


Research Article

Transcriptome Sequencing Reveals the Response to Acute Thermal Stress in the Pacific Abalone, *Haliotis discus hannai*

Zhou Wu,^{1,2} Yumin Yang,¹ Liqing Zhou ,¹ Changfeng Chi,² Xiujun Sun,¹ Biao Wu,¹ Zhihong Liu,¹ and Yan Wang¹

¹Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Chinese Academy of Fishery Sciences, Yellow Sea Fisheries Research Institute, Qingdao 266071, China

²National Engineering Research Center of Marine Facilities Aquaculture, National and Provincial Joint Laboratory of Exploration and Utilization of Marine Aquatic Genetic Resources, Zhejiang Ocean University, 1st Haidanan Road, Changzhi Island, Lincheng, Zhoushan 316022, China

Correspondence should be addressed to Liqing Zhou; zhoulq@ysfri.ac.cn

Received 10 November 2022; Revised 15 July 2023; Accepted 25 July 2023; Published 14 August 2023

Academic Editor: Pravesh Kumar

Copyright © 2023 Zhou Wu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The Pacific abalone is an economically important cold-water shellfish. With its widespread culture, high temperature has become a key abiotic factor for the high mortality of Pacific abalone in summer, particularly in the south of China. To understand the molecular regulatory mechanisms of thermal stress response in Pacific abalone for further analyzing its heat adaptive capacity, we subjected Pacific abalone to acute heat stress at 28°C for 6, 24, and 48 hr, respectively. A total of 2,213, 2,337, and 1,420 differentially expressed genes (DEGs) were identified in Pacific abalone after 6, 24, and 48 hr of thermal stress, respectively, as compared with the control group without heat stimulation. These DEGs were significantly enriched in protein folding and chaperone-mediated protein folding process, antigen processing and presentation, and protein processing in endoplasmic reticulum pathways. The expression of numerous molecular chaperones was significantly upregulated after thermal stress. Maintaining cellular homeostasis through the upregulated expression of molecular chaperones associated with endoplasmic reticulum-associated degradation (ERAD) pathways might be central to the defense of Pacific abalone against thermal stress. In addition, the expression of Toll-like receptor 6 (*TLR6*) and myeloid differentiation factor 88 (*MyD88*) genes of the TLR signaling pathway was also induced. Therefore, the Pacific abalone might activate the immune defense system to resist the invasion of pathogenic bacteria by upregulating immune-related genes after thermal challenges. Our results not only contribute to a deeper understanding of heat stress regulation in the Pacific abalone but also provide basic data for molecular genetic breeding of heat-resistant abalone.

1. Introduction

Temperature is one of the main physical factors affecting marine biological ecosystems [1]. Changes in seawater temperature can directly determine the distribution range and regulate all aspects of marine animals, including their growth and development [2–5], reproduction [6], metabolism, as well as immune responses [7–9]. The Pacific abalone *Haliotis discus hannai* is the main abalone species currently cultivated in China [10] and shows high economic and nutritional value. As a kind of cold-water shellfish, it is naturally distributed in the Korean Peninsula, the northern waters of Japan, the coastal areas of the Shandong Peninsula, and the Liaodong Peninsula of China.

With the vigorous development and continuous southward movement of the abalone aquaculture industry, Fujian Province has become the main region for cultivating Pacific abalone in southern China since 2000. According to statistics, the annual output of cultured abalone in China had reached 203,000 tons in 2020, including 155,000 tons from Fujian Province, accounting for 76.4% of the country's total output (China Fisheries Yearbook). However, the mass-scale culture of Pacific abalone in the south region of China has been facing the great challenge of high mortality in summer [11, 12]. This is because the temperature in these areas is significantly higher than that in the natural habitats of this species [13]. The optimum temperature on the north coast of

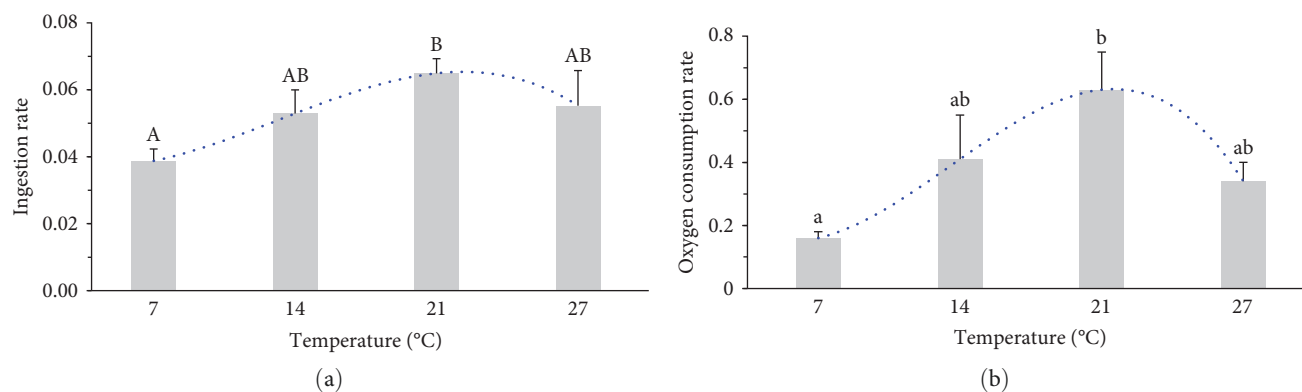


FIGURE 1: Ingestion rate (a) and oxygen consumption rate (b) of *Haliotis discus hannai* at different temperatures. Different letters among groups indicate significant differences ($P < 0.05$).

China was 4–26°C; when the temperature was higher than 26°C, the activity decreased and the growth stopped [14], while when the temperature was higher than 28°C, the mortality rate increased significantly [15]. Elevated seawater temperature not only increases the abundance of pathogens such as marine *Vibrio spp.* but also reduces the innate immunity of marine animals to *Vibrio spp.*, increasing their risk of infection and disease outbreaks [14, 16–18].

With the rapid development of next-generation sequencing technologies, low-cost high-throughput sequencing has become possible [19]. The transcriptome sequencing (RNA-seq) technology, which not only enables rapid generation of transcript data for studying species but also increases the depth and coverage of sequencing, has become a common method for studying nonmodel organisms lacking genomic information [20]. Recently, a growing number of economic aquatic organisms have been analyzed using this technique, elucidating the impact of external environmental changes on biological processes and the stress response of target species to various stimuli [21–23]. There have been several reports on the adaptation of shellfish to temperature changes. For example, transcriptome sequencing technology has been used to study the transcriptional responses of the Manila clam *Ruditapes philippinarum* [24] and the pearl oyster *Pinctada fucata martensii* [25] to cold stress to reveal the underlying mechanisms. Besides, the Pacific oyster *Crassostrea gigas* is often exposed to air and can adapt to severe temperature changes. Zhu et al. [26] have identified some key candidate genes, which encode heat shock proteins (*HSPs*) and apoptosis-related proteins by analyzing their transcriptome data under low- and high-temperature stress. During the hot summer months, the kuruma shrimp *Marsupenaeus japonicus* in the aquaculture system often suffers from mass die-offs. Zheng et al. [27] have conducted comparative transcriptome analysis of immune response, *HSP*, antioxidant system, and metabolic changes in kuruma shrimp under heat stress to explore the potential molecular mechanism of summer death.

Although RNA-seq technology has been widely used for research, the molecular mechanism of Pacific abalone's response to heat stress remains unclear. In recent studies, 26 *HSP* genes from four tissues (blood cells, gill, mantle, and muscle) of Pacific abalone were differentially expressed

under cold and heat stress conditions [28]. Yu et al. [29] found that under heat stress conditions, Pacific abalone in Dalian, northern China tends to maintain the synthesis and metabolism of macromolecules such as proteins in cells by activating transcription factors, thereby expressing various regulatory genes and protein folding-related genes. Shellfish, as invertebrates, lack an acquired immune system and only have a nonspecific immune system. Its hemolymph is a unique body fluid, and its functions are similar to human blood in many aspects, in which blood cells play an important functional role in hemolymph, and participate in immune responses as multifunctional hemolytic cells, mainly including phagocytosis, packaging, hemagglutination, blackening, etc. [30]. Hence, in this study, the hemolymph of Pacific abalone was selected as the target tissue, and the key candidate genes related to high-temperature stress response were screened by transcriptome sequencing of the hemolymph tissue after heat stress. This study can deepen the understanding of the molecular mechanism underlying the response of Pacific abalone to high temperature changes.

