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Research Article

Effects of temperature stress on genome-wide DNA methylation levels in the sea cucumber, *Apostichopus japonicus*

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Abstract

Sea cucumber *Apostichopus japonicus* lives in the lower temperature of the seawater and will enter the state of aestivation when the temperature is high. In recent years, the continuous high temperatures in summer have brought great loss to the production of sea cucumber in north China. To study the epigenetic regulating mechanism of *A. japonicus* and guide the temperature-controlled culture, the methylation level of *A. japonicus* genome was detected. Breeding imitation *A. japonicus* under different temperature conditions for a period of time, the difference of methylation level between the set temperature group and the control group was detected and verified. The methylation levels of *A. japonicus* in different temperature groups were detected. The methylation sites ranged from 84,766 to 96,225, and the sequencing depth was between 42.33 and 153.21. Under the temperature stress, more than 60% of the differentiated methylated genes in different groups were down-regulated, the HBW group had the most differentiated methylated genes (1,405), and the SBW group had the least differentiated methylated genes (643). Gene function classification and enrichment analysis were carried out, and a total of 3,512 differential genes were found, mostly related to immunity and metabolism. The results showed that temperature can change the gene methylation level of *A. japonicus*, affecting its expression level, and ultimately changing the metabolic and immune processes of *A. japonicus*.

Introduction

The sea cucumber, *Apostichopus japonicus*, is an echinoderm species with important biological characteristics and economic value. The production of *A. japonicus* in China in 2018 reached 174,340 tons (CFSY, 2018). The survival temperature range of *A. japonicus* is 0~25°C, however the optimal temperature for growth is 10~18°C. When the temperature exceeds the acceptable range, *A. japonicus* can physiology decline and even die (Wang *et al.*, 2015). In recent years, continuous high temperature in summer has brought great loss to the sea cucumber agriculture in Liaoning, China. Its adaptability to the environment is not only related to its genes but also to the epigenetic regulation mechanism (Jablonka and Lamb, 1995; Goldberg *et al.*, 2007). DNA methylation is an important form of DNA chemical modification and has become a common method for epigenetic studies (Bird, 1992; Li *et al.*, 1993; Robertson and Wolffe, 2000; Zemach *et al.*, 2010). There is a delicate balance between environment and DNA methylation. When environmental factors change beyond a certain limit, the epigenetic changes of some genes can lead to serious metabolism and behavior (Bird, 2002; Suzuki and Bird, 2008).

In recent years, more attention has been paid to how temperature affects gene methylation levels in aquatic animals. A small increase in ocean temperature causes periodic changes in DNA methylation and gene expression in European perch (Anastasiadi *et al.*, 2017). DNA methylation levels in Atlantic salmon embryos are decreased and the expression levels of *myogenin* are increased at high temperatures (Burgerhout *et al.*, 2017). A temperature increase of several degrees had a significant effect on microRNA expression profile in the embryo and larval development of *Atlantic cod* (Bizuayehu *et al.*, 2015). As the temperature increased, the genome-wide methylation level of Yesso scallops decreased, and the degree of reduction increased with the increase in temperature difference and duration (Jiang *et al.*, 2016). These studies suggested that temperature could change the genome methylation levels in organisms and regulate epigenetic behavior.

Previous studies on the methylation level of *A. japonicus* showed that the methylation level of the body wall tissues was higher than that of other tissues when the cucumber was in non-aestivation state. The methylation level of the whole genome of *A. japonicus* was upregulated in the aestivation state(Zhao *et al.*, 2015). To explore the mechanism of methylation modification in the body wall of *A. japonicus*, the genome-wide DNA methylation level of *A. japonicus* was measured in this study. MethylRAD method was used to determine the changes in DNA methylation levels in the body wall tissues of *A. japonicus* under continuous and stable temperature stimulation. According to gene annotation, candidate genes related to temperature stress were obtained and gene expression levels were measured. The relationship among temperature stress, genome-wide methylation and gene expression was studied to understand the effect of temperature stress on epigenetic inheritance of *A. japonicus*. This study combined epigenetic and functional genes to provide a reference for the epigenetic study of *A. japonicus*, and provide a theoretical basis for the protection of sea cucumber culture under high temperature.

Materials and methods

Experimental materials and rearing conditions

In this study, two-year-old adults of *A. japonicus* (100±15 g) were cultivated at the Key Laboratory of Mariculture and Stock Enhancement in the North China Sea. *A. japonicus* specimens were placed in a constant temperature tank (5m×0.3m×0.3m) with slow circulating water (14°C), and the temperature was changed by 1°C daily until the experimental temperature. Replenishing oxygen into the water (DO=5 mg L^{-1}), with seaweed mud feeding, and maintained a clean water environment. After the *A. japonicus* was cultured at experimental temperature for a period of time, 3 *A. japonicus* specimens were randomly selected and body wall tissue samples were collected. Inducing *A. japonicus* completely into an aestivation state is a slow process and the SBW group's breeding time was extended appropriately (Table 1).

Table 1: The breeding conditions of different groups of *Apostichopus japonicus*.

Group	Condition
LBW	temperature 10°C, held 10 days
NBW	temperature 16°C, held 10 days
HBW	temperature 21°C, held 10 days
XBW	temperature 26°C, held 10 days
SBW	temperature 26°C, held 20 days

Genome-wide DNA methylation assay

Genomic DNA was isolated from the body wall of *A. japonicus* by hexadecyl trimethylammonium bromide (Amresco, USA) and identified by agarose gel electrophoresis. Libraries were constructed using Methyl RAD technology (Li *et al.*, 2018a; Yang *et al.*, 2020), and high-throughput sequencing was performed on Illumina SE sequencing (50 bp) platform. DNA mass was calculated according to Phred quality score to obtain valid data. The calculation formula is as follows:

$$Q_{sanger} = -10\log_{10} p$$

End-pair sequencing data performed on PEAR (Zhang *et al.*, 2014), the filter deleted low-quality reads (more than 15% of bases with a quality value below 30) and sequences with too many N-bases (sequences containing less than 8% N) to obtain more accurate measurement results. The Methyl RAD sequencing data (Enzyme Reads) of each sample was obtained by filtering and deleting the tags that did not contain the expected digestion site. The methyl RAD data was analyzed using a reference based method (Li *et al.*, 2018a), and high quality reads were aligned to the reference genome of *A. japonicus* (Li *et al.*, 2008) using the SOAP method (version 2.21, with parameters set to -m4 - v2 - r0). The selected reference genome was: https://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/002/754/855/GCA_002754855.1_ASM275485v1/GCA_002754855.1_ASM275485v1_genomic.gff.gz. The cleavage sites of FspEI were (N)14CCGG(N)14 and (N)13CCHGG(N)13, and the length of the fragment was 32 bp and 31 bp, respectively. The degenerate base H represented the bases A, C, and T. When H is base C, the site is identified as CCGG, and FspEI is identified as CCGG and CCWGG, where

degenerate base W represents bases A and T. According to the consistency of equal-length sequence amplification efficiency, the sequencing depth of methylated labels can reflect the methylation level of the site, and the methylation sites with sequencing depth above 3 were counted (Korthauer *et al.*, 2019).

Genome-wide DNA methylation analysis

According to the consistency of equal-length sequence amplification efficiency, the sequencing depth of the methylated tag can reflect the methylation level of the site (CCGG/CCWGG). The quantitative value of methylation level of the locus in each sample was calculated in RPM (reads per million). The 2,000 kb segments of the upstream and downstream of TSS, and the gene body were selected to analyze the distribution trend of sequencing reads in the above segments and calculate Pearson's correlation coefficient between different samples.

