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Alterations to transcriptomic profile, histopathology, and oxidative stress in liver of pikeperch (*Sander lucioperca*) under heat stress

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ABSTRACT

Pikeperch (*Sander lucioperca*) is an economically important cool-water fish. In recent years, its cultivation has become threatened by higher temperatures in summer. We previously investigated the effects of heat stress on pikeperch liver under different temperatures, but the molecular mechanism of the heat-stress response is still unknown. This study applied consistent heat stress (29 °C, 0–48 h) to pikeperch juveniles, and a transcriptomic profile of pikeperch liver under heat stress (29 °C, 0 h) was performed by RNA-Seq. The antioxidant status, changes in liver histology, and antioxidant gene expression at different time points were examined. We identified 403 differentially expressed genes (DEGs), many of which were enriched in KEGG pathways, including protein processing in endoplasmic reticulum (ER), insulin signaling, and immune-related pathways. Among these, the most significant heat-stress-related pathway was protein processing in ER, indicating that this pathway is critical for the heat-stress response. After consistent heat stress at 29 °C, the total antioxidant capacity (T-AOC), the activities of total superoxide dismutase (T-SOD) and catalase (CAT), and the mRNA expression of manganese SOD (Mn-SOD), CAT, and glutathione peroxidase 1 and 7 (GPx1 and GPx7) in the treated groups showed the same trend of first increasing and then decreasing. Levels of malondialdehyde (MDA) content did not show significant differences between samples at 0 h and 3 h, but significantly increased by 6 h, and thereafter decreased. The liver tissue was normal at 0 h (29 °C); however, it suffered histological damage with increased duration of the heat stress. Above all, heat stress at 29 °C seemed to cause oxidative damage and dysfunction in pikeperch liver between 3 h and 48 h. The present results indicate that pikeperch have the capacity to defend against heat stress and maintain relative balance of oxidation-reduction reactions mainly through activating the antioxidant system, protein processing in ER, the insulin-signaling pathway, and immune-related pathways.

1. Introduction

Temperature is a critical environmental factor that can seriously affect the physiological state of fish [1]. Braz-Mota et al. [2] predicted that the average global surface temperature will increase by 6 °C by the end of the century. As ectothermic animals, fish are able to adapt to narrow temperature changes through behavioral, physiological and biochemical adjustments. However, if the temperature rises above a species' threshold limit it can cause physiological disturbances and may even lead to death of the fish [3,4].

Previous studies have proven various adverse effects of heat stress on fish, such as decreasing antioxidase activities [5], reducing metabolic activity [6], and activating apoptosis [7]. Heat stress also induces oxidative stress [8] and increases the production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), the hydroxyl radical OH, and singlet oxygen [9]. A normal level of ROS in an organism is vital for cell apoptosis, the immune response, and signaling transduction [10–12]. However, the generation of excessive ROS can cause various damage to the body, such as protein damage, lipid peroxidation, DNA damage, and impaired cellular functions [13]. First-line defense antioxidants include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH), as the antioxidant defense system is activated as a survival strategy to eliminate the extra ROS caused by heat stress [14]. Previous studies also have shown that these antioxidative factors could maintain relative balance of oxidation-reduction reactions, and consequently protect the animal's body from oxidative damage [15,16].

The pikeperch (*Sander lucioperca*) has a natural distribution across Eurasia and has come to be a valuable aquaculture species [17,18]. Due to the quality of its flesh and high commercial value [19], aquaculture

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production of this species has risen in the past decades; however, episodes of extreme summer heat can have serious negative effects on the growth and survival of pond-cultured pikeperch. Heat stress can affect the immune defense system of fish [8,20]. Persistent heat stress (30 °C, for 12–24 h) led to oxidative damage in the cyprinid *Onychostoma macrolepis* [21]. Our previous study of pikeperch [22] found that heat stress activated protective mechanisms that include the antioxidant defense system and expression of Hsc70; however, when water temperatures surpassed 32 °C the pikeperch suffered serious damage and dysfunction in the liver. In addition, our previous studies have found that the liver is the crucial metabolic organ associated with stress response [23]. However, data are still lacking on the physiological response of pikeperch liver subject to different durations of heat stress, and the underlying genetic basis of the response to heat stress remains unknown.

In the preliminary experiments, we found that some individuals of pikeperch could not survive for 48 h at 30 °C. Therefore, we chose 29 °C as the target heat stress temperature in this study. RNA-Seq technology was used to screen for a number of candidate genes and pathways that may be involved in the heat-stress response in pikeperch. Simultaneously, we investigated molecular indices of oxidative stress (T-AOC, T-SOD, CAT, GPx, GSH, and MDA), observed liver histology, and measured liver injury parameters (ALT/AST levels) after different durations of heat stress. Our research helps to uncover the molecular mechanism of the heat-stress response in pikeperch, and the results will contribute to theories of fish physiology under high-temperature conditions.

2. Materials and methods

2.1. Experimental animals

Juvenile pikeperch (average body weight 143.2 ± 27.9 g [mean ± standard deviation] and average total length 23.2 ± 1.4 cm) were provided by Suzhou Shajiabang East Lake Modern Fishery Science and Technology Development Co. Ltd., China. A total of 144 individuals were equally distributed among nine indoor tanks (16 fish per tank) and the size of each tank was 0.4 m^3. The water temperature was maintained at 23 °C, dissolved oxygen was 7.0 ± 0.5 mg l^{-1}, and pH was 8.0 ± 0.3.

2.2. Experimental procedures and sample collection

After one week's acclimatization under laboratory conditions, the water temperature was increased from 23 °C to 29 °C at increments of +1 °C per hour, and finally maintained at 29 °C for 48 h; controls were maintained at 23 °C. Experimental fish stressed at 29 °C were sampled at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h. Before sampling, fish were anesthetized with MS222, and 1.5 mL tail venous blood was sampled for hematological analysis. Then liver tissues from nine individuals per tank were collected at each sampling time. Part of the liver tissues of 3 individuals was fixed in 10% formalin for histological observation. The remaining fish liver samples were stored at −80 °C for transcriptome analysis, antioxidase activities analysis and qRT-PCR analysis.

Total RNA was extracted with a Total RNA Isolation Kit (Takara Bio Inc., Kyoto, Japan). cDNA was synthesized from RNA in liver tissue, using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA) for reverse transcription, and stored at −80 °C for qRT-PCR analysis. In this study, all animal work and animal protocols were approved by School of Biology and Basic Medical Sciences, Soochow University, China.

2.3. Liver tissue analysis

After fixation, the tissue samples were dehydrated in ethanol, embedded in paraffin, sliced to a thickness of 5 μm, and then stained with hematoxylin and eosin (HE). The prepared slides were sealed using neutral balsam and observed under a Leica DM750 microscope. The total number of cell nuclei was counted in five non-overlapping fields-of-view selected randomly under a 40× objective.