2. Materials and Methods

2.1. Pacific Abalone Samples. The Pacific abalone samples were collected on May 14, 2021 at Changqing Ocean Technology Co., Ltd. in Weihai City (Shandong Province, China). The average shell length of Pacific abalone was 49.07 ± 3.81 mm. After being transported to the laboratory, all abalones were temporarily cultured in a water tank, with the temperature set at 17°C and supply of oxygen and the salinity of the seawater is 35. They were fed with an appropriate amount of salted kelp every afternoon. In each next morning, a sewage pump was used to clean up the residual bait and feces and replace two-thirds of the water body with fresh one. Subsequent experiment was carried out after a week of temporary feeding.

2.2. Determination of Feeding Rate and Oxygen Consumption Rate of Pacific Abalone at Different Temperatures. To determine the optimal temperature for heat stress experiment, we measured the changes in feeding rate (FR) and oxygen consumption rate (OR) of abalone at 7, 14, 21, and 27°C (Figure 1). It was found that as the temperature exceeded the optimum growth temperature of the abalone, its FR and OR decreased.

Correlation fitting analysis found that FR, OR, and temperature (T) fit a cubic function relationship: $FR = (-1.2 \times 10^{-5})T^3 + 0.00047T^2 - 0.0038T + 0.046$ ($R^2 = 0.709$); $OR = (-2.8 \times 10^{-4})T^3 + 0.01166T^2 - 0.111T + 0.464$ ($R^2 = 0.850$). In summary, the final decision was made to set the temperature of the thermal stress group at 28°C and the control group at 17°C. The formulas for calculating feeding rate and oxygen consumption rate were as follows [31]:

$$F = \frac{F_1 - F_2}{t_1 \times N}, \quad (1)$$

$$FR = \frac{F}{G} \times 100\%, \quad (2)$$

$$OR = \frac{(DO_0 - DO_t) \times V}{W \times t_2}. \quad (3)$$

In the formula, F represents the food intake (g/(ind·d)), F_1 is the dry weight of the kelp fed (gram, g), F_2 is the dry weight of the remaining kelp (gram, g), t_1 is the feeding time (day, d), and N is the number of abalone in the experimental group; FR represents the feeding rate (%) and G is the average wet weight of the abalone in the experimental group (gram, g); OR stands for oxygen consumption rate per body weight (mg/(g)), DO_0 is the initial dissolved oxygen in water (mg/L), DO_t is the dissolved oxygen in the water after the experiment (mg/L), V is the water volume (liter, L) when measuring oxygen consumption, W is the dry weight of the abalone soft body (gram, g), and t_2 is the duration of the experiment (hour, hr).

2.3. Thermal Stress Treatment and RNA Extraction. After temporary culture, abalones were grouped and transferred to new breeding tanks with the tank temperatures set to 17°C (control group) and 28°C (thermal stress group), respectively. At 6, 24, and 48 hr of the experiment, a total of nine live abalones were randomly selected from each of two groups, respectively. The foot of each abalone was cut with a sterilized scalpel blade, and the hemolymph was extracted using a 1 ml syringe. The hemolymph of three abalone individuals was mixed in a 10 ml cryogenic tube and taken as one sample, and three biological replicates were set for each group. According to the sample processing requirements of Shanghai Biozeron Co., Ltd., the hemolymph and Trizol reagent (Invitrogen, Waltham, USA) were mixed at a ratio of 1 : 3, shaken with a vortex oscillator until the white floccs disappeared, and then immediately placed in liquid nitrogen for freezing. The frozen sample was then stored at -80°C for subsequent RNA extraction. Total RNAs were extracted from the hemolymph using a traditional Trizol reagent (Invitrogen, Waltham, USA) according to the manufacturer's instructions, and potential genomic DNA contamination was removed using DNase I (Takara, San Jose, USA) reagent. RNA degradation and contamination were detected by 1% electrophoresis gel. Then, the integrity of RNA was assessed by Agilent 2100 bioanalyzer, and the purity and concentration of RNA were detected by NanoDrop-2000 spectrophotometer.

High-quality RNA samples ($OD_{260/280} = 1.8-2.2$, $OD_{260/230} \geq 2.0$, $RIN \geq 6.5$, $28S:18S \geq 1.0$) with a concentration greater than $10 \mu\text{g}/\mu\text{l}$ were selected for the construction of transcriptome sequencing library.

2.4. Library Preparation and Illumina Hiseq Sequencing. RNA-seq transcriptome libraries were created using 1 g of total RNA and the TruSeq™ RNA Sample Preparation Kit from Illumina (San Diego, CA). Shortly, oligo (dT) beads were used to isolate messenger RNA, which was then fragmented with fragmentation buffer. Illumina's methodology was followed for cDNA synthesis, end repair, A-base addition, and ligation of the Illumina-indexed adaptors. Libraries were amplified for 15 cycles using Phusion DNA Polymerase (New England Biolabs, Ipswich, USA), and then screened 200–300 bp cDNA target fragment with 2% super-agarose gel. Paired-end libraries were sequenced by Illumina NovaSeq 6000 sequencing (150 bp * 2, Shanghai Biozeron Co., Ltd.) after being quantified by TBS 380.

2.5. Reads Quality Control and Mapping. Trimmomatic (version 0.36; <http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic>) was used to trim the raw paired-end readings for quality control (SLIDINGWINDOW:4:15 MINLEN:75). The clean reads were then aligned to the reference genome (<http://gigadb.org/dataset/100281>; file name: Haliotis.fa) using hisat2 (version 2.2.0; <https://daehwankimlab.github.io/hisat2/>) software in orientation mode. This software was used to map with default parameters. Then qualimap (version 2.2.1; <http://qualimap.conesalab.org/>) was used to analyze the data quality. Besides, htseq (version 2.0.2; <https://pypi.org/project/HTSeq/>) was used to count each gene read.

2.6. Differential Expression Analysis and Functional Enrichment. The expression level of each gene was estimated by calculating the fragments per kilobase of exon per million mapped reads (FRKM). The R statistical package edgeR (Empirical Analysis of Digital Gene Expression in R, version 3.40.2; <https://bioconductor.org/packages/release/bioc/html/edgeR.html>) was used to screen the differentially expressed genes (DEGs) and analyze their expression according to the fold change and P -value of the expression change. The selection standard is $|\log_2(\text{fold change})| > 2$, $P\text{-value} < 0.05$ and false discovery rate (FDR) < 0.05 . The analysis steps of Gene Ontology (GO) included mapping all DEGs to each term in the GO database, calculating the number of genes in each term, and finding the terms significantly enriched. Then, the DEGs contained in different levels of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were counted to determine the major metabolic pathways and signal transduction pathways involved. The DEGs between two samples were chosen according to the following criteria: logarithmic fold change of greater than 2 and FDR lower than 0.05. Goatools (<https://github.com/tanghaibao/Goatools>) and KOBAS (<http://bioinfo.org/kobas>) were used to perform GO functional enrichment and KEGG pathway analyses to better understand the functionalities of DEGs. When the Bonferroni-corrected P -value for DEGs was lower than

TABLE 1: The primer information of genes detected by qRT-PCR.