The sum of all methylation sites in a gene was used to represent the methylation level of the gene. Based on the sequencing depth information of each site in each sample, genes with inter-group differences of $p \leq 0.05$ and absolute value of $\log_2$ FC greater than 1 were calculated and screened out, and the differential genes were clustered to draw a heat map.

The number of genes in the Gene Ontology (http://geneontology.org/docs/ontology-documentation/) (Ashburner *et al.*, 2000) entry was counted and the significant differences were calculated, which were divided into three categories: molecular function, cellular component and biological function. The GO entries with the corresponding gene number greater than 2 in the three classifications were screened, and the 10 entries were sorted by the P value ($-\log_{10}$) corresponding to each entry. Combined with the KEGG annotation results (http://www.genome.jp/kegg/), Pathway analysis was conducted on the genes with differentially expressed sites, and the significance of gene enrichment in each Pathway entry was calculated.

Validation of methylated sequencing

Four methylation sites, numbered douj-1, douj-2, douj-3, douj-4, were randomly selected from the Methyl RAD sequencing results, and common PCR primers and pyrophosphate amplification primers were designed in primer5. Samples for Methyl RAD sequencing were placed in a bisulfite amplification kit, and the methylated sequencing results were verified by PCR amplification of biotin-labeled products.

To validate the results of differentially methylated genes determined by Methyl RAD sequencing, we used real-time fluorescence quantification to validate the expression of immunofunction-related *SMG1* and *NLRP3*. Total RNA was isolated from the collected samples using Trizol reagent (Li *et al.*, 2009), and RNA was reverse-transcribed into cDNA using Takara reagent (Luo *et al.*, 2007). Specific primers were designed for commercial synthesis in Primer5 based on CDS method, *Cytb* gene expression quantity for reference, and the candidate gene expression levels were calculated by using $2^{\Delta\Delta Ct}$ method.

Statistical analysis methods

Based on the sequencing depth information of each site in each sample, *P*-value of the differences and difference multiple (Log_2 FC) of methylation sites between groups were calculated. Enrichment analysis of genes differential methylation sites was performed with by combining *A. japonicus* genome annotation information (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/002/754/855/GCA_002754855.1_ASM275485v1/GCA_002754855.1_ASM275485v1_genomic.gff.gz). The number of genes included in each GO entry was counted, and the significance of the p value of difference of gene Enrichment in the GO entry and KEGG Pathway entry was calculated by hypergeometric distribution test. A small P value indicated that the gene Enrichment occurred in the GO entry, and the Enrichment score was calculated as follows:

$$\text{Enrichment score} = \frac{\text{m/n}}{M / N}$$

N: Number of genes with GO annotation in all genes; n: Number of genes with GO annotation in differentially expressed genes in N; M: Number of genes annotated as a specific GO term in all genes; m: Number of differentially expressed genes annotated with a specific GO term.

The correlation between the results of pyrosequencing and methylated sequencing was detected by regression analysis. Real-time fluorescence quantitative results were analyzed by one-way ANOVA and independent sample t-test for significant difference (p<0.05).

Results

Genome-wide methylation level analysis

In the whole genome methylation sites, 1.8×10^6 CCGG methylation sites and 0.8×10^6 CCWGG methylation sites were detected (Appendix Table 1, Table 2). The number of methylation sites was 84,766 ~ 96,225, and the sequencing depth was 42.33 ~ 153.21. The standard deviation of the number of methylation sites ranged from 222.56 to 5,126.18. LBW group had the most methylation sites (79,426), while HBW group had the least (8,126). HBW group showed the greatest change in sum of the two methylation sites, 16,688 less than that in NBW group. The total variation of the two methylated sequencing depths was the greatest in XBW group, which increased by 51.8% compared with NBW group. Standard deviation of the sequencing depth of methylation sites ranged from 3.91 to 88.86. The average sequencing depth of CCGG methylation sites in XBW group was the highest (153.21), while the average sequencing depth in LBW group was the lowest (42.33).

Table 2: Statistics of methylation site coverage depth in the whole genome of *Apostichopus japonicus*.

Group	CCGG		CCWGG	
	Number of sites	Average depth	Number of sites	Average depth
LBW	79,635	78.96	17,910	42.33
	79,192	91.35	13,415	56.08
	79,451	80.17	19,071	40.6
NBW	81,061	118.05	14,210	69.89
	82,391	112.48	18,495	57.38
	74,347	110.51	9,481	87.34
HBW	74,155	123.99	8,142	106.51
	67,248	73.86	7,876	63.51
	64,140	172.14	8,361	143.38
XBW	75,087	153.21	8,318	132.23
	79,739	99.76	13,412	56.58
	76,953	125.04	9,191	99.18
SBW	80,521	105.12	14,550	60.2
	71,992	257.38	8,173	226.32
	77,101	114.23	9,229	88.59

Appendix Table 1: Sample amount of sequencing data.

Sample	Raw Reads	Enzyme Reads	Mapping Reads	Ratio
LBW1-2	32,306,882	15,900,973	7,574,751	47.64%
LBW2-1	30,533,555	18,228,013	8,530,556	46.80%
LBW3-2	31,955,155	16,077,012	7,659,085	47.64%
NBW1-1	39,114,724	23,574,130	11,236,298	47.66%
NBW2-1	44,370,059	23,076,176	11,052,152	47.89%
NBW5-1	30,724,660	21,975,098	9,254,191	42.11%
HBW2-1	29,986,798	22,495,509	10,549,720	46.90%
HBW4-1	20,374,243	13,191,801	5,614,029	42.56%
HBW5-3	44,386,822	30,557,718	12,094,984	39.58%
SBW1-2	35,919,667	21,458,748	9,894,192	46.11%
SBW5-2	57,168,554	45,250,783	20,953,847	46.31%
SBW6-1	30,917,465	21,422,990	10,145,992	47.36%
XBW2-1	38,299,550	28,336,382	13,115,439	46.28%
XBW4-1	29,442,927	19,570,546	9,185,054	46.93%
XBW6-1	32,069,913	23,925,079	11,045,898	46.17%

(1) Sample: sample name;
(2) Raw reads: sequencing data;
(3) Enzyme reads: Reads containing the expected restriction sites after raw reads;
(4) Mapping reads: Enzyme Reads with unique alignment positions on the reference sequence;
(5) Ratio: the ratio of mapping reads to enzyme reads.

The distribution of methylation sites in different regions of genes

According to the distribution of methylation sites on different functional components of *A. japonicus*, the methylation amount in the intergenic region, intron and exon regions and the 2,000 bp region upstream of the transcription start site decreased successively, while no methylation sites

were detected in the utr5 and utr3 primers. Methylation sites of different functional elements in HBW group decreased by 9.51% on average compared with NBW group, while CCWGG sites in HBW, XBW, and SBW groups decreased by 41.41%, 23.30%, and 26.32% on average compared with NBW group (Fig. 1).

We calculated the distribution of methylation sites in the gene bodies. The results showed that gene bodies' methylation level was higher than the background level of the genome, the methylation levels peak before the transcription start site (TSS). The level of TSS methylation is lower than that at the transcription termination sites (TTS). Under different temperature stress, the peak value of HBW group was the largest before TSS, and there was an additional peak value in CCWGG locus of HBW group before TTS (Fig. 2).

