Genomics 111 (2019) 242–250

Contents lists available at ScienceDirect

Genomics

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

Original Article

Transcriptome profiling and histology changes in juvenile blunt snout bream (*Megalobrama amblycephala*) liver tissue in response to acute thermal stress

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ARTICLE INFO

Keywords:
Transcriptome
Thermal stress
Megalobrama amblycephala
RNA-seq
Liver

ABSTRACT

To understand the precise mechanism and the pathways activated by thermal stress in fish, we sampled livers from juvenile *Megalobrama amblycephala* exposed to control (25 °C) and test (35 °C) conditions, and performed short read (100 bp) next-generation RNA sequencing (RNA-seq). Using reads from different temperature, expression analysis identified a total of 440 differentially-expressed genes. These genes were related to oxidative stress, apoptosis, immune responses and so on. We used quantitative real-time reverse transcriptase PCR to assess the differential mRNA expression of selected genes that encode antioxidant enzymes and heat shock proteins in response to thermal stress. Fish exposed to thermal stress also showed liver damage associated with serum biochemical parameter changes. The set of genes identified showed regulatory modulation at different temperatures, and therefore could be further studied to determine how thermal stress damages *M. amblycephala* livers and the possible roles of reactive oxygen species in this process.

1. Introduction

Environmental stress disrupts homeostasis and can affect biological function [1]. One such environmental stress is temperature, which has profound effects on physical and chemical processes within biological systems [2]. Variations in environmental temperature affect many properties and functions of biomolecules and structural components of cells: including the assembly, folding, activity and stability of proteins [3]; structure and rigidity of lipids [4,5] and fluidity and permeability of cell membranes [6]. Fish cannot avoid these temperature fluctuations and therefore are likely to be exposed to stressful conditions. Any change in culture water temperature may influence the survival, physiological functions and immune defenses of fish, such as *Cyprinus carpio* [7], *Oncorhynchus mykiss* [8], *Oryzias latipes* [9].

Different techniques, including microarray and RNA sequencing, have been used to analyze changes in the genes expression in response to varying environmental conditions, including temperature. To date, these changes have been analyzed in various fish, including the common carp *Cyprinus carpio* [10], zebrafish *Danio rerio* [11,12], channel catfish *Ictalurus punctatus* [13], rainbow trout *Oncorhynchus mykiss* [14], large yellow croaker *Larimichthy crocea* [15], and olive flounder *Paralichthys olivaceus* [16]. However, no such studies have been carried out in juvenile blunt snout bream (*Megalobrama amblycephala*), which is farmed extensively in Chinese freshwater polyculture systems. Aquaculture of this species has increased rapidly during the last decade because of its excellent flesh quality, rapid growth performance and high larval survival rate [17–19]. Although a previous study reported that thermal stress could increase oxidative stress in *M. amblycephala* [20], there is little information on how its gene regulation mechanism responds to a high temperature challenge.

The optimal temperature for growth of *M. amblycephala* is 24–28 °C, but they commonly experience water temperatures of higher than 30 °C in summer. Such a high temperature may increase susceptibility to opportunistic bacterial pathogens and result in significant economic losses [21]. Since *M. amblycephala* is sensitive to thermal stress (Liu et al., 2016), the aim of the study was to identify heat-related pathways that would help our understanding of the mechanism of acute thermal stress. We measured several parameters including: (1) serum biochemical parameter changes, (2) gene expression profiles in the liver; (3) differential expression of mRNA transcripts by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR); and (4) histological changes in the liver. This is the first study to investigate how thermal stress induces transcriptome-wide gene expression changes in *M. amblycephala* and link these changes to biological processes.

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https://doi.org/10.1016/j.ygeno.2018.11.011
Received 1 February 2018; Received in revised form 21 September 2018; Accepted 11 November 2018
Available online 17 November 2018

2. Materials and methods

2.1. Ethics statement

All animals and experiments were conducted in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). All efforts were made to minimize suffering. All experimental procedures involving fish were approved by the institution animal care and use committee of the Chinese Academy of Fishery Sciences.

2.2. Experimental animals and thermal stress

We obtained 300 healthy *M. amblycephala* juveniles of consistent size (mean weight: 20.25 ± 2.24 g) from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, China. The fish were immediately transferred to the aquatic laboratory and held in three 500-L fiberglass tanks ($n = 100$ fish/tank). During acclimation, each tank was filled with pre-aerated municipal water at 25 ± 0.5 °C (pH 7.45 ± 0.08; dissolved oxygen 5.16–6.53 mg L^{-1}; total ammonia < 0.5 mg L^{-1}; baseline nitrite < 0.1 mg L^{-1}). Fish were kept in natural light and a photoperiod of a 14 h light: 10 h dark cycle. Fish were fed a commercial pelleted diet twice daily with 3% of total body weight. The water exchange rate was 33% per day and fecal matter was removed daily from the aerated tanks. After fasting for 24 h, fish were transferred from the holding tank to six plastic tanks, at a density of 50 fish/tank. Three tanks held fish that were subjected to thermal stress, and the other three held the control group, allowing the experiment to be repeated in triplicate. In the heat-treatment group, the water temperature was maintained at 35 °C for 72 h and the control maintained for the same time at 25 °C, and the blood samples of fish were sampled from two treatment groups at 0, 24, 48, and 72 h. At the end of the trial, all fish were quickly removed and anesthetized in diluted MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 100 mg L^{-1}. Blood samples were collected using heparinized syringes from the caudal peduncle. Serum was obtained by centrifugation at 4 °C and 1500 × *g* for 10 min and samples were stored at −80 °C until biochemical analysis. The livers from 3 blood-withdrawn fish per tank were dissected and fixed in 10% neutral-buffered formalin for the histological evaluation. Another three fish from each exposure group were sacrificed with anesthesia overdose to obtain liver samples that were stored at −80 °C until RNA extraction for RNA-seq analysis. MIxS descriptors are presented in Additional file 1.