Gene name		Primer sequence
Reference gene	<i>β-actin</i>	F: AGATGTTGCTGCGTTGGTTAT R: GATGGGGTACTTGAGGGTGAG
Heat shock protein 60	<i>HSP60</i>	F: GATGGTGTCACTGTGGCGAAGG R: TAGCAATGGCACGGGCAAGAAC
Heat shock protein 70 cognate 4	<i>HSC70-4</i>	F: GGTATCCTCAATGTATCCGCTGTGG R: TCTCGTCTCTGCCTTGTAGTTCTC
Heat shock protein 70 kDa	<i>HSP70A</i>	F: AACGACCAAGGTAACAGGACAACAC R: ACTTCTCCCGATCAGACGCTTAG
Heat shock protein 90	<i>HSP90A</i>	F: TTGTGCGTGGTGTGTAGACTCAG R: AGTTGTCCTTGTCTCCGTGATGTC
Calmodulin-dependent protein kinase	<i>CAMKK1</i>	F: GACGCTGTATCTTGAGTCGGATCTG R: GTGGGTTCTTCTGGGTCCTGTTTC
Calmodulin-dependent protein kinase	<i>CAMKK2</i>	F: TCCTCGGTTGTCTCCTCCTGATTC R: GCCTGCCTTGGACATGATGATCTG
Calreticulin	<i>CALR</i>	F: CGAGAAGGTGGAGAGCGGAGAG R: GGCTTGGTGTTCATCAGGGTCATC
Toll-like receptor 6	<i>TLR6</i>	F: ATGTTGGTCTGTCCTGCTGGTGTG R: ACTCGGGTCTTCTGTCAAATGG
Nuclear factor interleukin 3 regulated-like protein	<i>NFIL3</i>	F: GAGTGTGACATCGGAAGGAGAAGTG R: TGAACCCGTGTGATGAGCCAATG
Chaperonin containing T-complex polypeptide subunit eta	<i>CCT-eta</i>	F: GACAGCTATGAACTCCGTGGATGAC R: CCTTGACGAGAATGGTGACAGACTG
Cysteine aspartic acid-specific protease	<i>Caspases</i>	F: GTGGCTCCAGAGGCAGATTTCTTC R: TCCAGGTCATCACAGGAACCAGTC
Heme-binding protein 2-like	<i>HEBP2</i>	F: GTGTTCTCTTGGCAACCTCTGTCTC R: GGGCTGTCATGGCAATCTTCTTACC
Hexamethylene bisacetamide inducible 1-like protein	<i>HEXIM1</i>	F: CACCCTACAACACCACACAGTCC R: TTCCATTCCGTCCAAGTCCAACAC

TABLE 2: Analysis results of transcriptome sequencing data of the hemolymph of *Haliothis discus hannai*.

Sample	Raw reads	Clean reads	Mapped reads	Mapped rate (%)	Clean bases (G)
Thermal stress (6 hr)	148,380,238	127,129,600	109,323,878	85.99	17.32
Thermal stress (24 hr)	192,515,264	168,733,690	148,772,494	88.17	23.22
Thermal stress (48 hr)	129,161,202	110,991,322	970,537,42	87.44	15.17
Control	125,169,748	108,679,362	947,663,19	87.20	14.81

0.05, they were considered highly enriched in GO keywords and metabolic pathways.

2.7. Validation of RNA-Seq Data by Quantitative Real-Time PCR. Several interesting genes in the thermal stress group (6 hr), including *HSPs*, calreticulin (*CALR*), and immune-related genes, were selected for validation by quantitative real-time PCR (qRT-PCR), using the same samples used for RNA-seq. The cDNA of samples was synthesized using HiScript III RT Super Mix for qPCR (Vazyme, China) with 1 μg of total RNA, and the cDNAs were diluted 10 times for using as amplification template. qRT-PCR was performed on the Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) with ChamQ SYBR Color

qPCR Master Mix (Vazyme, China) according to the manufacturer's instructions. Three biological and three technical replicates were set, with *β-actin* [32] serving as the internal standardization reference. The specific primers for qRT-PCR are listed in Table 1. The PCR cycling conditions were as follows: 95°C for 30 s, then 40 cycles of 95°C for 10 s, and 60°C for 30 s. Finally, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each gene.

3. Results

3.1. Quality Control Evaluation and Reference Genome Alignment. A total of 595,226,452 raw reads were obtained by transcriptome sequencing (Table 2), and 515,533,974

clean reads were obtained after filtering the raw data. The total sequencing depth of this transcriptome was 276x. Among them, 17.32 G clean bases and 127,129,600 clean reads, 23.22 G clean bases and 168,733,690 clean reads, and 15.17 G clean bases and 110,991,322 clean reads were identified in the Pacific abalone in the three different stages of thermal stress (6, 24, 48 hr), respectively. Note that 14.81 G clean bases and 108,679,362 clean reads were obtained for the control group. The contents of Q20 and Q30 bases of clean reads of each group were calculated. It was found that the lowest contents of Q20 and Q30 bases were 97.29% and 90.08%, respectively. The clean reads after quality control were mapped to the genome of *Haliotis discus hannai*, and the mapping rate was in a range of 85.99%–88.17%.

3.2. Detection of Differentially Expressed Genes (DEGs). A total of 2,213, 2,337, and 1,420 DEGs (Figure 2(a)–2(c)) were detected between the control group and the thermal stress group at different stages (6, 24, and 48 hr), respectively. Specifically, 1,012 upregulated and 1,201 downregulated genes were observed after 6 hr of thermal stress (TH 6 hr group), 784 upregulated and 1,553 downregulated genes were observed after 24 hr of thermal stress (TH 24 hr group), and 540 upregulated and 880 downregulated genes were observed after 48 hr of thermal stress (TH 48 hr group). TH 6 hr group and TH 24 hr group showed similar number of DEGs as compared with control group but the number was significantly decreased in TH 48 hr group vs. control group. The Venn diagram shows that 926 genes were differentially expressed in both TH 6 hr group and TH 24 hr group, 605 genes were differentially expressed in both TH 24 hr group and TH 48 hr group, and 314 genes were differentially expressed in all three thermal stress groups as compared with those in control group (Figure 2(d)). Many DEGs of interest were used to draw heat maps to observe the expression changes of these genes in different heat stress stages (Figure 3).

3.3. GO and KEGG Enrichment Analysis of DEGs. GO and KEGG enrichment analysis of DEGs in Pacific abalone hemolymph after heat stress were carried out, respectively (Table 3). The GO enrichment analysis results of DEGs were consistent after 6, 24, and 48 hr of heat stress. The DEGs were mainly involved in cellular process (GO: 0009987), biological regulation (GO: 0065007), metabolic process (GO: 0008152), multicellular organismal process (GO: 0032501), response to stimulus (GO: 0050896), developmental process (GO: 0032502), and other biological processes that maintain cell homeostasis. The results of KEGG enrichment analysis showed that DEGs after 6 hr of thermal stress were significantly enriched in antigen presentation and processing, female signaling pathway, and endoplasmic reticulum protein processing pathway in genetic information processing in organic systems. The involved genes were heat shock protein 70 gene (*HSP70*), *HSP90*, glucose-regulated protein 78 gene (*GRP78*), endoplasmic reticulum protein 29 gene (*ERP29*), *CALR*, etc. In terms of metabolism, DEGs after 6 hr of thermal stress were significantly enriched in cysteine and methionine metabolic pathways. The KEGG enrichment analysis results of TH 48 hr group and TH 24 hr group were similar to those of TH 6 hr group. Many metabolism-related DEGs

were significantly enriched in tyrosine metabolic pathways after 24 hr of heat stress and were significantly enriched in cysteine and methionine metabolic pathways after 48 hr of heat stress. Additionally, immune-related Toll-like receptor 6 (*TLR6*) and myeloid differentiation factor 88 (*MyD88*) genes of Toll and Imd signaling pathways in organic systems were also observed to be significantly upregulated after 6 hr of thermal stress.

3.4. qRT-PCR Validation Analysis. The 13 candidate genes that were significantly upregulated or downregulated and involved in different biological processes were selected for qRT-PCR analysis to verify the accuracy of DEGs as revealed by RNA-seq (Figure 4). The qRT-PCR results showed that although the relative expression levels of the 13 candidate genes were slightly different from the transcriptome results, the overall upregulated and downregulated trend was consistent, which verified the accuracy of the transcriptome sequencing data.

4. Discussion

As a key abiotic factor for the high mortality of Pacific abalone in summer, high temperature restricts the development of abalone aquaculture industry in southern China. Therefore, it is necessary to understand the molecular regulatory mechanism of heat stress response in Pacific abalone and further analyze its thermal adaptation capacity. In this study, the transcriptome changes of Pacific abalone after acute heat stress at 28°C were explored by RNA sequencing in order to screen genes related to thermal stress response and the underlying regulatory mechanism. Here, 2,213, 2,337, and 1,420 DEGs were identified in the thermal stress group after 6, 24, and 48 hr, respectively, as compared with the control group without heat stimulation. Further, GO and KEGG enrichment analysis of DEGs suggested that Pacific abalone may maintain cellular homeostasis through the upregulated expression of molecular chaperones associated with the endoplasmic reticulum-associated degradation (ERAD) pathway. In addition, the expression of genes of TLR signaling pathways such as *TLR6* and *MyD88* was also induced. Thus, Pacific abalone may activate the immune defense system by upregulating immune-related genes to defend the invasion of pathogenic bacteria after heat stress.