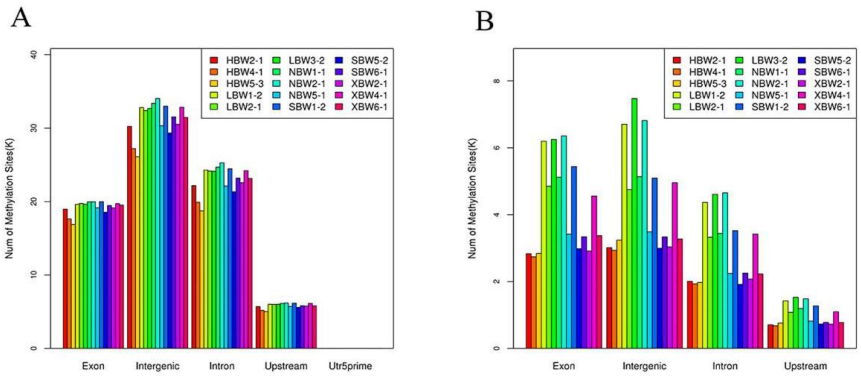

Figure 1: Distribution of methylation sites on different functional elements. Numbers of the two methylation sites, CCGG (A) and CCWGG (B), were counted (sequencing depth was greater than 3); horizontal axis represents different functional elements of the genome, and vertical axis represents the number of sites (K). The results showed that methylation sites were mainly concentrated in the intergenic segment, exon, and intron regions.

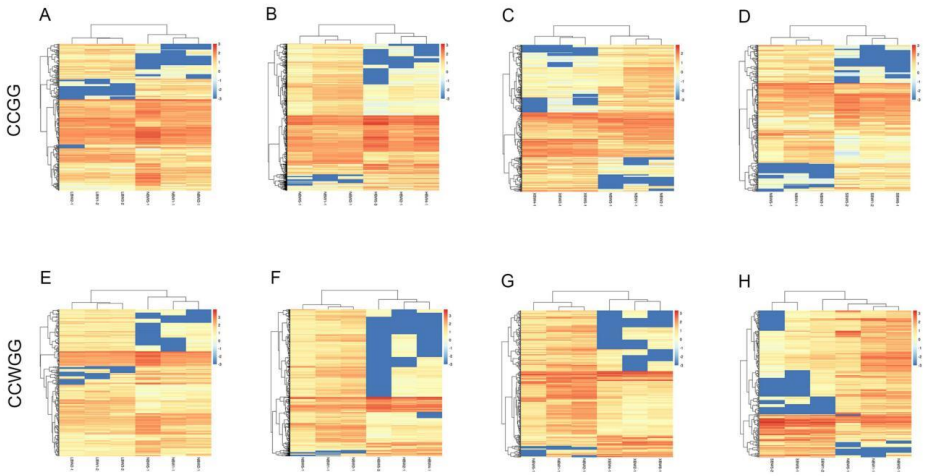

Figure 2: Distribution of methylation sites in different segments of the whole genome. The two characteristic sites, CCGG and CCWGG, were counted. The abscissa corresponds to the position information of the methylation site, and the ordinate corresponds to the relative level of methylation (RPM). For gene regions (1 and 4), each column indicates relative position of the locus on the gene. For the regions upstream and downstream of TSS (2 and 5), TTS (3 and 6), each column represents absolute position of the upstream and downstream. According to the content shown in the figure, there is a methylation peak at the positions of initiation and termination of gene transcription.

Comparison of different gene methylation levels between different groups

According to the comparison with NBW group, the methylation sites are divided into up-regulation sites and down-regulation sites. The methylation levels of *A. japonicus* genes changed to alter degrees under temperature stress, and the number of genes down-regulated by methylation level was higher than that of genes up-regulated. This indicated that the change in environmental temperature might promote the expression of *A. japonicus* genes. In the whole genome, 1,358 CCGG methylation differentially expressed genes and 1,554 CCWGG methylation differentially expressed genes were found. HBW group had the highest number of CCGG locus differentially expressed genes (830), while SBW group had the lowest number of CCWGG locus differentially expressed genes (291).

Compared with NBW group, HBW group had the highest number of differentiated methylated genes (1,405), among which 66.62% of the differentiated methylated genes were down-regulated. SBW group had the least number of differentially methylated genes (643), among which 63.25% decreased methylation (Fig. 3).

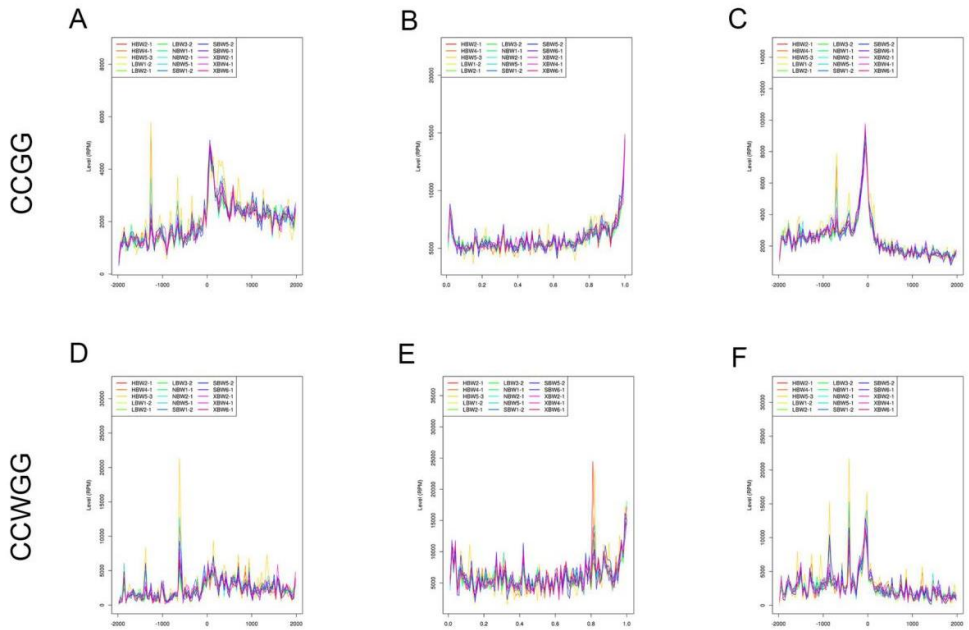

Figure 3: Cluster heat map of differential methylation genes in different temperature groups and NBW group. The differential genes of the CCGG locus and CCWGG locus in the different temperature groups were analyzed separately. The abscissa indicates the sample name, the ordinate indicates differential methylation gene, and the color indicates methylation level (blue→red, low methylation levels→high methylation levels). Depicted from left to right are the differential methylation genes of LBW group (1 and 5), HBW group (2 and 6), XBW group (3 and 7) and SBW group (4 and 8). The left 3 columns represent differential methylation genes in NBW group, and the 3 columns on the right represent differential methylation genes in different temperature groups. As shown in the figure, HBW group had the largest number of differential genes and most of them were down-regulated.

GO functional classification and enrichment analysis of whole genome differential methylation genes

GO functional classification and enrichment analysis were performed on differential methylation genes between different temperature groups and the NBW group. Functional analysis of the differential methylation genes showed that they were enriched in three categories: molecular function, cellular components, and biological functions. The differential methylation genes at CCGG sites were significantly enriched in terms related to nuclear-transcribed mRNA catabolic process (GO: 0000184), protein dimerization activity (GO: 0046983), and protein ubiquitination (GO: 0016567) (p<0.05). The differential methylation genes at CCWGG sites were extremely significantly enriched in terms related to protein ubiquitination (GO: 0016567), cytoplasmic vesicle membrane (GO: 0030659), and negative regulation of the extrinsic apoptotic signaling pathway (GO: 2001237) (p<0.01).