2.4. Liver injury parameters

Liver function was evaluated by measuring the serum levels of ALT and AST. Biochemical assays were performed using a clinical automatic chemistry analyzer (Type 7070, Hitachi, Japan).

2.5. Oxidative stress parameters in liver

For the detection of enzyme activities, 0.50 g of liver tissue was homogenized after adding 4.5 mL of 0.86% saline, for use in an IKA® T-18 electric homogenizer (Germany). Centrifugations were done with a refrigerated centrifuge (Primo R, Germany) at 4 °C, and the supernatant was used for measuring the enzymatic activity. The Coomassie brilliant blue staining method was used to measure the protein content in the enzyme fluid. The activities of T-SOD, CAT, and GPx, and the levels of T-AOC, GSH, and MDA in liver tissue were measured using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) following the instructions provided by the reagent company.

2.6. Transcriptome analysis

2.6.1. Illumina RNA-Seq with the HiSeq X Ten platform

Nine individuals from 23 °C (control) and 29 °C (0 h) group were sampled, respectively. RNA from three individuals of each group was pooled for constructing a sequencing library. Six cDNA libraries were finally constructed using an NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, USA), followed by paired-end sequencing on the Illumina Hi-Seq X Ten platform (Biomarker Technologies, Beijing, China). The RNA-Seq data was deposited into NCBI SRA database, and its accession is SRP221415.

2.6.2. Transcriptome assembly, annotation, and function enrichment

Initial raw reads were filtered using the SeqPrep program and ConDeTri v2.0 software to remove low-quality reads, sequencing adapters, reads with many ambiguous bases, and reads with a length of < 25 bp. All the clean reads were assembled by Trinity software with default parameters. Gene function was annotated based on several databases, including Nr (NCBI non-redundant protein sequences, e-value = 1e-5), Nt (NCBI non-redundant nucleotide sequences, e-value = 1e-5), Pfam (protein families, e-value = 0.01), KOG/COG (clusters of orthologous groups of proteins, e-value = 1e-3), Swiss-Prot (a manually annotated and reviewed protein sequence database, e-value = 1e-5), KO (KEGG Ortholog database, e-value = 1e-10), and GO (Gene Ontology, e-value = 1e-6), using BlastX software.

2.6.3. Analysis of quantification, differentially expressed genes (DEGs), and functional enrichment

The R package DESeq provides statistical routines for determining differential gene expression in digital gene expression data, using a model based on the negative binomial distribution. *P*-values were adjusted using the Benjamini–Hochberg procedure for decreasing the false discovery rate. Unigenes with fold changes of > 2 and an adjusted *P*-value of < 0.05 were assigned as differential expression genes. A functional-enrichment (GO and KEGG) analysis was performed using the package GOseq v1.16.2 and web server KOBAS, respectively, with significance set to $P < 0.05$. Candidate genes involved in the heat-stress response were identified from DEGs that significantly enriched GO terms and KEGG pathways.

Table 1
Primers used for qRT-PCR in pikeperch.

Primer name	Primer sequence (5′-3′)
Mn-SOD-F	TCATCCCCCTCCTCGGTAT
Mn-SOD-R	GACGCTCGCTCACATTCTC
Cu/Zn-SOD-F	TAAGTGCTGTGGGGAAACGAT
Cu/Zn-SOD-R	GATGACCTGGGAAAAGGGGGC
CAT-F	GGCAACAACACCCCCATT
CAT-R	CTCAGGCTCCAGAAGTCC
GPx1-F	CCAGGGATTACACCCAGATG
GPx1-R	CCAGGACGGAGGTACTTCAG
GPx3-F	TTCCATTTACATCACCCTTCTCG
GPx3-R	ACTCACCATTCTTGGCTTCCCTT
GPx7-F	TTCAGTAAAATCGCTGTCGTC
GPx7-R	CCAGAAATTCCAGTCAGGCTC
STAT1-F	TCACGAAGCAAAAAGATTAC
STAT1-R	AGGGCTGCCTCTCTACAACC
LDH-A-F	CAGCAAAGGAAAATGACA
LDH-A-R	GCAACAGGCGAAAGAAAC
RHEB-F	CTGCCACTCATCCTTTTTGT
RHEB-R	CTGCCAGTTCTCCTTCACCT
HES-1B-F	CCTAATGCAGCGTTTGCTCC
HES-1B-R	TCCACTTTTCACCAGGGTCG
LFNG-F	AGTTTGGTGAAGTACGCCGA
LFNG-R	CGAACGGAGAGAATACCGGG
PIK3R1-F	CGGGTAAGCTAGCGTCACAT
PIK3R1-R	CGTCATCAACAAGACCCCCT
GDF2-F	GTGGCGCCTCCAGAATATGA
GDF2-R	TGGATGAGGGCGTGTTTTGA
HMGB-F	CAGCAGTCAGACCCTTCCAT
HMGB2-R	TCGTAAGAAGAACCCCGAGC
GAPDH-F	CCGCCAAGTACGACGACATCAA
GAPDH-R	CGCCGTTGAAGTCTGTGGACAC

2.7. Gene expression analysis with qRT-PCR

The expression levels of Mn-SOD, Cu/Zn-SOD, CAT, GPx1, GPx3, GPx7, STAT1, LDH-A, RHEB, HES-1-B, LFNG, Pik3r1, GDF2, Hmgb-2 and GAPDH in liver of pikeperch were measured by fluorescence-based real-time quantitative PCR (qRT-PCR) with an A200 Gradient Thermal Cycler (Hangzhou, Langji Scientific Instrument Co., Ltd., China). Primers were designed based on sequencing data (Table 1). The relative expression of target genes was calculated with the comparative method $2^{-\Delta\Delta CT}$ and normalized to GAPDH.

2.8. Statistical analysis

All data were presented as mean ± standard deviation (SD) and subjected to one-way analysis of variance (one-way ANOVA) followed by multiple comparison (Tukey's test) to determine significant differences among the treatments and controls. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in the histological structure of pikeperch liver in response to heat stress

HE staining showed that the hepatic cells were regularly arranged with clear boundaries, and the hepatic sinusoids were normal in the control group (Fig. 1a). Hypochromatosis and the vacuolar degeneration of cells appeared in pikeperch liver subjected to 29 °C after 0 h (Fig. 1b). The vacuolar degeneration and hypochromatosis increased with duration of the consistent heat stress at 29 °C, with the most serious damage appearing at 48 h; the hepatic cell boundaries were not clear as of 48 h of heat stress (Fig. 1c and d).

