novel.2538	autophagy-related protein 27	up	6.62	2.03*
gene6868	metalloprotease TIKI1	down	−0.85	−3.07 *
novel.27316	OTU-like cysteine protease	up	−0.08	2.30 *
novel.35934	papain family cysteine protease	up	1.74	1.74 *
novel.37911	gag-polyprotein aspartyl protease	up	1.10	1.83 *

* *P*-value adjusted (padj) < 0.05.

enrichment analysis of DEGs revealed no significant GO terms and KEGG pathways in this comparison. In contrast, changes of gene expression between APR and JUL were comparatively obvious, with 1153 DEGs, 52 GO enrichment terms and 8 KEGG enrichment pathways. We discussed the immune response in the both conditions.

Among the 46 highlighted immunological genes, only a few genes were differentially expressed in JUN group. In the “heat shock” category, only hsp26 was up-regulated in this group. The result revealed that hsp26 was essential for the mild HSR in *A. japonicus*. Overexpressing the small HSPs seems to be more advantageous in mild stress environment [23]. Global gene response evaluated by microarray analyses revealed that hsp26 was also involved under mild heat stress in yeast *Saccharomyces cerevisiae* [24]. On the other hand, hsp70 showed no significant change in JUN group and was up-regulated only in JUL group, compared with APR group. The study in avian embryonic also suggested that moderate heat stress did not induce enhancements on hsp70 mRNA levels [25]. These results implied that hsp70 induction depended on temperature conditions, and it was vital for the severe HS instead of mild HS. The analysis revealed that myp1 and oatp1A4 were up-regulated in JUN group. The protein MYP1 belongs to the transferrin superfamily, due to its sequence similarity with transferrin protein and its iron binding ability [26]. OATPs play central roles in mediating the transmembrane transport of a wide range of organic compounds [27,28]. Collectively, our results implied a moderate change of immune response in JUN group.

The response in the JUL group was much more intense than the

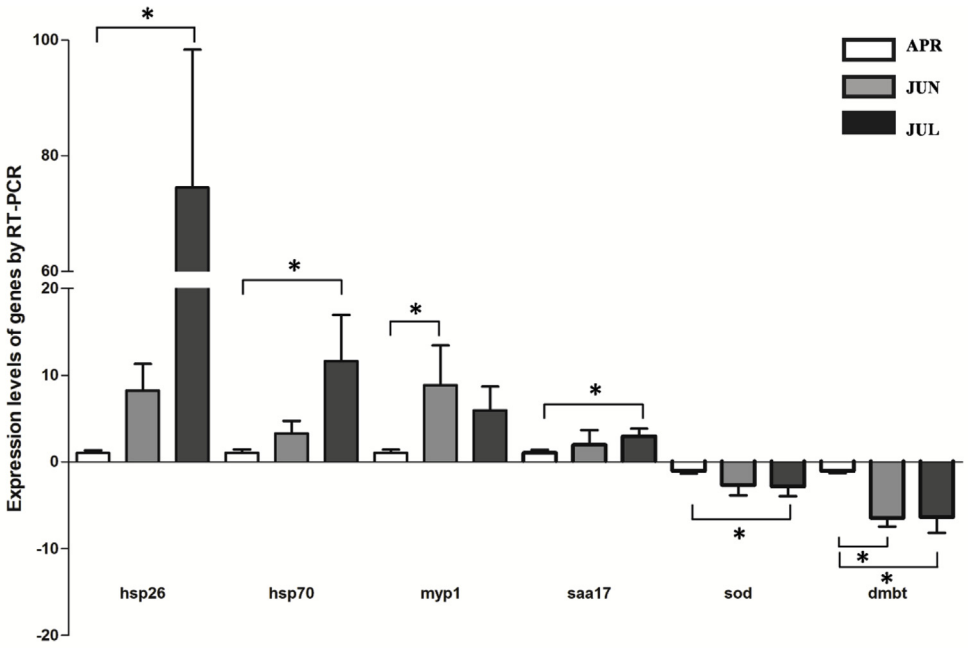

Fig. 4. Expression profiles of six genes in APR, JUN and JUL groups by real-time PCR. The $2^{-\triangle\triangle CT}$ method was used to analyze the data, and the symbol "*" indicate significant differences ($P < 0.05$). Values were represented as mean ± SD ($N = 5$).