2.3. Biochemical parameter analysis

Serum cortisol concentrations were determined using automatic biochemical analyzer by standard procedures based on manual Diagnostic Reagent kit (Diagnostic Biochem, Canada). The enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactic acid dehydrogenase (LDH) were measured following the modified methods (as per kit manufacturer's protocol) by Banaee et al. [22].

2.4. Library preparation and sequencing

Liver samples from three individuals were used for library preparation in control and treatment, respectively. In order to gain three biological replicates, only one liver sample from one fish to generate one library.

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analyzed by Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number > 7.0. Approximately 10 μg of total RNA representing a specific adipose type was subjected to isolate Poly (A) mRNA with poly-T oligo-attached magnetic beads (Invitrogen). Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. Then the cleaved RNA fragments were reverse-transcribed to create the final cDNA library in accordance with the protocol of the mRNASeq sample preparation kit (Illumina, San Diego, USA) to generate cDNA libraries. The libraries were size selected for cDNA target fragments of 200–300 bp on 2% low-range ultra-agarose. And then we performed the paired-end sequencing on an IlluminaHiseq2500 (LC Sceiences,USA) following the vendor's recommended protocol (2 × 100 bp read length).

2.5. Functional annotation of assembled contigs

The abundance of each tag was normalized to one transcript per million to allow comparison between samples. The raw data was trimmed and filtered to remove low quality sequences, including ambiguous nucleotides and adaptor sequences. The remaining clean reads were assembled using Trinity software [25] as described for *de novo* transcriptome assembly without a reference genome. The assembled transcriptome contigs were subjected to similarity searches against the NCBI non-redundant protein database using BLASTx with an e-value cutoff of.

1e-10. A gene name and description was assigned to each contig based on the BLASTx hit with the highest score. Gene ontology (GO) analysis was conducted on the assembled transcriptome using InterProScan (www.ebi.ac.uk/Tools/pfa/iprscan) and integrated protein databases with default parameters. The GO terms associated with transcriptome contigs were then obtained to describe their biological processes, molecular functions and cellular components. For pathway enrichment analysis, all differentially expressed genes (DEGs) were mapped to terms in the kyoto encyclopedia of genes and genomes (KEGG) database. Functional enrichment analyses, including GO and KEGG, were performed using an ultrageometric test to identify which DEGs were significantly enriched in terms of either GO (a *P*-value of ≤0.05) or metabolic pathways (a q-value of ≤0.05), compared with the whole transcriptome background [26,27].

2.6. Analysis of differentially expressed genes

All the cleaned reads were mapped to the assembled reference transcriptome using Bowtie [23]. RSEM was then used to quantify gene and isoform abundances according to the Trinity-assembled transcriptome. Finally, we used edgeR to normalize expression levels in each of these samples and to obtain differentially-expressed transcripts by pairwise comparisons [24]. EdgeR uses a negative binomial distribution method for differential expression analysis.

2.7. Analysis of gene expression by qRT-PCR

The expression of a selection of genes identified as being differentially expressed was analyzed by qRT-PCR. The sequences of the primer pairs (designed using Primer Express 3.0) can be found in Table 1. First, the RNA sample was reverse-transcribed using a PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China). Next, qRT-PCR was performed using a Bio-Rad iCycler iQ5 Real Time System (Biorad Inc.) using *β*-actin as the reference gene. The PCR temperature profile and reaction conditions referred to the instruction of the SYBR Premix Ex Taq (TaKaRa, Dalian, China). For the negative control, DEPC-water was used in place of the template. Each sample was run in triplicate along with an internal control gene. To ensure that only one PCR product was amplified and detected, dissociation curve analysis of the amplification products was performed at the end of each reaction. The amplification efficiency and threshold were automatically generated by standard curves [28]. The relative copy number of the specific gene's mRNA was calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method [29]. Statistical analysis was conducted using SPSS 14.0 (SPSS Inc.) and significant differences were tested using one-way ANOVA and *t*-test. A p value of < 0.05 was

Table 1
Genes and specific primers used in this study.