3.2. Changes in antioxidant enzyme activity and physiological injury parameters of pikeperch liver in response to heat stress

When the water temperature was increased from 23 °C (control) to 29 °C (0 h), the GPx activity and GSH contents decreased and the T-AOC activity increased significantly in pikeperch liver, while the activities of T-SOD and CAT and the MDA contents did not show significant changes. With consistent heat stress at 29 °C, T-AOC decreased. The

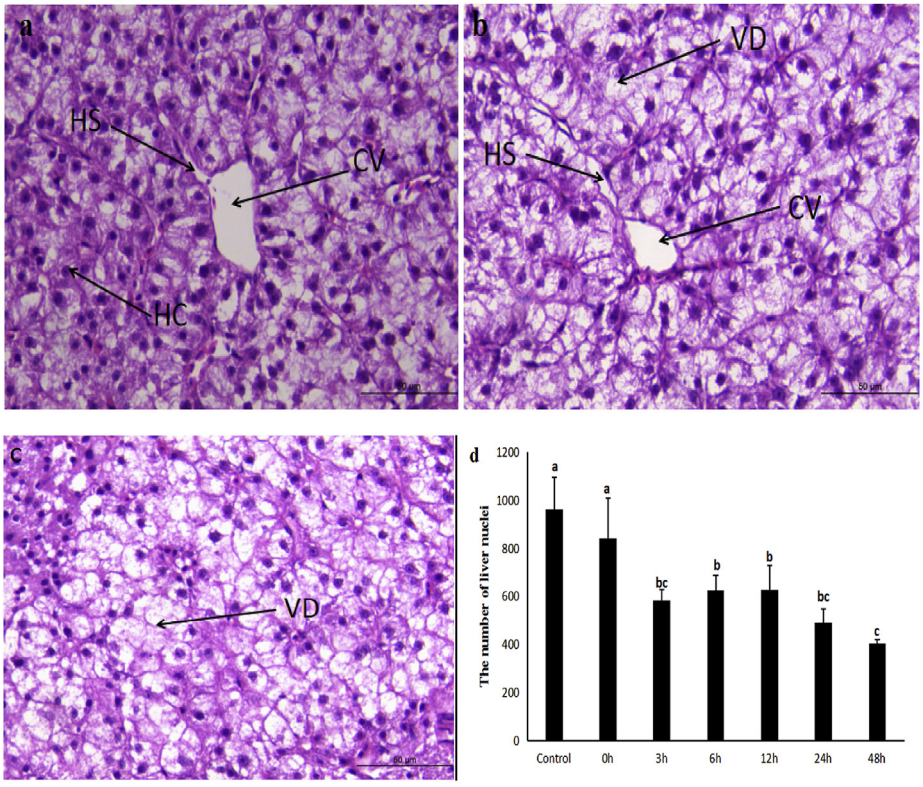

Fig. 1. Histological structure observation of the pikeperch liver under heat stress. a, b, c represents 23 °C (control), 29 °C (3 h) and 29 °C (48 h), respectively. Effect of heat stress on the number of nuclei (d) in the liver of pikeperch. HC means hepatic cells, HS means hepatic sinusoid, CV means central venous, VD means vacuolar degeneration in the liver of pikeperch.

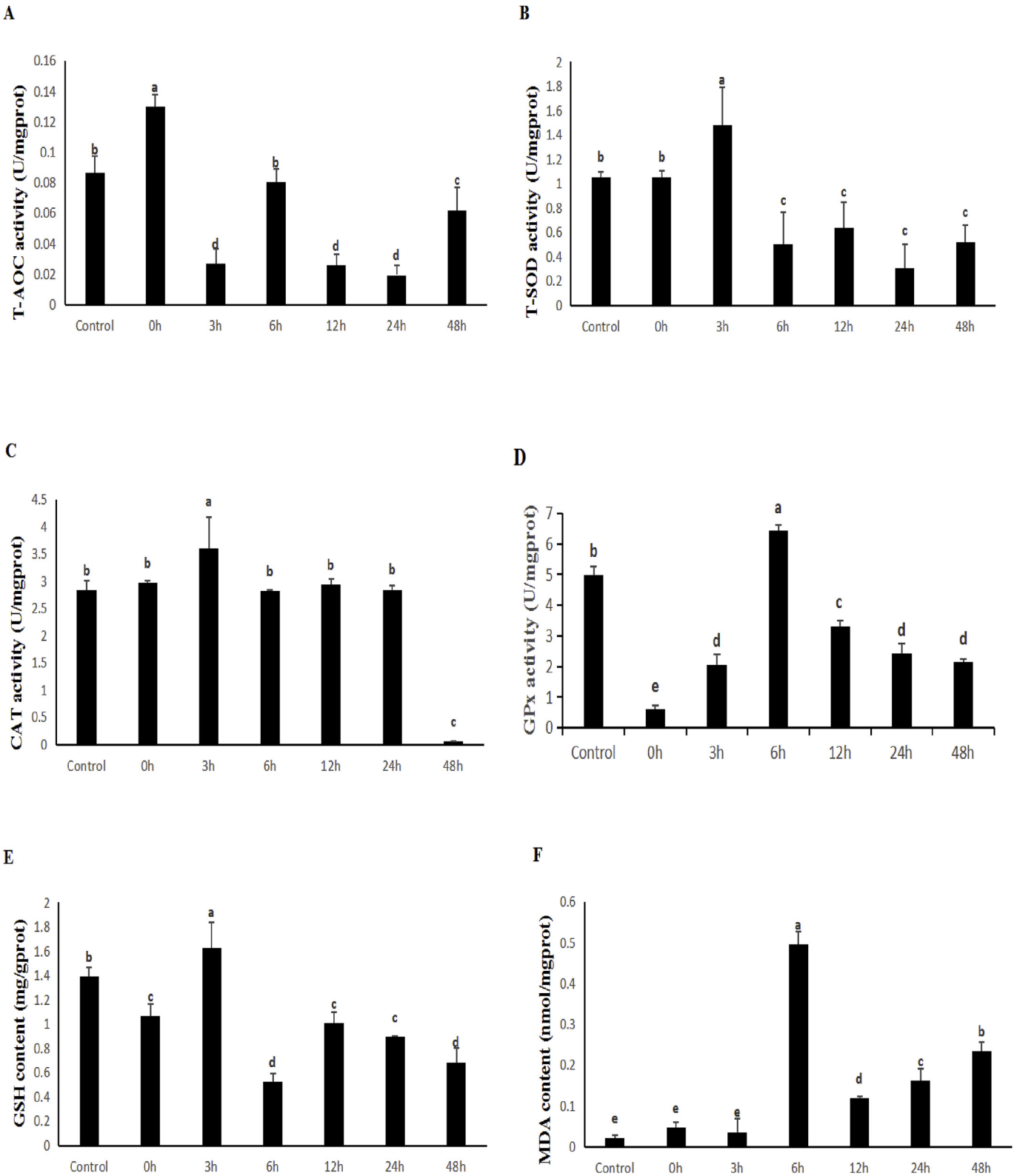

Fig. 2. Effect of heat stress on enzyme activities of T-AOC, T-SOD, CAT, GPx and the content of GSH and MDA in pikeperch liver. The values were expressed as mean ± SEM (n = 3). Mean values with different small letters in each group are significantly different ($P < 0.05$).

activities of T-SOD and CAT and the GSH content increased significantly ($P < 0.05$) as of 3 h, and thereafter decreased in samples from all remaining time points. The activities of GPx and T-AOC increased in samples taken up to 6 h, and thereafter decreased. The MDA contents did not significantly differ in the 3-h group, but had significantly increased in the 6-h group, and then showed a decrease with continuation of the heat stress ($P < 0.05$) (Fig. 2). In addition, compared with the control group, the activity of both ALT and AST significantly increased in the treatment groups as of 0 h and 3 h ($P < 0.05$) but then decreased with ongoing heat stress (Fig. 3).