Different genes were detected in translation, golgi apparatus and nucleic acid binding pathways in LBW, XBW and SBW groups, while the enriched genes in HBW group were different from those in the other groups. The differential methylation genes enrichment was highly significant, in terms related to nuclear-transcribed mRNA catabolic process (GO: 0000184) and gene expression (GO: 0010467) (p<0.01) in LBW group; protein dimerization activity (GO: 0046983) and negative regulation of extrinsic apoptotic signaling pathway (GO: 2001237) (p<0.01) in HBW group; protein dimerization activity (GO: 0046983) and cytoplasmic vesicle membrane (GO: 0030659) (p<0.01) in XBW group; and protein ubiquitination (GO: 0016567) and protein complex (GO: 0043234) (p<0.01) in SBW group (Fig. 4).

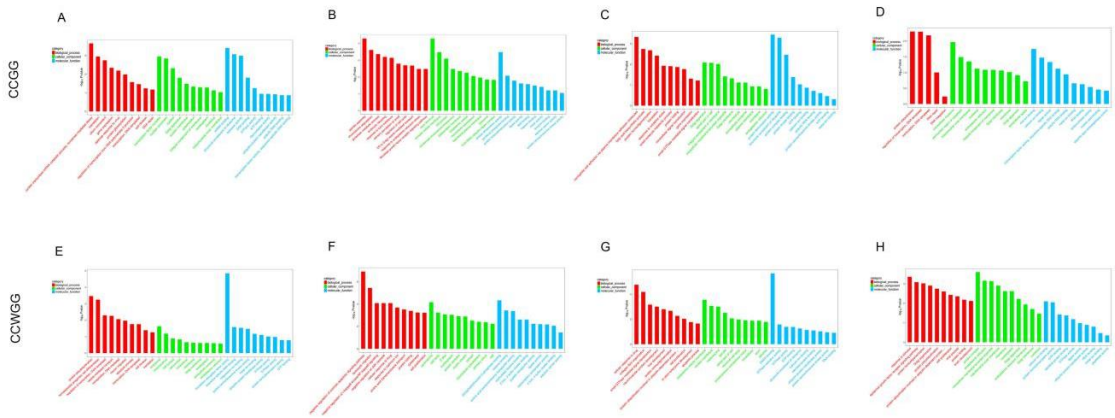

Figure 4: Comparison of ontology of differential methylation genes between different temperature groups and NBW group. Gene pathways of the differential genes of different groups of CCGG sites and CCWGG sites were counted separately. Horizontal axis represents the pathways and vertical axis indicates the gene numbers (-log10, P-value). From left to right differential methylation genes in LBW (1 and 5), HBW (2 and 6), XBW (3 and 7) and SBW groups (4 and 8) are shown. GO enrichment analysis classified the pathways of differential methylation genes into three categories: molecular function (blue), cellular components (green) and biological functions (red). GO items with corresponding gene numbers greater than 2 in the three categories were screened, and sorted according to -log10 (P-value) corresponding to each item, according to size from large to small.

Enrichment analysis of KEGG pathway of differentially methylated genes between groups

The KEGG pathway analysis was performed on differential methylation genes between different temperature groups and the NBW group. There were at least 9 to 20 terms with significant enrichment at CCGG sites in different temperature groups (p<0.05), while the enrichments in drug metabolism enzymes (ko: 00983), Leishmaniasis (ko: 05140), insulin resistance (ko: 04931) and ether lipid metabolism (ko: 00565) were highly significant (p<0.01). Most genes were enriched in the term of endocrine resistance (ko: 01522), with 65, including TM7SF3 (transmembrane 7 superfamily member 3), ELL-associated factor, and E3 ubiquitin protein ligase. There were 20 terms with significant enrichment at CCWGG sites (p<0.05), while enrichments in drug metabolism enzymes (ko: 00983), human cytomegalovirus infection (ko: 05163), insulin signaling term (ko: 04910), and synaptic vesicle cycle (ko: 04721) were highly significant (p<0.01). Most genes were enriched in the human cytomegalovirus infection term (ko: 05163), with 8 in total, including protein kinase C and inositol 1,4,5-trisphosphate receptor (Fig. 5).

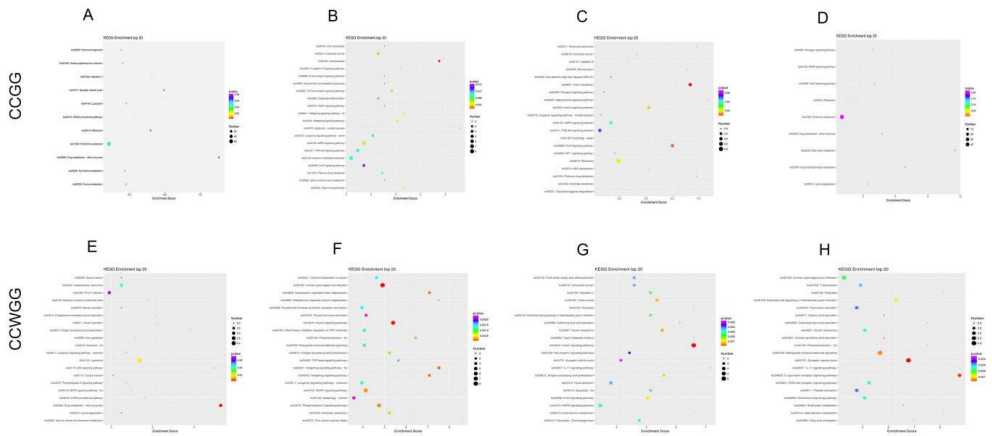

Figure 5: Comparison of KEGG enrichment of methylation genes between different temperature groups and NBW group. The differential methylation genes at CCGG sites and CCWGG sites were enriched separately. Horizontal axis represents the enrichment score and vertical axis indicates the pathways. From left to right differential methylation genes in LBW (1 and 5), HBW (2 and 6), XBW (3 and 7), and SBW groups (4 and 8) are shown. The larger the bubble, the more differential genes were contained within it, and the bubble color changes from red to blue to green to yellow as the enrichment P-value increases. KEGG enrichment analysis screened the top 20 path entries with the number of corresponding genes greater than 2 according to log10 (P-value) corresponding to each entry, sorted from large to small.

Verification of methylation levels by pyrosequencing

Since CCGG sites had smaller individual differences than CCWGG sites, 4 CCGG sites were randomly selected for pyrosequencing to verify methylated sequencing results (Table 3). According to the regression analysis results, the two sequencing results were consistent (Y=0.4042 x+92.618, r=0.9023), which proved the reliability of Methyl RAD seq sequencing results.

Table 3: Comparison of pyrosequencing results with Methyl RAD seq results.

Sample	douj-1	douj-2	douj-3	douj-4
Methyl RAD Seq results	0.85	20.95	14.31	8.13
Pyrosequencing results	92.12	100.00	99.66	96.57

Verification of gene transcription level

In order to verify the relationship between the methylation level and gene expression of *A. japonicus*, *SMG1* and *NLRP3* genes were selected for transcription testing, and the methylation level was compared with the transcription level. The results showed that the overall gene transcription level was negatively correlated with methylation level, indicating that temperature stress could change gene methylation level and thus regulate gene expression (Fig. 6). However, the transcription level of *SMG1* in XBW group and *NLRP3* in HBW group was positively correlated with methylation level.