4.1. Effects of Thermal Stress on Protein Folding and Degradation in Pacific Abalone. Maintenance of protein homeostasis is necessary for cells to respond to environmental stresses, and *HSPs* play an important role in this process. *HSPs* are molecular chaperones, which are highly conserved in terms of sequence among different species. When organisms are subjected to environmental stress, *HSPs* can promote the folding of cellular proteins and the degradation of misfolded proteins to prevent the accumulation of unfolded proteins and maintain cellular homeostasis, thus protecting the body from damage [33–35]. In recent years, there have been studies on the significant expression of *HSPs* in shellfish under heat stress. The induction of *HSPs* seems to be an important indicator for monitoring the state of intracellular

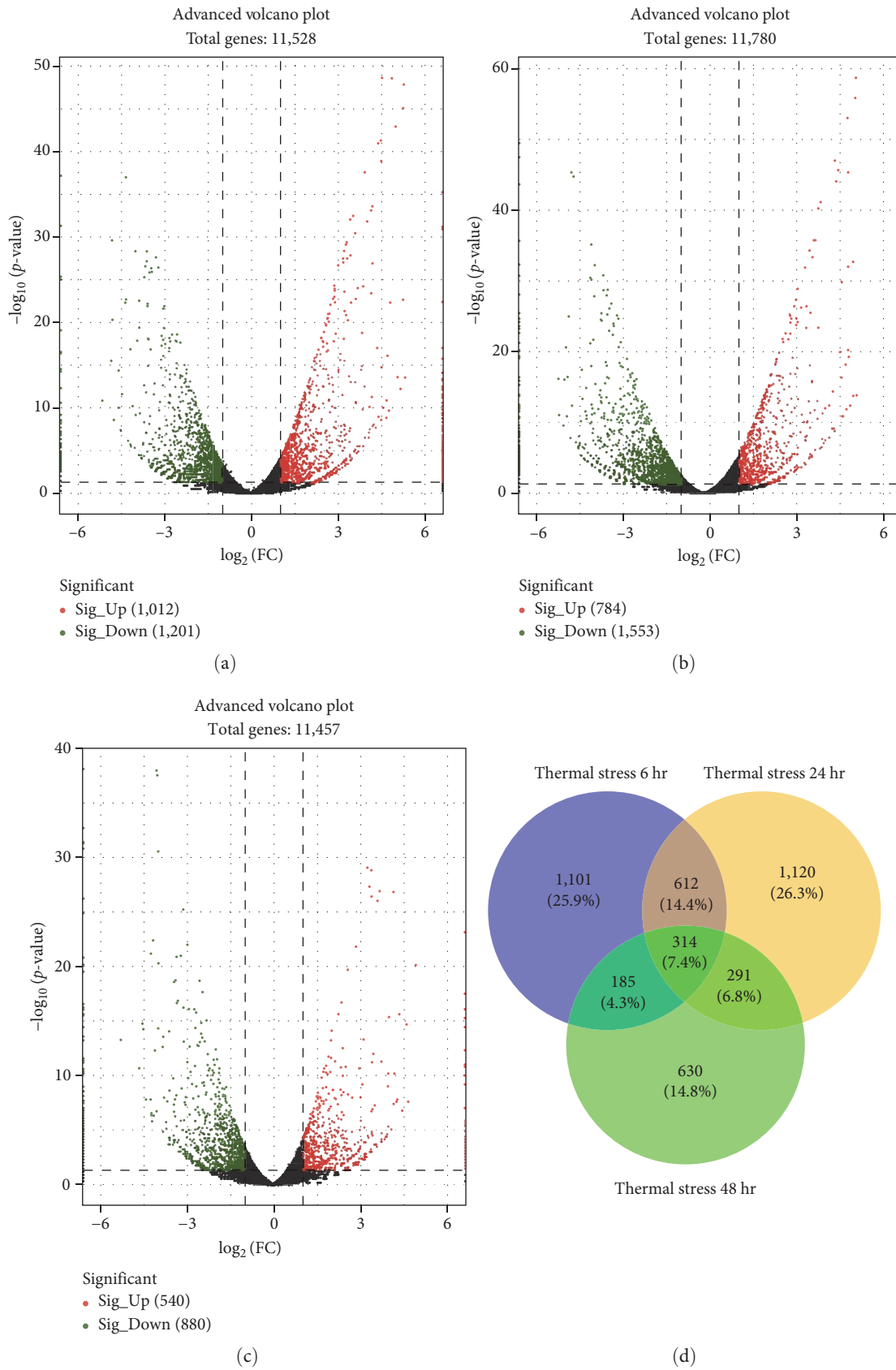


FIGURE 2: The volcano diagram and Venn diagram of DEGs in hemolymph of *Haliotis discus hannai* after thermal stress (FDR ≤ 0.05 , $|\log_2\text{FC}| > 1$). (a) The volcano diagram of DEGs after 6 hr of thermal stress. (b) The volcano diagram of DEGs after 24 hr of thermal stress. (c) The volcano diagram of DEGs after 48 hr of thermal stress. (d) The Venn diagram of DEGs in different stages after thermal stress.