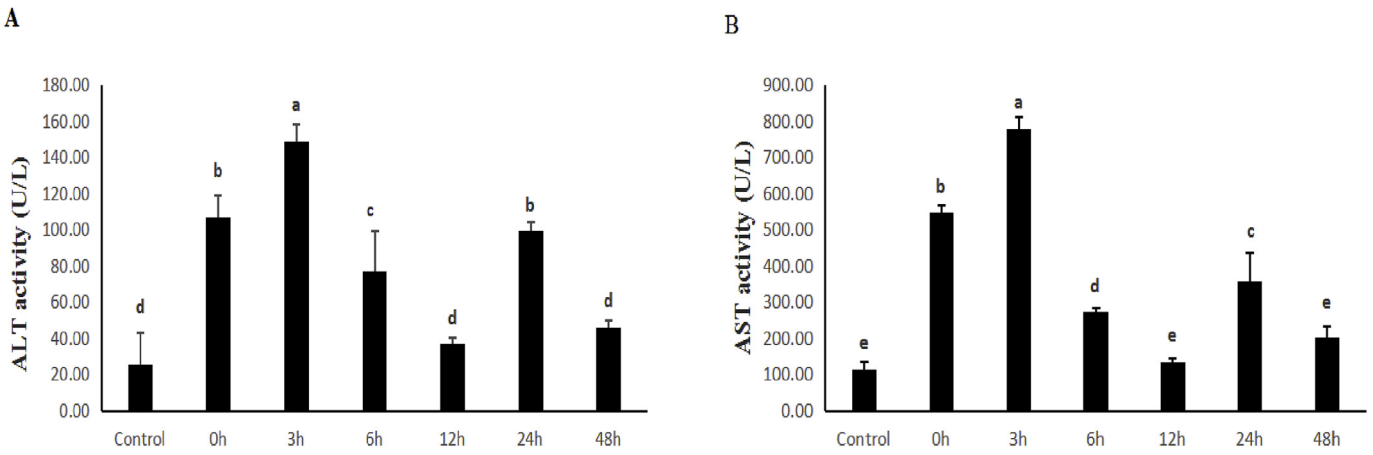

Fig. 3. Effect of heat stress on activity of ALT and AST in serum of pikeperch. The values were expressed as mean ± SEM (n = 3). Mean values with different small letters in each group are significantly different ($P < 0.05$).

3.3. Changes in mRNA expression of antioxidant enzymes

Compared with the controls (23 °C), the mRNA expression levels of Mn-SOD, CAT, GPx1, and GPx7 in pikeperch liver significantly increased once subjected to heat stress (29 °C, 0 h), but the mRNA expression levels decreased with duration of the heat stress ($P < 0.05$). mRNA expression of CAT and GPx1 significantly increased during the early stages of heat stress ($P < 0.05$), peaking at 3 h and 6 h, respectively. The GPx3 mRNA expression level did not experience obvious changes except for an increase in the 3-h group, while Cu/Zn-SOD mRNA expression decreased under heat stress in the 0-h group as compared with in the controls, then increased as of 6 h, and thereafter decreased (Fig. 4).

3.4. Gene ontology and KEGG analyses of DEGs in pikeperch liver under heat stress

A total of 100,871 unigenes (46.57 Gb) were acquired after assembly of six transcriptome libraries, of which 23,648 unigenes were identified in a public database. Under the heat stress in pikeperch liver, 403 unigenes were identified as heat-stress-related DEGs, with 125 downregulated and 278 upregulated (Table S1). The identified DEGs were classified into three major functional categories (biological processes, molecular function, and cellular components) and were enriched in 59 GO categories (Fig. 5). Among these GO categories, the 13 most-significant enriched GO categories included five categories of biological processes, two categories of molecular functions, and four categories involving cellular components. The five enriched subcategories belonging to biological processes were: cellular process, single-organism process, metabolic process, biological regulation, and response to stimulus. The two enriched subcategories of molecular function were: binding activity and catalytic activity. The four enriched subcategories for cellular components were: the cell, cell part, membrane, and membrane part.

A total of 96 DEGs were successfully assigned to 86 KEGG pathways (Fig. 6). Among these, 19 DEGs were identified in four folding, sorting and degradation-related pathways, including protein processing in ER, ubiquitin-mediated proteolysis, proteasome, protein export, and RNA degradation (Table 2); 18 of the 19 DEGs were upregulated, such as DNAJ homolog subfamily B member 1 (DNAJB1), DNAJ homolog subfamily C member 3 (DNAJC3), heat-shock protein HSP 90-alpha (HSP90α), and heat-shock protein 70 (HSP70), while the proteasome activator complex subunit 4B-like were downregulated. Furthermore, 8 DEGs were identified in the insulin-signaling pathway in the endocrine system, of which 6 DEGs were upregulated, including protein phosphatase 1 regulatory subunit 3E-like (PPP1R3E), fructose-1,6-bisphosphatase 1-like (FBPase1), solute carrier family 2, and facilitated glucose transporter member 4 (GLUT4), whereas glucose-6-phosphatase-like (G6Pase) and phosphatidylinositol 3-kinase regulatory subunit alpha isoform X1 (PIK3R1) were downregulated. In addition, 19 DEGs were also identified in seven immune-function-related pathways, involving phagosome, the Toll-like receptor signaling pathway, endocytosis, the NOD-like receptor signaling pathway, lysosomes, peroxisomes, and the intestinal immune network for IgA production. Of the 19 immune-related DEGs, 10 genes were upregulated, such as neutrophil cytosol factor 4 (NCF4), endoplasmin (GRP94), caspase-1-like (caspase-1), and signal transducer and activator of transcription 1 (STAT1), while complement 3 (C3), integrin beta-5 (ITGB5), phosphatidylinositol 3-kinase regulatory subunit alpha isoform X1 (PI3KR1), lysosomal acid phosphatase (ACP), and peroxisomal acyl-coenzyme A oxidase 3 isoform X1 (ACOX3) were downregulated. The primary KEGG pathways and DEGs are listed in Table 2.