The gene regulates methylation levels according to temperature stress. Some genes changed in different temperature groups, while most genes changed methylation levels under a single temperature stress. The methylation level of NOD-like receptor protein 3 (*NLRP3*) increased in different temperature groups, while the methylation level of serine/threonine protein kinase gene (*SMG1*) and Ankycorbin decreased. The methylation level of succinate-semialdehyde dehydrogenase (*SSAD*) decreased under high-temperature stress, but there was no significant change in low-temperature environment.

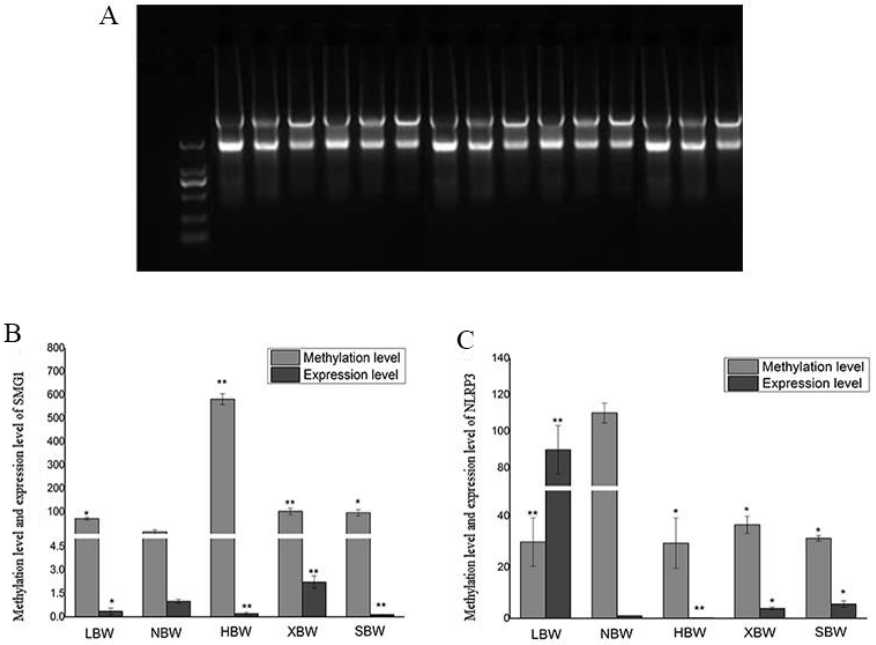

Figure 6: Comparison of methylation levels and expression levels of *SMG1* and *NLRP3* in different temperature groups. The methylation levels and transcriptional expression levels of *SMG1* (A) and *NLRP3* (B) genes were compared separately, with the horizontal axis representing different temperature groups and the vertical axis representing the relative levels of gene methylation (light) and expression (dark). As shown in the figure, there was a negative correlation between gene expression levels and methylation levels as a whole.

Discussion

CCGG was the main methylation pattern, which was consistent with the results of other eukaryotic genomes (Nai *et al.*, 2021; Villagómez-Aranda *et al.*, 2021). Continuous temperature stress had an effect on the methylation level of the whole genome of *A. japonicus*. Under different temperature conditions, the methylation level and methylation level of the whole genome of *A. japonicus* had different gene pathways. The methylated genes of HBW group showed the greatest difference, indicating the methylation level of the whole genome would be adjusted differently in response to different degrees of high-temperature stimulation. Previous studies have shown that under short-term high-temperature stress, the number of differentially methylated genes in *A. japonicus* increases with the extension of high-temperature stress time (Shen *et al.*, 2007). Under different high-temperature stimulation, in imitation of *A. japonicus* genome-wide methylation site first decreases after increasing, differences in gene number showed a trend of decrease, and cut in the number of gene methylation level than raising gene, suggesting that *A. japonicus* in water temperature rise, gene expression can be suppressed, and the gene expression by promoting into hibernation state part, specific regulatory mechanisms need to be researched (Wang *et al.*, 2013).

The differential methylation sites in *A. japonicus* genes were not evenly distributed and the methylation level of gene bodies was higher than that of the genome background. This pattern is similar to that observed in *Ciona intestinalis* and *Crassostrea gigas*. Similar to the results of PRMT6 and ATP genes, the methylation level of TSS gene was higher than that of other sites (Bouchard *et al.*, 2018). Like humans and *Crassostrea gigas* (Bonel *et al.*, 2013; Wang *et al.*, 2014), the TSS methylation level of the sea cucumber gene is higher, so it's speculated that sea cucumber can regulate its own expression by changing the methylation level (Gavery and Roberts, 2010; Deaton and Bird, 2011; Song *et al.*, 2017; Xu *et al.*, 2018).

The *NLRP3* gene plays a key role in the formation of *NLRP3* inflammation. It can sense stimuli and transduce signals and plays an important role in immune-related physiological processes (Franchi *et al.*, 2006; Schroder *et al.*, 2010). The *NLRP3* methylation level is decreased, probably to reduce the number of *NLRP3* inflammatory bodies and maintain a relatively healthy physiological state (Zhou *et al.*, 2011; Fan *et al.*, 2018; Yang *et al.*, 2019). *SMG1*, a serine/threonine protein kinase, is a key factor in nonsense-mediated decay (Henderson-Smith *et al.*, 2013). The *SMG1* showed high methylation levels in temperature stress, probably because the immunity of *A. japonicus* was reduced in adverse environment (Wang *et al.*, 2008; Chen *et al.*, 2014; Zhu *et al.*, 2016), cell activity was reduced, and apoptosis or even necrosis occurred (Byrum *et al.*, 2006; Li *et al.*, 2018b). *SSAD* is one of the common neurotransmitters and functions normally with the central nervous system in mammals. The methylation level of *A. japonicus SSAD* under high-temperature stress is down-regulated, and it is speculated that this change is related to signal regulation during adaptation to high temperature (Maneesh *et al.*, 2003; Perchat

et al., 2018). Ankyrin repeat proteins mediate specific protein-protein interactions and play an important role in maintaining the integrity of the cytoskeleton, regulating signaling pathways and inflammatory responses (Mosavi *et al.*, 2004; LaBella *et al.*, 2019). When the environment changes, the down-regulation of the methylation level of Ankyrin repeat proteins in *A. japonicus* may contribute to the stress response and maintain the stability of the body (Wang *et al.*, 2011; Bogdanović and Lister, 2017).

GO functional classification and enrichment analysis of differentially expressed genes showed that the level of gene methylation in the zinc ion binding pathway has the greatest effect on molecular function, as it may alter the damage caused by damaged cells and drugs in the body. The degree of binding (Bornens, 2012), which directly affects the drug management process and the possible survival probability after injury of the sea cucumber, affects the level of gene methylation in the centrosome pathway that plays an important role in the cell component. It can affect the cell division process and genomic stability (Radivojac *et al.*, 2010), which indicates that a temperature rise within a certain range can promote cell proliferation in *A. japonicus*, and has a positive effect on the growth of the individual. The genes in the ubiquitination pathway are involved in the regulation of transcription and the cell cycle (Parker and Armbrust, 2005), as the gene expression process of *A. japonicus* is inhibited during aestivation; this may be the reason why they shrink during summer.