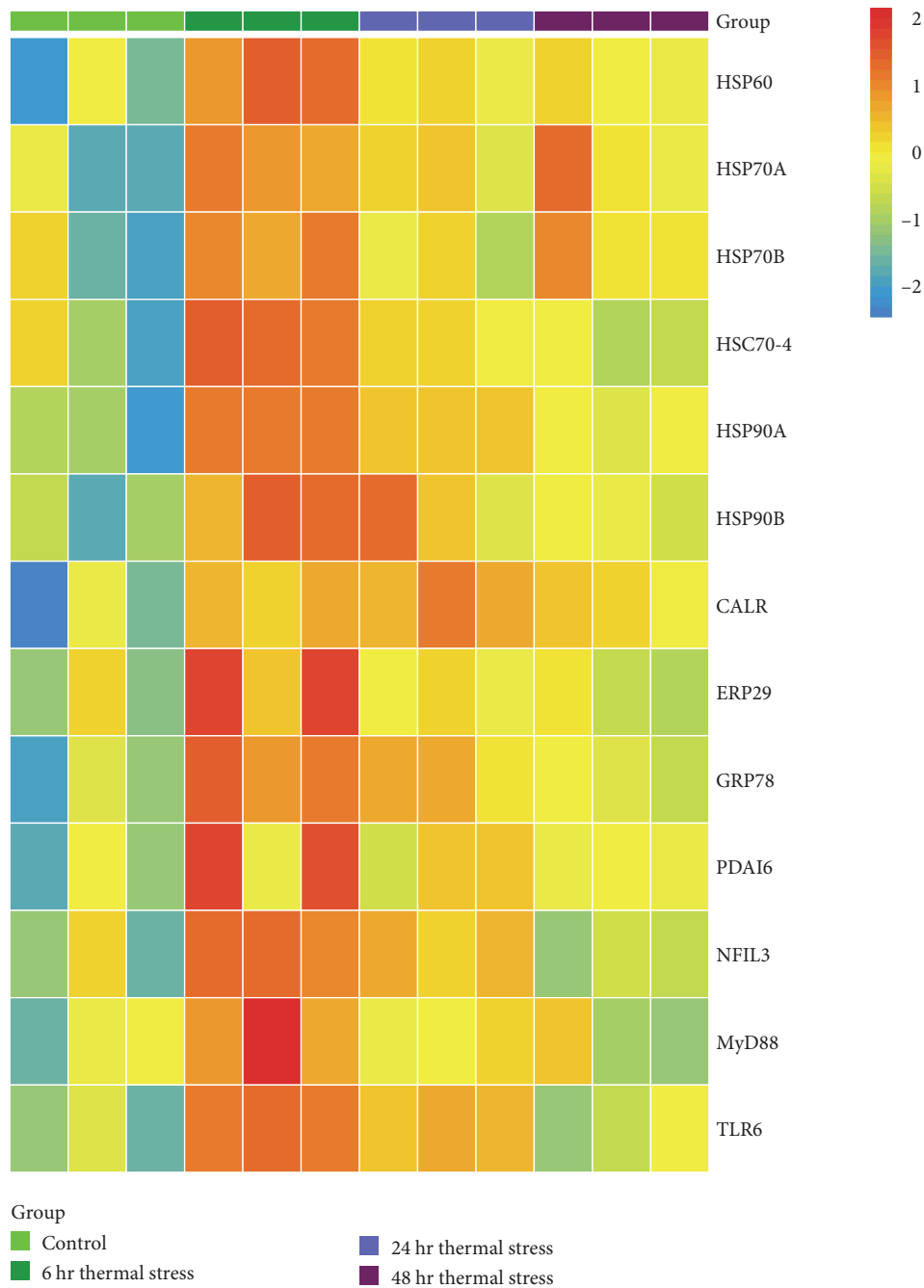


FIGURE 3: The heat map of the expression of many differentially expressed genes (DEGs) in *Haliotis discus hannai* hemolymph after thermal stress (6, 24, and 48 hr). The expression of these genes reached the highest value after thermal stress (6 hr) and then gradually decreased with the extension of thermal stress time.

heat stress. The studies on greenlip abalone *Haliotis laevis* [36], pearl oyster [37], Pacific oyster [38], Hong Kong oyster *Crassostrea hongkongensis* [39], Manila clam [40], Yesso scallop *Patinopecten yessoensis* [41], razor clam *Sinonovacula constricta* [42], Pacific abalone [28, 43], and other related studies have strongly proved that the induction of *HSPs* is a biological self-protection mechanism in response to heat stress, and the upregulation of *HSPs* can help cells resist damage and apoptosis caused [44]. The present study found that *HSPs* such as *HSP70A*, *HSP70B*, *HSP90A*, *HSP90B*, and

HSC70-4 (heat shock protein 70 cognate 4) were significantly upregulated after 6, 24, and 48 hr of thermal stress, indicating that *HSPs* are also the thermal stress markers of Pacific abalone.

Moreover, in the present study, the expression of genes related to endoplasmic reticulum protein-processing pathway (*GRP78*, *ERP29*, *CALR*, and *PDIA6*) in Pacific abalone hemolymph was significantly upregulated after heat stress. These genes play important roles in the unfolding protein response (UPR) and ERAD pathways and can perform

TABLE 3: Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the hemolymph of *Haliotis discus hannai* under thermal stress.

Pathways	KO ID	Gene number	P-value
Thermal stress (6 hr)			
Cysteine and methionine metabolism	KO00270	7	0.0058
FoxO signaling pathway	KO04068	7	0.0118
Antigen processing and presentation	KO04612	6	0.0177
MAPK signaling pathway	KO04010	9	0.0191
Flavonoid biosynthesis	KO00941	2	0.0200
Stilbenoid, diarylheptanoid, and gingerol biosynthesis	KO00945	2	0.0200
Protein processing in endoplasmic reticulum	KO04141	14	0.0210
Dorsoventral axis formation	KO04320	3	0.0225
Longevity regulating pathway	KO04211	6	0.0242
Adipocytokine signaling pathway	KO04920	4	0.0277
Thermal stress (24 hr)			
Antigen processing and presentation	KO04612	8	0.0006
Estrogen signaling pathway	KO04915	8	0.0025
Tyrosine metabolism	KO00350	6	0.0031
Calcium signaling pathway	KO04020	8	0.0168
Protein processing in endoplasmic reticulum	KO04141	14	0.0191
Osteoclast differentiation	KO04380	3	0.0391
Phenylalanine metabolism	KO00360	2	0.0530
Sesquiterpenoid and triterpenoid biosynthesis	KO00909	2	0.0530
Styrene degradation	KO00643	2	0.0530
Necroptosis	KO04217	11	0.0628
Thermal stress (48 hr)			
Estrogen signaling pathway	KO04915	7	0.0031
IL-17 signaling pathway	KO04657	4	0.0040
Antigen processing and presentation	KO04612	6	0.0055
Th17 cell differentiation	KO04659	3	0.0115
Adipocytokine signaling pathway	KO04920	4	0.0120
Osteoclast differentiation	KO04926	3	0.0211
Relaxin signaling pathway	KO04380	3	0.0211
Cysteine and methionine metabolism	KO00270	5	0.0331
T-cell receptor signaling pathway	KO04660	3	0.0339
B-cell receptor signaling pathway	KO04662	2	0.0343

protein quality control and maintain ER protein homeostasis [45]. High-temperature treatment results in ER stress that activates the UPR pathway. UPR enhances protein folding ability by increasing the synthesis of endoplasmic reticulum protein chaperones [46]. ERAD pathway can identify, locate, and eliminate unfolded or misfolded proteins [47], maintaining the dynamic balance of protein folding in the ER. As the dominant player of UPR in normal intracellular environment, *GRP78* could protect cells from damage caused by harmful chemicals, oxidative stress, calcium depletion, programmed cell death, etc. by forcing unfolded proteins to refold or degrade through cellular degradation mechanisms [48]. Besides, *GRP78* may play an important role in the defense of the giant tiger prawn to environmental stress [49], as well as in response of the giant river prawn to high temperature and pathogen stress [50]. *ERP29* has been speculated to be a chaperone protein, which is mainly involved in the processing and secretion of secreted proteins [51]. *CALR* is the binding partner of calcium

ions in the ER, mainly involved in calcium storage and signal transduction [52]. *PDIA6* is a member of the protein disulfide isomerase (*PDI*) family, which acts as a molecular chaperone in the folding and repair of faulty proteins during stress responses [53]. It has been observed that the expression of *PDIA6* is significantly upregulated after heat stress in the Pacific oyster [54] and razor clam [42]. In conclusion, maintaining cellular homeostasis through the upregulated expression of molecular chaperones associated with ERAD pathways may be central to the defense of Pacific abalone against thermal stress.

4.2. Inducing Immune Defense System after Thermal Stress in Pacific Abalone. Rising seawater temperature not only leads to increase in the abundance of pathogens such as marine *Vibrio spp.*, but also reduces the natural immunity of marine animals, resulting in easier bacterial infection. It has been reported that thermal stress negatively affects the immune

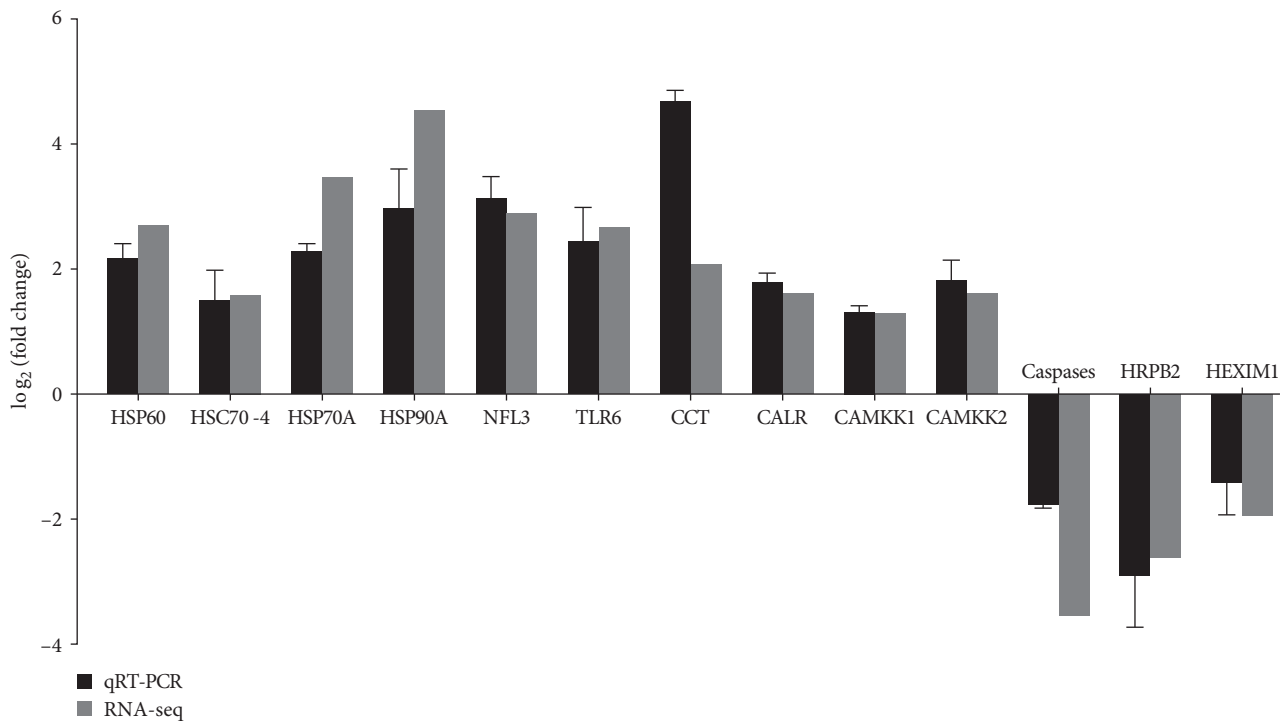


FIGURE 4: The relative expression levels of 13 genes as revealed by qRT-PCR to confirm the accuracy of transcriptome data.