3.5. qRT-PCR validation of transcriptome data

The relative mRNA expression levels in pikeperch liver under heat stress (29 °C, 0 h) of eight stress-related genes identified by DEG analysis (i.e. STAT1, LDH-A, RHEB, HES-1B, LFNG, PIK3R1, GDF2, and HMGB2) were further measured by qRT-PCR. The expression patterns of these genes were consistent with the RNA-Seq results (Fig. 7). In addition, the trends in mRNA expression of antioxidant enzymes (Mn-SOD, Cu/Zn-SOD, CAT, GPx1, GPx3 and GPx7) in pikeperch liver under heat stress (29 °C, 0 h) by qRT-PCR in Fig. 4 were also consistent with the RNA-Seq results (Table S2).

4. Discussion

The liver carries out vital physiological functions, such as metabolism, excretion and detoxification, and its state in an organism can best reflect the nutritional physiology and pathological state of the body [24]. Many studies have shown that various environmental stresses can cause changes in liver structure, and even affect its function. For example, nitrite exposure could lead to nuclear hypertrophy and sinusoid dilatation in the liver of bighead carp (Hypophthalmichthys nobilis) [14], and exogenous histamine from diets could cause bleeding and inflammatory cell infiltration in the liver of yellow catfish (*Tachysurus fulvidraco*) [25]. In this study, we found that persistent heat stress led to the liver damage in pikeperch, which included vacuolar degeneration and hypochromatosis. Moreover, at 29 °C, the liver suffered histological damage as of 3 h and was most serious at 48 h.

Malondialdehyde (MDA) or thiobarbituric acid-reactive substances (TBARS) are products of the reaction between ROS and unsaturated

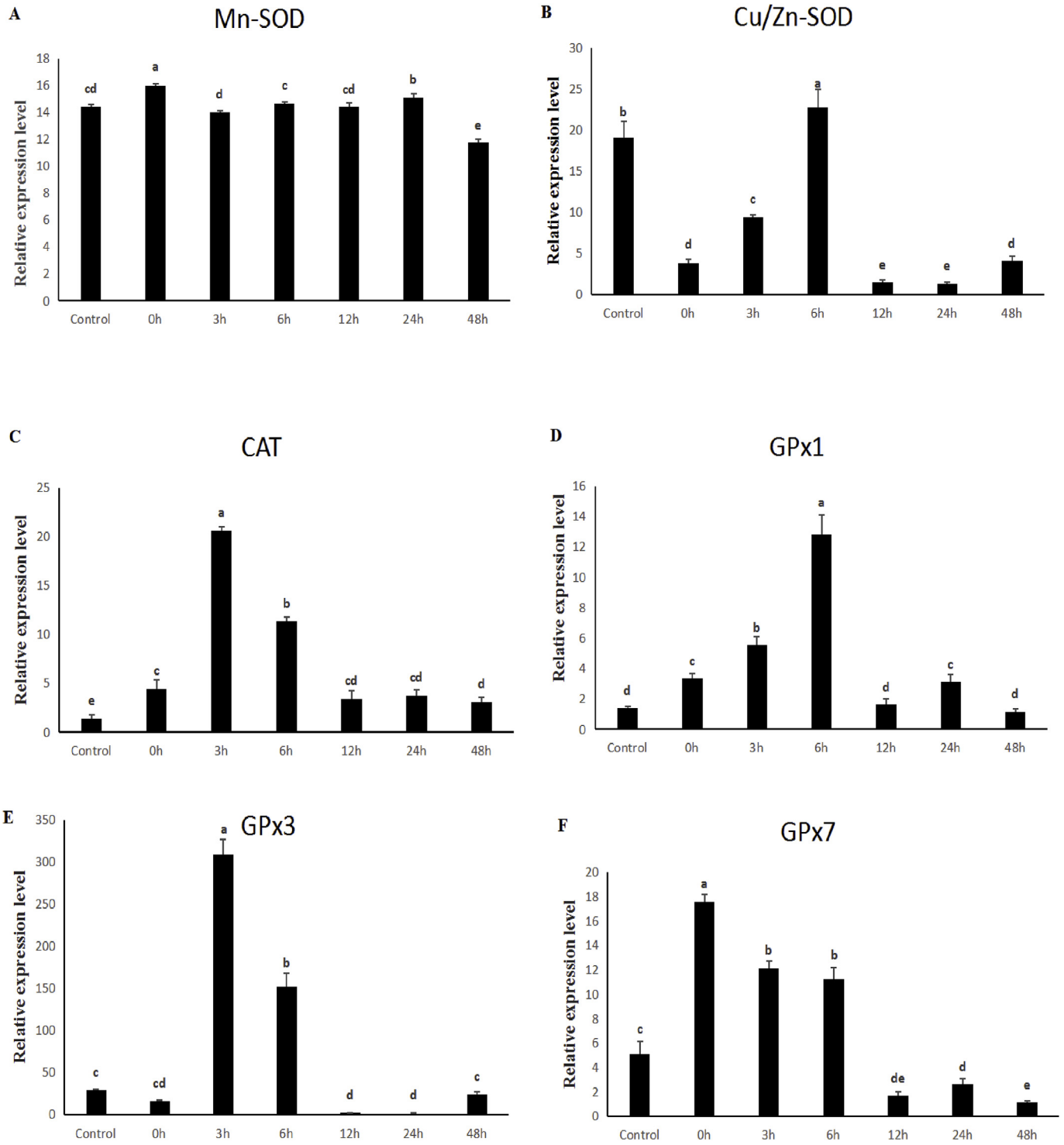

Fig. 4. Effect of heat stress on mRNA level of Mn-SOD, Cu/Zn-SOD, CAT, GPx1, GPx3 and GPx7 in pikeperch liver. The values were expressed as mean ± SEM (n = 3). Mean values with different small letters in each group are significantly different ($P < 0.05$).

fatty acids in the cell membrane, and alterations of their contents in tissues indirectly reflects the degree of oxidative stress and the damage to cellular membranes caused by excess ROS [22]. In this study, there was no significant change in MDA contents up to 3 h of heat stress at 29 °C; this finding may be because of the elimination of ROS by the antioxidant system in the pikeperch liver. However, the significant increase in the MDA level at 6 h (29 °C) may have been caused by extra oxidative stress. In addition to the MDA contents, changes in the activities of ALT and AST in serum often reflect cell damage in liver [26]. In this study, the activities of ALT and AST increased with up to 3 h of heat stress, suggesting that the liver of pikeperch had suffered damage to some extent. Furthermore, we speculate that the decreased activities of AST and ALT after 3 h may have been a result of dysfunction of the liver tissue [22].

