Journal of Thermal Biology 85 (2019) 102404

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Effects and transcriptional responses in the hepatopancreas of red claw crayfish *Cherax quadricarinatus* under cold stress

Donglei Wu[a,1], Youhui Huang[a,1], Qiang Chen[a], Qichen Jiang[b], Yiming Li[a], Yunlong Zhao[a,c,*]

[a] *School of Life Science, East China Normal University, Shanghai, 200241, China*
[b] *Freshwater Fisheries Research Institute of Jiangsu Province, Nanjing, Jiangsu, 210017, China*
[c] *State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai 200241, China*

ARTICLE INFO

Keywords:
Cherax quadricarinatus
Low temperature response
Enzyme activity
Ultrastructure
RNA-Seq
Expression analysis

ABSTRACT

The red claw crayfish, *Cherax quadricarinatus*, is an economically important freshwater crustacean that cannot tolerate low temperature, which diminishes survival via unknown mechanisms. Herein, physiological regulation of *C. quadricarinatus* was investigated following exposure to low temperature stress at 9 ± 2 °C for 4 weeks. Hepatopancreas tissue was tested for nonspecific enzyme activity, histological structure, and transcriptome sequencing analyses. The results showed that the activities of nonspecific enzymes were inhibited following low temperature stress. Ultrastructural observation revealed that the hepatopancreas structure was oxidatively damaged at low temperature, with numerous autophagic vesicles visible. Apoptosis in the hepatopancreas was significantly increased in the cold stress group, indicating diminished function. Transcriptome sequencing identified 2615 differentially expressed genes (DEGs) following low temperature stress, of which 1147 and 1468 were up- and down-regulated, respectively. Functional analysis of DEGs indicated involvement in substance metabolism, antioxidant defences, signal transduction, and immune responses. Therefore, chronic cold stress can suppress metabolism and cause oxidative damage and immune deficiency in crayfish. The findings provide fundamental molecular information for further study of the regulatory mechanisms of cold tolerance in red claw crayfish.

1. Introduction

Temperature is a one of the most important abiotic factors affecting the growth, development, reproduction and geographical distribution of aquatic poikilotherms (Burel et al., 1996; Hennig and Andreatta, 1998; Lesniak et al., 2015; Qiu et al., 2011; Yuan et al., 2017). Temperature affects many chemical and biological processes, including the velocity of chemical reactions and metabolic pathways, the activities of metabolic enzymes, aerobic respiration, signal transduction, and resistance to toxic substances, parasites and disease (Fadhlaoui and Couture, 2016; Gracey et al., 2004; Huang et al., 2017; Pernet et al., 2015; Vinagre et al., 2007). In particular, fluctuating environmental temperature caused by seasonal changes and dramatic daily temperature swings can impose stress on aquatic poikilotherms (Guschina and Harwood, 2006).

The red claw crayfish, *Cherax quadricarinatus*, is a freshwater decapod crustacean native to Australia that has been economically important for both aquaculture and aquarium trade industries worldwide since the 1990s, including in China (Hennig and Andreatta, 1998; Karplus et al., 1998; Saoud et al., 2013). Temperature plays a key role in maximising the growth of red claw crayfish. As a typical tropical species, the optimal temperature for growth is 24–30 °C. Although there are reports that this species of crayfish can resist low ambient winter temperatures in some temperate regions, when the temperature is below 10 °C *C. quadricarinatus* exhibits arrested growth and high mortality for a period of 2–3 months, and at 6–7 °C they become immobile and can only survive for a few days (Diaz et al., 2004; Karplus et al., 1998; GarcíaGuerrero et al., 2013). In addition, pH and salinity also affect the survival and growth of *C. quadricarinatus* (Meade et al., 2002; López-López et al., 2003). However, little is known about the physiological regulatory mechanisms of *C. quadricarinatus* during stress, especially the gene expression level.

The hepatopancreas is an important metabolic organ in crustaceans responsible for the digestion, absorption and storage of nutrients. It is

---

[*] Corresponding author. Laboratory of Crustacean Development Biology and Macrobenthic Ecology, School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China.,.

E-mail address: ylzhaoecnu@163.com (Y. Zhao).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jtherbio.2019.102404
Received 11 March 2019; Received in revised form 5 August 2019; Accepted 25 August 2019

also an important immune organ, the epithelial cells of which are major sources of immune molecules such as lectins, hemocyanin, ferritin, antibacterial and antiviral proteins, proteolytic enzyme, and nitric oxide (Rőszer, 2014). Many previous studies on *C. quadricarinatus* have examined enzymes, histopathology and transcriptome changes in the hepatopancreas (Li et al., 2012; Sacristán et al., 2016; Yudkovski et al., 2007), but knowledge of the biological impact of cold stress on red claw crayfish remains limited and conflicting.

In recent years, high-throughput RNA sequencing (RNA-Seq) has been increasingly applied for investigating the transcriptomic responses of aquatic animals to various biotic and abiotic stresses as a strategy for understanding stress mechanisms (Yu et al., 2019; Zhang et al., 2018). Transcriptome sequencing can identify changes in the expression of numerous genes in response to different stimuli, reveal the most significant metabolic pathways affected, and provide a foundation for understanding the molecular mechanisms involved in environmental stress factors. In previous studies on *C. quadricarinatus*, RNA-Seq technology has been conducted to investigate the expression of genes involved in various environmental factors and developmental processes, such as pathogen infection, pH and salinity (Ali et al., 2015; Zhao et al., 2016). However, profile information related to the regulation of gene expression in *C. quadricarinatus* at low temperatures remains lacking. In the present study, the activities of non-specific immune enzymes, changes in morphology, and apoptosis were investigated in the hepatopancreas of *C. quadricarinatus* following low-temperature stress. RNA-Seq and subsequent bioinformatic analyses identified key genes/proteins and signalling pathways related to the responses to cold stress. The findings provide a theoretical basis for understanding the regulatory mechanisms operating in red claw crayfish in response to low temperature stress.

2. Materials and methods

2.1. Experimental animals and samples

Healthy adult red claw crayfish (initial weight = 22.56 ± 1.25 g) were obtained from the Caojing Aquaculture Demonstration Garden Hatchery (Shanghai, China). Before sample collection, all crayfish were kept in a greenhouse at a temperature of 23–25 °C. Before the experiment, all crayfish were acclimated under laboratory conditions for 1 week in aerated water at a water temperature of 25 ± 1 °C, a pH of 7.6 ± 0.5, and a dissolved oxygen (DO) content of 6.2 ± 0.6 mg/L, measured using an HQ 40D portable multi-parameter water quality meter (Hach, Loveland, Colorado, USA) each morning and afternoon. A commercial shrimp diet was provided twice per day, and each feeding amount was 5% of the crayfish body weight. After acclimation, 72 healthy animals were selected and grouped randomly into two groups, with three replicates in each group. All animals were cultured in an open room matching the outdoor temperature (9 ± 2 °C). The water temperature in the control group was 25 ± 1 °C, and water temperature was controlled using submerged heaters with thermostats. A heater was not included in the cold stress group, hence the water temperature varied according to room temperature (9 ± 2 °C) from December to January in Shanghai, China. One-third of the water in each tank was changed every day using water at the same temperature. Water temperature was measured twice per day, and crayfish in each group were fed a commercial shrimp feed (Tongwei Feed Company, Sichuan, China) containing 35% crude protein and 8% lipid at 8:00 and 18:00 (Fig. 1).

Before the end of the 4-week cold stress period, all crayfish were starved for 24 h, 12 crayfish within the interphase of the molting period in each group were selected, hepatopancreas tissue was dissected and washed with pre-cooled distilled water, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent experiments. Complete and intact hepatopancreas tips from control and cold stressed groups were fixed with 2.5% glutaraldehyde and 4% paraformaldehyde, respectively.