functions in mollusks, such as the striped venus clam *Chamelea gallina* [55], surf clam *Macrta veneriformis* [56], the Taiwan abalone (*Haliotis diversicolor supertexta*) [57], and Pacific abalone [58]. In this study, DEGs were found significantly enriched in immune-related signaling pathway of antigen processing and presentation in hemolymph tissue after high-temperature challenge. *HSP* family genes, including *HSP90A*, *HSP90B*, *HSP70A*, and *HSP70B*, were involved in this pathway. In addition to acting as molecular chaperones, *HSP90* proteins are also necessary for the assembly, activation, and maintenance of nucleotide-binding oligomerization domain-like receptor (NLR) protein complexes [59]. These genes can participate in the turnover control of NLRs to maintain proper NLR protein levels and avoid immune deficiency caused by excessive accumulation of immune receptors [60]. *HSP70* proteins have been confirmed to be induced by pyrogen and *Vibrio* and play a crucial role in immune response and regulation of TLR signaling pathway [61]. The results were consistent with those reported in the Pacific oyster [26] and razor clam [42], in which the upregulation of *HSP70* and *HSP90* family genes has been also observed after heat treatment.

In addition, the expression of other immune-related genes such as *TLR6* and *MyD88* gene of TLR signaling pathway was also induced in the Pacific abalone under thermal stress. Since *TLRs* were first discovered to play an important role in the immune response against fungal infection in *Drosophila* [62], a large number of *TLRs* have been identified as important genes for recognizing pathogen structures [63]. *TLRs* play a crucial role in activating the immune system by sensing pathogen-associated molecular patterns of viruses and *Vibrio spp.* [64] and their family members are

conservative during evolution [65]. As a member of *TLRs*, *TLR6* plays an important role in the immune defense of mollusks. Studies on the Pacific oyster [66] and Pearl oyster [67] have shown that *TLR6* expression is markedly upregulated by bacterial infection. *MyD88*, as a key connector protein in the TLR signal transduction pathway, can stimulate downstream signal transduction and induce the expression of proinflammatory cytokines or antiviral genes, thereby participating in the innate immune process. *MyD88* and *TLRs* can interact each other [68]. Research on miiuy croaker (*Miichthys miiuy*) has found that *TLR2* plays an indispensable role in the transcription of *MyD88* [69]. *MyD88* has been considered to represent the intracytoplasm partner of *TLRs*, since the expression of *MyD88* and *TLR1* is both upregulated in Mediterranean mussel (*Mytilus galloprovincialis*) after injection with filamentous fungus (*Fusarium oxysporum*) [70]. In the present study, *HSP70*, *TLR6*, and *MyD88* were highly expressed in the hemolymph of Pacific abalone after thermal stress, indicating that the Pacific abalone might upregulate some TLR signaling pathway genes to resist pathogen infection after temperature stress. In the study of Ren et al. [61], the expression of *MyD88* and *TLR13* is significantly reduced after silencing *HSP70* by RNA interference, confirming that *HSP70* plays an important role in regulating the TLR signaling pathway. These results may support that thermal stress could activate the immune defense system in the Pacific abalone.

5. Conclusions

In this study, the transcriptome of Pacific abalone under thermal stress was sequenced and analyzed. A total of

2,213, 2,337, and 1,420 DEGs were identified in Pacific abalone after 6, 24, and 48 hr of thermal stress in 28°C, respectively, as compared with the control group. We found *HSPs* such as *HSP70A*, *HSP70B*, *HSC70-4*, *HSP90A*, and *HSP90B* were significantly upregulated after thermal stress, while the expression of genes related to endoplasmic reticulum protein-processing pathway (*GRP78*, *ERP29*, *CALR*, and *PDIA6*) was significantly upregulated. It indicated that Pacific abalone against thermal stress by maintaining cellular homeostasis through the upregulated expression of molecular chaperones associated with ERAD pathways. In addition, DEGs were found significantly enriched the immune-related signaling pathway of antigen processing and presentation in hemolymph tissue after high-temperature challenges. The immune-related genes like *HSP70*, *TLR6*, and *MyD88* were highly expressed in the hemolymph of Pacific abalone after thermal stress. This revealed that the Pacific abalone might activate the immune defense system to resist the invasion of pathogenic bacteria by upregulating immune-related genes after thermal challenges. This study preliminarily explored the molecular mechanism of thermal stress adaptation in Pacific abalone and provided a reference for further study of the response patterns of related genes and pathways in Pacific abalone under high-temperature stress.

Data Availability

The *Haliotis discus hannai* transcriptome sequencing data generated in this study have been submitted to the China National GeneBank DataBase (CNGBdb; <https://db.cngb.org/>) under accession number CNP0003705.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zhou Wu: conceptualization, methodology, sample collection, formal analysis, software, and writing; Yumin Yang: conceptualization, methodology, formal analysis, data curation, software, and writing; Liqing Zhou: conceptualization, methodology, funding acquisition, and writing-review and editing; Changfeng Chi, Xiujun Sun, Biao Wu, Zhihong Liu, and Yan Wang: conceptualization and methodology. All authors have read and approved the final manuscript for publication.

Acknowledgments

This work was supported by the National Key Research and Development Program of China (grant number 2018YFD0900803).