Gene name	Primer name	Primer sequence (5′–3′)
β-actin	β-actin-F	TCGTCCACCGCAAATGCTTCTA
	β-actin-R	CCGTCACCTTCACCGTTCCAGT
Caspase-8	Caspase-8-F	AGAGGCTTGGGGAAGACAACC
	Caspase-8-R	CAAGCGAGGCAACAGAAGAGC
Caspase-9	Caspase-9-F	ACGGATTCTTCAGCGGCACAG
	Caspase-9-R	ATGATGCCCAGTCTCACGCAG
Caspase-3	Caspase-3-F	ATGAACCAACGCAATGGCAC
	Caspase-3-R	TTCCCACTAGTGATGGGCAG
Heat shock protein 70	Heat shock protein 70-F	CTTTATCAGGGAGGGATGCCAGC
	Heat shock protein 70-R	CCCTGCAGCATTGAGTTCATAAG
Heat shock protein 90β	Heat shock protein 90β-F	TGGTGTGGGATTCTACTC
	Heat shock protein 90β-R	TGACTGTGAAAGAGCCGC
Glutathione S-transferase	Glutathione S-transferase-F	TTGCCCTACCTAGTGGATGGT
	Glutathione S-transferase-R	GTCCATCGCCTGATTCTCCAA
Glutathione reductase	Glutathione peroxidase-F	CTTTTGTCCTTGAAGTATGTCC
	Glutathione reductase-R	CTTGAGGAAGACGAAGAGAGGG
Alpha-2-macroglobulin	alpha-2-macroglobulin-F	GTCTTTGGTGTTGTACCAGCC
	alpha-2-macroglobulin-R	GGCTTCCTGAAGAGCGGAT

considered to be significant.

2.8. Light and transmission electron microscopy

To prepare the livers for light microscopy, they were removed and preserved in 10% neutral-buffered formalin for 48 h, then rinsed in 70% ethanol and stored until further processing. Once required, the livers were dehydrated in isopropanol, cleared in xylene, embedded in paraffin and then sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin and examined with a light microscope.

In preparation for transmission electron microscopy (TEM), small pieces of liver were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). Sections were embedded in epoxy resin (Epon 812) and cut in an RMC PowerTome XL microtome (Boeckeler Instruments, Tucson, AZ, USA) to a thickness of 60 nm. Finally, sections were stained with uranyl acetate and lead citrate. The ultrastructure was examined with a Hitachi H-7650 TEM in the School of Life Sciences at the East China Normal University.

3. Results

3.1. Biochemical parameters

Serum cortisol levels in fish exposed to 35 °C were significantly higher ($P < .05$) than that of control group from 24 h to 72 h (Fig. 1 A). In terms of the influence of the thermal stress, relatively similar trends of changes were observed in AST and ALT fluctuations as for the cortisol levels (Fig. 1 B & C). In this regard, highest AST and ALT activities were observed in the fish exposed to 35 °C high temperature for 48–72 h. With the exception of 0–24 h, LDH levels were significantly ($P < .05$) increased in fish exposed to high temperature at other sampling time points in comparison with the control groups (Fig. 1 D).

3.2. RNA-seq of the liver transcriptome

In total, six cDNA library preparations were sequenced from *M. amblycephala* livers from both groups. Raw sequencing data is available through the NCBI (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86405). A total of 398,100,330 paired-end reads were generated from six samples with a read length of 101 bp. After removal of ambiguous nucleotides, low-quality sequences (those with Phred quality scores of < 20), contaminated microbial sequences and ribosomal RNA sequences, a total of 265,294,865 cleaned reads were harvested for further analysis. The cleaned sequences in each sample ranged from 64.4 to 66.8 million reads, confirming the stability and consistency of sampling, library preparation and sequencing methodologies. Using the Trinity assembly program, we generated a total of 35,281 unigenes. The average length was 904 bp and the N50 length was 1474 bp (Table 2). The length distribution of unigenes is shown in Fig. 2.

3.3. Differentially expressed genes

To avoid noise signals from high-throughput sequencing, genes with average transcripts per million values of < 1 were excluded. In the present study, an absolute fold-change of ≥ 2 and a false discovery rate (FDR) of $q < 0.05$ were used to define each DEG. According to this definition, 440 genes were differentially expressed in the control and treatment groups, and there were 207 and 233 up-regulated genes in the control and treatment groups, respectively (Additional file 2). We generated a list of 20 candidate DEGs for control and treatment, with 10 genes identified from previous transcriptomic studies in fish [30–33]. All of these genes are involved in three target biological processes (protein folding, immune responses and oxidative stress responses). From this list we observed that genes for heat shock proteins hsp60s, hsp70s and hsp90s were up-regulated in the liver tissues of *M. amblycephala* in response to thermal stress (Table 3).

For both groups, the 440 DEG genes were assigned to various KEGG pathways relating to distinct biological processes including: proteolysis, response to stress, apoptosis, transport and carbohydrate metabolic progress (Fig. 3). A high temperature was found to up-regulate a broad range of pathways, including those linked to fish immunity such as the complement and coagulation cascade and endocytosis; it also up-regulated metabolic pathways such as pyruvate metabolism. The top ten pathways based on *p*-value are listed in Table 4.

3.4. Analysis of differentially expressed genes in response to thermal stress

The qRT-PCR results were identical to those obtained by DEG profiling (Fig. 4). Of the eight transcripts selected for qRT-PCR, expression of seven genes was significantly higher in the high temperature group than that of control group. This up-regulation was seen in the genes that encoded caspase-3 (CASP3), caspase-8 (CASP8), caspase-9 (CASP9), glutathione reductase (GR), glutathione S-transferase (GST), heat shock protein 70 (HSP70) and heat shock protein 90β (HSP90β). Expression of alpha-2-macroglobulin (A2M) was significantly lower in the high temperature group than that of control group.