2.2. Biochemical enzyme activity analysis

Hepatopancreas tissue from each crayfish was homogenised in ice-cold 0.86% sodium chloride solution using an automatic rapid grinding machine (Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China) to prepare 10% (w:v) homogenates. Homogenates were centrifuged at 3500 rpm for 15 min at 4 °C. Supernatants were immediately transferred to new sterile tubes for enzymatic analysis. Acid phosphatase (ACP), alkaline phosphatase (AKP) and lysozyme (LSZ) enzyme activities were measured using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions with a microplate spectrophotometer (BioTek Instruments, Inc, USA). Phenoloxidase (PO) activity was measured as described previously (Ashida, 1971) with modifications by recording the formation of dopachrome produced from the levodopa (Sigma, St. Louis, MO, USA).

2.3. Morphological observation

Hepatopancreas tissue prefixed in 2.5% glutaraldehyde for 24 h was post-fixed in 1% osmium tetroxide (OsO4) for 1 h, dehydrated using an alcohol gradient, embedded in Epon 812 epoxy resin, sliced into sections (70 nm thickness) using an RMC PowerTome XL microtome (Boeckeler Instruments, Tuscon, AZ), stained with uranyl acetate and lead citrate, and examined under a Hitachi H-7650 transmission electron microscope (TEM) at 80 kV. For detection of apoptosis in hepatocytes, hepatopancreas tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 4 μm sections. TdT-mediated dUTP Nick-End Labeling (TUNEL) staining was performed using a dedicated kit (Catalog No. 11684817910; Roche, Basel, Switzerland) to assess apoptosis as described previously (Cui et al., 2015). Sections were observed under a BX51 fluorescence microscope (Olympus BX53F, Olympus Corporation; Tokyo, Japan) and images were collected. Apoptosis rates was measured using Image-pro plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The apoptotic index was expressed as the percentage of total cells scored.

2.4. Total RNA extraction, library construction and sequencing analysis

Total RNA was extracted from isolated hepatopancreas tissues of nine individuals in cold stress and control groups using a commercial assay kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's protocol. RNA quantity and quality were examined using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis, respectively. RNA used to construct the sequencing library was also evaluated using a 2100 Bioanalyzer (Agilent, California, USA). cDNA libraries were constructed using a Truseq RNA sample prep Kit (Illumina, USA). The resulting DNA template was enriched by PCR amplification (15 cycles), and amplicons were collected and purified by Certified Low Range Ultra Agarose gel electrophoresis (Bio-Rad, USA). Before sequencing, DNA libraries were quantified using a TBS-380 microfluorimeter with Picogreen reagent (Invitrogen, USA). Clone clusters were generated with an Illumina cBot using a Truseq PE Cluster Kit v3-cBot-HS, and high-throughput sequencing was performed on an Illumina Miseq sequencer using Truseq SBS Kit v3-HS with 200 cycles at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China).

2.5. Sequence data processing and de novo assembly

Raw paired-end reads were trimmed and checked for quality using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) by removing reads with adaptors, reads with a 10% Q value < 20 bases (those with a base quality < 20) and

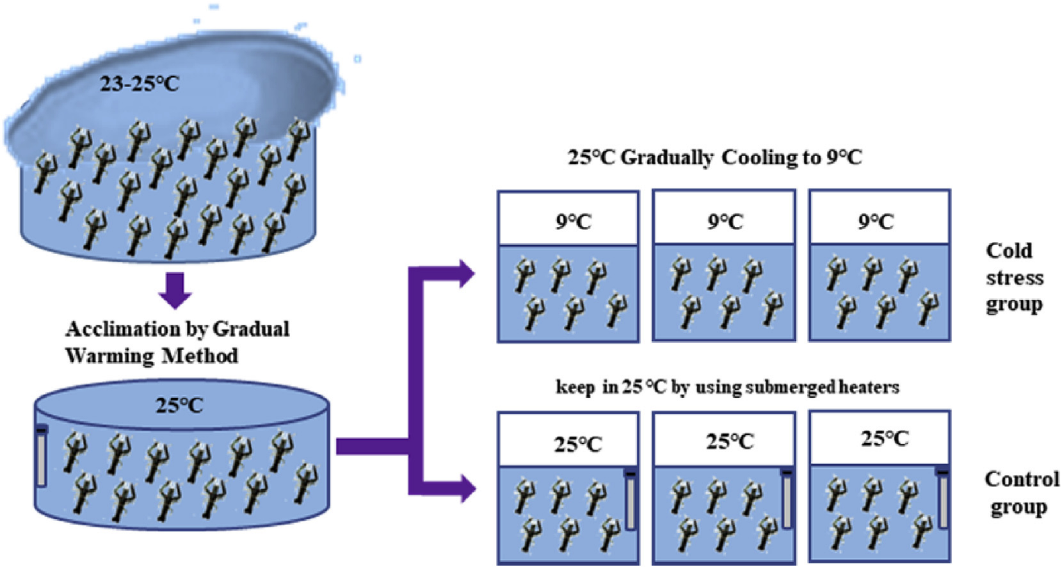

Fig. 1. Establishment of cold response in Cherax quadricarinatus.

low-quality sequences (reads with 'N' ambiguous bases). Clean data from all samples were used to perform *de novo* assembly with Trinity (version trinityrnaseq_r20140413; Grabherr et al., 2011) with default parameters. Inchworm, Chrysalis, and Butterfly software modules of Trinity were used for data analysis in series with default parameters. Inchworm builds a k-mer graph (K = 25) from the original sequence and finds all paths in the graph through a comprehensive approach. The k-mer with the highest frequency is then chosen to build contigs, and the process is repeated until the k-mer library is empty. Chrysalis was used to cluster contigs with variable cleavage sites, and for reads containing a (k-1) mer repeat in the contig, or reads in the original sequence spanning different contigs, these contigs were considered to be derived from the same gene and assigned to the same group. After grouping, each group was re-established with its own de Bruijn graph for later assembly. Butterfly was used to merge contigs with continuous nodes to form longer sequences and rate contigs using a dynamic programming algorithm to delete error bifurcation supported by few reads. The assembled *de novo* transcriptome was used as the reference database.

2.6. Sequence assembly and annotation of transcriptome data

After assembly, the open reading frame (ORF) of each unisequence was predicted using Transdecoder software (Haas et al., 2013). Unisequences were used for BLAST searching (Camacho et al., 2009) and annotation against the non-redundant (NR) NCBI protein NR (McGinnis and Madden, 2004), SwissProt (Boeckmann et al., 2003), String (Szklarczyk et al., 2016), KEGG (Kanehisa and Goto, 2000), and Pfam (Finn et al., 2013) databases with a typical E-value cut-off < 1e-5 to identify proteins sharing high sequence similarity with unigenes, and their functions were retrieved. Functional annotation based on Gene Ontology (Consortium, 2004) terms was performed using BLAST2GO software (Conesa et al., 2005).

2.7. Identification and validation of differentially expressed genes (DEGs)

The edgeR program (version 2.12 (Robinson et al., 2010); was employed for differential expression analysis using gene read count data based on the negative two item distribution model. False discovery rate FDR < 0.05 and $|\log2FC| \geq 1$ were used as thresholds for judging significant differences in transcript expression. DEGs across samples were further annotated using GO and KEGG pathway analyses.

Functional enrichment analysis was performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at *p*-value ≤ 0.05 using DEGs as input and the complete annotated *C. quadricarinatus* gene set as the background. KOBAS (Xie et al., 2011) and Goatools (Klopfenstein et al., 2018) were used for KEGG and GO enrichment analyses, respectively.

A total of 15 DEGs related to antioxidants, energy metabolism and immunity were selected for qRT-PCR validation of the RNA-Seq data. Quantitative real-time PCR (qRT-PCR) was performed to verify the accuracy of the RNA-Seq data. Total RNA (500 ng) was extracted from hepatopancreas tissue from each group as described above and reverse-transcribed into cDNA using a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) according to the manufacturer's protocol. After checking the quality by reverse transcription PCR (RT-PCR), all cDNA samples were diluted 1:1 with ultra-pure water was used as qRT-PCR template, and qRT-PCR was performed using a CFX96 Multicolor Real-time PCR Detection System (Bio-Rad). The reaction volume was 20 μL, comprising 10 μL 2 × SYBR Premix ExTaq (Trans, Beijing, China), 1.0 μL diluted cDNA template (diluted two-fold), 9.5 μL PCR-grade water, and 1.0 μL of each primer (10 μM). Thermal cycling included an initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and 57 °C for 30 s. This was followed by melting curve analysis to confirm single PCR products. Each sample was run in triplicate, and the housekeeping gene18S rRNA (AF235966) was employed for normalisation of mRNA levels, since this has been validated as a good control gene in *C. quadricarinatus* (Wang et al., 2013). Primers for 18S rRNA and other sequencing reactions are listed in Supplementary Table 1. Each real-time PCR assay was repeated three times. Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method (Livak and Schmittgen, 2001). Means and standard deviation (SD) were calculated from triplicate experiments.