T-AOC includes enzymatic antioxidants (e.g. SOD, CAT, GPx) and non-enzymatic antioxidants (e.g. GSH, hypotaurine, ascorbate), and reflects the antioxidant capacity of the organism [27,28]. Increasing T-AOC activity at 29 °C (0 h) showed that the overall antioxidant ability of

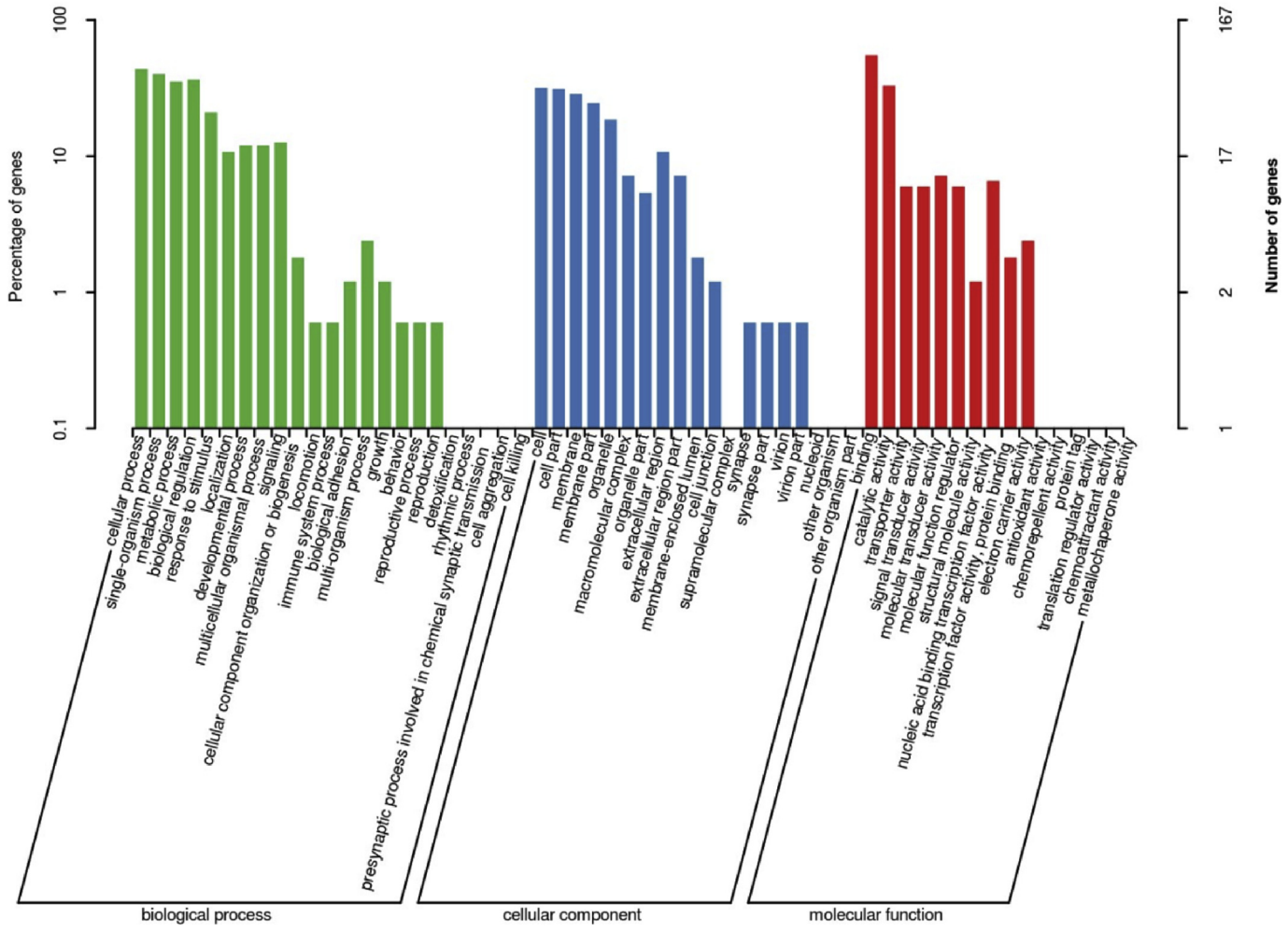

Fig. 5. Gene ontology (GO) classifications of DEGs between the treatment groups and control groups.

pikeperch was improved. Heat-induced antioxidant defenses are closely involved in changes in the activity of antioxidant enzymes [7]. Of the antioxidant enzymes, SOD is considered to be vital in first-line defense against oxidative stress [29]. In general, the elevated activities of antioxidant enzymes play a predominant role in an animal's adaption to oxidative stress, and improved antioxidant defenses can help minimize ROS generation [9,30]. In this study, the activities of T-SOD, CAT, and GPx in pikeperch liver increased during the initial stage of heat stress, which helps explain why the MDA content was not significantly changed in the early stages. In contrast, the activities of T-SOD, CAT, and GPx decreased after 0 h, 3 h, and 6 h of heat stress, respectively. This result suggests that continuous heat stress could reduce the activity of antioxidant enzymes in the fish, similar to the findings of Yu et al. [21] for the cyprinid *Onychostoma macrolepis*. We propose that the decreased activity of the antioxidant enzymes may be due to ROS production that surpasses the threshold of certain antioxidant enzymes, and the overproduction of ROS possibly leads to damage of DNA and protein structure and may even degrade the antioxidant enzymes themselves [31]. In the present study, GPx activity in liver of treated fish decreased significantly at 0 h (29 °C) as compared with the controls, indicating that heat stress of the higher level could inhibit the GPx activity to a certain degree [32]. As one of the most important antioxidants in cells [33], the decrease in the GSH level from 23 °C to 29 °C (0 h) was likely caused by GPx and GST to remove GSH and excessive H_2O_2 from the cells, thus maintaining the normal function and metabolism of liver [14].

Antioxidant gene expression is also considered an accurate estimation of fish antioxidant capacity, where interference of biochemical origin is not involved [34]. Zheng et al. [29] reported that the mRNA level of antioxidant enzymes was inconsistent with the enzyme activity level in large yellow croaker exposed to low concentrations of Zn. Craig et al. [35] also found no association between mRNA expression levels and enzyme activities of antioxidant enzymes in zebrafish under Cu exposure. In our study, there was an increase in the mRNA expression of CAT before 6 h, which might explain the upregulated activities of CAT before 6 h under heat stress. However, the levels of mRNA expression of most of the tested antioxidant enzymes did not exactly reflect their activities. This could have various causes. First, the mRNA level of each specific isoenzyme might not reflect the enzyme's activity level because of the presence of multiple gene copies. For example, in pikeperch, the GPx gene has three isozymes (GPx1, GPx3 and GPx7), while the SOD gene has two isozymes (Mn-SOD and Cu/Zn-SOD). Thus, the mRNA transcription level was limited to one subtype of the SOD and GPx genes encoding a single isoenzyme. Second, antioxidant enzyme activity might also be modulated at post-translational levels [35]. Finally, most eukaryotic genes have introns, which require multiple processing, including splicing, modification and tailing, to form mature mRNA. Moreover, transcription is generally carried out in the nucleus, and translation is carried out in the cytoplasm. mRNA is transported from the nucleus to the cytoplasm, so it is difficult to synchronize in time [36]. The most significantly enriched KEGG pathway in the liver of pikeperch in response to heat stress was protein processing in ER, with 14 genes upregulated as compared that found in the control group. Protein processing in ER was also observed in the