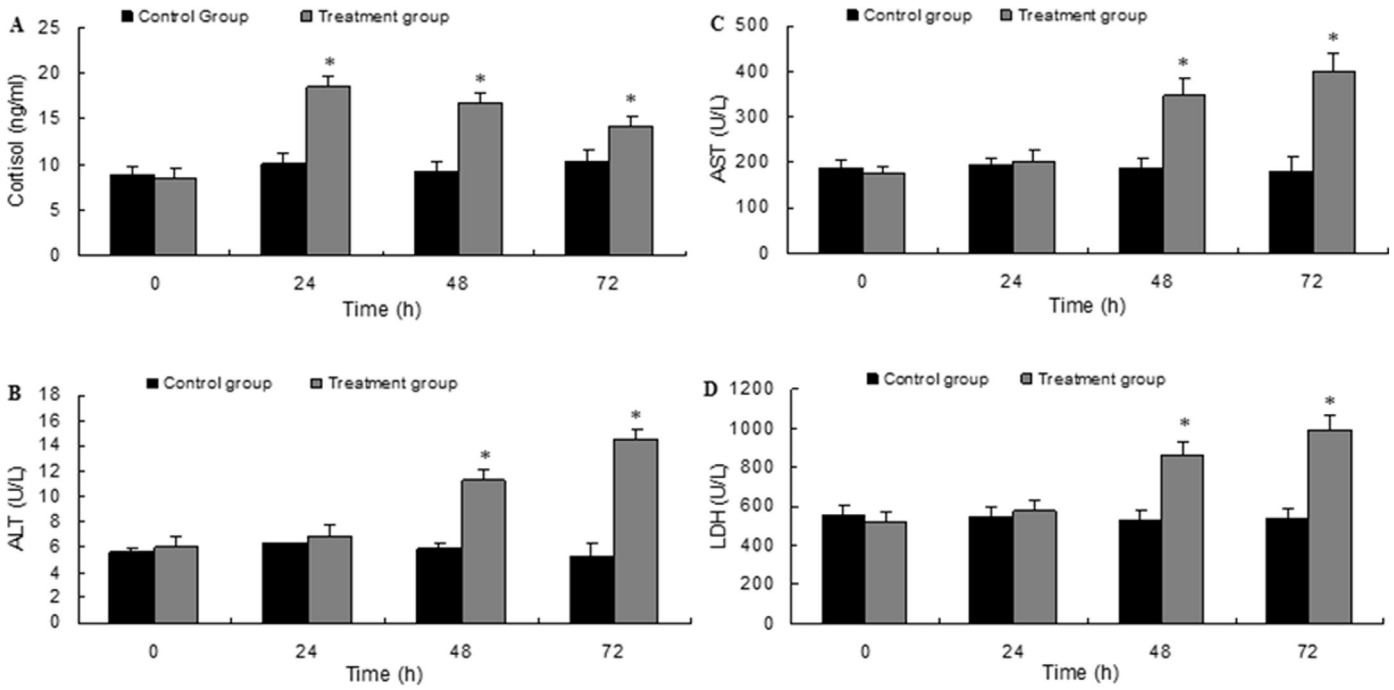

Fig. 1. (A) cortisol, (B) aspartate aminotransferase, (C) alanine aminotransferase, and (D) lactic acid dehydrogenase in the serum of *Megalobrama amblycephala* exposed to thermal stress for different time periods. Significant differences between treatment and control group are indicated with asterisks. Each bar represents the mean ± SD (n = 3).

Table 2
Assembly statistics of reads.

Parameter	Numbers
Number of unigenes	35,281
Total bases of unigenes (bp)	31,924,437
Unigene mean length (bp)	904
Minimum length (bp)	201
Max length (bp)	15,116
N50 length (bp)	1474

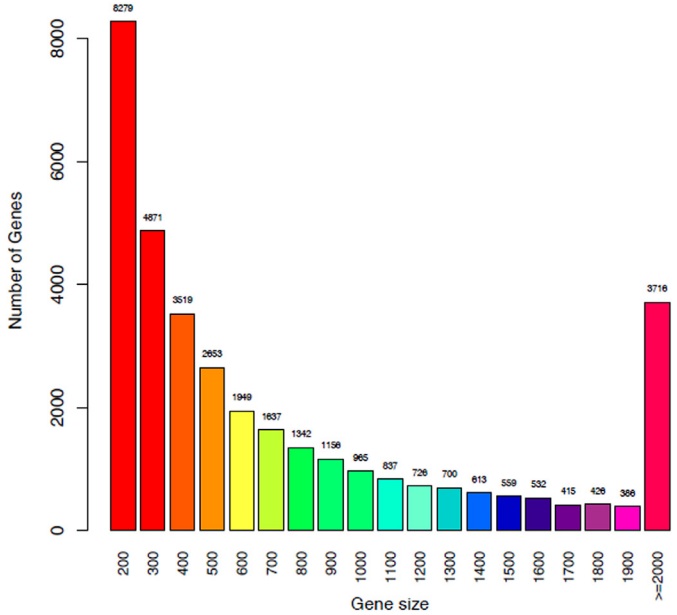

Fig. 2. Length distribution of assembled transcriptome unigenes from *Megalobrama amblycephala*. The x-axis shows contig size and the y-axis shows the number of unigenes.