2.8. Statistical analysis

Enzyme activity and apoptotic rate analysis data are presented as mean ± SD. One-way analysis of variance (ANOVA) and Tukey 's tests were used to determine statistical significance ($p < 0.05$) between control and treatment groups. All statistical analyses were performed using SPSS 19.0.

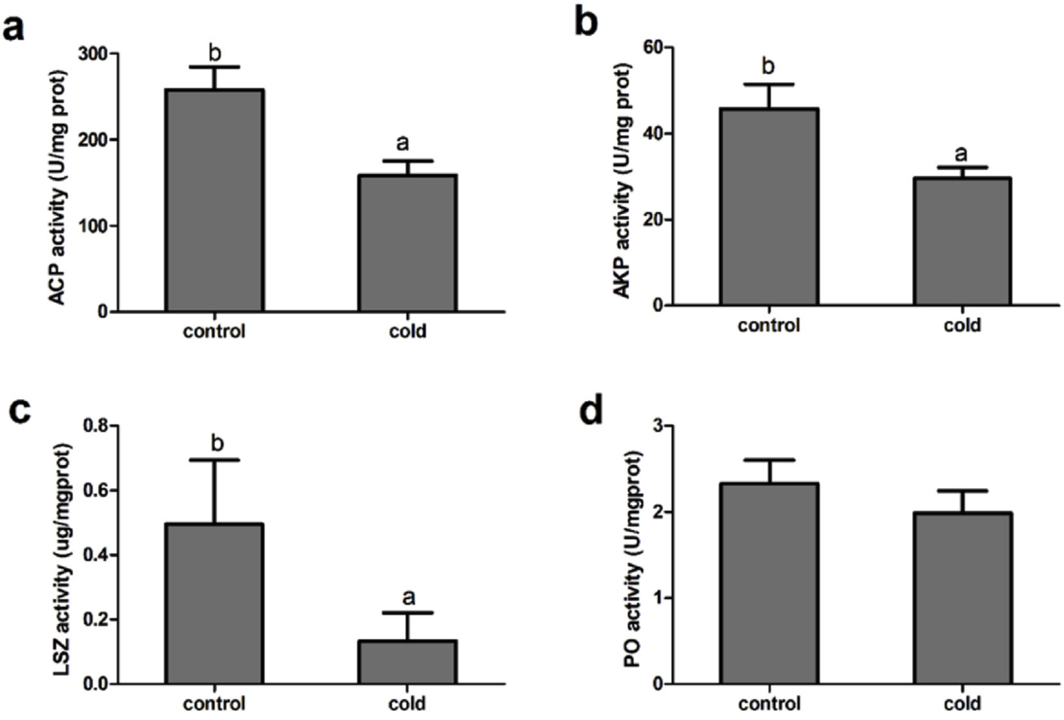

Fig. 2. Effects of low temperature acclimation on acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LSZ) and phenoloxidase (PO) enzyme activities in the hepatopancreas of red claw crayfish *C. quadricarinatus*. Values are presented as means ± standard deviation (SD). Significant differences are indicated with different letters ($p < 0.05$).

3. Results

3.1. Effects of cold stress on immune enzyme activity in the hepatopancreas of C. quadricarinatus

We measured the activity of four immune-related enzymes in the hepatopancreas (Fig. 2): acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LSZ) and phenoloxidase (PO). ACP (ANOVA, F = 30.118, d. f. = 1,4, $p = 0.005$), AKP (ANOVA, F = 19.643, d. f. = 1,4, $p = 0.011$) and LSZ (ANOVA, F = 11.162, d. f. = 1,4, $p = 0.016$) were significantly inhibited following cold temperature stress compared with the control group. The activity of PO (ANOVA, F = 0.861, d. f. = 1,4, $p = 0.389$) was also lower than the control group, but not significantly.

3.2. Effects of cold stress on hepatopancreas morphology

To investigate whether cold stress causes apoptosis, we examined ultrastructural changes in the hepatopancreas. In the control group (Fig. 3A − C), the cytoplasm was dense, with intact mitochondria, clear inner ridges, and food vacuoles evident, indicating normal metabolism. By contrast, a loss of endoplasm was observed in the cold stress treatment group (Fig. 3D), mitochondria were hazy with a damaged structure (Fig. 3E), and broken mitochondria in the process of being removed by the membrane structure were observed. In addition, numerous autophagic vacuoles were observed in the cold stress treatment group (Fig. 3F).

The results of TUNEL staining showed that the percentage of apoptotic hepatopancreatic cells was significantly increased in the low temperature treatment group compared with the control group (Fig. 4), while the apoptotic rate of the low temperature stress group (34.72%) was significantly higher than that of the control group (3.11%; ANOVA, F = 13.988, d. f. = 1,4, $p = 0.020$).

3.3. Overview of sequence assembly

A total of 273, 918, 136 and 238, 106, 534 raw reads with a length of 150 bp were generated from control and cold stress groups, respectively, and have been submitted to the NCBI SRA database under BioProject ID PRJNA493678. Raw reads were filtered to obtain high-quality reads, which were *de novo* assembled using Trinity software (Grabherr et al., 2011) (see Table 1).

After assembly, transcripts were annotated to explore their putative biological functions. The assembly yielded 48,035 unigenes with an average length of 1115.42 bp, a minimum length of 201 bp, and a maximum length of 30,385 bp, with an N_{50} length of 1782 bp (Table 2).

3.4. Annotation of unigenes

All unigenes were subjected to annotation by matching sequences against NR, SwissProt, String, KEGG, and Pfam databases using BLAST searches with an E-value < 0.00001. As shown in Table 3, 16,592 sequences (34.54%) were matched using NR, 11,880 (24.73) were matched using SwissProt, 4145 (8.63%) were matched using String, 9550 (19.88%) were matched using KEGG, and 10,334 (21.51%) were matched using Pfam databases.

3.5. Identification of differentially expressed genes

Genes significantly differentially expressed between cold stress and control groups were identified. The results showed that 2615 genes were significantly differently expressed, including 1147 up- and 1468 down-regulated genes (Table S2, Supplementary material).

A total of 338 DEGs were mapped to the KEGG database, of which 139 were up-regulated and 199 were down-regulated (Table S3, Supplementary material). A total of 332 DEGs were mapped to the GO database, of which 161 were up-regulated and 171 were down-regulated (Fig. S1, Supplementary material). Details about the up- and down-regulated genes annotated in the GO database can be found in