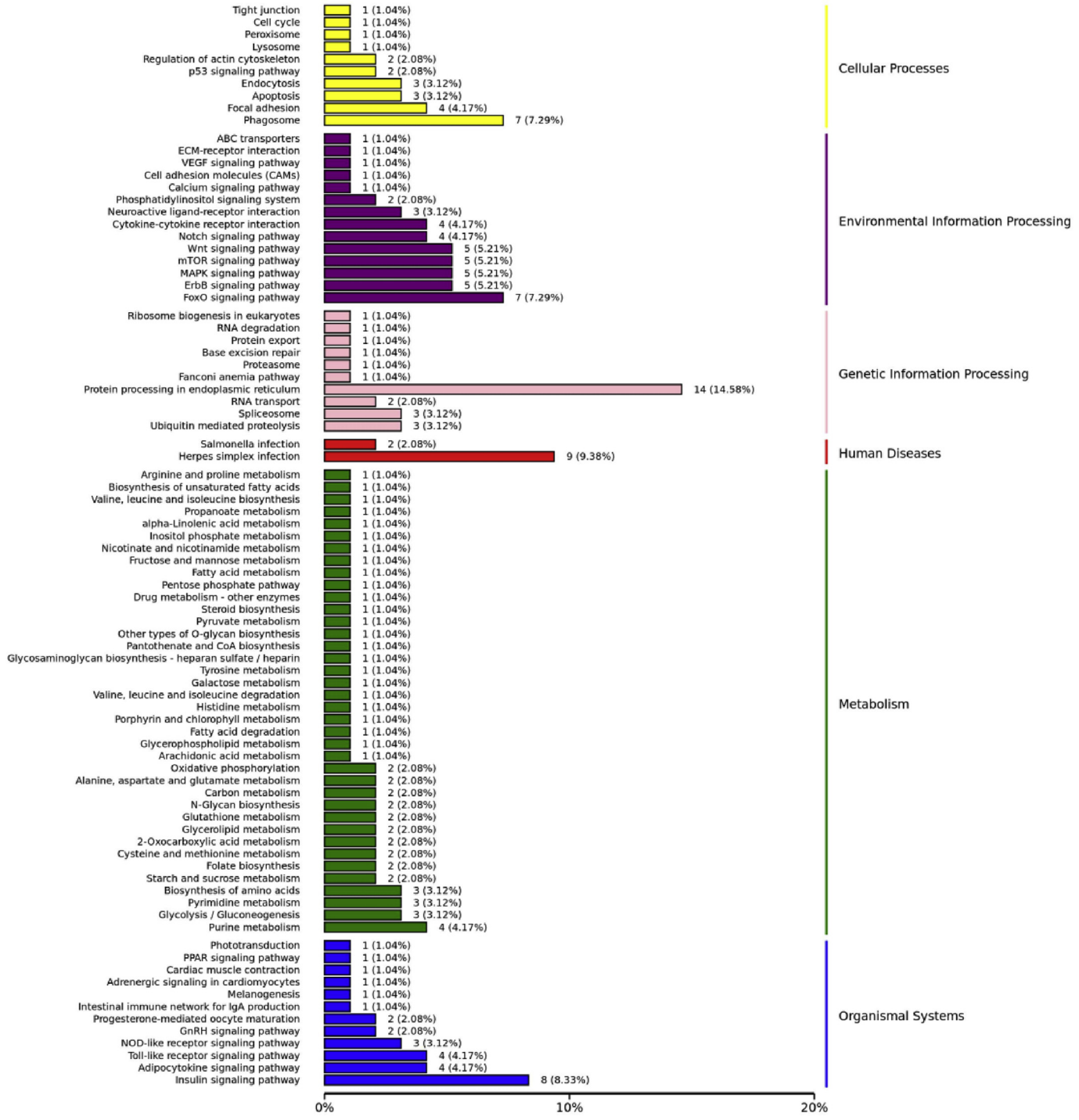

Fig. 6. KEGG annotation of DEGs. Six major pathways were enriched: cellular processes, environmental information processing, genetic information processing, human disease, metabolism, and organismal systems.

liver of rainbow trout under heat stress [23]. In the protein processing in ER pathway, proteins undergo strict quality control to ensure correct folding; terminally misfolded proteins bind to GRP78 and are directionally degraded through the proteasome in a process called ER-associated degradation (ERAD) [37]. In this study, the expression levels of HSP40, HSP70 and HSP90, which participate in the ERAD process in pikeperch, were upregulated under heat stress (29 °C), indicating that the ratio of misfolded proteins had increased. These misfolded proteins in the ER lumen are recognized by chaperones, which deliver misfolded proteins to dislocons at the ER membrane for ubiquitin-dependent degradation and maintaining protein homeostasis [38]. In protein processing in ER, most of the annotated DEGs are heat-shock proteins (Hsps), which not only help to stabilize and correctly refold the denaturing proteins but also facilitate removal of damaged proteins that cannot be properly folded [39,40]. HSP40 proteins can regulate the activity of HSP70 proteins by stimulating their ATPase activity and by stabilizing their interactions with substrate proteins [41]. HSP70 and HSP90 are the most broadly studied proteins in the HSP families and their expression can be induced in response to a series of stress conditions, including heat stress [1,42]. In this study, the upregulation of HSP genes, including HSP40 (DNAJA1, DNAJB1, DNAJB6, DNAJC3), HSP70 (HSPA8, GRP78, HSP70), and HSP90 (GRP94, HSP90α),

Table 2
List of the genes of a few significant changed KEGG pathways under heat stress (29 °C) in pikeperch. "Up" represents the up-regulated genes, "Down" represents the down-regulated genes.