changes under mild HS in JUN group. Particularly, 45 of these 46 genes in the 10 categories mentioned above represented distinct expression in JUL group. Generally, we found that increasing immune genes were involved in "heat shock", "coagulation cascades", "antigen procession and presentation", "Inflammatory response" and "transporter activity" categories while the declining immune genes were included in "Immunoglobulin", "lectin C", "cell adhesion" and "ROS scavenging" categories. The category "apoptosis and autophagy" consisted of both up-regulated and down-regulated genes. It should be emphasized that immunoglobulin were down-regulated and some antigens were up-regulated in JUL group, which suggested a susceptible state to bacterial of sea cucumbers [15]. It had been previously suggested that immune systems were significantly changed with seasons in other species [29,30]. Results in sliders indicated that bactericidal capacity of plasma, delayed-type hypersensitivity and total immunoglobulin levels varied significantly across seasons, but each measure had a different pattern of variation [31]. Seasonal temperature variation could also affect both the innate and adaptive immune systems in fish [32,33]. Besides, an increase in the photoperiod can also have a positive relation with the activity of lysozyme and the circulating levels of IgM [34]. The expression characteristics of immune genes are complicated along with seasons [35,36]. We suppose that temperature is the critical reason for the observed seasonal variation of immune response, though may be not the sole one.

Skin ulceration syndrome, one of the most common diseases for *A. japonicus*, is caused by bacterial pathogens of *Pseudoalteromonas* [37]. Although our data revealed that various immune genes, such as hsps, were up-regulated with the increasing temperature, a diversity of immune genes showed declining expression, especially genes associated with ROS scavenging. The bacterial infections is usually quicker at the higher temperature. Therefore, more attention should be paid to the prevention and control of infection of the diseases in summer.

4.2. Comparison of immune response under acute and seasonal heat stress

We studied the genes expression profiles of short-term acute HS in our previous studies [17,38]. The temperature of HS was set at around 26 °C, which was close to that in JUL group. The difference was that the heating process was faster, with a heating rate of 2 °C/h carried by the heating rod. We found that the response to short-term acute HS and prolonged seasonal HS shared some similarities and differences.

Unsurprisingly, members of hsps were involved in both conditions. Specifically, hsp26, hsp70, hsp90 and aha1 responded the acute HS significantly after 6 h exposure. However, their expression levels returned to the control level after 48 and 192 h [17]. The results suggested that acute HS resulted in rapid initiation of hsps synthesis and dramatic changes in gene expression. Regarding to the gradual seasonable temperature elevation, hsp26, hsp70 and aha1 were also up-regulated in JUL group compared with APR group. The results implied the vital role of hsps (hsp26 and hsp70 in particular) during both chronic and acute HS. Except hsps, DEGs under acute and chronic HS shared some other immune genes. For example, myp1 and phospholipase C was up-regulated while various lectin C genes were down-regulated in both two studies.

GSH metabolism forms antioxidant systems and provides important defense from many kinds of stressors [39,40]. In our previous acute HS study, three glutathione S-transferases and phospholipid hydroperoxide glutathione peroxidase and were up-regulated after acute HS in *A. japonicus* [38]. Conversely, genes involved in GSH metabolism showed decreased expression under prolonged HS in the present study, which may be on account of adaptation and energy budget. Environmental stressors have also been shown to alter the activity of SOD, but with inconsistent characteristics [11]. For example, SOD activity was shown to decrease with increasing anoxia in the clam *Chamelea gallina* [41], while increased with increasing temperature in the scallop *Chlamys farreri* [42]. Additionally, Chen et al. showed that SOD activity was not affected by temperature stress in the scallop *C. farreri* [43]. The results of SOD activity in *A. japonicus* under HS indicated that SOD activity markedly increased only at the 0 day at 25 °C, and then declined to normal level after 3 days [44]. In the present study, we found that the mRNA expression of sod decreased in JUL group in this study, while no obvious change in acute HS in our previous study [17]. Therefore, our results suggested that ROS scavenging system might play a more important role under short-term acute HS rather than the seasonal prolonged HS [45].

5. Conclusion

This study provides a global view of the differentially expressed genes in seasons with gradually increasing water temperature in *A. japonicus*. Compared with the APR group, changes of expression profiles were more significant in JUL group than that in JUN group. Particularly, a total of 46 immune DEGs involved in 10 categories were highlighted, including 27 up-regulated and 19 down-regulated genes. The study will offer deep insights of the molecular mechanisms underlying the physiological responses to seasonal high temperature in *A. japonicus*. Particularly, knowledge about the immunological effects of seasonal temperature on the species will be provided, which is critical for the optimal management practices for both wild and aquaculture populations.

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Appendix A. Supplementary data

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