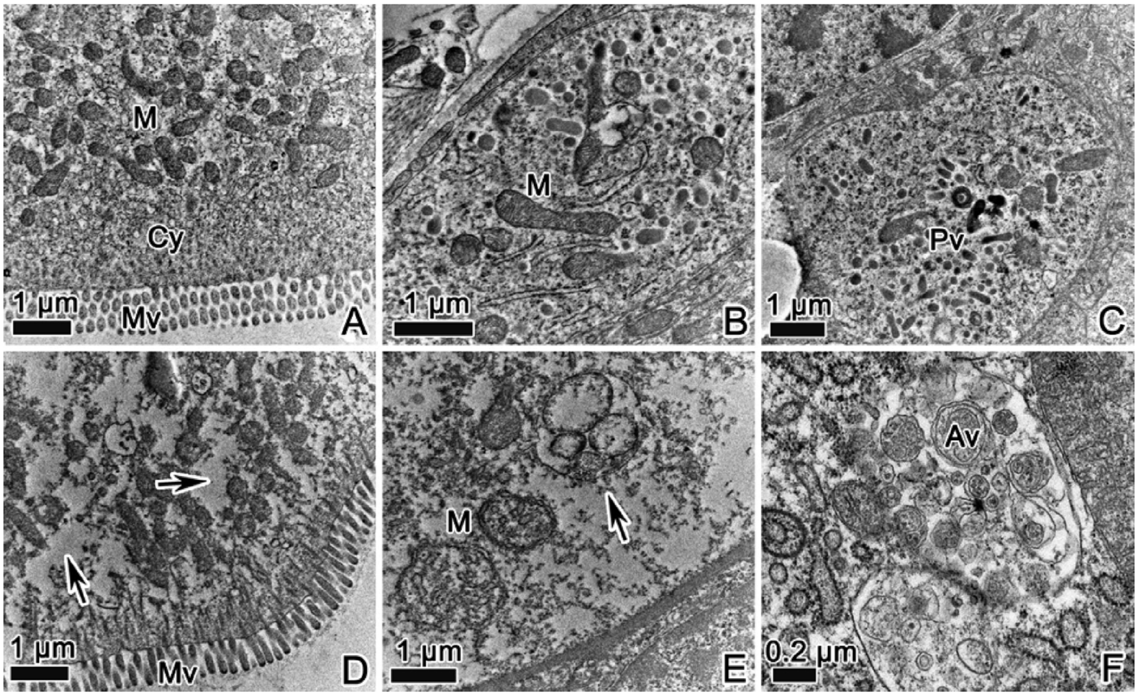

Fig. 3. Effects of low temperature acclimation on the ultrastructure of the hepatopancreas in *C. quadricarinatus*. A − C, Ultrastructure of the hepatopancreas in the control group. D − E, Ultrastructure of the hepatopancreas in the cold stress group. M, mitochondria; Cy, cytoplasm; Mv, microvillus; Pv, food vacuole; Av, autophagic vacuole.

Table S4 and Table S5 (Supplementary material).

KEGG enrichment analysis showed that 27 pathways (Table 4) were significantly enriched ($p < 0.05$). The top three enrichment pathways were metabolism (13 pathways), human diseases (five pathways) and environmental information processing (four pathways; Table S6, Supplementary material), which implies that these three categories of pathways are important for the cold stress response of *C. quadricarinatus*.

GO enrichment analysis showed that 51 GO terms were significantly enriched ($p < 0.05$, Table S7, Supplementary material), implying that a broad spectrum of biological processes is involved in the cold stress response of *C. quadricarinatus*. Among these enrichment pathways, 16 KEGG pathways (Table 4) and 19 GO terms (Table S7, Supplementary material) were significantly enriched ($p < 0.01$), which indicates that these pathways may play more important roles in resistance to cold temperature.

Genes related to the process of energy metabolism, immunity and diseases, antioxidant defences, and apoptosis with $|\log2\ \text{fold-change}| \geq 2$ and FDR < 0.05 were selected (Table S8, Supplementary material) since they may play key roles in the acclimation of poikilotherms to cold stress.

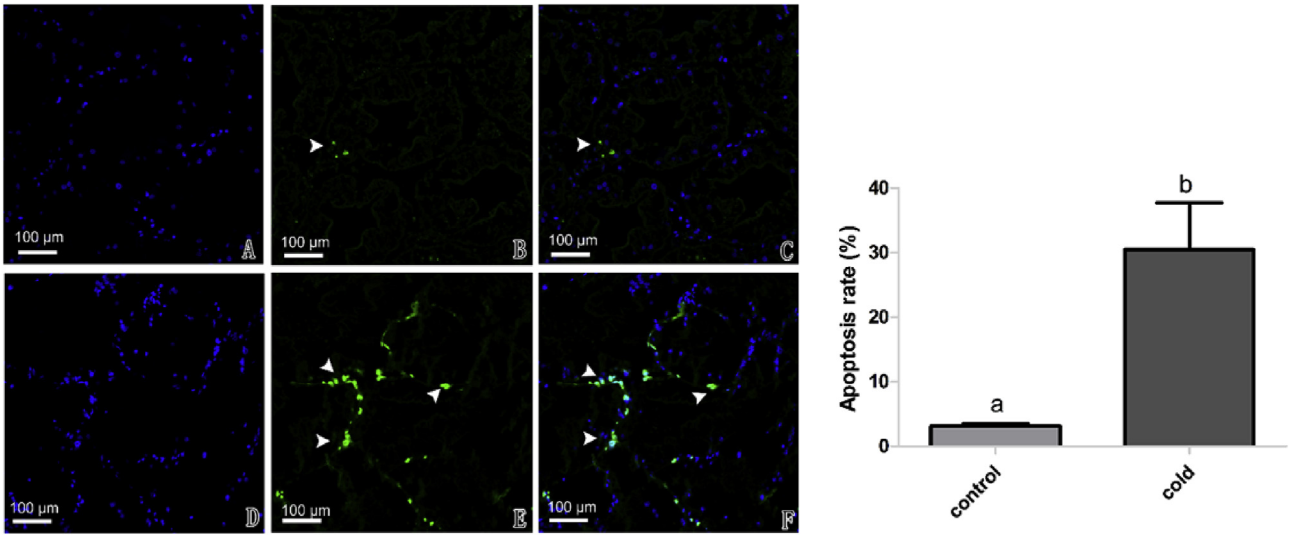

Fig. 4. Results of TUNEL method for detecting hepatopancreas apoptosis rate. A − C, Apoptosis in the hepatopancreas of the control group. D − F, Apoptosis in the hepatopancreas of the cold temperature group. Blue fluorescence in A and D indicate nucleus. Green fluorescence in B and E indicate apoptotic cells. C and F show merged results.

Table 1
Quality of sequencing.

Sample	Raw reads	Raw bases	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
cold1	53,231,864	7.98E+09	51,950,814	7.63E+09	0.0096	98.54	96.04	42.86
cold2	88,945,782	1.34E+10	85,944,020	1.26E+10	0.0138	97.67	93.33	43.91
cold3	1.32E+08	1.99E+10	1.27E+08	1.85E+10	0.0144	97.44	92.72	45.41
Control1	53,148,254	7.97E+09	51,864,322	7.63E+09	0.0097	98.52	95.99	42.68
Control2	66,076,340	9.98E+09	63,674,684	9.23E+09	0.0152	97.14	91.86	42.94
Control3	1.19E+08	1.8E+10	1.14E+08	1.66E+10	0.0144	97.4	92.68	44.66

Table 2
Summary of assembled RNA-seq results.

Type	Unigenes	Transcripts
Total number of sequences	48,035	63,328
Total number of nucleotides	53,579,050	83,905,433
Percentage GC	40.63	41.14
Longest sequence	30,385	30,385
Shortest sequence	201	201
Average sequence length	1115.42	1324.93
N_{50} value	1782	2165

Table 3
Summary of database search results.

Database	Unigenes	Total unigenes	Percentage of unigenes
Pfam	10,334	48,035	21.51
String	4145	48,035	8.63
KEGG	9550	48,035	19.88
SwissProt	11,880	48,035	24.73
NR	16,592	48,035	34.54

3.6. *Validation of RNA-Seq results by qRT-PCR*

To confirm the accuracy and reliability of the RNA-Seq expression analysis. Results showed that the expression patterns of the selected DEGs were consistent with the RNA-Seq data (Fig. 5). Thus, expression profiling of DEGs by RNA-Seq was reliable and accurate (R = 0.932).

4. Discussion

Temperature is one of the most important environmental factors affecting the survival, growth and development of warm-water aquatic animals, especially those not tolerant of low temperatures, such as tilapia and Pacific white shrimp *Penaeus vannamei* (Li et al., 2002; Souza et al., 2016). Sudden temperature drops caused by seasonal changes and cold currents can have adverse effects on life processes, such as disruption to energy metabolism, loss of membrane perm-selectivity, and collapse of ion gradients, as well as loss of neuromuscular coordination, which may cause tissue damage, and even death (Hayward et al., 2014). Therefore, studying the physiological and biochemical changes occurring in aquatic animals under low temperature stress has important theoretical and practical significance for aquaculture. Studies have shown that low temperature can cause apoptotic reactions in the crustacean hemolymph, which in turn affects innate immunity (Gomez-Jimenez et al., 2000; Li et al., 2014). In our present study, we found that low temperature stress also inhibited the activity of hepatopancreas immune enzymes. Analysis of the hepatopancreas ultrastructure revealed oxidative damage, loss of endoplasm, and breakage of mitochondrial cristae and vacuolisation of mitochondria in hepatopancreas tissues in the low temperature stress group. In addition to destruction of mitochondrial structure, we also observed a large number of autophagic vacuoles and multilayer membrane structures in the hepatopancreas sections, indicating that besides the apoptotic response,

Table 4
KEGG pathways significantly altered ($p < 0.05$) in *C. quadricarinatus* following chronic cold stress.