KEGG pathway enrichment analysis showed that the methylation genes were enriched in different pathways in groups cultured at different temperatures. In the LBW group, the methylation levels of genes associated with transcription, such as ELL-associated factor and E3 ubiquitin-protein ligase were increased, probably because of changes in gene transcription level at low temperatures in *A. japonicus* regulated gene expression and adjusted energy distribution in the body (Thyssen *et al.*, 2011). In the HBW group, the methylation levels of genes associated with cell cycle regulation, such as tuberin, E3 ubiquitin-protein ligase (UBR5) were increased, probably because the increased temperature could inhibit cell cycle progression (Wang and Wahl, 2014). In the XBW group, the methylation levels of genes associated with insulin signal transduction, such as stress-activated protein kinase (JNK) and serine/threonine-protein kinase A-Raf, were increased, probably because changes in the insulin level at high-temperature alter water-carbohydrate metabolism in the sea cucumber (Park and Kwak, 2014). In the SBW group, the methylation levels of genes associated with endocrines, such as peroxisomal bifunctional enzyme and septin-5 were decreased, probably because high-temperature could change protein secretion and affect hormone levels in the sea cucumber (Sun *et al.*, 2011; Abdi *et al.*, 2018). The differential methylation genes in different temperature groups were enriched in different functional pathways, which indicated that *A. japonicus* could undergo different physiological changes to

adapt to the environment as the temperature changes.

Four gene loci were randomly selected and the methylation results were verified by pyrophosphate to obtain r=0.9023. The expression levels of *SMG1* and *NLRP3* genes showed that there was a negative correlation between gene methylation level and expression level. The methylation levels and expression levels of the *SMG1* gene in XBW group and the *NLRP3* in HBW group showed a positive correlation, indicating that gene expression was not completely regulated by gene methylation levels, consistent with the previous research results (Zhou *et al.*, 2011; Berner *et al.*, 2017; Vafadar-Isfahani *et al.*, 2017). Probably due to reduced immune tolerance in *A. japonicus* at high temperatures, the body was placed in the process of immune regulation, and the immune metabolites were temporarily increased (An *et al.*, 2009; Lv *et al.*, 2017; Vafadar-Isfahani *et al.*, 2017; Ding *et al.*, 2018).

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Confilicts of interests

The authors declare no conflicts of interest.

References

Abdi, A., Khabazi, A., Sakhinia, E., Alipour, S., Talei, M. and Babaloo, Z., 2018. Evaluation of SOCS1 methylation in patients with Behcet's disease. *Immunology Letters*, 203, 15-20. DOI: 10.1016/j.imlet.2018.07.001

An, Z.H., Dong, Y.W., Dong, S.L., 2009. A high-performance temperature-control scheme: growth of sea cucumber *Apostichopus japonicus* with different modes of diel temperature fluctuation. *Aquaculture International*, 17(**5**), 459-467. DOI: 10.1007/s10499-008-9216-4

Anastasiadi, D., Díaz, N. and Piferrer, F., 2017. Small ocean temperature increases elicit stage-dependent changes in DNA methylation and gene expression in a fish, the *European sea bass*. *Scientific Reports*, 7(1), 12401. DOI: 10.1038/s41598-017-10861-6

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Traver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M. and Sherlock, G., 2000. Gene ontology: Tool for the unification of biology. *Nature Genetics*, 25(**1**), 25-29. DOI: 10.1038/75556

Berner, D., Zenkel, M., Pasutto, F., Hoja, U., Liravi, P., Gusek-Schneider, G.C., Kruse, F.E., Schödel, J., Reis, A. and Schlötzer-Schrehardt, U., 2017. Posttranscriptional regulation of LOXL1 expression via alternative splicing and nonsense-mediated mRNA decay as an adaptive stress response. *Investigative Ophthalmology and Visual Science*, 58(13), 5930-5940. DOI: 10.1167/iovs.17-22963

Bird, A., 1992. The essentials of DNA methylation. *Cell*, 70(1), 5-8. DOI: 10.1016/0092-8674(92)90526-i

Bird, A., 2002. DNA methylation patterns and epigenetic memory. *Genes and Development*, 16(1), 6-21. DOI: 10.1101/gad.947102

Bizuayehu, T.T., Johansen, S.D., Puvanendran, V., Toften, H. and Babiak, I., 2015. Temperature during early development has long-term effects on microRNA expression in Atlantic cod. *BMC Genomics*, 16(1), 305. DOI: 10.1186/s12864-015-1503-7

Bogdanović, O. and Lister, R., 2017. DNA methylation and the preservation of cell identity. *Current Opinion in Genetics and Development*, 46, 9-14. DOI: 10.1016/j.gde.2017.06.007

Bonel, N., Sampson, J., Burn, J., Wilson, I.J., McGrown, G., Margison, G.P., Thorncroft, M., Crossbie, P., Povey, A.C., Santibanez-Koref, M. and Walters, K., 2013. Bayesian inference supports a location and neighbour-dependent model of DNA methylation propagation at the MGMT gene promoter in lung tumours. *Journal of Theoretical Biology*, 336, 87-95. DOI: 10.1016/j.jtbi.2013.07.019

Bornens, M., 2012. The centrosome in cells and organisms. *Science*, 335(6067), 422-426. DOI: 10.1126/science.1209037

Bouchard, C., Sahu, P., Meixner, M., Nötzold, R.R., Rust, M.B., Kremmer, E., Feederle, R., Hart-Smith, G., Finkernagel, F., Bartkuhn, M., Pullmasetti, S.S., Nist, A., Stiewe, T., Philipsen, S. and Bauer, U.M., 2018. Genomic location of PRMT6-dependent H3R2 methylation is linked to the transcriptional outcome of associated genes. *Cell Reports*, 24(12), 3339-3352. DOI: 10.1016/j.celrep.2018.08.052

Burgerhout, E., Mommens, M., Johnsen, H., Aunsmo, A., Santi, N. and Andersen, Ø., 2017. Genetic background and embryonic temperature affect DNA methylation and expression of myogenin and muscle development in Atlantic salmon (*Salmo salar*). *PloS One*, 12(6), e0179918. DOI: 10.1371/journal.pone.0179918

Byrum, C.A., Walton, K.D., Robertson. A.J., Thomason, R.T., Coffman, J.A. and McClay, D.R., 2006. Protein tyrosine and serine-threonine phosphatases in the sea urchin, *Strongylocentrotus purpuratus*: identification and potential functions. *Developmental Biology*, 300(1), 194-218. DOI: 10.1016/j.ydbio.2006.08.050

Chen, S., Zhang, G., Shao, C., Huang, Q., Liu, G., Zhang, P., Song, W., An, N., Chalopin, D., Volfi, J.N., Hong, Y., Li, Q., Sha, Z., Zhou, H., Xie, M., Yu, Q., Liu, Y., Xiang, H., Wang, N., Wu, K., Yang, C., Zhou, H., Liao, X., Yang, L., Hu, Q., Zhang, J., Meng, L., Jin, Y., Tian, Y., Lian, J., Yang, J., Miao, G., Liu, S., Liang, Z., Yan, F., Yangzhen, L., Sun, B., Zhang, H., Zhang J., Zhu, Y., Du, M., Zhao, Y., Schartl, M., Tang, Q. and Wang J., 2014. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nature Genetics*, 46(3), 253-260. DOI: 10.1038/ng.2890

China Fishery Statistical Yearbook (CFSY), 2018. China Agriculture Press,

China. 23P. (In Chinese)

Deaton, A.M. and Bird, A., 2011. CpG islands and the regulation of transcription. *Genes and Development*, 25(10), 1010-1022. DOI: 10.1101/gad.2037511