Pathway/Gene Id	Gene Title	log_2(FC)	Up/Down
ko04141/Protein processing in endoplasmic reticulum			
DNAJB1	DNAJ homolog subfamily B member 1	2.30	Up
HSPA8	Heat shock cognate 71 kDa protein isoform X3	2.67	Up
Glu2β	Glucosidase 2 subunit beta	1.30	Up
GRP94	endoplasmin	1.20	Up
DDOST	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit	1.11	Up
DNAJC3	DNAJ homolog subfamily C member 3	1.45	Up
GRP78	Glucose-regulated protein 78 kDa	2.36	Up
HSP90α	Heat shock protein HSP 90-alpha	3.97	Up
HERPUD1	Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein isoform X2	1.04	Up
DNAJB6	DNAJ homolog subfamily B member 6 isoform X2	1.03	Up
DNAJC3X1	DNAJ homolog subfamily C member 3-like isoform X1	1.05	Up
DNAJC3	DNAJ homolog subfamily C member 3-like	1.44	Up
DNAJA1	DNAJ homolog subfamily A member 1-like	1.04	Up
HSP70	Heat shock protein 70	4.48	Up
ko04910/Insulin signaling pathway			
PPP1R3E	Protein phosphatase 1 regulatory subunit 3E-like	1.74	Up
FBPase1	Fructose-1,6-bisphosphatase 1-like	1.49	Up
eIF4E-1A	Eukaryotic translation initiation factor 4E-1A-like	1.06	Up
RHEB	GTP-binding protein Rheb	1.16	Up
G6Pase	Glucose-6-phosphatase-like	−1.22	Down
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha isoform X1	−1.07	Down
GLUT4	Solute carrier family 2, facilitated glucose transporter member 4	1.43	Up
GLUT4	PREDICTED: solute carrier family 2, facilitated glucose transporter member 4	1.59	Up
ko04120/Ubiquitin mediated proteolysis			
HERC5	E3 ISG15–protein ligase HERC5-like	1.37	Up
UBA1	Ubiquitin-like modifier-activating enzyme 1	1.10	Up
ko04620/Toll-like receptor signaling pathway			
TFAP1	Transcription factor AP-1-like	1.64	Up
c-Jun	C-Jun	1.46	Up
STAT1	Signal transducer and activator of transcription 1	1.03	Up
PI3KR1	Phosphatidylinositol 3-kinase regulatory subunit alpha isoform X1	−1.07	Down
ko04144/Endocytosis			
CXCR4	C-X-C chemokine receptor type 4	1.69	Up
HSPA8	Heat shock cognate 71 kDa protein isoform X3	2.67	Up
HSP70	Heat shock protein 70	4.48	Up
ko04621/NOD-like receptor signaling pathway			
GRP94	Endoplasmin	1.20	Up
HSP90α	Heat shock protein HSP 90-alpha	3.97	Up
caspase-1	Caspase-1-like	1.14	Up
ko04142/Lysosome			
ACP	Lysosomal acid phosphatase	−1.00	Down
ko04146/Peroxisome			
ACOX3	Peroxisomal acyl-coenzyme A oxidase 3 isoform X1	−1.86	Down
ko04672/Intestinal immune network for IgA production			
CXCR4	C-X-C chemokine receptor type 4	1.69	Up

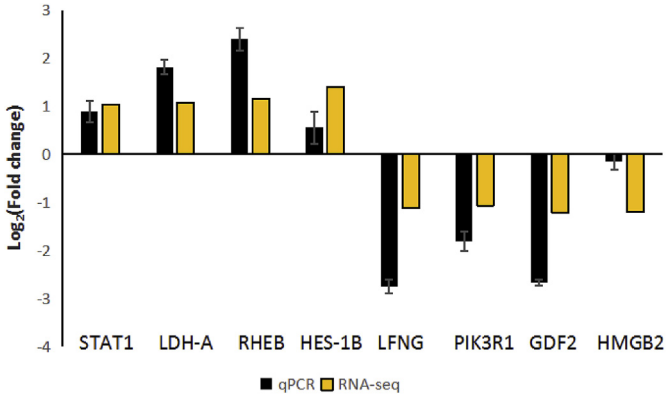

Fig. 7. Comparison of gene expression levels determined by RNA-seq and qRT-PCR methods in pikeperch liver.

suggests that HSP genes were induced significantly to maintain protein homeostasis and protect the pikeperch from heat shock.

The insulin-signaling pathway may play an essential role in the adaption of pikeperch to varying temperatures. The insulin-signaling pathway mainly controls the balance of glucose regulation in the organism [43]. FBPase-1, a key gluconeogenic enzyme, was significantly elevated in the insulin-signaling pathway, reflecting that gluconeogenesis may be enhanced under heat stress to some extent. When insulin binds to the receptor, it triggers a series of responses, which cause the GLUT4-rich vesicles to move to the outer membrane of the cell, and the vesicle membrane fuses with the outer cellular membrane. GLUT4 translocates to the outer membrane, where it has the best activity, and binds to glucose to then transports glucose into the cell [44]. In this study, the expression of GLUT4 gene in liver of pikeperch was significantly increased, indicating that the rate of glucose uptake had increased. We speculate that the heat stress increased energy demands, which required the fish to adopt metabolic adjustments, such as changes in gluconeogenic enzyme activities.

In the present study, the seven enriched and immune-related pathways identified were phagosome, the Toll-like receptor signaling pathway, endocytosis, the NOD-like receptor signaling pathway, lysosomes, peroxisomes, and the intestinal immune network for IgA production. Genes involved in these pathways included ITGB5, NCF4, C3, ACP, STAT1, HSP90 family, and caspase-1. The digestion of

microorganisms in phagosomes is a complicated process, in which nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) plays an important role. NADPH oxidase can kill bacteria by generating superoxide, and NCF4 is a component of NADPH oxidase [45]. Our data showed that NCF4 was significantly downregulated under heat stress, suggesting that the phagosome pathway was inhibited. The complement system in fish is important in immune defense against bacterial invasion and inflammation. C3 is a key component of both classical and lectin pathways [46], and has been found to be downregulated under many stressors [47]. In this study, C3 mRNA expression was downregulated at 29 °C (0 h), suggesting that the complement system was also inhibited by heat stress. Lysosomes fuse with phagosomes during maturation, and kill and digest invading pathogenic microorganisms by releasing products [48]. Lysosomal ACP is a typical hydrolase involved in pollution detoxification and the elimination of toxins, and also plays a key role in the immune system [49]. The decrease of mRNA expression of ACP in this study indicated that the immune defense of pikeperch was weakened by heat stress. STAT1 can induce the synthesis of caspase precursors [50] and inhibit the expression of anti-apoptotic proteins Bcl-2 and Bcl-X [51]. In our study, the STAT1 gene was significantly upregulated, suggesting the heat stress might cause apoptosis. The activation of caspase-1 can induce a pro-inflammatory response and perform the processing of pro-inflammatory interleukins, such as IL-1β, IL-18, and IL-33 [52]. In this study we observed significant downregulation of caspase-1. This variation suggests that heat stress may lead to inflammation in pikeperch. Above all, our results indicated that the immunity of the pikeperch liver may be inhibited under heat stress. In addition, gene ontology (GO) annotations revealed that the annotated DEGs were primarily assigned to various terms of the biological-processes categories, which is consistent with the results of research with tilapia and grass carp under stress [53,54]. In this study, the GO terms were mainly enriched in binding and catalytic activity for the molecular-function domain; cellular process and single-organism process for the biological-process domain; and cell and cell part for the cellular-component domain—which are all directly or indirectly connected with the immune response in fish [55].

In summary, short-term heat stress can cause oxidative stress and mild damage to pikeperch liver, weakening the fish's immune defense system. Pikeperch liver suffered histological damage and dysfunction under consistent heat stress at 29 °C after 3–48 h, with the most serious damage observed as of 48 h. In addition, the antioxidant system, protein processing in ER, and the insulin-signaling pathway appear to play important roles in the heat-stress response in pikeperch liver. Our results provide information on the molecular mechanisms of the heat-stress response in pikeperch, and contribute to our understanding of the transcriptomic and physiological responses to heat stress in fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.11.014.

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