Pathway	KEGG ID	Typel	Mapping number	Background number	*p*-valve
Lysosome	ko04142	Cellular Processes	34	164	2.67E-09
Sphingolipid metabolism	ko00600	Metabolism	14	61	4.89E-05
Glycosaminoglycan degradation	ko00531	Metabolism	8	24	0.000255
Other glycan degradation	ko00511	Metabolism	10	39	0.000271
AMPK signalling pathway	ko04152	Environmental Information Processing	13	80	0.001632
Drug metabolism - other enzymes	ko00983	Metabolism	11	65	0.002746
Glutathione metabolism	ko00480	Metabolism	13	89	0.003744
Metabolism of xenobiotics by cytochrome P450	ko00980	Metabolism	10	58	0.003745
NF-kappa B signalling pathway	ko04064	Environmental Information Processing	7	31	0.004111
Proximal tubule bicarbonate reclamation	ko04964	Organismal Systems	6	23	0.004266
Adipocytokine signalling pathway	ko04920	Organismal Systems	8	41	0.0048
p53 signalling pathway	ko04115	Cellular Processes	8	44	0.006935
Insect hormone biosynthesis	ko00981	Metabolism	4	11	0.007512
Cytokine-cytokine receptor interactions	ko04060	Environmental Information Processing	4	11	0.007512
Drug metabolism - cytochrome P450	ko00982	Metabolism	9	56	0.008654
Chemical carcinogenesis	ko05204	Human Diseases	10	67	0.009091
Small cell lung cancer	ko05222	Human Diseases	10	70	0.011,787
Pentose and glucuronate interconversions	ko00040	Metabolism	11	83	0.013,628
Pathways in cancer	ko05200	Human Diseases	21	212	0.017,409
Transcriptional misregulation in cancer	ko05202	Human Diseases	11	88	0.019,478
Glucagon signalling pathway	ko04922	Organismal Systems	10	79	0.023,514
Tyrosine metabolism	ko00350	Metabolism	5	26	0.02532
PI3K-Akt signalling pathway	ko04151	Environmental Information Processing	17	169	0.026,841
Toxoplasmosis	ko05145	Human Diseases	10	85	0.034,976
Glycerolipid metabolism	ko00561	Metabolism	7	53	0.044,825
Arginine biosynthesis	ko00220	Metabolism	5	31	0.044,972
Isoquinoline alkaloid biosynthesis	ko00950	Metabolism	3	12	0.045,497

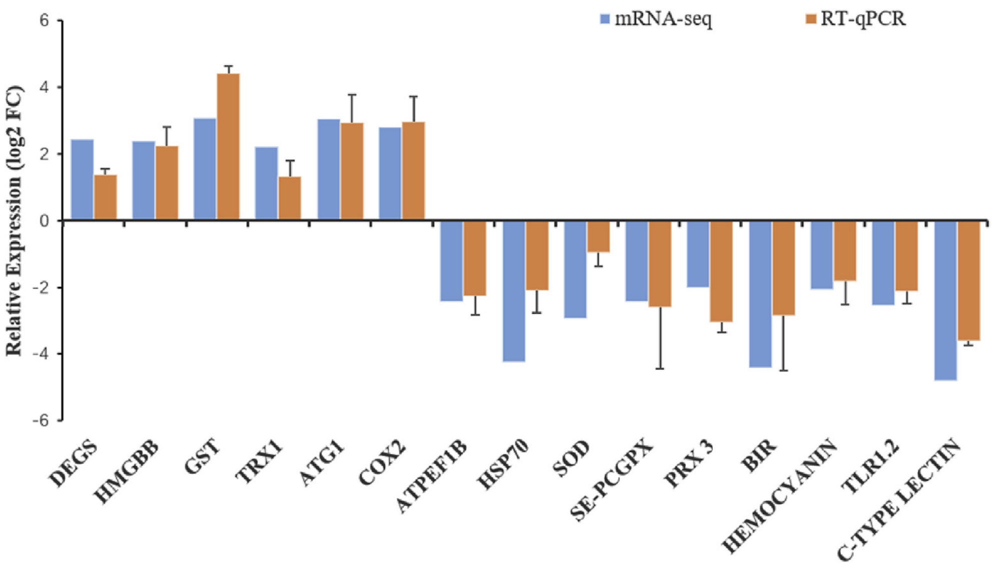

Fig. 5. Validation of RNA-Seq results by RT-qPCR. Transcript expression levels of selected genes were normalised against the 18S internal control. Log2FC refers to the log2 fold-change between control and cold groups.

autophagy also occurred. One of the reasons for autophagy may be a decrease in energy intake at low temperatures, with cells responding by providing energy to the body through autophagy. Second, disruption of the organelle structure may induce autophagy, which in turn clears damaged organelles. Additionally, reactive oxygen species (ROS) can also induce autophagy, hence increased ROS levels at low temperatures may also cause autophagy in cells. We also found that the apoptotic rate of hepatopancreas cells was increased significantly after low temperature stress, indicating that cold stress can induce apoptotic responses in the hepatopancreas of *C. quadricarinatus*. Together, the above results indicate that chronic exposure to a low temperature environment can exert stress on the red claw crayfish, which has an adverse effect on tissue structure and immune performance.

To further investigate the effects of low temperature stress at the molecular, we sequenced and analysed the *C. quadricarinatus* hepatopancreas transcriptome using an Illumina platform, since RNA-Seq is a powerful method for evaluating the transcriptomic responses of aquatic animals to cold stress (Hu et al., 2014; Long et al., 2013; Wang et al., 2014; Ju et al., 2002).

The biochemical and molecular mechanisms operating under temperature stress in fish have been thoroughly investigated (Gracey et al. 2004; Yong et al., 2013; Yang et al., 2015), and some aspects of the molecular mechanisms in shrimp have been elucidated (Huang et al., 2017). In the present study, we explored the effects of chronic cold stress on the hepatopancreas transcriptome of *C. quadricarinatus* to identify candidate cold tolerance-related and cold change-related genes affected following chronic cold stress. We identified 48,035 unigenes using RNA-Seq, of which 2615 were expressed differentially between cold stress and control groups, including 1147 up- and 1468 down-regulated genes. In total, 27 KEGG pathways and 51 GO terms were significantly enriched. The identified DEGs are involved in many crucial processes including basic metabolism, antioxidant defences, immune responses and apoptosis. The results will prove useful for further investigation of cold response genes and pathways in shrimp.

Previous studies showed that energy is important for enabling poikilotherms to maintain metabolic homeostasis under cold stress. Animals possessing a high capacity for ATP synthesis at reduced temperatures also display good tolerance to cold stress (Wang et al., 2014; Lu et al., 2017). Poikilotherms can also increase their capacity for glycolytic gluconeogenesis to produce more energy under cold temperatures (Zhao et al., 2015; Wang et al., 2018). However, in zebrafish larvae, basic metabolism processes such as steroid biosynthesis, peroxisomes, and drug metabolism are reportedly suppressed at low temperatures (Yong et al., 2013). In the present study, metabolic pathways were the most enriched among KEGG pathways, and several key genes were significantly altered under cold stress. For example, expression of 6-phosphogluconate dehydrogenase and phosphoenolpyruvate carboxykinase (PEPCK) was up-regulated, suggesting the capacity for increasing the pentose phosphate pathway and gluconeogenesis metabolism in *C. quadricarinatus* at low temperatures. This indicates that energy produced by oxidative phosphorylation may be insufficient for *C. quadricarinatus* under cold stress, but further studies are needed to confirm this hypothesis. Down-regulation of NADH dehydrogenase, mitochondrial ATP synthase subunit beta, and other suppressed metabolic pathways involved in producing ATP, such as the citric acid cycle and the electron transport chain, also indicate a shortage of energy. These results may also explain the weak tolerance of *C. quadricarinatus* to cold stress.