Ding, J., Li, L.X., Liu, X.H. and Chang, Y., 2018. Molecular characterization and expression of AjNLRP3 in the antibacterial host defense of the sea cucumber (*Apostichopus japonicus*). *Invertebrate Survival Journal*, 14(**1**), 423-431. DOI: 10.1016/j.gene.2018.06.072

Fan, Y., Diao, J., Yu, X.Q., Li, L., Liu, E.F., Li, T.B., Ye, H.B. and Ma, D.P., 2018. Effect of *Codonopsis pilosula* on immune and digestion in sea cucumber, *Apostichopus japonicus*. *Russian Journal of Marine Biology*, 44(1), 51-57. DOI: 10.1134/S1063074018010042

Franchi, L., McDonald, C., Kanneganti, T.D., Amer, A. and Núñez, G., 2006. Nucleotide-binding oligomerization domain-like receptors: intracellular pattern recognition molecules for pathogen detection and host defense. *Journal of Immunology*, 177(6), 3507-3513. DOI: 10.4049/jimmunol.177.6.3507

Gavery, M.R. and Roberts, S.B., 2010. DNA methylation patterns provide insight into epigenetic regulation in the Pacific oyster (*Crassostrea gigas*). *BMC Genomics*, 11(1), 483. DOI: 10.1186/1471-2164-11-483

Goldberg, A.D., Allis, C.D. and Bernstein, E., 2007. Epigenetics: a landscape takes shape. *Cell*, 128(4), 635-638. DOI: 10.1016/j.cell.2007.02.006

Henderson-Smith, A., Chow, D., Meechoovet, B., Aziz, M., Jacobson, S.A., Shill, H.A., Sabbagh, M.N., Caviness, J.N., Adler, C.H., Driver-Dunckley, E.D., Beach, T.G., Yin, H. and Dunckley, T., 2013. SMG1 identified as a regulator of Parkinson's disease-associated alpha-synuclein through siRNA screening. *Plos One*, 8(10), e77711. DOI: 10.1371/journal.pone.0077711

Jablonka, E. and Lamb, M., 1995. *Epigenetic inheritance and evolution: The Lamarckian dimension.* Oxford University Press, Oxford, UK. 346 P.

Jiang, W., Li, J., Gao, Y., Mao, Y., Jiang, Z., Du, M., Zhang, M. and Fang J., 2016. Effects of temperature change on physiological and biochemical responses of Yesso scallop, *Patinopecten yessoensis*. *Aquaculture*, 451, 463-472. DOI: 10.1016/j.aquaculture.2015.10.012

Korthauer, K., Chakraborty, S., Benjamini, Y. and Irizarry, R.A., 2019. Detection and accurate false discovery rate control of differentially methylated regions from whole genome bisulfite sequencing. *Biostatistics*, 20(3), 367-383. DOI: 10.1093/biostatistics/kxy007

LaBella, M.L., Hujber, E.J., Moore, K.A., Rawson, R.L., Merrill, S.A., Allaire, P.D., Ailion, M., Hollien, J., Bastiani, M. and Jorgensen, E.M., 2019. Casein Kinase 1δ stabilizes mature axons by inhibiting transcription termination of ankyrin. *Developmental Cell*, 52(**1**), 88-103. DOI: 10.1016/j.devcel.2019.12.005

Li, E., Beard, C. and Jaenisch, R., 1993. Role for DNA methylation in genomic imprinting. *Nature*, 366(6453), 362-265.

DOI: 10.1038/366362a0

Li, R., Li, Y., Kristiansen, K. and Wang, J., 2008. SOAP: short oligonucleotide alignment program. *Bioinformatics*, 24(5), 713-714. DOI: 10.1093/bioinformatics/btn025

Li, D., Ren, W., Wang, X., Wang, F., Gao, Y., Ning, Q., Han, Y., Song, T. and Lu, S., 2009. A modified method using TRIzol reagent and liquid nitrogen produces high-quality RNA from rat pancreas. *Applied Biochemistry and Biotechnology*, 158(**2**), 253-261. DOI: 10.1007/s12010-008-8391-0

Li, Y.Q., Wang, R.J. and Li, Y.L., 2018a. Genome-wide profiling of DNA methylation in *Apostichopus japonicas* based on Methyl RAD-Seq. *Periodical of Ocean University of China*, 48(09), 41-50. DOI: 10.16441/j.cnki.hdxb.20170242 (In Chinese)

Li, S., Yang, F.J., Fu, X., Dong, L., Zhou, D.Y., Sun, L.M. and Gong, Z., 2018b. Ultraviolet-ray-induced sea cucumber (*Stichopus japonicus*) melting is mediated by the caspase-dependent mitochondrial apoptotic pathway. *Journal of Agricultural and Food Chemistry*, 66(1), 45-52. DOI: 10.1021/acs.jafc.7b03888

Luo, Y., Gong, X., Xu, L. and Li, S., 2007. Isolation of RNA and RT-PCR, cloning, and sequencing of noncoding RNAs from fungi. *Biochemistry and Molecular Biology Education*, 35(**5**), 355-358. DOI: 10.1002/bmb.76

Lv, Z., Wei, Z., Zhang, Z., Li, C., Shao, Y., Zhang, W., Zhao, X., Li, Y., Duan, X. and Xoing, J., 2017. Characterization of NLRP3-like gene from *Apostichopus japonicus* provides new evidence on inflammation response in invertebrates. *Fish and Shellfish Immunology*, 68, 114-123. DOI: 10.1016/j.fsi.2017.07.024

Maneesh, G., Hogema, B.M., Grompe, M., Bottiglieri, G., Concas, A., Biggio, G., Sogliano, C., Rigamonti, A.E., Pearl, P.L., Snead, O.C., Jakobs, C. and Gibson, K.M., 2003. Murine succinate semialdehyde dehydrogenase deficiency. *Annals of Neurology*, 54(**Suppl 6**), S81-S90. DOI: 10.1002/ana.10625

Mosavi, L.K., Cammett, T.J., Desrosiers, D.C. and Peng, Z.Y., 2004. The ankyrin repeat as molecular architecture for protein recognition. *Protein Science*, 13(**6**), 1435-1448. DOI: 10.1110/ps.03554604

Nai, Y.S., Huang, Y.C., Yen, M.R. and Chen, P.Y., 2021. Diversity of fungal DNA methyltransferases and their association with DNA methylation patterns. *Frontiers in Microbiology*, 11, 616922. DOI: 10.3389/fmicb.2020.616922

Park, K. and Kwak, I.S., 2014. The effect of temperature gradients on endocrine signaling and antioxidant gene expression during *Chironomus riparius* development. *Science of the Total Environment*, 470-471, 1003-1011. DOI: 10.1016/j.scitotenv.2013.10.052

Parker, M.S. and Armbrust, E.V., 2005. Synergistic effects of light, temperature, and nitrogen source on transcription of genes for carbon and nitrogen metabolism in the centric diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Journal of*

Phycology, 41(6), 1142-1153. DOI: 10.1111/j.1529-8817.2005.00139.x

Perchat, N., Saaidi, P.L., Darii, E., Pellé, C., Petit, J.L., Besnard-Gonnet, M., de Beardinis, V., Dupont, M., Gimbernat, A., Salanoubat, M., Fischer, C. and Perret, A., 2018. Elucidation of the trigonelline degradation pathway reveals previously undescribed enzymes and metabolites. *Proceedings of the National Academy of Sciences of the United States of America*, 115(19), E4338-4367. DOI: 10.1073/pnas.1722368115