3.5. Histological changes

No abnormality was seen in livers from the control group (Fig. 5 A). In contrast, the light microscopy images of livers from the high temperature group showed increases in the sinusoidal space and deformation of hepatocytes after 72 h (Fig. 5 B). Under TEM, no abnormality was seen in livers from the control group (Fig. 5 C), hepatocytes from the high temperature group showed signs of vacuolization and the appearance of pyknotic nuclei and lipid droplets (Fig. 5 D).

4. Discussion

Climate change and other anthropogenic impacts are likely to increase pressure on water for ecosystems and human uses globally. Freshwater ecosystems have of all ecosystems the highest proportion of species threatened by increasing in water temperatures and changing in water quality due to climate change and increased human impacts [34,35]. Freshwater fish are commonly exposed to seasonal and daily fluctuations in water temperatures in their aquatic environment. The primary stress responses in fish are characterized by elevated levels of stress hormones, catecholamine and cortisol in the blood. Cortisol is the most commonly used indicator of stress in fish [36]. The present results of this study revealed rise in the cortisol levels when the fish were exposed to thermal stress. ALT and AST are both very important transaminases that are transformed between catalytic amino acid and ketonic acid. In our study, when the activity of ALT and AST both increase under thermal stress for 48–72 h, the damage by high temperature to the fish's liver cells may be cause by the emission of ALT and AST, which is similar to the previous studies on catfish *Horabagrus brachysoma* [37] and rainbow trout *Oncorhynchus mykiss* [38]. In the present study, when the temperature reaches its high for 48–72 h, the activity of LDH in their body increases, a reasonable explaination is that the glycometabolism of fish body changed from oxygen to anaerobic glycolysis area to improve the activity of LDH, thus we choose thermal stress 72 h to identifying gene expression profiles and histological changes of liver tissue by next-generation sequencing technologies

Table 3
List of the 20 differentially-expressed genes (DEG)[a] involved in major processes associated with response to high temperatures, based on gene ontology (GO) categorization.

GO term	Gene identifier	Gene description	Log_2FC (control/treated)	q-Value
Reactive oxygen species	comp15346_c0	Superoxide dismutase	−6.62	0.00
	comp3468_c0	Glutathione peroxidase	−2.56	0.00
	comp5818_c0	Glutathione reductase	−6.95	0.00
	comp11633_c0	Glutathione S-transferase	−4.88	0.00
	comp451323_c0	L-ascorbate peroxidase	−1.75	0.00
Response to stress	comp9959_c0	Heat shock protein 70	−7.12	0.00
	comp7496_c0	Heat shock protein 90α	−4.92	0.00
	comp19087_c0	Heat shock protein 90β	−4.91	0.05
	comp24726_c0	Heat shock protein 27	−6.57	0.00
Immune response	comp16845_c0	Complement component 3	10.61	0.00
	comp17603_c0	Complement component 4	5.89	0.00
	comp13453_c0	Alpha-2-macroglobulin	5.39	0.00
	comp243836_c0	Immunoglobulin heavy chain	3.46	0.00
Proteolysis	comp278700_c0	ATP-dependent metalloprotease	−5.48	0.00
	comp116781_c0	Ubiquitin-conjugating enzyme E2 C	3.05	0.00
	comp27420_c0	26S proteasome regulatory subunit N11	−4.44	0.00
Apoptosis	comp3690_c0	Caspase-8	−1.72	0.00
	comp15561_c0	Caspase-3	−6.6	0.00
	comp12078_c0	Caspase-9	−7.91	0.00
	comp18236_c0	Caspase-1	−2.79	0.04

[a] DEG was filtered by using a threshold of false discovery rate of q ≤ 0.05 and an absolute value of Log_2(fold change) ≥ 1.

[39].

A set of previously-identified DEG genes that code for three biologically significant functions allows them to be used as markers of thermal stress in future studies, such as protein folding, oxidative stress, immune response and energy metabolism, which was similar with previous studies on liver transcriptome responses to thermal stress in rainbow trout *Oncorhynchus mykiss* [40], half-smooth tongue sole *Cynoglossus semilaevis* [41], crimson spotted rainbowfish *Melanotaenia duboulayi* [33], redband trout *Oncorhynchus mykiss gairdneri* [42], snow trout *Schizothorax richardsonii* [43] and large yellow croaker *Larimichthy crocea* [15]. The present results of this study also showed that the up-regulated apoptosis-related gene expression in *M. amblycephala* in responding to thermal stress, suggesting *M. amblycephala* is a heat-sensitive species. It is unsurprising to find that genes relating to apoptosis are among the most highly regulated in temperature stress, when liver tissue damage of *M. amblycephala* under thermal stress. Further experimental studies are required to determine whether these candidate genes play homologous roles among different species.