The second group of identified genes are related to disease and immune processes. The immune system is affected by temperature changes and temperature stress. For example, in the fruit fly *Drosophila melanogaster*, expression levels of immune-related genes are increased after cold stress stimulation (Bösner et al., 2009), and similar results have been reported for other species (Fan et al., 2016; Wentworth et al., 2018). However, in some species, immunity is suppressed under low temperatures, manifesting as a decrease in immune enzyme activity and a reduction in phagocytosis. Differences in immunity between different species following low temperature stress may be related to cold resistance. In the present study, we found that many immune genes were down-regulated following cold stress, including lectin 3, a lectin A isoform, C-type lectin, toll-like receptor Tlr1.2-like, cathepsin L, and hemocyanin. These genes play important roles in the innate immune process, such as pathogen detection in crustaceans, the presentation of antigens and antiviral responses, and a decline in the expression of these genes at low temperature indicates immunosuppression. The results are consistent with the down-regulation of immune enzymes, and with previous results on *Portunus trituberculatus* and *Carcinus maenas* (Chisholm and Smith, 1994; Zhao et al., 2015). Immune responses are usually accompanied by inflammatory reactions. In the present study, we also observed the up-regulation of some inflammatory factors including COX2, TGFBR1 and HMGB1, indicating that cold stress triggers inflammatory reactions in *C. quadricarinatus*, which may diminish survival and growth.

Low temperature stress usually causes an increase in ROS levels, which can induce up-regulation of antioxidant enzymes, but this is not sustainable under periods of prolonged stress (Kong et al., 2012; Wu et al., 2018). When ROS cannot be removed in time, oxidative damage occurs (Fan et al., 2013; Kong et al., 2012; Souza et al., 2014). In our present study, we also found that the expression of genes encoding antioxidant enzymes were significantly differentially expressed. Among them, glutathione S-transferase and thioredoxin were up-regulated under cold stress, but glutathione peroxidase, copper/zinc superoxide dismutase and thioredoxin-dependent peroxide reductase (mitochondrial-like isoform X1) were down-regulated. A decrease in antioxidant enzyme activity will reduce the ROS scavenging capacity, which in turn causes a large accumulation of ROS and hence oxidative damage (Xian et al., 2010, 2011). Heat shock proteins (HSPs) are important molecular chaperones that play a key role in maintaining cellular homeostasis. They are also indicative of temperature changes because they are often induced following a temperature shift. In the flesh fly and the saltwater clam *Ruditapes philippinarum*, expression of HSP70 is increased after cold shock (Rinehart et al., 2000; Wang et al., 2018), whereas induction is transient in channel catfish (Ju et al., 2002). In the present study, expression of HSP70 was down-regulated by cold stress, probably due to reduced basic metabolism caused by low temperature. The down-regulation of HSP70 may in turn increase the number of misfolded proteins, resulting in the failure of cellular defences under cold stress conditions. The up-regulation of E3 ubiquitin-protein ligase indicates an increase in damaged proteins under cold stress (Gracey et al., 2004).

The third group of differentially expressed genes included genes that are involved in environmental information processing. To adapt to either biotic or abiotic stress, the sensing and intracellular transduction of stress signals is critical for the adaptation and survival of organisms under various environmental stresses. In the L. *vannamei*, the serine/threonine protein kinase signalling pathway in muscle is activated following a change to low temperature, suggesting that it may play an important role in the response to low temperature stress (Huang et al., 2017). In zebrafish (*Danio rerio*), MAPK kinase, P53 and PPAR signalling pathways undergo significant changes at low temperature, and these pathways are related to environmental stress (Long et al., 2013). In catfish, changes in calcium-dependent protein kinase-mediated signalling pathways occur at low temperatures (Ju et al., 2002). Significant changes in NO-cGMP and Ras/MAPK signalling pathways have also been observed at low temperatures (Hu et al., 2014). In the present study, we also found that AMPK, NF-kappa B, P53 and PI3K-Akt signalling pathways were markedly affected by low temperature stress. The AMPK signalling pathway is associated with energy metabolism; when energy levels in the body are insufficient, this pathway is activated, resulting in a decrease in anabolism and an increase in catabolism to maintain the energy supply under nutritional deficiency or environmental stress conditions (Lu et al., 2017). NF-kappa B and PI3K-Akt signalling pathways are also activated by stress responses, leading to apoptosis, autophagy and other reactions. Changes in signalling pathways are usually accompanied by activation of serine/threonine protein kinases. In plants, low temperature signals are reportedly first perceived by membranes, which in turn opens calcium channels, causing an increase in intracellular calcium concentration. This stimulates the activity of downstream calcium-dependent protein kinases, activates downstream signalling pathways, and elicits cold responses (Blatt, 2000). In the present study, we found that many serine/threonine protein kinases were up-regulated under cold stress, including MARK, ATG1, SIK3 and death-associated protein kinase. Together, these results showed that serine/threonine protein kinases might play important roles in the response to cold stress by regulating downstream signalling pathways. Further studies are needed to explore the regulatory mechanisms of each pathway.

Environmental temperatures are known to affect the physiology and biochemistry of ectothermic organisms, including the lipid composition of membrane systems (Hazel, 1973). Fish species can activate many genes involved in membrane metabolism to maintain membrane fluidity and function at low temperature (Ju et al., 2002), which is an important factor for increasing cold tolerance. Previous studies found that ectothermic organisms use different mechanisms to alter the cell membrane response to cold stress. In cold-tolerant transgenic zebrafish, sphingolipid metabolism and steroid biosynthesis were un-regulated at low temperature, which are major pathways for cell membrane construction, and play important roles in maintaining membrane fluidity and function (Wang et al., 2014). In flounder, genes involved in steroid biosynthesis and cholesterol transport are also up-regulated to stabilise lipid fluidity and plasma lipoproteins via the enrichment of cholesterol in cell membranes (Hu et al., 2014). In carp, the expression of acyl-CoA 9-desaturase is up-regulated in all tissues after cold stress, which increases the unsaturation of the cell membrane to maintain fluidity under cold temperature conditions (Gracey et al., 2004). Several studies in crustaceans also indicate that cold shock results in rapid changes in cell membrane fatty acids (Cuculescu et al., 1995; Ronges et al., 20122012). In the present study, genes associated with high unsaturated fatty acid biosynthesis such as *degs* were up-regulated in the cold temperature group, indicating that membrane metabolism in *C. quadricarinatus* is also stimulated at low temperature, and presumably enhances tolerance to cold conditions.

5. Conclusion

Herein, we measured the activity of hepatopancreas immune enzyme, observed the hepatopancreas ultrastructure, and evaluated the apoptotic rate of red claw crayfish *C. quadricarinatus* in response to cold stress. The results showed that the structure and function of the hepatopancreas were significantly affected under low temperature stress. At the molecular level, transcriptomic analysis demonstrated that the metabolism of substances in the hepatopancreas was slowed down at lower temperatures, which in turn decreases the energy supply, thereby inducing autophagy in the hepatopancreas. Additionally, activity of antioxidant-related enzyme was reduced, which in turn prevents the removal of excess ROS, resulting in oxidative damage in the hepatopancreas, consistent with an increase in apoptosis in this tissue following low temperature stress. Expression of numerous immune-related genes in the hepatopancreas was significantly down-regulated, which could explain the decreased immune enzyme activity in this tissue. In addition, low temperature stress stimulated serine/threonine protein kinase activity, which in turn alters downstream signalling pathways, and supports low temperature stress responses. The potential dysfunction of the immune response and energy metabolism under cold stress might contribute to the mass mortality of *C. quadricarinatus* during cold winter months.