Radivojac, P., Vacic, V., Haynes, C., Cocklin, R.R., Mohan, A., Heyen, J.W., Goebl, M.G. and Iakoucheva, L.M., 2010. Identification, analysis, and prediction of protein ubiquitination sites. *Proteins*, 78(2), 365-380. DOI: 10.1002/prot.22555

Robertson, K.D. and Wolffe, A.P., 2000. DNA methylation in health and disease. *Nature Reviews Genetics*, 1(1), 11-19. DOI: 10.1038/35049533

Schroder, K., Zhou, R. and Tschopp, J., 2010. The NLRP3 inflammasome: a sensor for metabolic danger? *Science*, 327(5963), 296-300. DOI: 10.1126/science.1184003

Shen, L., Guo, Y., Chen, X., Ahmed, S. and Issa, J.P.J., 2007. Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. *Biotechniques*, 42(1), 48-58. DOI: 10.2144/000112312

Song, K., Li, L. and Zhang, G., 2017. The association between DNA methylation and exon expression in the Pacific oyster *Crassostrea gigas*. *PloS one*, 12(9), e0185224. DOI: 10.1371/journal.pone.0185224

Sun, L., Chen, M., Yang, H., Wang, T., Liu, B., Shu, C. and Gardner, D.M., 2011. Large scale gene expression profiling during intestine and body wall regeneration in the sea cucumber *Apostichopus japonicus*. *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics*, 6(2), 195-205. DOI: 10.1016/j.cbd.2011.03.002

Suzuki, M.M. and Bird, A., 2008. DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics*, 9(6), 465-476. DOI: 10.1038/nrg2341

Thyssen, M., Ferreyra, G., Moreau, S., Schloss, I., Denis, M. and Demers, S., 2011. The combined effect of ultraviolet B radiation and temperature increase on phytoplankton dynamics and cell cycle using pulse shape recording flow cytometry. *Journal of Experimental Marine Biology and Ecology*, 406(1-2), 95-107. DOI: 10.1016/j.jembe.2011.06.015

Vafadar-Isfahani, N., Parr, C., McMillan, L.E., Sanner, J., Yeo, Z., Saddington, S., Peacock, O., Cruickshanks, H.A., Meehan, R.R., Lund, J.N. and Tufarelli, C., 2017. Decoupling of DNA methylation and activity of intergenic LINE-1 promoters in colorectal cancer. *Epigenetics*, 12(6), 456-475. DOI: 10.1080/15592294.2017.1300729

Villagómez-Aranda, A.L., Barcia-Ortega, L.F., Torres-Pacheco, I. and Guevara-González R.G., 2021. Whole-genome DNA methylation analysis in hydrogen peroxide overproducing transgenic tobacco resistant to biotic and abiotic stresses. *Plants*, 10(1), 10010178. DOI: 10.3390/plants10010178

Wang, F., Yang, H., Gabr, H.R. and Gao, F., 2008. Immune condition of *Apostichopus japonicus* during aestivation. *Aquaculture*, 285(1-4), 238-243. DOI: 10.1016/j.aquaculture.2008.08.033

Wang, T., Yang, H., Zhao, H., Chen, M. and Wang, B., 2011. Transcriptional changes in epigenetic modifiers associated with gene silencing in the intestine of the sea cucumber, *Apostichopus japonicus* (Selenka), during aestivation. *Chinese Journal of Oceanology and Limnology (CJOL)*, 29(6), 1267. DOI: 10.1007/s00343-011-0143-2

Wang, Q.L., Dong, Y.W., Qin, C.X., Yu, S.S., Dong, S.L. and Fang, W., 2013. Effects of rearing temperature on growth, metabolism and thermal tolerance of juvenile sea cucumber, *Apostichopus japonicus* Selenka: critical thermal maximum (CTmax) and *hsps* gene expression. *Aquaculture Research*, 44(10), 1550-1559. DOI: 10.1111/j.1365-2109.2012.03162.x

Wang, X. and Wahl, R., 2014. Responses of the insulin signaling pathways in the brown adipose tissue of rats following cold exposure. *PloS One*, 9(6), e99772. DOI: 10.1371/journal.pone.0099772

Wang, X., Li, Q., Lian, J., Li, L., Jin, L., Cai, H., Xu, F., Qi, H., Zhang, L., Wu, F., Meng, J., Que, H., Fang, X., Guo, X. and Zhang, G., 2014. Genome-wide and single-base resolution DNA methylomes of the Pacific oyster *Crassostrea gigas* provide insight into the evolution of invertebrate CpG methylation. *BMC Genomics*, 15(1), 1119. DOI: 10.1186/1471-2164-15-1119

Wang, T., Sun, L. and Chen, M., 2015. *Aestivation and regeneration*. In: The sea cucumber *Apostichopus japonicus,* history, biology and aquaculture. Yang H., Hamel F. and Mercier A. editors. Developments in Aquaculture and Fisheries Science, 39, Elsevier, Amsterdam, Netherlands. 177-209. DOI: 10.1016/b978-0-12-799953-1.00011-8

Xu, D., Zhou, S. and Sun, L., 2018. RNA-seq based transcriptional analysis reveals dynamic genes expression profiles and immune-associated regulation under heat stress in *Apostichopus japonicus*. *Fish and Shellfish Immunology*, 78, 169-176. DOI: 10.1016/j.fsi.2018.04.037

Yang, Y., Wang, H., Kouadir, M., Song, H. and Shi, F., 2019. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. *Cell Death and Disease*, 10(2), 128. DOI: 10.1038/s41419-019-1413-8

Yang, Y., Luo, Z., Wang, Q., Zhang, Q., Zhang, X., Xiang, J. and Li, F., 2020. Development of high throughput SNP genotyping approach using target sequencing in Pacific white shrimp and its application for genetic study. *Aquaculture*, 528, 735549. DOI: 10.1016/j.aquaculture.2020.735549

Yin, Y.H., Hu, W., Zhu, Z.Y., Han, M., Xie, Z.H. and Bi, J.Z., 2020. The *SOST* (Sclerostin) Gene +219 site methylation is the risk factor of acute cerebral ischemia in the Han Chinese population: A case-control study. *Biomedical and Environmental Sciences*, 33(9), 708-712. DOI: 10.3967/bes2020.092

Zemach, A., McDaniel, I.E., Silva, P. and Zilberman, D., 2010. Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science*, 328(5980), 916-919. DOI: 10.1126/science.1186366

Zhang, J., Kobert, K., Flouri, T. and Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End read mergeR. *Bioinformatics*, 30(5), 614-620. DOI: 10.1093/bioinformatics/btt593

Zhao, Y., Chen, M., Storey, K.B., Sun, L. and Yang, H., 2015. DNA methylation levels analysis in four tissues of sea cucumber *Apostichopus japonicus* based on fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) during aestivation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 181, 26-32. DOI: 10.1016/j.cbpb.2014.11.001

Zhou, R., Yazdi, A.S., Menu, P. and Tschopp, J., 2011. A role for mitochondria in NLRP3 inflammasome activation. *Nature*, 469(7329), 221-225. DOI: 10.1038/nature09663

Zhu, A., Chen, M., Zhang, X. and Storey, K.B., 2016. Gene structure, expression, and DNA methylation characteristics of sea cucumber cyclin B gene during aestivation. *Gene*, 594(1), 82-88. DOI: 10.1016/j.gene.2016.09.006