An organism's response to environmental and physiological stressors follows a highly ordered set of events that often results in rapid changes in gene expression, followed by synthesis of the proteins involved in adaptation [44]. In most cases, the over-production of reactive oxygen species (ROS) is considered an important signal of oxidative damage since these can result in significant damage to the cell structure [45]. To counteract the damaging effects of activated ROS, cells have evolved defense mechanisms that act at different levels to prevent or repair such damage [46]. As an important component of antioxidant defense, antioxidant enzymes (including SOD, GST and GR) have been found to coordinate cellular defenses against stress and inflammation [47]. In the present study, we demonstrated thermal effects on genes that encode

Table 4
The top 10 enriched KEGG pathways under thermal stress.

Number	Pathway	P-value	Pathway ID
1	Oxidative phosphorylation	0.03546	ko00190
2	MAPK signaling pathway	0.04752	ko04010
3	Glycolysis/gluconeogenesis	0.03758	ko00010
4	Purine metabolism	0.01992	ko00230
5	Toll-like receptor signaling pathway	0.02987	ko04620
6	p53 signaling pathway	0.04987	ko04115
7	Apoptosis	0.03650	ko04210
8	Cell adhesion molecules	0.02688	ko04514
9	Fatty acid metabolism	0.04860	ko00071
10	Cell cycle	0.03875	ko04111

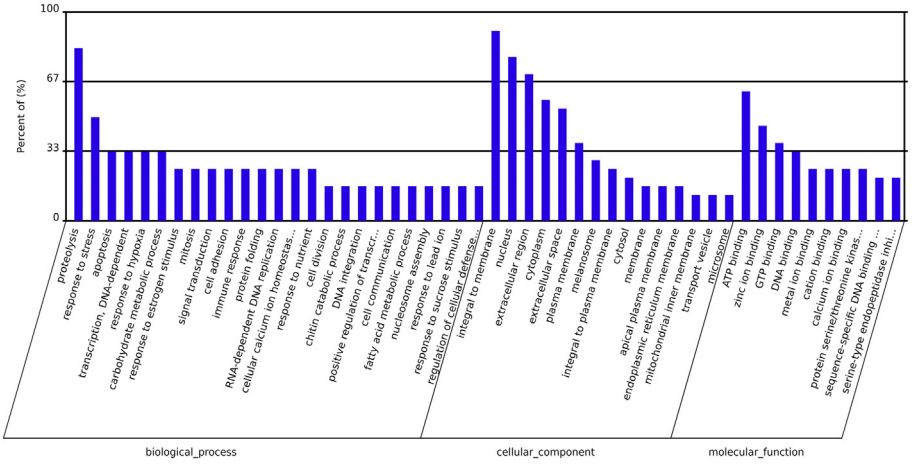

Fig. 3. The distribution of GO terms assigned to the *Megalobrama amblycephala* transcriptome.

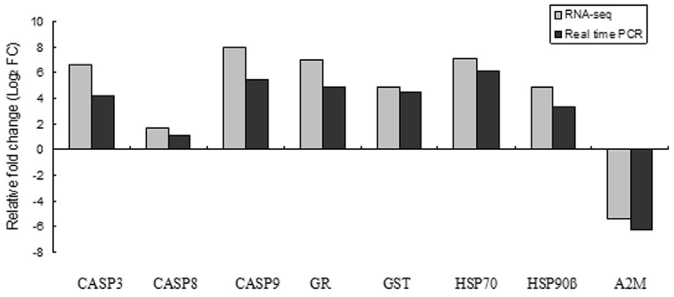

Fig. 4. Validation results of RNA-seq profiles by qPCR. The expression of the following proteins was detected by was detected by RNA-seq (gray column) and real-time qPCR (black column): caspase-3 (CASP3), caspase-8 (CASP8), caspase-9 (CASP9), glutathione reductase (GR), glutathione peroxidase (GST), heat shock protein 70 (HSP70), heat shock protein 90β (HSP90β) and alpha-2-macroglobulin (A2M).

antioxidant enzymes and stress-responsive proteins, suggesting that these genes can serve as biomarkers for thermal stress. Among these genes, those coding for the enzymes GR and GST showed a particularly high sensitivity. As a non-enzymatic antioxidant, glutathione plays a key role in fish immunity by forming glutathione disulfide when it reacts with ROS [48], the main function of GR is to maintain cellular homeostasis, both in terms of the reduced glutathione (GSH): oxidized glutathione (GSSG) ratio and in the redox balance of the cell [49].

ROS production and the accumulation of denatured proteins may subsequently trigger expression of heat shock proteins (HSPs) [50–52]. HSPs are involved in the immune response under stress since they play a critical role in preventing protein aggregation and refolding of stress-denatured proteins [53]. In the present study, the thermal stress up-regulated expression of HSP60, HSP70 and HSP90. Similar results have been found in aquatic animals such as the grass carp and zebrafish, where thermal stress induced expression of HSP70 and HSP90 [12,54]. In addition, HSPs may be involved in preventing apoptosis [55] since increased production of HSPs has been linked to cell resistance to apoptotic cell death [56]. In the current study, thermal stress resulted in increased production of HSPs, suggesting that HSPs may be involved in protecting *M. amblycephala* from oxidative stress.