Acknowledgements

This research was funded by the National Natural Science Foundation of China (Grant Number: 31572221), the Science Foundation for Young Scientists of Jiangsu in China (Grant Number: 20171093) and the Shanghai Science and Technology Commission Scientific Research Project (Grant Number:18DZ1204802). We would like to thank the native English speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.102404.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

References

Ali, M.Y., Pavasovic, A., Mather, P.B., Prentis, P.J., 2015. Transcriptome analysis and characterisation of gill-expressed carbonic anhydrase and other key osmoregulatory genes in freshwater crayfish Cherax quadricarinatus. Data in brief, vol. 5. pp. 187–193.

Ashida, M., 1971. Purification and characterization of pre-phenoloxidase from hemolymph of the silkworm Bombyx mori. Arch. Biochem. Biophys. 144, 749–762.

Blatt, M.R., 2000. Ca(2+) signalling and control of guard-cell volume in stomatal movements. Curr. Opin. Plant Biol. 3, 196–204.

Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M.-C., Estreicher, A., Gasteiger, E., Martin, M.J., Michoud, K., O'Donovan, C., Phan, I., 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res. 31, 365–370.

Bösner, S., Haasenritter, J., Hani, M.A., Keller, H., Sönnichsen, A.C., Karatolios, K., Schaefer, J.R., Baum, E., Donnerbanzhoff, N., 2009. Gender differences in presentation and diagnosis of chest pain in primary care. BMC Fam. Pract. 10, 1–9.

Burel, C., Gaumet, F., Roux, A.L., Boeuf, G., 1996. Effects of temperature on growth and metabolism in juvenile turbot. J. Fish Biol. 49, 678–692.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., Madden, T.L., 2009. BLAST+: architecture and applications. BMC bioinformatics 10, 421.

Chisholm, J., Smith, V., 1994. Variation of antibacterial activity in the haemocytes of the shore crab, carcinus maenas, with temperature. J. Mar. Biol. Assoc. U. K. 74, 82–979.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676.

Consortium, G.O., 2004. The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res. 32, D258–D261.

Cuculescu, M., Hyde, D., Bowler, K., 1995. Temperature acclimation of marine crabs: changes in plasma membrane fluidity and lipid composition. Journal of Thermal Biology 20, 207–222.

Cui, J., Bai, X.Y., Sun, X., Cai, G., Hong, Q., Ding, R., Chen, X., 2015. Rapamycin protects against gentamicin-induced acute kidney injury via autophagy in mini-pig models. Sci. Rep. 5, 11256.

Diaz, F., Re, A.D., Sierra, E., Amador, G., 2004. Behavioural thermoregulation and critical limits applied to the culture of red claw crayfish Cherax quadricarinatus (Von Martens). Freshwater Crayfish 14, 90–98.

Fadhlaoui, M., Couture, P., 2016. Combined effects of temperature and metal exposure on the fatty acid composition of cell membranes, antioxidant enzyme activities and lipid peroxidation in yellow perch (Perca flavescens). Aquat. Toxicol. 180, 45–55.

Fan, L., Wang, A., Wu, Y., 2013. Comparative proteomic identification of the hemocyte response to cold stress in white shrimp, Litopenaeus vannamei. J. Prot. 80, 196–206.

Fan, L., Wang, A., Miao, Y., Liao, S., Ye, C., Lin, Q., 2016. Comparative proteomic identification of the hepatopancreas response to cold stress in white shrimp, Litopenaeus vannamei. Aquaculture 454, 27–34.

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Heger, A., Hetherington, K., Holm, L., Mistry, J., 2013. Pfam: the protein families database. Nucleic Acids Res. 42, D222–D230.

GarcíaGuerrero, M., HernándezSandoval, P., OrduñaRojas, J., CortésJacinto, E., 2013. Effect of temperature on weight increase, survival, and thermal preference of juvenile redclaw crayfish Cherax quadricarinatus. Hidrobiológica: [revista del Departamento de Hidrobiología] 23 (1), 73–81.

Gomez-Jimenez, S., Uglow, R.F., Gollas-Galvan, T., 2000. The effects of cooling and emersion on total haemocyte count and phenoloxidase activity of the spiny lobster Panulirus interruptus. Fish Shellfish Immunol. 10, 631–635.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Xian, A., Fan, L., Raychowdhury, R., Zeng, Q., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644.

Gracey, A.Y., Fraser, E.J., Li, W., Fang, Y., Taylor, R.R., Rogers, J., Brass, A., Cossins, A.R., 2004. Coping with cold: an integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. Proc. Natl. Acad. Sci. U.S.A. 101, 16970–16975.

Guschina, I.A., Harwood, J.L., 2006. Mechanisms of temperature adaptation in poikilotherms. Febs Lett. 580, 5477–5483.

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nat. Protoc. 8, 1494.

Hayward, S.A., Manso, B., Cossins, A.R., 2014. Molecular basis of chill resistance adaptations in poikilothermic animals. J. Exp. Biol. 217, 6.

Hazel, J.R., 1973. The Regulation of Cellular Function by Temperature-Induced Alterations in Membrane Composition. pp. 55–67.

Hennig, O.L., Andreatta, E.R., 1998. Effect of temperature in an intensive nursery system for Penaeus paulensis (Pérez Farfante, 1967). Aquaculture 164, 167–172.

Hu, J., You, F., Wang, Q., Weng, S., Liu, H., Wang, L., Zhang, P.J., Tan, X., 2014. Transcriptional responses of olive flounder (Paralichthys olivaceus) to low temperature. PLoS One 9, e108582.

Huang, W., Ren, C., Li, H., Huo, D., Wang, Y., Jiang, X., Tian, Y., Luo, P., Chen, T., Hu, C., 2017. Transcriptomic analyses on muscle tissues of Litopenaeus vannamei provide the first profile insight into the response to low temperature stress. PLoS One 12, e0178604.

Ju, Z., Dunham, R.A., Liu, Z., 2002. Differential gene expression in the brain of channel catfish (Ictalurus punctatus) in response to cold acclimation. Mol. Genet. Genom. 268, 87–95.

Kanehisa, M., Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28, 27–30.

Karplus, I., Zoran, M., Milstein, A., Harpaz, S., Eran, Y., Dan, J., Sagi, A., 1998. Culture of the Australian red-claw crayfish (Cherax quadricarinatus) in Israel : III. Survival in earthen ponds under ambient winter temperatures. Aquaculture 166, 259–267.

Klopfenstein, D., Zhang, L., Pedersen, B.S., Ramírez, F., Vesztrocy, A.W., Naldi, A., Mungall, C.J., Yunes, J.M., Botvinnik, O., Weigel, M., 2018. GOATOOLS: a Python library for Gene Ontology analyses. Sci. Rep. 8, 10872.

Kong, X., Wang, G., Li, S., 2012. Effects of low temperature acclimation on antioxidant defenses and ATPase activities in the muscle of mud crab (Scylla paramamosain). Aquaculture s 370–371, 144–149.

Lesniak, T.C., Schirmer, A.E., Knapp, C.R., 2015. Evaluating the role of temperature in the reproduction of the yellow stingray (Urobatis jamaicensis). Zoo Biol. 34, 33–39.

Li, J., Henry, E., Wang, L., Delelis, O., Wang, H., Simon, F., Françoise, P., Brochon, J.-C., Zhao, Y., Deprez, E., 2012. Comparative study of the fatty acid binding process of a new FABP from cherax quadricarinatus by fluorescence intensity, lifetime and anisotropy. PloS One 7, e51079.

Li, S., Li, C., Dey, M., Gagalac, F., Dunham, R., 2002. Cold tolerance of three strains of Nile tilapia, Oreochromis niloticus, in China. Aquaculture 213, 123–129.

Li, B., Xian, J.A., Guo, H., Wang, A.L., Miao, Y.T., Ye, J.M., Ye, C.X., Liao, S.A., 2014. Effect of temperature decrease on hemocyte apoptosis of the white shrimp Litopenaeus vannamei. Aquacult. Int. 22, 761–774.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Long, Y., Song, G., Yan, J., He, X., Li, Q., Cui, Z., 2013. Transcriptomic characterization of cold acclimation in larval zebrafish. BMC Genomics 14, 612.