References

- [1] O. Hoegh-Guldberg and J. F. Bruno, "The impact of climate change on the world's marine ecosystems," *Science*, vol. 328, no. 5985, pp. 1523–1528, 2010.
- [2] J. A. Crisp, G. J. Partridge, F. M. L. D'Souza, J. R. Tweedley, and N. R. Moheimani, "Effects of temperature and salinity on larval survival and development of the western school prawn *Metapenaeus dalli*," *International Aquatic Research*, vol. 9, pp. 1–10, 2017.
- [3] M. S. Azaza, M. N. Dhraïef, and M. M. Kraïem, "Effects of water temperature on growth and sex ratio of juvenile Nile tilapia *Oreochromis niloticus* (Linnaeus) reared in geothermal waters in southern Tunisia," *Journal of Thermal Biology*, vol. 33, no. 2, pp. 98–105, 2008.
- [4] P. L. Munday, M. J. Kingsford, M. O'callaghan, and J. M. Donelson, "Elevated temperature restricts growth potential of the coral reef fish *Acanthochromis polyacanthus*," *Coral Reefs*, vol. 27, pp. 927–931, 2008.
- [5] M. P. Acquafredda, D. M. Munroe, L. M. Ragone Calvo, and M. De Luca, "The effect of rearing temperature on the survival and growth of early juvenile Atlantic surfclams (*Spisula solidissima*)," *Aquaculture Reports*, vol. 13, Article ID 100176, 2019.
- [6] N. W. Pankhurst and P. L. Munday, "Effects of climate change on fish reproduction and early life history stages," *Marine & Freshwater Research*, vol. 62, no. 9, pp. 1015–1026, 2011.
- [7] D. Xu, S. Zhou, and H. Yang, "Carbohydrate and amino acids metabolic response to heat stress in the intestine of the sea cucumber *Apostichopus japonicus*," *Aquaculture Research*, vol. 48, no. 12, pp. 5883–5891, 2017.
- [8] M. Perrigault, S. F. Dahl, E. P. Espinosa, L. Gambino, and B. Allam, "Effects of temperature on hard clam (*Mercenaria mercenaria*) immunity and QPX (quahog parasite unknown) disease development: II. Defense parameters," *Journal of Invertebrate Pathology*, vol. 106, no. 2, pp. 322–332, 2011.
- [9] V. T. Dang, P. Speck, and K. Benkendorff, "Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*," *Fish & Shellfish Immunology*, vol. 32, no. 5, pp. 732–740, 2012.
- [10] W. You, X. Luo, and C. Ke, "Progress in genetics and breeding of abalone," *Journal of Xiamen University Natural Science*, vol. 60, no. 2, pp. 417–424, 2021.
- [11] C. Ke, "Current situation and prospect of abalone aquaculture industry in China," *China Fisheries*, vol. 1, pp. 27–30, 2013.
- [12] S. Liang, X. Luo, W. You, L. Luo, and C. Ke, "The role of hybridization in improving the immune response and thermal tolerance of abalone," *Fish & Shellfish Immunology*, vol. 39, no. 1, pp. 69–77, 2014.
- [13] J. Li, Q. He, H. Sun, and X. Liu, "Acclimation-dependent expression of heat shock protein 70 in pacific abalone (*Haliotis discus hannai* Ino) and its acute response to thermal exposure," *Chinese Journal of Oceanology and Limnology*, vol. 30, pp. 146–151, 2012.
- [14] M. Zhang, Z. Wang, and X. Gao, "Comparison on adaptability of abalone *Haliotis discus hannai* Ino to temperatures at different growing stages," *Journal of Fishery Sciences of China*, vol. 12, no. 6, pp. 720–725, 2005.
- [15] S. Wei, D. Luo, C. Xu et al., "Stress and early warning threshold of several environmental factors on *Haliotis discus hannai* Ino," *Marine Sciences*, vol. 42, no. 12, pp. 47–54, 2018.
- [16] C. D. Harvell, C. E. Mitchell, J. R. Ward et al., "Climate warming and disease risks for terrestrial and marine biota," *Science*, vol. 296, no. 5576, pp. 2158–2162, 2002.
- [17] J. Tout, N. Siboni, L. F. Messer et al., "Increased seawater temperature increases the abundance and alters the structure of natural *Vibrio* populations associated with the coral *Pocillopora damicornis*," *Frontiers in Microbiology*, vol. 6, Article ID 432, 2015.
- [18] M.-A. Travers, O. Basuyaux, N. L. Goïc et al., "Influence of temperature and spawning effort on *Haliotis tuberculata*

- mortalities caused by *Vibrio harveyi*: an example of emerging vibriosis linked to global warming,” *Global Change Biology*, vol. 15, no. 6, pp. 1365–1376, 2009.
- [19] A. Grada and K. Weinbrecht, “Next-generation sequencing: methodology and application,” *The Journal of Investigative Dermatology*, vol. 133, no. 8, pp. 1–4, 2013.
- [20] J. Qin, Z. Huang, J. Chen, Q. Zou, W. You, and C. Ke, “Sequencing and *de novo* analysis of *Crassostrea angulata* (fujian oyster) from 8 different developing phases using 454 GSFLx,” *PLOS ONE*, vol. 7, no. 8, Article ID e43653, 2012.
- [21] S. Xue, J. Lin, Q. Zhou, H. Wang, and Y. Han, “Effect of ammonia stress on transcriptome and endoplasmic reticulum stress pathway for common carp (*Cyprinus carpio*) hepatopancreas,” *Aquaculture Reports*, vol. 20, Article ID 100694, 2021.
- [22] X. Zhuo, Y. Qin, P. He et al., “Transcriptomic analysis of *Litopenaeus vannamei* hepatopancreas under cold stress in cold-tolerant and cold-sensitive cultivars,” *Gene*, vol. 764, Article ID 145090, 2021.
- [23] W. Zhang, X. Xu, J. Li, and Y. Shen, “Transcriptomic analysis of the liver and brain in grass carp (*Ctenopharyngodon idella*) under heat stress,” *Marine Biotechnology*, vol. 24, pp. 856–870, 2022.
- [24] H. Nie, L. Jiang, Z. Huo, L. Liu, F. Yang, and X. Yan, “Transcriptomic responses to low temperature stress in the Manila clam, *Ruditapes philippinarum*,” *Fish & Shellfish Immunology*, vol. 55, pp. 358–366, 2016.
- [25] Q. Wang, Y. Liu, Z. Zheng, Y. Deng, Y. Jiao, and X. Du, “Adaptive response of pearl oyster *Pinctada fucata martensii* to low water temperature stress,” *Fish & Shellfish Immunology*, vol. 78, pp. 310–315, 2018.
- [26] Q. Zhu, L. Zhang, L. Li, H. Que, and G. Zhang, “Expression characterization of stress genes under high and low temperature stresses in the pacific oyster, *Crassostrea gigas*,” *Marine Biotechnology*, vol. 18, pp. 176–188, 2016.
- [27] J. Zheng, J. Cao, Y. Mao, Y. Su, and J. Wang, “Comparative transcriptome analysis provides comprehensive insights into the heat stress response of *Marsupenaeus japonicus*,” *Aquaculture*, vol. 502, pp. 338–346, 2019.
- [28] D. Kyeong, J. Kim, Y. Shin et al., “Expression of heat shock proteins in thermally challenged pacific abalone *Haliotis discus hannai*,” *Genes*, vol. 11, no. 1, Article ID 22, 2020.
- [29] F. Yu, Y. Shen, W. Peng et al., “Metabolic and transcriptional responses demonstrating enhanced thermal tolerance in domesticated abalone,” *Science of the Total Environment*, vol. 872, Article ID 162060, 2023.
- [30] P. Jiravanichpaisal, B. L. Lee, and K. Söderhäll, “Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization,” *Immunobiology*, vol. 211, no. 4, pp. 213–236, 2006.
- [31] D. Jiaoyang, L. Hui, C. Siqing et al., “Temperature effects on feeding and oxygen consumption rate of *Haliotis discus hannai* Ino,” *Chinese Agricultural Science Bulletin*, vol. 36, no. 27, pp. 153–157, 2020.
- [32] Y.-L. Chen, W.-Y. Li, J.-J. Hu et al., “Nucleus-translocated matrix metalloprotease 1 regulates innate immune response in pacific abalone (*Haliotis discus hannai*),” *Fish & Shellfish Immunology*, vol. 84, pp. 290–298, 2019.
- [33] R. J. Roberts, C. Agius, C. Saliba, P. Bossier, and Y. Y. Sung, “Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review,” *Journal of Fish Diseases*, vol. 33, no. 10, pp. 789–801, 2010.
- [34] M. E. Feder and G. E. Hofmann, “Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology,” *Annual Review of Physiology*, vol. 61, no. 1, pp. 243–282, 1999.
- [35] D. Liu and Z. Chen, “The expression and induction of heat shock proteins in molluscs,” *Protein & Peptide Letters*, vol. 20, no. 5, pp. 602–606, 2013.
- [36] B. P. Shiel, N. E. Hall, I. R. Cooke, N. A. Robinson, and J. M. Strugnell, “De novo characterisation of the greenlip abalone transcriptome (*Haliotis laevigata*) with a focus on the heat shock protein 70 (HSP70) family,” *Marine Biotechnology*, vol. 17, pp. 23–32, 2015.
- [37] J. Li, Y. Zhang, Y. Liu, Y. Zhang, S. Xiao, and Z. Yu, “Co-expression of heat shock protein (HSP) 40 and HSP70 in *Pinctada martensii* response to thermal, low salinity and bacterial challenges,” *Fish & Shellfish Immunology*, vol. 48, pp. 239–243, 2016.
- [38] H.-J. Lim, B.-M. Kim, I. J. Hwang et al., “Thermal stress induces a distinct transcriptome profile in the pacific oyster *Crassostrea gigas*,” *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, vol. 19, pp. 62–70, 2016.
- [39] J. Li, Y. Zhang, F. Mao et al., “Characterization and identification of differentially expressed genes involved in thermal adaptation of the Hong Kong oyster *Crassostrea hongkongensis* by digital gene expression profiling,” *Frontiers in Marine Science*, vol. 4, Article ID 112, 2017.
- [40] H. Nie, L. Liu, Z. Huo et al., “The HSP70 gene expression responses to thermal and salinity stress in wild and cultivated Manila clam *Ruditapes philippinarum*,” *Aquaculture*, vol. 470, pp. 149–156, 2017.
- [41] W. Jiang, F. Lin, J. Fang et al., “Transcriptome analysis of the Yesso scallop, *Patinopecten yessoensis* gills in response to water temperature fluctuations,” *Fish & Shellfish Immunology*, vol. 80, pp. 133–140, 2018.
- [42] X. Kong, L. Lv, J. Ren, Y. Liu, Z. Lin, and Y. Dong, “Comparative transcriptome analyses unravel the response to acute thermal stress in the razor clam, *Sinonovacula constricta*,” *Aquaculture Reports*, vol. 23, Article ID 101079, 2022.
- [43] K. Park, J. S. Lee, J.-C. Kang, J. W. Kim, and I.-S. Kwak, “Cascading effects from survival to physiological activities, and gene expression of heat shock protein 90 on the abalone *Haliotis discus hannai* responding to continuous thermal stress,” *Fish & Shellfish Immunology*, vol. 42, no. 2, pp. 233–240, 2015.
- [44] D. D. Mosser, A. W. Caron, L. Bourget, C. Denis-Larose, and B. Massie, “Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis,” *Molecular and Cellular Biology*, vol. 17, no. 9, pp. 5317–5327, 1997.
- [45] E. Tiffany-Castiglioni and Y. Qian, “ER chaperone-metal interactions: links to protein folding disorders,” *Neurotoxicology*, vol. 33, no. 3, pp. 545–557, 2012.
- [46] M. Schröder, “Endoplasmic reticulum stress responses,” *Cellular and Molecular Life Sciences*, vol. 65, pp. 862–894, 2008.
- [47] B. Meusser, C. Hirsch, E. Jarosch, and T. Sommer, “ERAD: the long road to destruction,” *Nature Cell Biology*, vol. 7, pp. 766–772, 2005.
- [48] I. M. Ibrahim, D. H. Abdelmalek, and A. A. Elfiky, “GRP78: a cell’s response to stress,” *Life Sciences*, vol. 226, pp. 156–163, 2019.
- [49] L. Li, P. Wang, C. Zhao, and L. Qiu, “The anti-stresses capability of GRP78 in *Penaeus monodon*: evidence from in vitro and in vivo studies,” *Fish & Shellfish Immunology*, vol. 72, pp. 132–142, 2018.