Although apoptosis can be triggered by different stimuli, apoptotic pathways are mainly classified into the extrinsic death receptor pathway and intrinsic mitochondrial pathway [45]. Both of these pathways are regulated by the caspase family of proteins [57], although certain caspases are associated with a particular pathway. For example, activation of caspase-8 is associated with the extrinsic death receptor pathway whereas activation of caspase-9 triggers the intrinsic mitochondrial pathway. Caspase-3 is considered an effector caspase in both pathways [58]. In this study, the thermal stress led to an increased expression of caspase-3, caspase-8 and caspase-9, suggesting that thermal stress can potentially both activate the intrinsic/extrinsic apoptosis pathways (Fig. 6). However, the activation mechanism of apoptotic pathways requires further study.

Since the liver is the main organ for detoxification, it is highly susceptible to damage and therefore changes in the liver may serve as markers to indicate prior exposure to environmental stressors [59,60]. The histological changes in the liver tissue showed a trend to progressively worsen over the week, indicating that prolonged high temperature could have an increasingly deleterious effect, which also was confirmed by changes in enzymes activities, such as ALT and AST. It is likely that these enzymes can discharge from cells because of injury to the cell membrane and, in some cases, injury to organelles and subsequent discharge from cytosol to the cell membrane [61,62]. The histological observation of liver tissues showed several changes, including vacuolization and the appearance of pyknotic nuclei and lipid droplets in hepatocytes. These observations are consistent with previous studies on juvenile *M. amblycephala* [20,63,64]. In addition, microscopy images showed changes to mitochondria including irregular clustering, loss of cristae and matrix and structural damage to the outer and the inner membrane. This observation suggested that exposure to

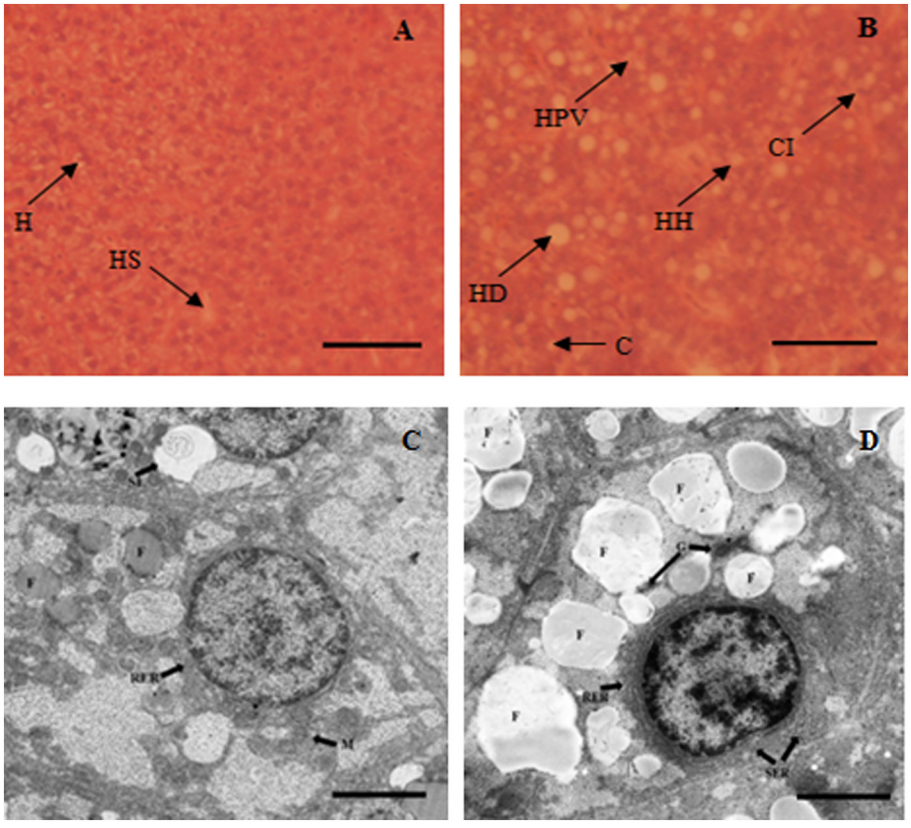

Fig. 5. Light microscopy images of livers stained with hematoxylin and eosin from *Megalobrama amblycephala* juveniles in response to thermal stress. The control group is shown in Fig. A and the heat-treated group is shown in Fig. B.
H: Hepatocytes. HS: Sinusoids. HH: Hypertrophy of hepatocytes. C: Cytolysis. HPV: hepatocellular vacuolization. HD: hydropic degeneration. CI: cellular outline indistinguishable. Scale bar = 20 μm. Magnification × 100. Hepatic cell ultrastructure images from *M. amblycephala* juveniles in response to thermal stress. The control group is shown in Fig. C and the heat-treated group is shown in Fig. D. M: mitochondria. NM: nuclear membrane. RER: rough endoplasmic reticulum. SER: smooth endoplasmic reticulum. L: lysosome. G: glycogen. F: fat. Scale bar = 2 μm. Magnification × 1000.