López-López, S., Nolasco, H., Vega-Villasante, F., 2003. López-López, Silverio Nolasco, Héctor Vega-Villasante, Fernando. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 135, 337–347.

Lu, Y., Nie, M., Wang, L., Xiong, Y., Wang, F., Wang, L., Xiao, P., Wu, Z., Liu, Y., You, F., 2017. Energy response and modulation of AMPK pathway of the olive flounder Paralichthys olivaceus in low-temperature challenged. Aquaculture 484, 205–213.

McGinnis, S., Madden, T.L., 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 32, W20–W25.

Meade, M.E., Doeller, J.E., Kraus, D., Watts, S.A., 2002. Effects of temperature and salinity on weight gain, oxygen consumption rate, and growth efficiency in juvenile red-claw crayfish Cherax quadricarinatus. J. World Aquacult. Soc. 33, 188–198.

Pernet, F., Tamayo, D., Petton, B., 2015. Influence of low temperatures on the survival of the Pacific oyster (Crassostrea gigas) infected with ostreid herpes virus type 1. Aquaculture 445, 57–62.

Qiu, J., Wang, W.N., Wang, L.J., Liu, Y.F., Wang, A.L., 2011. Oxidative stress, DNA damage and osmolality in the Pacific white shrimp, Litopenaeus vannamei exposed to acute low temperature stress. Comparative biochemistry and physiology. Toxicol. Pharmacol. : CBP 154, 36–41.

Rinehart, J.P., Yocum, G.D., Denlinger, D.L., 2000. Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, Ssarcophaga crassipalpis. Insect Biochem. Mol. Biol. 30, 515–521.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140.

Ronges, D., Walsh, J.P., Sinclair, B.J., Stillman, J.H., 2012. Changes in extreme cold tolerance, membrane composition and cardiac transcriptome during the first day of thermal acclimation in the porcelain crab Petrolisthes cinctipes. Journal of Experimental Biology 215, 1834–1836.

Rőszer, T., 2014. The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism. Cell Tissue Res. 358, 685–695.

Sacristán, H.J., Ansaldo, M., Franco-Tadic, L.M., Gimenez, A.V.F., Greco, L.S.L., 2016. Long-Term starvation and posterior feeding effects on biochemical and physiological responses of midgut gland of Cherax quadricarinatus juveniles (Parastacidae). Plos One 11, e0150854.

Saoud, I.P., Ghanawi, J., Thompson, K.R., Webster, C.D., 2013. A review of the culture and diseases of redclaw crayfish Cherax quadricarinatus (von Martens 1868). J. World Aquac. Soc. 44, 1–29.

Souza, D.M.D., Martins, Á.C., Jensen, L., W.W. Jr., , Monserrat, J.M., Garcia, L.D.O., 2014. Effect of temperature on antioxidant enzymatic activity in the Pacific white shrimp Litopenaeus vannamei in a BFT (Biofloc technology) system. Mar. Freshw. Behav. Physiol. 47, 1–10.

Souza, D.M.D., Borges, V.D., Furtado, P., Romano, L.A., Jr, W.W., Monserrat, J.M., Garcia, L.D.O., 2016. Antioxidant enzyme activities and immunological system analysis of Litopenaeus vannamei reared in biofloc technology (BFT) at different water temperatures. Aquaculture 451, 436–443.

Szklarczyk, D., Morris, J.H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork, P., 2016. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Res. 45, D362–D368.

Vinagre, A.S., Ap, N.D.A., Ribarcki, F.P., Fraga, d.S.E., Périco, E., 2007. Seasonal variation of energy metabolism in ghost crab Ocypode quadrata at Siriú Beach (Brazil). Comp. Biochem. Physiol., A 146, 514–519.

Wang, Lan-mei, Zuo, Di, Wei-wei, Liu, A-jing, Zhao, Yunlong, 2013. Characterization of Cdc2 kinase in the red claw crayfish (Cherax;quadricarinatus): evidence for its role in regulating oogenesis. Gene 515, 258–265.

Wang, Q., Liu, Y., Zheng, Z., Deng, Y., Jiao, Y., Du, X., 2018. Adaptive response of pearl oyster Pinctada fucata martensii to low water temperature stress. Fish shellfish immunology 78, 310–315.

Wang, Q., Tan, X., Jiao, S., You, F., Zhang, P.J., 2014. Analyzing cold tolerance mechanism in transgenic zebrafish (Danio rerio). PLoS One 9, e102492.

Wentworth, S.A., Thede, K., Aravindabose, V., Monroe, I., Thompson, A.W., Molyneaux, N., Owen, C.L., Burns, J.R., Gonzalez-Vicente, A., Garvin, J.L., Packer, R.K., 2018.

Transcriptomic analysis of changes in gene expression of immune proteins of gill tissue in response to low environmental temperature in fathead minnows (Pimephales promelas). Comparative biochemistry and physiology. Part D Genom. Prot. 25, 109–117.

Wu, D.L., Liu, Z.Q., Huang, Y.H., Lv, W.W., Chen, M.H., Li, Y.M., Zhao, Y.L., 2018. Effects of cold acclimation on the survival, feeding rate, and non-specific immune responses of the freshwater red claw crayfish (Cherax quadricarinatus). Aquacult. Int. 26, 557–567.

Xian, J.A., Wang, A.L., Ye, C.X., Chen, X.D., Wang, W.N., 2010. Phagocytic activity, respiratory burst, cytoplasmic free-Ca(2+) concentration and apoptotic cell ratio of haemocytes from the black tiger shrimp, Penaeus monodon under acute copper stress. Comp. Biochem. Physiol., C 152, 182–188.

Xian, J.A., Wang, A.L., Chen, X.D., Gou, N.N., Miao, Y.T., Liao, S.A., Ye, C.X., 2011. Cytotoxicity of nitrite on haemocytes of the tiger shrimp, Penaeus monodon, using flow cytometric analysis. Aquaculture 317, 240–244.

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J., Dong, S., Kong, L., Gao, G., Li, C.-Y., Wei, L., 2011. Kobas 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res. 39, W316–W322.

Yang, C., Jiang, M., Wen, H., Tian, J., Liu, W., Wu, F., Guo, G., 2015. Analysis of differential gene expression under low-temperature stress in Nile tilapia (Oreochromis niloticus) using digital gene expression. Gene 564, 134–140.

Yong, L., Song, G., Yan, J., He, X., Li, Q., Cui, Z., 2013. Transcriptomic characterization of cold acclimation in larval zebrafish. BMC Genomics 14, 612.

Yu, J., Ji, X., Wang, X., Li, T., Wang, H., Zeng, Q., 2019. Identification and characterization of differentially expressed genes in hepatopancreas of oriental river prawn Macrobrachium nipponense under nitrite stress. Fish Shellfish Immunol. 87, 144–154.

Yuan, Q., Wang, Q., Zhang, T., Li, Z., Liu, J., 2017. Effects of water temperature on growth, feeding and molting of juvenile Chinese mitten crab Eriocheir sinensis. Aquaculture 468, 169–174.

Yudkovski, Y., Shechter, A., Chalifa-Caspi, V., Auslander, M., Ophir, R., Dauphin-Villemant, C., Waterman, M., Sagi, A., Tom, M., 2007. Hepatopancreatic multi-transcript expression patterns in the crayfish Cherax quadricarinatus during the moult cycle. Insect molecular biology 16, 661–674.

Zhang, D., Liu, J., Qi, T., Ge, B., Liu, Q., Jiang, S., Zhang, H., Wang, Z., Ding, G., Tang, B., 2018. Comparative transcriptome analysis of Eriocheir japonica sinensis response to environmental salinity. PLoS One 13, e0203280.

Zhao, Q., Pan, L., Ren, Q., Wang, L., 2015. Identification of genes differentially expressed in swimming crab Portunus trituberculatus response to low temperature. Aquaculture 442, 21–28.

Zhao, M.-r., Meng, C., Xie, X.-l., Li, C.-h., Liu, H.-p., 2016. Characterization of microRNAs by deep sequencing in red claw crayfish Cherax quadricarinatus haematopoietic tissue cells after white spot syndrome virus infection. Fish Shellfish Immunol. 59, 469–483.