- [50] P. Srisapoome, T. Ju-Ngam, and R. Wongpanya, "Characterization, stress response and functional analyses of giant river prawn (*Macrobrachium rosenbergii*) glucose-regulated protein 78 (Mr-grp78) under temperature stress and during aeromonas hydrophila infection," *Animals*, vol. 11, no. 10, Article ID 3004, 2021.
- [51] N. N. Barak, P. Neumann, M. Sevvana et al., "Crystal structure and functional analysis of the protein disulfide isomerase-related protein ERp29," *Journal of Molecular Biology*, vol. 385, no. 5, pp. 1630–1642, 2009.
- [52] H. M. V. Udayantha, G. I. Godahewa, S. D. N. K. Bathige et al., "A molluscan calreticulin ortholog from *Haliotis discus discus*: molecular characterization and transcriptional evidence for its role in host immunity," *Biochemical and Biophysical Research Communications*, vol. 474, no. 1, pp. 43–50, 2016.
- [53] J. Groenendyk, Z. Peng, E. Dudek et al., "Interplay between the oxidoreductase PDIA6 and microRNA-322 controls the response to disrupted endoplasmic reticulum calcium homeostasis," *Science Signaling*, vol. 7, no. 329, Article ID ra54, 2014.
- [54] C. Yang, Q. Gao, C. Liu et al., "The transcriptional response of the pacific oyster *Crassostrea gigas* against acute heat stress," *Fish & Shellfish Immunology*, vol. 68, pp. 132–143, 2017.
- [55] M. Monari, V. Matozzo, J. Foschi, O. Cattani, G. P. Serrazanetti, and M. G. Marin, "Effects of high temperatures on functional responses of haemocytes in the clam *Chamelea gallina*," *Fish & Shellfish Immunology*, vol. 22, no. 1-2, pp. 98–114, 2007.
- [56] J. H. Yu, J. H. Song, M. C. Choi, and S. W. Park, "Effects of water temperature change on immune function in surf clams, *Macra veneriformis* (Bivalvia: Mactridae)," *Journal of Invertebrate Pathology*, vol. 102, no. 1, pp. 30–35, 2009.
- [57] W. Cheng, I.-S. Hsiao, C.-H. Hsu, and J.-C. Chen, "Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*," *Fish & Shellfish Immunology*, vol. 17, no. 3, pp. 235–243, 2004.
- [58] J. Ding, L. Li, F. Wu, and G. Zhang, "Effect of chronic temperature exposure on the immunity of abalone, *Haliotis discus hannai*," *Aquaculture Research*, vol. 47, no. 9, pp. 2861–2873, 2016.
- [59] K. Shirasu, "The HSP90-SGT1 chaperone complex for NLR immune sensors," *Annual Review of Plant Biology*, vol. 60, pp. 139–164, 2009.
- [60] S. Huang, J. Monaghan, X. Zhong et al., "HSP90s are required for NLR immune receptor accumulation in Arabidopsis," *The Plant Journal*, vol. 79, no. 3, pp. 427–439, 2014.
- [61] Y. Ren, H. Pan, Y. Yang, B. Pan, and W. Bu, "Molecular cloning, characterization and functional analysis of a heat shock protein 70 gene in *Cyclina sinensis*," *Fish & Shellfish Immunology*, vol. 58, pp. 663–668, 2016.
- [62] B. Lemaitre, E. Nicolas, L. Michaut, J.-M. Reichhart, and J. A. Hoffmann, "The dorsoventral regulatory gene cassette *spätzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults," *Cell*, vol. 86, no. 6, pp. 973–983, 1996.
- [63] S. Akira, K. Takeda, and T. Kaisho, "Toll-like receptors: critical proteins linking innate and acquired immunity," *Nature Immunology*, vol. 2, pp. 675–680, 2001.
- [64] K.-H. Lim and L. M. Staudt, "Toll-like receptor signaling," *Cold Spring Harbor Perspectives in Biology*, vol. 5, Article ID a011247, 2013.
- [65] H. Liu, M. Yang, X. Tang et al., "Molecular insights of a novel fish Toll-like receptor 9 homologue in *Nibea albiflora* to reveal its function as PRRs," *Fish & Shellfish Immunology*, vol. 118, pp. 321–332, 2021.
- [66] W. Wang, T. Zhang, L. Wang et al., "A new non-phagocytic TLR6 with broad recognition ligands from pacific oyster *Crassostrea gigas*," *Developmental & Comparative Immunology*, vol. 65, pp. 182–190, 2016.
- [67] W. U. Yu-Yuan, Z. Guo, H. Liang, L. Lin, R. Hao, and Y. Jiao, "Cloning, sequence analysis and expression studies on *PmTLR6* gene from *Pinctada fucata martensii*," *Journal of Fisheries of China*, vol. 41, no. 11, pp. 1687–1698, 2017.
- [68] X. Tang, M. Yang, J. Liu et al., "Identification, functional characterization and expression pattern of myeloid differentiation factor 88 (MyD88) in *Nibea albiflora*," *Fish & Shellfish Immunology*, vol. 124, pp. 380–390, 2022.
- [69] D. Tang, Y. Gao, R. Wang, Y. Sun, and T. Xu, "Characterization, genomic organization, and expression profiles of MyD88, a key adaptor molecule in the TLR signaling pathways in miiuy croaker (*Miichthys miiuy*)," *Fish Physiology and Biochemistry*, vol. 38, pp. 1667–1677, 2012.
- [70] M. Toubiana, M. Gerdol, U. Rosani, A. Pallavicini, P. Venier, and P. Roch, "Toll-like receptors and MyD88 adaptors in *Mytilus*: complete cds and gene expression levels," *Developmental & Comparative Immunology*, vol. 40, no. 2, pp. 158–166, 2013.