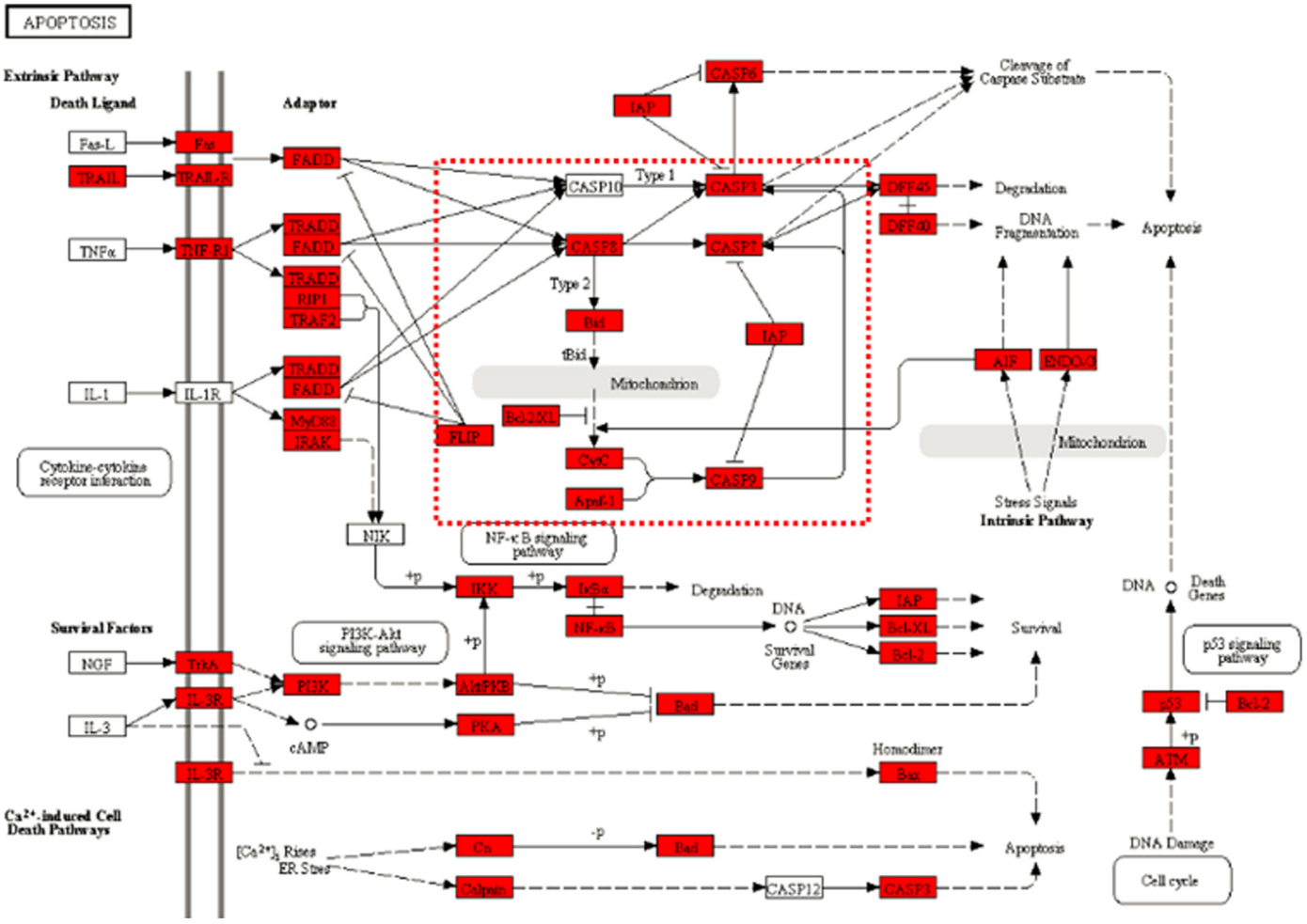

Fig. 6. Differentially expressed genes (DEG) were mapped to a portion of the apoptosis pathway (http://www.genome.jp/kegg-bin/show_pathway?ko04210). Red box represents differentially expressed genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thermal stress damaged the mitochondria, further increasing production of ROS, which were subsequently released into the cytosol and caused oxidative stress [65,66].

5. Conclusion

Overall, this study demonstrated that thermal stress increased relative gene expression of antioxidant enzyme, apoptosis, immune responses and the metabolism of proteins and fats. The results suggest that HSP and GST mRNA transcript levels could be a useful molecular biomarker that is capable to yield valuable information on thermal stress. Furthermore, changes in blood biochemical parameters as well as liver histological disorders were evident in the high temperature-exposed fish. Functional analysis of potentially important pathways identified in *M. amblycephala* and further studies of chronic exposure to temperature elevation will be helpful for understanding molecular and physiological adaptation or thermotolerance mechanisms in this species.

Acknowledgments

This work was supported by the China Central Governmental Research Institutional Basic Special Research Project from Public Welfare Fund (2017JBFM02), the Natural Science Foundation of Jiangsu Pvovince, China (BK20151103), the Modern Agriculture Industrial Technology System Special Project-the National Technology System for Conventional Freshwater Fish Industries (CARS-45), and the National "Twelfth Five-Year" Plan for Science & Technology Support (2012BAD25B07).

Conflict of interest

We declare that we have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2018.11.011.

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